ABSTRACT

Functional proteomics provides a powerful approach to screen for alterations in protein expression and posttranslational modifications under conditions of human disease. In this study, we use protein screening to examine markers of melanoma progression, by profiling melanocyte versus melanoma cell lines using two-dimensional electrophoresis and mass spectrometry. Eight candidate markers were identified as differentially regulated in transformed cells. In particular, hepatoma-derived growth factor (HDGF) and nucleophosmin B23 were strongly correlated with melanoma. Nucleophosmin B23 is a nuclear and centrosome-associated protein, which has been implicated as a target for cyclin E/cyclin-dependent kinase 2 (CDK2) in modulating centrosome duplication and cell cycle control. Western blotting of one-dimensional and two-dimensional gels showed that the form of nucleophosmin B23 that is up-regulated in melanoma represents a posttranslationally modified form, most likely reflecting enhanced phosphorylation in the tumor-derived cells. In contrast, Western analysis of HDGF demonstrated increased expression of all forms in melanoma cell lines compared with melanocytes. Immunohistochemical analysis of human tissue biopsies showed strong expression of HDGF in early and late stage melanomas and low expression in melanocytes and nontumorigenic nevi. Interestingly, biopsies of nevi showed a graded effect in which HDGF immunoreactivity was reduced in nevoid nests penetrating deep into the dermis compared with nests at the epidermal-dermal junction, suggesting that HDGF expression in nevi is dependent on epidermal cell interactions. In contrast, biopsies of melanoma showed strong expression of HDGF throughout the tumor, including cells located deeply within dermis. Thus, expression of this antigen likely reports a reduced dependence of protein expression on epidermal interactions.

INTRODUCTION

Cutaneous malignant melanoma is among the fastest increasing malignancies in humans, featuring a capacity to occur in young persons and a refractoriness to therapy once metastases have occurred. The paradigm is that melanoma tumors develop from melanocytes after progressive stages. In this model, sunlight induced mutations of melanocytes produce nontumorigenic nevi; these may form atypical or dysplastic nevi, with relatively high probability for transition to primary melanoma. Early primary melanomas are characterized by horizontal growth confined to the epidermis (RGP), but can develop into advanced primary melanomas characterized by more aggressive growth and dermal invasion (VGP). Late stage VGP tumors are almost always associated with metastases (1).

The process of melanocyte transformation is also characterized by reduced anchorage dependence, contact inhibition, and dendricity and increased surface mobility (2). Important goals are to identify proteins preferentially expressed during melanoma progression, and to develop markers for molecular pathology. In particular, markers that distinguish early melanoma from benign melanocytic nevi as well as markers that distinguish RGP melanomas from later malignant stages would be useful diagnostically. Several molecular markers of melanoma progression have been identified using different approaches, including antibody screening (3), differential or subtractive cloning procedures (4), and microarrays (5, 6).

Proteomics is a powerful screening method for alterations in protein expression and posttranslational modifications. Recent protein profiling in human tumors has been successful in identifying proteins differentially expressed between hepatoma and normal liver cell lines (7), as well as markers that define the degree of differentiation of bladder squamous cell carcinoma (8). In this study, we assess melanoma progression by profiling proteins in melanocyte versus melanoma cell lines using 2-DE and mass spectrometry, from which eight candidate markers are identified. Two candidate markers were nucleophosmin/B23 and HDGF, both of which were strongly up-regulated in melanoma, and cathepsin D which was down-regulated in melanoma cell lines. Follow-up studies of HDGF expression in human tissue biopsies showed behavior similar to those of cell lines, where HDGF immunoreactivity discriminates between nontumorigenic nevus/melanocytes versus later stage melanoma tumors. Thus, functional proteomics successfully reveals potential markers of tumor initiation.

MATERIALS AND METHODS

Cell Culture. All melanoma cell lines originated from the laboratory of Meenhard Herlyn, Wistar Institute, except for A375 and HS294-T, which were obtained from American Type Culture Collection. Melanoma cells were cultured in 75-cm² T-flasks containing 25 ml 10% fetal bovine serum, RPMI 1640, 5 μg/ml insulin. At 80% confluence, cells were washed once with PBS and incubated for 24 h in 0.05% fetal bovine serum-RPMI 1640. Primary melanocyte lines FOM 71 and FOM 78 were explanted from patients and cultured as described (9). For harvesting, cells were washed twice with 13 ml of PBS, thoroughly drained, and lysed with 700 μl of lysis buffer containing 7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 1% immobilized pH gradient buffer (pH 4–7; Amerham), 1 mM DTT, 1 mM benzamidine, 25 μg/ml leupeptin, 20 μg/ml pepstatin A, 10 μg/ml aprotinin, 1 mM sodiumorthovanadate, and 1 μM microcystin. Protein concentrations were determined using the Coomassie Plus reagent (Pierce).

2-DE. Chemicals and reagents used were analytical grade or better. Methods for separation of proteins by 2-DE were performed as detailed in previous publications (10, 11). Isoelectric focusing was carried out using commercial Immobiline Dry Strips (18 cm; pH 4–7) rehydrated overnight in presence of 150 μg of protein lysate and run on a Multiphor II flatbed electrophoresis unit with an EPS 3500 XL power supply (Amersham). Electrofocusing was performed for 28 h at a maximum current of 2 mA and maximum power of 7 W using the following gradient: Ramp 0--300 V over 0.05 h; constant 300 V for 1 h; ramp 300--3500 V over 9 h; constant 3500 V for 18 h. Before the second dimension, each immobilized pH gradient strip was reduced in 1% DTT for 15 min and alkylated in 4% iodoacetamide for 15 min. Second-dimension SDS-PAGE was performed using 20 × 20 cm slab gels cast and run on a Protean

Received 3/27/03; revised 7/24/03; accepted 7/31/03.

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2 Supported by NIH Grant R01 CA87648 (K. A. R.) and a postdoctoral fellowship from the Association pour la Recherche contre le Cancer (K. R. B.).

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1 The abbreviations used are: RGP, radial growth phase; VGP, vertical growth phase; 2-DE, two-dimensional electrophoresis; 1-DE, one-dimensional electrophoresis; HDGF, hepatoma-derived growth factor; pl, isoelectric point; ACN, acetonitrile; DAB, 3,3'-diaminobenzidine; TBS, Tris-buffered saline; CDK, cyclin-dependent kinase.
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II xi system (Bio-Rad) for 16 h at 29 mA/gel. Analytical gels were silver stained as described by Blum et al. (12), whereas preparative gels were silver stained using a low fixation protocol (13). Gels were dried between sheets of cellophane and scanned at 300 dots per inch (UMAX PowerLook II) in transmission mode.

2-DE gels were analyzed using Melanie III software (GeneBio). After spot detection, percent volume intensities (% vol) were determined by integrating digitized staining intensities over the areas of individual features, normalizing by total intensity of all features, and multiplying by 100. Comparison of melanocytic, RGP, VGP, and metastatic classes of cell lines was assessed using the nonparametric Kolmogorov test, the parametric Student t test, and the nonparametric Student t test. Results were considered significant when statistical scores were >0.99 for the Student t test, 2.7 for Wilcoxon, and 0.75 for Kolmogorov-Smirnoff.

Mass Spectrometry. In-gel digestions were performed on silver-stained proteins as described (14). Proteins were excised from multiple wet gels or from dried gels followed by rehydration with deionized water. Gel pieces corresponding to one protein were pooled and washed with 100 mm ammonium bicarbonate (NH₄HCO₃) for 20 min, and 50% ACN-0.1% trifluoroacetic acid for 20 min. The gel pieces were dehydrated with 50% ACN-0.1% trifluoroacetic acid for 20 min, and 50% ACN-0.1% trifluoroacetic acid for 20 min in a sonicating water bath. Supernatants were pooled and dried in a Speedvac (Heto), reswelled in 10–30 μl of sequencing grade porcine trypsin (Promega) in 25 mm NH₄HCO₃, to a final concentration of 20 ng/μl trypsin, and incubated overnight at 37°C. Supernatants were removed and transferred to separate tubes, and gel pieces were reextracted twice with 50% (v/v) with concentrate peptides before mass spectrometry.

Peptide digests were analyzed by matrix-assisted desorption ionization-time of flight mass spectrometry using an ABI Voyager DE-STR instrument (Applied Biosystems) with delayed extraction. Tryptic digestions were mixed 1:1 (v/v) with α-cyano-4-hydroxy-trans-cinnamic acid (Hewlett Packard) and spotted onto gold-plated matrix-assisted desorption ionization sample planchets. Mass spectra were summed over 150 acquisitions (20 kV accelerating voltage, 10 V guide wire voltage, 100 ns delay) using Voyager 5.0 software, and spectra were processed using Data Explorer. Internal calibrations were performed using tryptic autolysis fragments at 842.51, 1045.56, 2211.10 Da, and masses from contaminating keratin ions were eliminated. Peptide fingerprinting data were matched against the National Center for Biotechnology Information nonredundant database using ProFound (http://www.expasy.ch/tools/pi_tool.html), with mass tolerance of 0.1 Da and allowing 1 missed cleavage. Artifactual modification of peptides by acrylamide adducts with cysteine and methionine oxidation was considered during database searching. Evaluation of results considered the number of peptides matched to identified proteins, percent coverage of protein sequence, and apparent mass/pl of proteins.

Peptides in tryptic digestes were then sequenced using a Q-STAR Pulsar q-time of flight mass spectrometer (Applied Biosystems) equipped with an o-matrix-assisted desorption ionization interface. Accurately measured masses of tryptic peptides and fragment ions were used to search for protein candidates in the NCBInr database using the program MS-Tag in Protein Prospector (http://prospector.ucsf.edu).

Immunoblotting. Cell lysates were separated by 1-DE or 2-DE, then transferred to polyvinylidene difluoride (PVDF) membranes, and blots were probed with donkey antimouse, goat, or rabbit secondary antibodies diluted into Tris-borate-saline, 0.1% Tween 20, and 5% nonfat dried milk. Blots were then probed with donkey antimouse, goat, or rabbit secondary antibodies (1:5,000) for 1 h at room temperature, and Vector Red Alkaline Phosphatase Substrate Kit II (Vector Laboratories) was used in place of Fast Red, which allowed examination by both bright-field and fluorescence microscopy.

Immunohistochemistry. Sections from formalin-fixed, paraffin-embedded patient biopsies were analyzed. Samples were deparaffinized and rehydrated, and antigen was retrieved using antigen retrieval solution (DAKO Cytomation) in a decloaking chamber (Biocare Medical) first at 120°C for 30 s and then at 85°C for 10 s. Slides were then cooled to 25–30°C and rinsed with tap water. Sections were either double stained for HDGF and S100 or S100B (to distinguish melanocytes) or single-stained for HDGF alone. Primary antibody dilutions used were 1:1200 for S100 (polyclonal; DAKO Cytomation), 1:25 for S100B (DAKO Cytomation), and 1:750 for HDGF [affinity purified polyclonal; Everett et al. (16)]. The order in which sections were double stained with S100/S100B + HDGF + Vector Red were varied to verify consistent results. Negative controls (mouse, rabbit; DAKO Cytomation) were used to verify low background staining. Double staining for S100/S100B and HDGF was performed using the DAKO EnVision Doublestain System (DAKO Cytomation) according to the manufacturer’s directions except that before incubation with HDGF, sections were treated with Powerblock Universal Blocking Reagent (BioGenex). HDGF was incubated for 30 min at room temperature, and Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories) was used in place of Fast Red, which allowed examination by both bright-field and fluorescence microscopy. For immunohistochemistry with anti-HDGF antibody alone, samples were blocked with Powerblock,
rinsed twice for 3 min with TBS (DAKO Cytomation) with 0.05% Tween 20 (Sigma), and incubated for 30 min at room temperature with anti-HDGF antibody in dilution buffer (TBS with 1% BSA, 0.01% sodium azide). Specimens were rinsed 3×3 min in TBS with 0.05% Tween 20 and then incubated with DAKO Envision Alkaline Phosphatase (DAKO Cytomation) for 30 min. After TBS-Tween 20 rinses, the sections were treated with Vector Red Alkaline Phosphatase Substrate Kit 1 according to the manufacturer's directions.

All sections (single or double stained) were counterstained with hematoxylin and azure B (2.5 mg/ml Azure B; Sigma; 8.5 mM acetic acid, 1.5 mM sodium acetate, and 12.5% acetone), the latter which stains melanin green, and then incubated in bluing reagent (Richard Allan Scientific) for 1 min.

RESULTS

Profiling of Melanocyte versus Melanoma Cell Lines by 2-DE and Mass Spectrometry. Whole cell lysates were made from 2 primary melanocyte cell strains and 12 melanoma cell lines (Table 1). The latter included representative cell lines cultured from RGP, VGP, and metastatic tumors that were catalogued with respect to tumor pathology as well as tumorigenicity and invasiveness of cells in culture and in nude mice (17–20). Extracts from each cell line were analyzed in replicate by 2-DE, using pH 4–7 focusing gradients in the first dimension and 8–18% polyacrylamide gradients in the second dimension. Proteins were visualized by silver staining, which yields highest sensitivity but limited dynamic range because of saturation. Therefore at least four well-resolved gels from three different experiments were analyzed for each cell line, resolving ~2000 distinct protein spots on each gel. A representative gel (Fig. 1) shows resolution of proteins from an early primary melanoma cell line (WM35).

The 14 selected cell lines were assigned into melanocyte, RGP, VGP, and metastatic classes based on tumor pathology and in vitro soft agar growth. Protein features were clustered into the four classes based on intensity, normalized to the sum of intensities of all spots on the gel, and quantified as percentage volume by gel image analysis software. Data for individual spots were then averaged over replicate gels, and results from automated imaging were confirmed by direct visual inspection of gels. Eight protein spots (Fig. 1) were identified in this screen as candidate markers, based on differences in averaged normalized intensity between classes as described in “Materials and Methods.”

Each of the spots was processed by in-gel digestion and analyzed by peptide mass fingerprinting and tandem mass spectrometry. All eight proteins (M1–M8) were matched to entries in the human non-redundant database. The relative normalized intensities within different cell lines for these proteins are indicated in Fig. 2, and characteristics of the proteins are summarized in Table 2.

Biochemical Characterization of Candidate Melanoma Markers. Inspection of Fig. 2 illustrates that candidate markers, HDGF (M1) and nucleophosmin/B23 (M2), showed significant and reproducible differences between melanocytes and all of the melanoma cell lines, whereas cathepsin D (M3, M7) and glutathione S-transferase-ω (M6) showed differences between melanocytes and more than one-half of the tumor lines. Other markers, such as quinolinate phospho-
ribosyltransferase (M4) and 14-3-3γ (M8) varied between different melanoma stages and are candidates for distinguishing RGP versus VGP cell lines. Further biochemical characterizations of HDGF, cathepsin D, and nucleophosmin/B23 were performed on the cell lines used in the screen as well as five additional melanoma cell lines (Table 1).

Nucleophosmin/B23 Is Posttranslationally Modified in Melanoma Cells. An important advantage of functional proteomic analysis by 2-DE is the ability to reveal changes in protein covalent modifications. This was illustrated by the behavior of nucleophosmin/B23 during melanoma progression. Silver-stained 2-DE gels showed an increase in a single spot (M2) corresponding to nucleophosmin/B23, which was located immediately adjacent to two higher intensity spots that were invariant with cell type (M2b, M2c; Fig. 3A). Mass spectrometry/mass spectrometry analysis identified the two invariant spots as nucleophosmin/B23, indicating that M2 represents an acidic isoform increased in melanoma cells. This was corroborated by immunoblotting 2-DE gels, which showed immunoreactivity of all three spots as well as an obvious increase in immunoreactivity of the form with acidic pI (Fig. 3B). Examination of 19 cell lines by 1-DE immunoblotting showed variable expression of nucleophosmin/B23 in different melanoma cell lines, compared with melanocytes (Fig. 3C). Thus, increased posttranslational modification of nucleophosmin/B23, rather than overall protein expression, correlated with melanoma progression.

Cathepsin D (Molecular Mass 34 kDa) is Overexpressed in Melanocytes. Cathepsin D is a ubiquitous lysosomal aspartyl endoproteinase which is processed from an inactive 52-kDa procathepsin D to an active 48-kDa single-chain molecule in late endosomes and early lysosomes (21). The 48-kDa chain is then further processed in lysosomes to a 14-kDa light chain, derived from the NH₂ terminus, and a 34-kDa heavy chain, derived from the COOH terminus. In the 2-DE screen, cathepsin D was observed as two silver-stained protein spots (Fig. 4A, M3, M7). Both had masses corresponding to the 34-kDa form and showed reduced intensities in melanoma cell lines compared with melanocytes. Immunoblots of 2-DE gels, probed with anticathepsin D antibodies directed against the COOH terminus revealed two and five different forms in different cell lines, each with various pI values but all with similar mass. In some cell lines (e.g., SBCL2 and 1205Lu), the reduced total amount of the 34-kDa heavy chain correlated with increases in acidic isoforms (Fig. 4B). Thus, multiple forms of cathepsin D were present in various cell lines, but only spots...
corresponding to M3 and M7 were consistently down-regulated in melanoma compared with melanocytes. Immunoblots of 1-DE gels confirmed that the 34-kDa form was present in melanocytes and significantly reduced in 15 of 19 melanoma cell lines (Fig. 4C). The 34-kDa heavy chain was the major immunoreactive form in both melanocytes and melanoma (data not shown).

**HDGF Is Overexpressed in Melanoma.** HDGF is an intracellular protein that is mitogenic in several cell types, including endothelial and smooth muscle cell systems. Although this protein was identified by silver staining as a single protein spot in the initial screen (Fig. 5A), immunoblotting of 2-DE gels revealed that the antigen resolved into multiple forms each with the same apparent molecular mass, indicating covalent modification of this marker (Fig. 5B). Nevertheless, immunoblotting after 1-DE revealed that all forms of HDGF showed elevated expression in 19 melanoma cell lines compared with melanocytes (Fig. 5C), indicating strong correlation of expression with early stage melanoma.

Additional experiments were performed to investigate the expression of HDGF in human melanoma biopsies. Tissue sections of primary melanomas at various stages of progression were immunostained with anti-HDGF, counterstained with hematoxylin-azure B, and visualized by light microscopy. Nuclear reactivity with anti-HDGF was observed within the epidermal layer in keratinocytes, which is consistent with the previous characterization of this antigen as a nuclear protein that is expressed in epithelial cells (Fig. 6A and B). Intense staining was also observed in all stages of melanomas, which included in situ melanomas, level II tumors confined within the epidermis (in which three of four biopsies were lentigo maligna melanomas), as well as level IV tumors that were strongly invasive into the dermal layer (Fig. 6, A–F). The speci-
ficity of the antibody is indicated by peptide competition controls (Fig. 6, O and P).

In contrast, melanocytes within the epidermal layer, which were identifiable by their hyperchromogenicity and dissociation from basal keratinocytes, were either negative or weakly stained (Fig. 7). Furthermore, nests of nontumorigenic melanocytes forming benign or atypical/dysplastic nevi also showed reduced staining with anti-HDGF, compared with the melanomas (Fig. 6, G–L). Staining intensities in nevi revealed a graded effect, in which cells within junctional nests were more positive for HDGF reactivity than cells deep within the dermal layer (Fig. 6, G and I). Interestingly, Spitz nevi were variably reactive, where many biopsies showed staining intensities comparable with advanced melanoma (Fig. 6, M and N). Of 24 nevus and tumor samples examined immunohistochemically, 54% of cells within benign nevoid nests were positively reactive, whereas 78–90% of cells within melanoma tumors were reactive (Table 3). Although the differences between melanocyte/nevi versus melanoma were not absolute, quantitative differences in the number of immunoreactive cells were nevertheless observed. In addition, the level of staining within positively reactive cells differed substantially between transformed and nontransformed tissue, showing weak reactivity in nevoid cells and strong reactivity in tumor cells. The results indicate that HDGF expression in human tissues is correlated with early stages of melanoma, in a manner that is recapitulated by expression behavior in cell lines.
DISCUSSION

In this study, candidate molecular markers of melanoma progression were identified using 2-DE and mass spectrometry. The proteomic approach allowed comparative analysis of many gene products, using a unique set of melanoma cell lines that were carefully catalogued with respect to tumor staging and included cells grown from early primary melanoma (22). Most of the proteins identified showed variations between melanocyte and melanoma cell lines and may reflect changes occurring on tumor initiation. In two examples (quinolinate phosphoribosyltransferase and 14-3-3γ), changes in intensity appeared to correlate with progression from RGP to VGP tumors. Most striking were the abundances of HDGF and an acidic form of nucleophosmin/B23, which were elevated in all melanoma cell lines compared with melanocytes, and are thus good candidates for the development of diagnostic probes.

Most of the tumor-deregulated proteins identified in the screen were sample specific, as reported in earlier studies of prostate cancer (23) and breast cancer (24, 25). In a study that systematically investigated the genetic profiles of primary tumors and corresponding metastases in a number of melanoma patients, the most frequent genetic alteration detected during metastatic progression was the loss of p16INK4a, which was detected in only 4 of 14 cases (26). In another study examining tumoral heterogeneity by 2-DE in ovarian cancer, a large degree of intertumoral heterogeneity observed, whereas intratumoral heterogeneity was very low (27). There are
various reasons why observed protein changes were not more consistent within each class of melanoma cell lines in the study. First, the molecular complexity of melanoma tumor biology may result in few alterations in common between tumors. In addition, cell lines derived from tumors likely accumulate further mutations and protein changes during long-term culture. Together, these results support the hypothesis that early and fundamental mutations occur in key genes to drive malignancy, followed by accumulation of further mutations that are not specifically found in all tumors.

Our study illustrates an advantage of expression profiling at the proteomic level in that it revealed a correlation in melanoma lines with a posttranslationally modified form of nucleophosmin/B23. Although the M2 protein spot showed intensity that increased in a manner strongly correlated with melanoma, analysis of total nucleophosmin/B23 protein expression by 1-DE immunoblotting showed incomplete correlations. Further analysis of neighboring silver-stained spots by mass spectrometry as well as 2-DE immunoblotting revealed at least three spots that represented differentially modified forms of nucleophosmin/B23. The spot with most acidic pI was up-regulated in the initial screen, indicating that covalent modification of nucleophosmin/B23 is elevated in melanoma, an event that would not have been observed in experiments profiling mRNA or total protein.

Nucleophosmin/B23 localizes to centrosomes as well as nucleoli and regulates centrosome duplication in a cell cycle-dependent manner (15). It is phosphorylated at several residues by various protein kinases, including CDK1-cyclin B (28), casein kinase II (29), and CDK2-cyclin E (15), the latter event which promotes centrosome duplication during S-G2. Furthermore, overexpression of nucleophosmin/B23 decreases the susceptibility of human leukemia HL-60 cells to retinoic acid-induced differentiation and apoptosis (30), and in-
increased stability of nucleophosmin/B23 protein is involved in the antiapoptotic effect of ras during serum deprivation (31). Thus, phosphorylation of nucleophosmin/B23 is correlated with cell cycle progression and cell survival, and this likely reflects its corresponding regulation and function in melanoma. Phosphorylation of nucleophosmin/B23 has not been specifically described in any tumor type, although elevated nucleophosmin protein and mRNA expression have been associated with breast cancer, colorectal cancer, and leukemia (32–34). It will be important to identify the posttranslational modification(s) relevant to melanoma and develop probes to the modified residues to test their potential relevance to disease progression and to provide useful reagents for the diagnosis and characterization of early melanoma.

Cathepsin D has been previously characterized as a marker of malignant cancers, including melanoma, breast, colorectal, and squamous cell carcinomas (35). This antigen has been shown to be a prognostic factor in breast cancer in a number of studies (36). However, in malignant melanoma, the results in different studies are conflicting, where the protein has been found to be either positively or negatively correlated with advanced stages of tumorigenesis (37–40). The appearance of acidic isoforms has been described in two breast cancer cell lines in which the amino acid sequence of cathepsin D was normal, but its glycosylation state appeared to vary (21). Our results indicate that there are several isoforms for cathepsin D with a mass of 34 kDa. In two cell lines, SBCL2 and 1205Lu, we also observed the appearance of additional acidic isoforms.

HDGF is a heparin-binding growth factor, originally purified from conditioned media of the human hepatoma-derived cell line, HuH-7 (41). HDGF has only recently been characterized, and changes in expression have never been systematically investigated in any tumor type, although it promotes hepatocyte proliferation and is correlated with reduced radioresistance in esophageal cancers (42–44). The translated cDNA sequence of HDGF reveals little homology with known growth factors but instead shows sequence similarity to non-DNA-binding domains of the chromatin-associated protein, HMG1. HDGF is mitogenic and localizes to both cytosol and nucleus when expressed in cultured cells, and nuclear targeting of HDGF via a consensus nuclear localization sequence is required for stimulation of DNA replication in vascular smooth muscle cells (42). When overexpressed, HDGF can be purified from conditioned media and can act as a mitogen when added exogenously to fibroblasts, HuH-7, aortic endothelial cells, and smooth muscle cells. The mechanism for extrusion to conditioned media is unknown, and it is curious given the nuclear localization of the expressed protein, but the association with growth regulation suggests that HDGF may be causal for transforming behavior. Nevertheless, suppression of HDGF in melanoma cell lines using RNA interference had little effect on extracellular signal-regulated kinase activation or cell growth rate.

In our screen, HDGF expression was highly correlated with melanoma in all cell lines examined. This was mainly attributable to intracellular expression, although the protein was also detected in conditioned media of at least one cell line tested (WM35). Several forms of HDGF with the same apparent mass were observed by 2-DE immunoblotting, suggesting the existence of posttranslationally modified forms. These most likely correspond to differentially phosphorylated forms.

Examination of human biopsies showed a similar expression pattern, in which HDGF was absent or weakly present in nontumorigenic melanocytes and was expressed at a low level in 54% of cells within nevoid tissues. In contrast, HDGF showed high reactivity in both early and late melanoma, and highest expression in advanced malignant tumors (Table 3). The expression of HDGF was graded with progression. Thus, although representation of HDGF in benign nevi was significantly lower than Clark’s IV melanoma (54% versus 90%, respectively), atypical and Spitz nevi showed higher percentages of positive cells than benign nevi. Furthermore, early in situ melanoma and Clark’s II (mostly lentigo) melanoma showed lower percentages of positive cells than advanced malignant tumors. This analysis suggests that HDGF is expressed in benign nevi, but increases its frequency of expression during the transitions into atypical nevi, early melanoma, and late melanoma. Such a protein may be a useful marker for examining properties of nevoid cells that eventually undergo selection for conversion into melanoma.

Staining patterns in nevoid tissue showed heterogeneous reactivity, in which cells within junctional nests showed higher expression of HDGF compared with interdermal nests. In contrast, early and advanced melanoma tissue showed high reactivity toward HDGF that was observed homogeneously throughout the tumor and was independent of depth of dermal penetration. This pattern may reflect a selective process, in which a subset of junctional cells with high HDGF expression preferentially progress into melanoma. Alternatively, the heterogeneity in nevi may reflect processes in which HDGF expression in nevoid and melanocyte cells are subject to control through their interactions with neighboring epidermal cells. For example, melanocyte and nevoid cell interactions with keratinocytes may lead to elevated HDGF expression within junc-

<table>
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<tr>
<th>Stage</th>
<th>No. of biopsies</th>
<th>% of positive cells for HDGF</th>
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<tr>
<td>Benign nevi</td>
<td>4</td>
<td>53.7 ± 12</td>
</tr>
<tr>
<td>Nevi with atypia</td>
<td>6</td>
<td>65 ± 5</td>
</tr>
<tr>
<td>Spitz nevi</td>
<td>4</td>
<td>72 ± 11</td>
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<td>Melanoma in situ</td>
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<tr>
<td>Clark’s II</td>
<td>4</td>
<td>78 ± 8</td>
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<tr>
<td>Clark’s IV</td>
<td>4</td>
<td>90 ± 7</td>
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Table 3 Expression of HDGF in human melanoma biopsies*  

* Biopsies examined for immunoreactivity with anti-HDGF + Vector Red. For each biopsy, 350–450 cells within defined nevoid nests or tumors were scored for reactivity and percentages of positive cells were reported.

4 K. Bernard and N. Ahn, unpublished data.

5 A. Everett, unpublished results.

Fig. 7. Melanocytes are negatives for HDGF. The paraffin section was prepared and stained with anti-HDGF + Vector Red and anti-S100 + DAB, as described in “Materials and Methods.” The picture is from the outside edge of a benign nevi biopsy where normal melanocytes are present (arrows).
tional layers, whereas loss of epithelial-melanocyte interactions may lead to reduced HDGF expression within interdermal nevoids. This is consistent with the low expression of this marker in melanocytes in culture, where epithelial cell interactions are absent. Likewise, the depth independence of HDGF reactivity observed in biopsies of early and advanced melanoma may report independence from transcriptional regulatory processes controlled by tissue interactions in transformed cells. Future studies are needed to investigate the relevance of the melanocyte-melanoma microenvironment in this process and test the hypothesis that HDGF expression observed in early melanoma cell lines may also reflect a reduced requirement for tissue cell-cell interactions in transformation.

In summary, this study illustrates the use of functional proteomics to identify relevant molecular markers of cancer progression. These results may provide a better understanding of tumor biology and may eventually lead to the design and implementation of new cancer diagnostics and therapies.

ACKNOWLEDGMENTS

We thank Dr. Meenhard Herlyn, Wistar Institute, for providing us with melanocyte and melanoma cell lines used in this study. We are also grateful to Norma Aumen, University of Colorado Health Sciences Center, Denver, CO, for expert assistance in preparing paraffin slides of human biopsies.

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