N-Cadherin-mediated Intercellular Interactions Promote Survival and Migration of Melanoma Cells

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ABSTRACT

During melanoma development, loss of functional E-cadherin accompanies gain of expression of N-cadherin. The present study was carried out to investigate the functional significance of N-cadherin in melanoma cells. N-Cadherin mediated homotypic aggregation among melanoma cells as well as heterotypic adhesion of melanoma cells to dermal fibroblasts and vascular endothelial cells. Blocking of N-cadherin-mediated intercellular interaction by N-cadherin-specific antibodies increased the number of cells undergoing apoptosis. N-Cadherin-mediated cell adhesion-activated antiapoptotic protein Akt/PKB and subsequently increased β-catenin and inactivated the proapoptotic factor Bad. Furthermore, N-cadherin promoted migration of melanocytic cells over dermal fibroblasts, suggesting that N-cadherin may also play a role in metastasis. Together, these results indicate that the cadherin subtype switching from E- to N-cadherin during melanoma development not only frees melanocytic cells from the control by keratinocytes but also provides growth and possibly metastatic advantages to melanoma cells.

INTRODUCTION

The incidence of cutaneous melanoma has risen rapidly in the last several decades (1–3). Unlike many other cancer types, melanoma affects a relatively younger population and is notorious for its propensity to metastasize and for its poor response to current therapeutic regimens. Usually, the morbidity is attributable to metastatic disease and not to the primary cancer. Thus, understanding the progression of tumors to the metastatic state and the changes occurring in highly malignant cells are important in the development of new approaches to diagnose, assess, and treat progressive malignancies. The transition from benign lesions to invasive, metastatic cancer occurs through a stepwise process involving changes in expression and function of oncoproteins or tumor suppressor genes, as well as changes that enable tumor cells to overcome cell-cell adhesion and host microenvironmental controls and to invade surrounding tissues and translocate to remote tissues and organs.

Tumor development is a complex phenomenon influenced collectively by various environmental factors such as growth factors and cytokines. Cross-talk between benign precursor cells, malignant cells, and surrounding host cells also influences tumor development. Among the molecules involved in the intercellular communication are cadherins, which have been shown to play a critical role in tumor formation and progression. Cadherins form a family of cell surface glycoproteins that function in promoting calcium-dependent homotypic and heterotypic cell-cell adhesion and serve as the transmembrane components of cell-cell adherens junctions (4). The subtypes of classical cadherins, E-, N-, P-cadherin, are expressed in a cell-, tissue-, and development-specific manner (4). E-Cadherin is the major cadherin in polarized epithelial cells, whereas N-cadherin is expressed mainly by mesenchymal cells, such as myocytes and fibroblasts.

Cadherins appear to determine the location of melanocytes in the skin (5). In mouse development, melanocytes migrate into the epidermis at stage E11.5, where E-cadherin expression increases ~200-fold. E-Cadherin expression then decreases, and the melanocytes leave the epidermis to migrate to hair follicles and dermis. In the hair follicle, melanocytes expressed high levels of P-cadherin but little or no E-cadherin, whereas in the dermis, melanocytes expressed only N-cadherin. In normal human skin, E-cadherin is expressed on the cell surface of keratinocytes and melanocytes and is the major adhesion molecule between epidermal melanocytes and keratinocytes (6–8), whereas N-cadherin is expressed by fibroblasts and endothelial cells. During melanoma development, a progressive loss of E-cadherin expression has been observed; superficial compartments of nevi show heterogeneous membrane E-cadherin immunoreactivity, junctional nevus cell nests display heterogeneous or diffuse cytoplasmic staining (9), whereas melanoma cells, with few exceptions, do not express E-cadherin (8, 10, 11). Disruption of E-cadherin-mediated cell adhesion frees the melanocytic cells from microenvironmental control by keratinocytes, whereas restoration of E-cadherin expression in melanoma cells results in keratinocyte-mediated growth control and downregulated expression of invasion-related adhesion receptors (12).

Despite the loss of E-cadherin expression by melanoma cells, these cells express high levels of N-cadherin in vitro (6, 8, 10) and in vivo (8, 13). The switching of cadherin subtypes during melanoma development might enable melanoma cells to interact directly with other N-cadherin-expressing cells, such as fibroblasts and vascular endothelial cells, thus affecting tumor-host cell adhesion, tumor cell invasion and migration, and gene expression. Indeed, N-cadherin expression in melanoma cells allows communication with N-cadherin-expressing fibroblasts through gap junctions (14), and anti-N-cadherin antibodies can delay the transendothelial migration of melanoma cells (15, 16).

In the present studies, we addressed the potential role of N-cadherin in the development and progression of melanoma. N-Cadherin mediates homotypic aggregation among melanoma cells as well as heterotypic adhesion of melanoma cells to dermal fibroblasts and vascular endothelial cells, which may improve their ability to migrate through stroma and enter the vasculature. We demonstrated that N-cadherin-mediated cell adhesion activates antiapoptotic protein Akt/PKB and subsequently stabilizes β-catenin and inactivates proapoptotic factor Bad. N-Cadherin also promotes migration of melanoma cells over dermal fibroblasts, suggesting that N-cadherin may play a role in metastasis. However, its in vivo involvement remains to be proven. This study provides a basis for further characterization of the molecular mechanism of N-cadherin-mediated metastasis in melanoma, which could yield potential insights for diagnostic or therapeutic applications.

MATERIALS AND METHODS

Cell Culture. Human melanoma cells were isolated and cultured as described (17). After establishment of continuous growth, cells were maintained in medium W489, a 4:1 mixture of MCDB153 and L15, supplemented with 2 mm Ca²⁺, heat-inactivated FBS (2%), and insulin (5 μg/ml) in a 37°C, 5%
CO2 atmosphere at constant humidity. Primary human dermal fibroblasts were initiated as explant cultures from trypsin-treated and epidermis-stripped neonatal foreskin and maintained in DMEM with 10% FBS. Human melanocytes were isolated from foreskin and maintained in MCD153 medium supplemented with 2% FBS, endothelin-3, and stem cell factor. Transcomplementing 293 cells were maintained in DMEM with 10% FBS. All tissue culture reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and Life Technologies, Inc. (Gaithersburg, MD). HUVEC3 were grown on gelatin-coated plastic dishes in M199 medium supplemented with 10% FCS, 150 μg/ml endothelial cell growth factor, and 5 units/ml heparin (18).

**Antibodies.** Mouse mAb against N-cadherin (A-CAM clone GC-4; Sigma Chemical Co.) was used in blocking experiments for inhibition of N-cadherin-mediated adhesion, immunostaining, and flow cytometry. Another mouse anti-N-cadherin mAb (33-3900; Zymed Laboratories; San Francisco, CA) was used for Western blotting and immunoprecipitation. Mouse anti-E-cadherin mAb SH-E78-7 (Zymed) was used for Western blotting and flow cytometry as well as control antibody in N-cadherin blocking experiments. Anti-α-, anti-β-catenin, and anti-γ-catenin (plakoglobin) mAbs were from Transduction Laboratories, Inc. (Lexington, KY). Anti-Akt, anti-phospho-Akt (Ser-473), anti-Bad, and anti-phospho-Bad antibodies were from New England Biolabs, Inc. (Beverly, MA). Mel-5, a mouse mAb against the melanocytic marker tyrosinase-related protein-1 was from Signet (Dedham, MA). FITC- and Cy3-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

**RT-PCR and DNA Sequencing.** Total RNA was isolated from the cell lines using Trizol (Life Technologies, Inc.). Reverse transcription was carried using the Superscript system (Life Technologies, Inc.). PCR primers for amplification of β-catenin exon 3 were: forward primer, 5’-GCT GAT TTG ATG GAG TTG GA-3’; and reverse primer, 5’-ACT TCT TGT TGA GTG AA-3’. PCR reactions were performed using the Expand High Fidelity PCR system (Boehringer Mannheim, Mannheim, Germany). PCR products were precipitated in ethanol and dissolved in pH 8.0 TE buffer (10 mM Tris-HCl, 1 mM EDTA). Sequences were determined using an ABI automatic DNA sequencer (PerkinElmer).

**Construction and Production of Replication-deficient N-Cadherin Adenoviral (N-cad/Ad5) Vector.** Full-length human N-cadherin cDNA was obtained by RT-PCR. cRNA was derived from normal human dermal fibroblasts by Trizol reagent (Life Technologies, Inc.) and reverse-transcribed into cDNA using Superscript II (Life Technologies, Inc.). The forward primer was 5’-TCG TCT TCG AGC TCT CCT CCA TCT GTC CCC GG-3’, and the reverse primer was 5’-AAG GAT CAC CTG AAG TAC AGT CAT CAC-3’. The PCR product was cloned into pCR2.1 vector (Invitrogen, Groningen, the Netherlands) and sequenced. The adenoviral vector was constructed according to described methods developed by He et al. (19). Briefly, full-length human N-cadherin cDNA was digested with BamHI and EcoRV and inserted into the multiple cloning sites (BglII and EcoRV) of pShuttle-CMV (from Dr. Bert Vogelstein, Howard Hughes Medical Institute and The Johns Hopkins Oncology Center). The resulting shuttle vector (pShuttle-CMV-N-cad) was linearized by Pmel digestion and cotransformed with adenovirus backbone vector pAd-easy-I into recombination-competent Escherichia coli strain JS183. Recombinant adenovirus vector containing N-cadherin coding sequence was identified by enzyme digests and PCR. The resulting vector, pAd-CMV-N-cad, was transfected into 293 cells using the calcium phosphate coprecipitation method. For overexpression of N-cadherin in melanocytes, subconfluent melanocytes were infected with 20 pfu/cell N-cad/Ad5 for 4 h at 37°C in a 96-well microtiter plate (2 μl/well TUNEL label) or in 50 μl/well TUNEL label as negative control. After 60 min at 37°C incubation, cells were transferred onto slides and visualized by fluorescence microscopy (excitation, 480 nm; detection, 515–565 nm).

**Immunoblotting, Immunofluorescence, and Immunoprecipitation.** For immunoblotting, cells were washed with PBS and harvested in RIPA buffer (50 mM NaHPO4 pH 7.2, 1% deoxycholate, 1% Triton X-100, 0.5% SDS, 150 mM NaCl, 2 mM EDTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM PMSF). Total protein concentrations were measured using the bicinchoninic acid assay (Pierce Chemical Co. Rockford, IL). Samples (15–20 μg/lane) were separated on an 8% SDS/PAGE gel, transferred onto polyvinylidene difluoride membrane and probed with specific primary antibodies. To detect the signal, peroxidase-conjugated secondary antibody was added, followed by exposure using enhanced chemiluminescence (Amersham, Arlington Heights, IL). Some of the immunoblots were quantified using NIH image software. For immunofluorescence assay, melanoma cells or melanoma/fibroblast mixtures (ratio, 1:1) were seeded in 8-well chamber slides (Lab-Tek, Nunc, Inc., Naperville, IL). After 2 days, cells were washed with PBS, fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, blocked with 5% BSA, and incubated sequentially with antibodies GC-4 (IgG1, 10 μg/ml), biotin-conjugated goat antimouse IgG1, Cy3-conjugated streptavidin, Mel-5 (IgG2a), and FITC-conjugated horse antimouse IgG2a. As a negative control, normal mouse serum (dilution, 1:100) was used instead of primary antibody. All incubations were performed at room temperature for 30 min with at least three washings. Cells were mounted in anti-fade medium Gel Mount (Biomeda Corp.) and examined by fluorescence microscopy. For immunoprecipitation, confluent cells were scraped off, washed with PBS, and extracted in PBS containing 1% Triton X-100, 1% NP40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors (leupeptin, aprotinin, and pepstatin). After immunoprecipitation with anti-N-cadherin mAb or nonimmune mouse IgG at a final concentration of 1 mg/ml for 1 h at 4°C with shaking, protein A-Sepharose CL-4B beads (Pharmacia Biotech, Uppsala, Sweden) were added and incubated for another 4 h. Samples were washed three times with lysis buffer, boiled in Laemmli buffer containing β-mercaptoethanol, and subjected to electrophoresis on an 8% SDS-polyacrylamide gel. Separated

3 The abbreviations used are: HUVEC, human umbilical vein endothelial cell; mAb, monoclonal antibody; RT-PCR, reverse transcription-PCR; CMV, cytomegalovirus; TUNEL, terminal deoxynucleotidyltransferase-mediated nick end labeling; Dil, 1’1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate.

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proteins were transferred onto polyvinylidene difluoride membrane and immunoblotted with anti-catenin mAbs and peroxidase-conjugated secondary antibody. Signals were detected using enhanced chemiluminescence.

**Cell Fractionation.** Cells were harvested in Versene at different times after treatment and washed with cold PBS, resuspended in cold hypotonic lysis buffer (10 mM Tris (pH 7.4), 10 mM NaCl, 1.5 mM MgCl₂, 1 mM sodium orthovanadate, 0.5 mM DTT, leupeptin, pepstatin, and aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride), and kept on ice for 2 min. A Dounce homogenizer was used to break the cells while keeping the nuclei intact. Subcellular fractions (nuclear fraction and non-nuclear fraction) were obtained by centrifugation, and the purity was checked under the light microscope.

**Intercellular Migration Assay.** To test the migration of melanocytic cells on the surface of the fibroblast monolayer as well as the extracellular matrix formed by fibroblasts, dermal fibroblasts were seeded into four-well chamber slides (5000 cells/well) and allowed to form a monolayer over 3 days. Melanoma cells or melanocytes (~4000 cells) prelabeled with DiI were seeded on top of the fibroblast monolayer using a sloped surface so that the seeded cells settled on one side of the chamber (Fig. 6A). After 50% of the seeded cells attached, unattached cells were removed by extensive washes with PBS. Cells were cultured in regular medium. For blocking assays, antibody GC-4 was added to the medium at 40 μg/ml for the first day and at 20 μg/ml on each additional day. Cells migrating into a preselected region were counted under a fluorescence microscope. Each migration assay was repeated six times. Student t test P < 0.05 was considered significant.

**Disruption and Reestablishment of N-Cadherin-mediated Intercellular Interaction.** This assay was performed as described (21) with modifications. Briefly, cells were cultured in regular medium until confluent, medium was replaced with W489 (serum and growth factor free), and cells were grown for 12 h. Four mM EGTA (Sigma Chemical Co.) was added, and 30 min later, medium was replaced with fresh W489 (serum and growth factor free), with or without blocking antibody GC-4 (40 μg/ml). Cells were collected at different times, lysed, and immunoblotted.

**RESULTS**

N-Cadherin Is Expressed in Melanoma Cells and Fibroblasts, Forming Adherens Junctions at Cell-Cell Contact Sites. Previous studies (6, 8, 13) showed that melanoma cells lose expression of E-cadherin but gain expression of N-cadherin. However, it is not clear whether N-cadherin expressed in melanoma cells leads to formation of adherens junctions. To address this question, total cell lysates at equal amounts from two representative melanoma cell lines, WM278 proteins were transferred onto polyvinylidene difluoride membrane and immunoblotted with anti-catenin mAbs and peroxidase-conjugated secondary antibody. Signals were detected using enhanced chemiluminescence.

**Cell Fractionation.** Cells were harvested in Versene at different times after treatment and washed with cold PBS, resuspended in cold hypotonic lysis buffer (10 mM Tris (pH 7.4), 10 mM NaCl, 1.5 mM MgCl₂, 1 mM sodium orthovanadate, 0.5 mM DTT, leupeptin, pepstatin, and aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride), and kept on ice for 2 min. A Dounce homogenizer was used to break the cells while keeping the nuclei intact. Subcellular fractions (nuclear fraction and non-nuclear fraction) were obtained by centrifugation, and the purity was checked under the light microscope.

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**Cell Fractionation.** Cells were harvested in Versene at different times after treatment and washed with cold PBS, resuspended in cold hypotonic lysis buffer (10 mM Tris (pH 7.4), 10 mM NaCl, 1.5 mM MgCl₂, 1 mM sodium orthovanadate, 0.5 mM DTT, leupeptin, pepstatin, and aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride), and kept on ice for 2 min. A Dounce homogenizer was used to break the cells while keeping the nuclei intact. Subcellular fractions (nuclear fraction and non-nuclear fraction) were obtained by centrifugation, and the purity was checked under the light microscope.

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(primary VGP melanoma) and 1205Lu (metastatic melanoma), together with melanocytes, fibroblasts, and HUVECs were analyzed by immunoblotting with antibodies against N-cadherin, E-cadherin, α-catenin, β-catenin, and γ-catenin (plakoglobin; Fig. 1A). Cell surface expression of N-cadherin and E-cadherin in those cells was confirmed by flow cytometry analysis (data not shown). Both immunoblotting and flow cytometry indicated high levels of N-cadherin and no detectable E-cadherin in both melanoma cell lines tested. As expected (22), dermal fibroblasts expressed high levels of N-cadherin, although trace levels of E-cadherin were detected. No correlation between expression of α-, β-, and γ-catenin (plakoglobin) expression levels and melanoma progression was observed. Amplification of β-catenin exon 3 by RT-PCR, followed by sequence analysis, revealed no mutation in the melanoma cell lines tested (in addition to WM278 and 1205Lu, 22 other melanoma cell lines from different progression stages were also tested), consistent with the observation that an activating mutation is rare in melanoma (23). Furthermore, β-catenin and α-catenin were coimmunoprecipitated with N-cadherin from melanoma cells (Fig. 1B), demonstrating the integrity of the junctional complexes in these cells. Immunostaining of melanoma cells cocultured with dermal fibroblasts indicated localization of N-cadherin in the cell-cell contact regions (Fig. 1C and D). Therefore, although melanoma cells lose expression of E-cadherin, they switch on N-cadherin and form N-cadherin-mediated intercellular interactions between themselves and with dermal fibroblasts.

N-Cadherin Mediates Cell-Cell Adhesion and Aggregation. In cell aggregation assays using melanoma cell lines, aggregates were observed in the presence of the control mAb but not in the presence of the N-cadherin blocking mAb GC-4 (Fig. 2A). The calculated index of aggregation differed significantly ($P < 0.01$) between the control and blocking antibody-treated cells (Fig. 2B). Similarly, N-cadherin-mediated adhesion between melanoma cells and dermal fibroblasts (Fig. 3A) and between melanoma cells and vascular endothelial cells (Fig. 3B) was observed. Furthermore, WM39 cells, which express no N-cadherin, did not adhere to either fibroblasts or vascular endothelial cells (data not shown), suggesting that the interaction was N-cadherin specific.

N-Cadherin Promotes Melanoma Cell Survival under Anchorage-independent Conditions. In the cell aggregation experiments, after extended overnight incubation with shaking, phase-contrast mi-

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![Fig. 3. N-Cadherin mediates adhesion of melanoma cells to fibroblasts (A) and to vascular endothelial cells (B). Melanoma cells were labeled with red fluorescent dye DiI. For blocking experiments, cells were preincubated with mAb GC-4 (40 μg/ml) or control antibodies at 4°C for 30 min. About 5000 cells were added to a dermal fibroblast monolayer (A) or a HUVEC monolayer (B) in eight-well chamber slides and allowed to adhere for 30 min. After removal of nonadherent cells, slides were examined under a fluorescence microscope. The light dots show the DiI-labeled melanoma cells.](image)

![Fig. 4. N-Cadherin promotes melanoma cell survival under anchorage-independent conditions.](image) Cells were cultured in 24-well culture plates coated with 1% agarose with control or blocking mAb (40 μg/ml). Cells were harvested at different time points as indicated. A, cell viability determined in triplicate trypan blue exclusion assays. B, cell cycle distribution and apoptotic DNA profiles of cells determined by propidium iodide staining in the presence of RNase A, followed by flow cytometry. Results are for cells cultured under anchorage-independent conditions for 4 h; bars, SD. The percentage in each panel represents the proportion of sub-G1 cells. Insets, representative TUNEL staining results.
crossscopy revealed a greater number of dead cells in the antibody-blocked groups than in the controls. Trypan blue exclusion analysis of WM278 and 1205Lu cells cultured in suspension in agarose-coated dishes, with or without blocking antibody, showed that with time in anchorage-free culture, survival rates were significantly lower ($P < 0.05$) in the presence of the N-cadherin-specific blocking antibody (Fig. 4A). Propidium iodide staining, followed by flow cytometry, showed that at 4 h after antibody addition, a substantial proportion of cells had died from apoptosis (Fig. 4B). This finding was confirmed by TUNEL assay (see insets).

**N-Cadherin-mediated Intercellular Interaction Promotes Survival by Activating the Akt/PKB Pathway.** The Akt/PKB pathway is reportedly involved in E-cadherin-mediated cell survival (21). To determine whether N-cadherin engagement leads to Akt/PKB activation, confluent cells were serum starved overnight and treated with EGTA (final concentration, 4 mM) for 30 min to disrupt Ca$^{2+}$-dependent, N-cadherin-mediated adhesion (24). The medium was then replaced with serum-free medium containing 2 mM calcium. Cells were collected at different time points after calcium restoration and subjected to lysis and immunoblotting with antibodies against Akt/PKB and phospho-Akt/PKB (Ser-473). The calcium restoration led to activation (25, 26) in melanoma cell lines WM278 and 1205Lu (Fig. 5A). Kinetic studies showed that activation occurred as early as 5 min after calcium restoration, with different peak times in the two cell lines. To test whether Akt/PKB activation was attributable to reestablishment of N-cadherin-mediated interaction after calcium restoration, N-cadherin blocking antibody was used to inhibit N-cadherin-mediated adhesion in WM278 and 1205Lu cells treated with EGTA and then Ca$^{2+}$ restored. In both cell lines, N-cadherin blocking antibody inhibited Akt/PKB phosphorylation. The effect was more dramatic in WM278 than in 1205Lu cells (Fig. 5B).

Upon activation, Akt/PKB translocates into nucleus (Fig. 5B; Ref. 21). Because the proapoptotic protein Bad (27) is one of the targets of Akt/PKB activity (26, 28, 29), we also determined the status of Bad upon Akt/PKB activation. Of the potential phosphorylation sites in Bad (28, 30) at Ser-112 and Ser-136 (27, 29), only phosphorylation of Ser-112 was observed in concert with the activation of Akt/PKB. Analysis of catenin levels in WM278 and 1205Lu cells revealed increased levels of β-catenin upon Akt/PKB activation. Interestingly, a band corresponding to a smaller molecular size ($M_\text{r} \sim 60,000$) showed reactivity to β-catenin antibody when Akt/PKB activation was blocked by anti-N-cadherin antibody GC-4. This band might represent a degradation product of β-catenin, possibly generated by caspase in cells undergoing apoptosis (31). Overall, the results suggest that N-cadherin promotes cell survival by activating Akt/PKB, which subsequently inactivates the proapoptotic protein Bad and stabilizes the antiapoptotic protein β-catenin.

**N-Cadherin Promotes Migration of Melanoma Cells and Melanocytes on Fibroblasts.** DiI-labeled melanoma cells were seeded onto monolayers of dermal fibroblasts as described in “Materials and Methods” (Fig. 6A). These cells migrated faster than their counterparts seeded without fibroblasts (Fig. 6B). N-Cadherin blocking antibody partially, but significantly, inhibited migration of melanoma cells over fibroblasts (Fig. 6B), suggesting that N-cadherin promoted intercellular migration.

The role of N-cadherin in normal melanocytes was analyzed using transduction with an adenovirus construct to express wild-type N-cadherin on the cell surface. Normal melanocytes express high levels of E-cadherin and little, if any, N-cadherin (Figs. 1A and Fig. 7B). Expression of N-cadherin in N-cad/Ad5-transduced melanocytes was confirmed by both immunoblotting (Fig. 7A) and flow cytometry (Fig. 7B). The two bands shown in Fig. 7A may represent different post-translational modifications, possibly glycosylation. Overexpression of N-cadherin had no effect on the expression level of endogenous E-cadherin (Fig. 7B). When transduced cells were assayed under the same migration conditions as in Fig. 6, an increase in the number of migrating cells was observed in the N-cad/Ad5-transduced cells, and this increase was abrogated by including blocking antibody in the assay (Fig. 7C). In vitro proliferation assay of N-cad/Ad5-transduced melanocytes revealed no significant change in tritium incorporation (data not shown), suggesting that N-cad promotes migration by a mechanism other than that regulating cell proliferation.
DISCUSSION

Previous studies have documented the switching of cadherin profiles of melanocytic cells during melanoma development (8, 13). Here, we provide evidence that functional N-cadherin expressed in melanoma cells not only mediates cell-cell homophilic interactions between adjacent cancer cells and stromal fibroblasts and endothelial cells but also promotes survival and migration of melanoma cells. Specifically, our results demonstrate that: (a) N-cadherin expressed in melanoma cells confers new adhesion and communication properties, which may allow malignant cells to adapt to a new micromilieu once they leave the primary site; (b) N-cadherin-mediated intercellular interaction promotes survival by activating the Akt/PKB pathway, resulting in inactivation of Bad and accumulation of steady-state β-catenin; (c) N-cadherin promotes melanoma cell migration on fibroblasts; and (d) forced expression of N-cadherin in melanocytes promotes migration.

N-Cadherin-mediated adhesion between melanoma cells and vascular endothelial cells may facilitate transmigration of cancer cells through the vascular endothelium during metastasis. The two major cadherins of vascular endothelial cells are N-cadherin and VE-cadherin (32). VE-cadherin predominantly promotes the homotypic interaction between endothelial cells, whereas N-cadherin is responsible for the interaction between endothelial cells and other N-cadherin-expressing cells (32). Our data suggest that expression of N-cadherin in melanoma cells increases their adherence to the endothelium, thus promoting a critical step in the breaching of blood vessels by tumor cells (33).

Fig. 6. N-Cadherin promotes migration of melanoma cells on fibroblast monolayer. A, schematic representation of intercellular cell migration assay. Dermal fibroblasts were seeded onto chamber slides and allowed to form monolayers. a, melanoma cells prelabeled with DiI were seeded on top of the fibroblast monolayer. b, the surface was placed at a slope so that seeded cells settled on one side of the chamber. c, after 50% of the seeded cells attached, unattached cells were removed by extensive washes with PBS. d, cells were cultured in regular medium for 2 or more days. For blocking assay, antibody GC-4 was added to the medium (final concentration, 40 μg/ml) for the first day, followed by adding fresh GC-4 at 20 μg/ml for each additional day. Migrating cells were counted in preselected areas (shaded box in c and d), where no seeding occurred at the beginning. B, WM278 and 1205Lu were tested for N-cadherin-mediated migration in the presence or absence of a fibroblast monolayer, as well as in the presence or absence of N-cadherin-specific blocking antibody. Migrating cells were counted in four preselected regions for each treatment after 3 days of coculture (P < 0.05); bars, SD.

Fig. 7. Forced expression of N-cadherin in N-cadherin-negative melanocytes promotes migration on fibroblast monolayers. A, immunoblotting of total cell lysates of nontransduced (Lane 1), LacZ/Ad5-transduced (Lane 2), or N-cad/Ad5-transduced (Lane 3) melanocytes. B, flow cytometry analyses of cell surface expression of N- and E-cadherin molecules. C, melanocytes transduced with either LacZ/Ad5 or N-cad/Ad5 were tested for N-cadherin-mediated intercellular migration in the presence or absence of N-cadherin-specific blocking antibody. Migrating cells were counted in four preselected regions for each treatment after 2 days of coculture (P < 0.05); bars, SD.
ROLE OF N-CADHERIN IN MELANOMA

Our data provide evidence that melanoma cells may benefit from N-cadherin-mediated aggregation with increased viability and resistance to apoptosis. N-Cadherin promoted anchorage-independent survival by activation of Akt/PKB and inactivation of the proapoptotic protein Bad. Moderate stabilization of β-catenin was also observed, although it is not clear whether this stabilization reflects inactivation (phosphorylation) of glycogen synthase kinase 3β by activated Akt/ PKB or involves an alternative pathway. The mechanism(s) whereby cadherins stimulate those biochemical routes is not well understood; however, our findings indicate that N-cadherin can initiate outside-in signal transduction pathways that ultimately benefit melanoma cells.

The biological function of individual molecules should be investigated within the context of a microenvironment, because cross-talk between cells and communication between cells and the extracellular matrix exert profound effects on function. It remains unknown how disseminating tumor cells reconcile their requirements for variations in cell adhesion, i.e., down-regulation of E-cadherin activity to break away from the primary tumor mass, followed by increased involvement of other adhesion molecules in cell-substrate and cell-cell interactions during increased motility. These diverse requirements might be met as a consequence of interactions between the different classes or subclasses of adhesion receptors. It is possible that differences in signaling capabilities of the individual cadherins, in addition to the distinct affinity and specificity in mediating adhesion, contribute to the difference in phenotype. Understanding the difference between E-cadherin- and N-cadherin-mediated interactions and the mechanism by which N-cadherin promotes malignancy might provide a step toward development of treatments that decrease the survival and invasiveness of malignant cells.

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