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 mediates 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 oncogenes 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 membranous 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). HUVECs 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 the melanocytic marker tyrosinase-related protein-1 was from Signet (Vollum, KY). Anti-Akt, anti-phospho-Akt (Ser-473), anti-Bad, and anti-phospho-Bad 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 H2O 8.0 TE buffer (10 mM Tris-HCl, 1 mM EDTA). Sequences were determined using an ABI automatic DNA sequencer (Perkin-Elmer).

**Construction and Production of Replication-deficient N-Cadherin Adenoviral (N-cad/Ad5) Vector.** Full-length human N-cadherin cDNA was obtained by RT-PCR. mRNA 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 CCG CCT CCA TGT GCC GG-3' and the reverse primer was 5'-AAG GAT CAC CTG AGG ATC 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 BJ5183. Recombinant adenovirus vector containing N-cadherin coding sequence was identified by enzyme digestions 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 protein-free W489 medium. After 4 h, the virus-containing medium was replaced by regular medium. Cells were used 48 h after infection.

**Flow Cytometry.** Cultured cells were detached with Versene, washed once with PBS, and stained for 40 min with primary antibody at 4°C with shaking at 150 rpm. Cells were then washed and incubated with FITC-conjugated secondary antibody. After enzymatic washing, cells were analyzed by flow fluorescence-activated cell sorting. Unrelated mouse IgG was used as a negative control.

**Cell Aggregation, Adhesion, and Survival Assays.** Melanoma cells were washed with PBS, detached with 0.02% trypsin containing 2 mM CaCl₂, which protects cadherins from digestion, and resuspended in growth medium. Cells were allowed to aggregate for 30 min or overnight at 37°C with constant shaking at 30 rpm. In blocking experiments, mAb GC-4 and control antibodies were added to the medium at a final concentration of 40 μg/ml. Cell aggregates were fixed in 4% paraformaldehyde. The extent of cell aggregation (index) was calculated as: Index = (Nₐ - Nᵢ)/Nᵢ, where Nᵢ and Nₐ are the number of particles before and after aggregation, respectively (20). Each treatment was repeated five times. For cell adhesion assay, melanoma cells were prelabeled with the red fluorescent dye Dil (10 mg/ml; Molecular Probes, Eugene, OR) for 2 h, washed with HBSS, and harvested by treatment with 0.02% trypsin in HBSS containing 2 mM calcium for 30 min at 37°C. For blocking experiments, cells were incubated with mAb GC-4 (40 μg/ml) or control antibodies at 4°C for 30 min, washed with HBSS, and resuspended in assay medium containing mAb GC-4. About 5000 cells of dermal fibroblast or HUVEC monolayers in gelatin-coated, eight-well chamber slides and allowed to adhere for 30 min. After removal of nonadherent cells, slides were fixed. The number of adherent cells per high-power field in triplicate wells was counted under a fluorescence microscope. For anchorage-independent cell viability assay, cells were cultured in 1% agarose-coated dishes with or without blocking antibody treatment. Viability was determined in triplicate samples by trypan blue exclusion assay, and the survival index was calculated as: Survival index = Number of live cells/Total number of cells. Cell cycle distribution and apoptotic DNA profiles of cells were determined by propidium iodide (Sigma Chemical Co.) staining in the presence of RNaseA and flow cytometry. Apoptotic cells were also measured by TUNEL assay. TUNEL enzyme and TUNEL label were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Because the cells to be tested were cultured under anchorage-independent conditions, the assay protocol was adapted from the manufacturer’s suggested procedure for cell suspensions. Briefly, cells were collected and washed in PBS twice and then transferred into U-bottomed, 96-well microtiter plate (2 × 10⁵ cells/ml, 100 μl/well). Cells were fixed in 4% paraformaldehyde for 60 min at room temperature, washed in PBS, and permeabilized in 0.1% sodium citrate containing 0.1% Triton X-100 for 2 min on ice. Cells were then washed in PBS and resuspended in 50 μl/well TUNEL reaction mixture (5 μl of TUNEL enzyme mixed with 45 μl 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 (Amer sham, 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-G 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...
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 Dil 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 Inter cellular 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

![Fig. 1. Characterization of cadherins and cadherin-containing complexes in melanoma cells.](image-url)

A, immunoblotting analyses of N-cadherin, E-cadherin, α-catenin, β-catenin, and γ-catenin (plakoglobin) expression in melanoma cell lines WM278 (primary VGP) and 1205Lu (metastatic), dermal fibroblasts, melanocytes, and HUVECs. B, immunoprecipitation of the N-cadherin/catenin complex from WM278 melanoma cells. The same pattern was obtained with 1205Lu cells. C and D, indirect immunofluorescence of N-cadherin expression in melanoma cells cocultured with fibroblasts. Fluorescent staining (Cy3) in C shows the localization of N-cadherin in the region of cell-cell contact between melanoma cells (left arrowhead) and between melanoma cells and fibroblasts (right arrowhead). Fluorescent staining (FITC) in D of the same field as in C was used to identify melanoma cells in coculture by staining melanocytic cell-specific protein tyrosinase-related protein-1.

![Fig. 2. N-Cadherin mediates aggregation of melanoma cells.](image-url)

A, phase contrast microscopy. Single cells were prepared by treatment with 0.02% trypsin containing 2 mM CaCl₂ and resuspended in regular medium. Cells were allowed to aggregate for 30 min at 37°C with constant shaking at 30 rpm. In blocking experiments, blocking antibody GC-4 and control antibodies were added to the medium at a final concentration of 40 μg/ml. B, the index of cell aggregation was calculated as: Index = (N₀ - N₂)/N₀, where N₀ and N₂ are the number of particles before and after aggregation, respectively. Data are given as mean of five independent experiments; bars, SD. *, significant difference from control (P < 0.01).
and 1205Lu (metastatic melanoma), together with melanocytes, fibroblasts, and HUVECs were analyzed by immunoblotting with antibodies against N-cadherin, E-cadherin, \( \alpha \)-catenin, \( \beta \)-catenin, and \( \gamma \)-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 \( \alpha \)-, \( \beta \)-, and \( \gamma \)-catenin (plakoglobin) expression levels and melanoma progression was observed. Amplification of \( \beta \)-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, \( \beta \)-catenin and \( \alpha \)-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-
croscopy 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. Immunoblots were quantified using NIH Image 1.62 software. Levels of Akt/PKB activation at individual time points were calculated as the ratio of the amount of phosphorylated Akt/PKB to that of total Akt/PKB. The calculated ratio at time 0 min was used as control. 

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, ~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. Dil-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.
ROLE OF N-CADHERIN IN MELANOMA

N-Cadherin-mediated adhesion between melanoma cells and the stromal fibroblasts may facilitate dynamic processes such as cell migration and outgrowth. Cell migration depends on a delicate balance of cell adhesion and detachment. Under different physiological conditions and microenvironments, cell adhesion molecules can either promote or inhibit migration (34). Our studies show that N-cadherin mediates migration of melanoma cells on fibroblasts, and that forced expression of N-cadherin in otherwise E-cadherin-positive, N-cadherin-negative melanocytes promotes migration relative to fibroblasts. N-Cadherin has been postulated to promote both stable and labile cellular interactions (35–37). N-Cadherin-expressing cells retained their adhesive properties in short-term aggregation assays and possibly in the apparently tightly clustered metastases in vivo (38–40), suggesting that reduced cell adhesion does not underlie N-cadherin-induced invasiveness. Rather, it is likely that N-cadherin promotes a state of dynamic adhesion that allows both attachment and detachment of cells from the primary tumor and selective association with tissues such as the stroma or the endothelium.

In vivo observations showed that tumor cells predominantly invade the surrounding tissue as coherent clusters or cell nests (39). For example, the invasion front of colon carcinomas consists of compact tumor glands preceded by scattered tumor cell clusters or a few single cells (39). Cohort migration is a major form of migration in melano-

![Image](62x483 to 279x742)

**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.

**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 microenvironment 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).

![Image](308x120 to 561x464)

**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.

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noma. 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 $\beta$-catenin was also observed, although it is not clear whether this stabilization reflects inactivation (phosphorylation) of glycogen synthase kinase 3$\beta$ 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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## N-Cadherin-mediated Intercellular Interactions Promote Survival and Migration of Melanoma Cells

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