Transendothelial Migration of Melanoma Cells Involves N-Cadherin-mediated Adhesion and Activation of the β-Catenin Signaling Pathway

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Cancer metastasis is a multistep process involving many types of cell-cell interactions, but little is known about the adhesive interactions and signaling events during extravasation of cancer cells. Transendothelial migration of cancer cells was investigated using an in vitro assay, in which melanoma cells were seeded on top of a monolayer of endothelial cells. Attachment of melanoma cells on the endothelium induced a twofold increase in N-cadherin expression in melanoma cells and the redistribution of N-cadherin to the heterotypic contacts. Transendothelial migration was inhibited when N-cadherin expression was repressed by antisense RNA, indicating a key role played by N-cadherin. Whereas N-cadherin and β-catenin colocalized in the contact regions between melanoma cells and endothelial cells during the initial stages of attachment, β-catenin disappeared from the heterotypic contacts during transmigration of melanoma cells. Immunolocalization and immunoprecipitation studies indicate that N-cadherin became tyrosine-phosphorylated, resulting in the dissociation of β-catenin from these contact regions. Concomitantly, an increase in the nuclear level of β-catenin occurred in melanoma cells, together with a sixfold increase in β-catenin-dependent transcription. Transendothelial migration was compromised in cells expressing a dominant-negative form of β-catenin, thus supporting a regulatory role of β-catenin signaling in this process.

INTRODUCTION

Cancer metastasis is a complex multistep process that involves the detachment of cancer cells from the primary tumor mass, intravasation, extravasation, and the establishment of new foci in a remote organ (Fidler, 2003; Pantel and Brakenhoff, 2004). All these steps involve intricate interactions between different types of cells and it is, therefore, evident that cell adhesion molecules (CAMs) play an important role in cancer metastasis. Changes in CAM profile are often associated with the disruption of normal cell-cell interactions and the establishment of new interactions, both of which are essential to metastasis formation (Christofori, 2003).

One of the least known steps in cancer metastasis is extravasation. Unlike leukocytes, only a few cancer cell types undergo rolling on the endothelial surface under flow conditions in vitro assays (Giavazzi et al., 1993; Brenner et al., 1995; Aigner et al., 1998). On the other hand, intravital microscopy shows that initial arrest of cancer cells occurs primarily by size restriction in the capillaries (Chambers et al., 1992) and rolling has not been observed (Orr et al., 2000). To investigate the mechanism of transendothelial migration, we have established an in vitro assay by depositing melanoma cells on top of a monolayer of microvascular endothelial

cells cultured on Matrigel (Sandig et al., 1997b; Voura et al., 1999). Adhesion among endothelial cells is mediated by several major CAMs, including VE-cadherin and PECAM-1 (Vestweber, 2002; Ilan and Madri, 2003). The attachment of melanoma cells on an endothelial monolayer has been found to induce localized dissolution of both VE-cadherin and PECAM-1 in the endothelial junction (Sandig et al., 1997b; Voura et al., 2000). Thus, neither VE-cadherin nor PECAM-1 appears to be involved in the transendothelial migration of melanoma cells.

Transendothelial migration is a dynamic process that involves the constant breaking and remaking of intercellular contacts and is accompanied by drastic changes in cell shape and cytoskeletal reorganization in both the tumor cell and its neighboring endothelial cells (Voura et al., 1999; Brandt et al., 1999). We have found that the cell adhesion molecule L1 and integrin αvβ3 play a role in the formation of heterotypic contacts between melanoma cells and endothelial cells (Voura et al., 2001). However, antibody and peptide inhibition studies suggest the involvement of multiple CAMs. A potential candidate is N-cadherin, because transendothelial migration can be retarded by antibodies against N-cadherin (Sandig et al., 1997b).

Tumor cells from prostate, breast, and skin have been found to express high levels of N-cadherin (Islam et al., 1996; Nieman et al., 1999; Tomita et al., 2000; Li et al., 2001). In the case of melanoma progression, metastasis is accompanied by the down-regulation of E-cadherin and the up-regulation of N-cadherin expression, which facilitate the separation of melanoma cells from adjacent E-cadherin-expressing keratinocytes and the invasion of the dermal tissue through interactions with the N-cadherin-expressing fibroblasts (Li and Herlyn, 2000). Because blood vessel endothelial cells
also express N-cadherin (Salomon et al., 1992; Navarro et al., 1998), it is likely that N-cadherin-dependent interactions may contribute to the adhesive events during intravasation and extravasation of melanoma cells.

N-cadherin and several members of the cadherin family mediate cell-cell adhesion via homophilic binding and the stability of cadherin-mediated cell adhesion depends on the association of β-catenin with the cadherin cytoplasmic domain. β-catenin binds α-catenin, which links the cadherin complex to the actin cytoskeleton (Takeichi, 1995; Nagafuchi, 2001). Another catenin, p120*cn, binds to the juxtamembrane region of the cadherin cytoplasmic domain (Anastasiadis and Reynolds, 2000).

In addition to its role in cell-cell adhesion, β-catenin is a key signal transducer in the Wnt signaling pathway, which plays an important role in many biological processes such as embryogenesis, tumorigenesis, and cancer metastasis (Smalley and Dale, 1999; Peifer and Polakis, 2000; Nelson and Nusse, 2004). Activation of Wnt signaling leads to the stabilization and translocation of β-catenin into the nucleus, where it binds the TCF/LEF family of transcription factors to induce gene transcription (Behrens et al., 1996). To investigate the potential role of N-cadherin during cancer cell extravasation, immunofluorescence staining was used to examine the distribution of N-cadherin during transendothelial migration. A novel type of N-cadherin adhesion complex that lacked β-catenin was discovered in the heterotypic contact. Further studies indicate that N-cadherin became tyrosine-phosphorylated during the transmigration process, resulting in the dissociation of β-catenin, which then translocated to the nucleus of melanoma cells to activate gene transcription.

### MATERIALS AND METHODS

#### Antibodies

Mouse mAbs against human N-cadherin (clone 32), E-cadherin, VE-cadherin, β-catenin, γ-catenin, and p120*cn, and anti-phospho-tyrosine monoclonal antibody (mAb; PY-20) were purchased from Transduction Laboratories (Lexington, KY). Rabbit anti-human β-catenin antibody, mouse anti-human N-cadherin mAb (GC4), mouse anti-Protein-A mAb, and rabbit PAP (peroxidase-antiperoxidase) antibody were purchased from Sigma (St. Louis, MO). Mouse mAb against the dephosphorylated form of β-catenin was purchased from AG Scientific (San Diego, CA) for immunostaining of nuclear β-catenin. For immunoprecipitation studies, rabbit anti-human N-cadherin antibody (H-36) was purchased from R&D Systems (Minneapolis, MN). The Alexa488- or Alexa568-conjugated goat anti-mouse and goat anti-rabbit antibodies were purchased from Molecular Probes (Eugene, OR). Phycoerythrin (PE)-conjugated goat anti-mouse F(ab’2) was purchased from CalTag Laboratories (Burlingame, CA).

#### Cell Lines

Human lung microvascular endothelial cells (HMVEC) were purchased from Clonetics (San Diego, CA) and cultured in endothelial medium (EGM-2 MV; Clonetics) supplemented with penicillin-streptomycin (100 U/ml and 100 μg/ml, respectively). Melanoma cells were routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin-streptomycin.

#### cDNA Constructs

The pPUR vector that contains an N-cadherin antisense sequence was a kind gift from Dr. Keith Johnson (University of Toledo, Toledo, OH; Islam et al., 1996). The original TAP-tag construct PBS1761 was obtained from Dr. Bertrand Seraphin (CNRS, France; Rigaut et al., 1990). The β-catenin cDNA was obtained by RT-PCR using WM239 total RNA. The PCR product was cloned into the XbaI/KpnI sites of the pBSK(+) vector, which was used as the PCR template to produce the truncation mutants of β-catenins (ΔN β-catenin with deletion of the N-terminal 1–148; ΔN ΔC β-catenin with deletion of amino acids 1–148 and 665–781). The PCR products were subcloned into the XbaI/KpnI sites of the pcDNA3.1 (+) vector that contained an N-terminal TAP-tag. All the cDNA constructs were confirmed by DNA sequencing.

### Cell Transfection

Plasmid DNA was transfected into WM239 melanoma cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The selection media were applied after 48 h of transfection (1 mg/ml G418 for the pcDNA vector; 5 μg/ml puromycin for the pPUR vector). Stable colonies were expanded and screened by immunoblot using the PAP antibody for TAP-tag transfection and the N-cadherin mAb for N-cadherin antisense transfection.

### Transendothelial Migration Assay

The in vitro transendothelial migration assay was carried out as described previously (Sandig et al., 1997b; Voura et al., 2001). HMVEC cells (1.5 × 10⁵ cells; passages 5–8) in 200 μl of endothelial medium were placed on top of a Matrigel-coated glass coverslip (12-mm diameter) and allowed to settle for 5–6 h. Coverslips were then transferred to a new 24-well plate, and incubated in 0.5 ml of endothelial medium containing 10 ng/ml TNF-α (Sigma) for 12 h to stimulate the expression of cell adhesion molecules, which in turn promote the attachment of melanoma cells (Lucinskas et al., 1995; Orr et al., 2000). The monolayer was washed three times and then incubated in 0.5 ml of fresh endothelial medium without TNF-α. Melanoma cells were labeled with 10 μg/ml DiI (Molecular Probes) for 5 min at 37°C. After washing, cells were resuspended at 2.5 × 10⁵ cells/ml in the endothelial medium. Melanoma cells (5 × 10⁴ cells) were added to each HMVEC monolayer. Cocultures were fixed at different time points with paraformaldehyde and stained for F-actin. Quantitative analysis of transmigration was carried out using a Wild Leitz Orthoplan confocal microscope (Leica Microsystems, Richmond Hill, Ontario, Canada). For each coverslip, three sets of random fields for a total of 45 fields were scored for transmigrated cells. Each set of 15 fields contained 100–200 melanoma cells and >1000 melanoma cells were routinely scored for each experimental condition.

In inhibition studies, cocultures were carried in the presence of genistein (10 μM), calphostin C (1 μM) or H8 (10 μM). In antibody inhibition studies, melanoma cells were preincubated with the anti-N-cadherin function-blocking antibody GC4 (1/10 dilution; Sigma) for 30 min before adding to a HMVEC monolayer. In all inhibition studies, the total number of melanoma cells associated with the endothelial monolayer was estimated to ensure that any reduction in the number of transmigrated cells was not due to a reduction in cell attachment.

#### Immunofluorescence Staining

Melanoma cells were labeled with Cell Tracker Orange (Molecular Probes) and then deposited on a monolayer of HMVEC. Cocultures were fixed by immunoblot using the PAP antibody for TAP-tag transfection and the N-cadherin mAb for N-cadherin antisense transfection.

#### FACs Analysis of N-cadherin Expression in Cocultures

Melanoma cells (5 × 10⁵) were labeled with Cell Tracker Green (Molecular Probes) before coculture with an endothelial monolayer (3 × 10⁵ cells) for 5 h. The cells were dissociated with 5 mM EDTA, incubated with N-cadherin mAb (GC4), followed by phycoerythrin-conjugated goat anti-mouse F(ab’2), for 15 min each, and fixed in 3.5% paraformaldehyde. FACs analysis was performed to examine the level of N-cadherin on the cell surface of melanoma cells and endothelial cells.

#### Immunoprecipitation, Subcellular Fractionation, and Immunoblot Analysis

For biochemical analysis, 4 × 10⁶ endothelial cells were cultured on a Matrigel-coated 60-mm plate in endothelial medium containing 10 ng/ml TNF-α for 12 h. The cells were washed three times and then incubated in fresh endothelial medium. An equal number of melanoma cells was deposited on the endothelial monolayer and cocultured for 5 h before lysing. To prepare samples for 0 h of coculture, melanoma cells and endothelial cells were cultured on Matrigel and collected separately. The cell lysates were mixed together before further analysis. Subcellular fractionation was performed to obtain membrane and cytosolic fractions or nuclear and postnuclear fractions according to published protocols (Dignam et al., 1983; Thoreson et al., 2000). For immunoprecipitation, samples were solubilized in lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1% NP-40, and a protease inhibitor cocktail). The supernatant was incubated overnight with 3 μg of primary antibody at 4°C and then incubated with protein A–Sepharose (1:1000) for 1 h at 4°C. The beads were washed three times with lysis buffer and boiled for 5 min before SDS-PAGE. For immunoblot analysis, protein samples were separated by SDS-PAGE and transferred to nitrocellulose membrane. Blots were probed with different antibodies and developed with ECL reagents. For quantification, the
blots were analyzed in a Fluor-S imager (Bio-Rad, Hercules, CA) and quantification was based on the pixel value of each band after background subtraction.

Luciferase Reporter Assay

The TCF reporter plasmid kit, which contained the TOPflash, FOPflash, and TK vectors, was purchased from Upstate Biotechnology (Lake Placid, NY). Melanoma cells were cotransfected transiently with TOPflash (or FOPflash) and TK vectors at a ratio of 30:1 using Lipofectamine 2000. Two days later, melanoma cells (3 x 10^5) were seeded on top of a HMVEC monolayer (3 x 10^5 cells) in a Matrigel-coated 24-well plate. Cells were collected at different times and analyzed using the Promega's dual luciferase reporter assay system (Madison, WI). Luminescence was measured using a Berthold Lumat LB luminometer. The ratio between firefly luciferase activity (TOPflash or FOPflash) and Renilla luciferase activity (TK vector) was used to estimate changes in β-catenin-mediated transcription. The fold increase in luciferase activity was calculated by normalizing the data to that at 0 h of coculture.

RNA Isolation and Northern Blot Analysis

Melanoma cells (4 x 10^6 cells) were cultured on an endothelial monolayer (4 x 10^6 cells). Total RNA was isolated from 0- or 5-h cocultures using the RNeasy Mini Kit (Qiagen, Mississauga, Ontario, Canada). Equal amounts of total RNA (~3 μg per lane) were separated in 1.2% agarose gel containing 2% formaldehyde and then transferred onto positively charged nylon membranes. DIG-labeled RNA probes against CD44, N-cadherin, and MT1-MMP were generated using RT-PCR and in vitro transcription. RNA blots were probed using the DIG-detection starter kit of Roche (Indianapolis, IN) according to the manufacturer’s protocol.

RESULTS

N-cadherin Is Up-regulated and Concentrated in Heterotypic Contacts during Transendothelial Migration of Melanoma Cells

To determine whether N-cadherin was involved in transendothelial migration, labeled melanoma cells were deposited on a monolayer of endothelial cells. As previously reported (Voura et al., 1998), melanoma cells attached on the endothelium and sent out membrane protrusions around their contact regions within 1 h. Pseudopodia were used to invade the endothelial junction and melanoma cells became intercalated within the endothelial cells by 3 h. More than 50% of melanoma cells were spreading on the Matrigel by 5 h and they were scored as transmigrated cells.

The cocultures were fixed and stained with antibodies against different cadherins. VE-cadherin staining demarcated the endothelial junction, but it was distinctly absent from the heterotypic contact between endothelial cells and melanoma cells (Figure 1).
melanoma cells (Figure 1a, b). N-cadherin was the major cadherin species in the heterotypic contact during transendothelial migration (Figure 1c). N-cadherin was diffusely distributed on the surface of endothelial cells and absent from the endothelial junction as reported by Salomon et al. (1992). E-cadherin showed only background level of staining and was not detectable in heterotypic contacts (Figure 1d).

Attachment of melanoma cells on the endothelium quickly induced redistribution of N-cadherin to the cell-cell contact regions (Figure 1e). Initial cell-cell interactions stimulated the melanoma cells to form membrane blebs and pseudopodia around the contact regions and N-cadherin staining was observed at the tips of these protrusions (Figure 1f). As the melanoma cell began its transmigration process by inserting pseudopodia into the endothelial junction, intense staining of N-cadherin was present around the pseudopodium (Figure 1g). Subsequently, melanoma cells became intercalated between endothelial cells and N-cadherin staining was observed along the leading edges of surrounding endothelial cells which began to spread on top of the melanoma cell (Figure 1h).

To investigate whether cell-cell interactions had any effect on the expression of N-cadherin during transendothelial migration, melanoma cells were cultured on an endothelial monolayer. Immunoblots showed a time-dependent increase in N-cadherin level, with an averaged 1.6-fold increase at 5 h (Figure 2A). Melanoma cells and endothelial cells were also cultured separately on Matrigel to determine whether Matrigel had any effect on N-cadherin synthesis. The results show that Matrigel did not induce N-cadherin expression in either cell type. To determine which cell type up-regulated N-cadherin expression during coculture, endothelial cells and dye-labeled melanoma cells were cocultured for 5 h. Cells were collected and then probed with an anti-N-cadherin antibody. FACS analysis was carried out to determine the cell surface level of N-cadherin for each cell population. A twofold increase was observed in melanoma cells, but little change was detected in endothelial cells (Figure 2B). The data suggest that cell-cell interactions during transendothelial migration stimulated the expression of N-cadherin in melanoma cells, but not in endothelial cells.

Knockdown of N-cadherin Expression in Melanoma Cells Inhibits Transendothelial Migration

The above results suggest that N-cadherin may play a role in transendothelial migration of melanoma cells. To assess the importance of N-cadherin in this process, the expression of N-cadherin in WM239 melanoma cells was repressed by stable transfection with an N-cadherin antisense construct (Figure 3A). Clones (A35, A36, and A2), which showed 90, 60, and 0% reduction in N-cadherin expression, respectively, were subjected to the transendothelial migration assay. Maximum inhibition was observed for A35 cells, with 60% inhibition at 3 h and 45% inhibition at 5 h of coculture, whereas A36 cells had an intermediate level of inhibition and A2 cells underwent transendothelial migration as efficiently as WM239 cells. The results show a dose-dependent relationship between N-cadherin level and transendothelial migration (Figure 3B). To block the residual amounts of N-cadherin expressed on A35 and A36 cells, melanoma cells were incubated with the function-blocking anti-N-cadherin mAb GC4 before deposition on the endothelial monolayer. Transendothelial migration was inhibited by another 10–15% in both A35 and A36 cells (Figure 3C). The data support a key role for N-cadherin in the transendothelial migration of melanoma cells.

\( \beta \)-catenin Dissociates from N-cadherin and Translocates into the Nucleus of Transmigrating Melanoma Cells

Because actively transmigrating melanoma cells must continuously “break” and “remake” contacts with the endothelial surface, immunofluorescence studies were carried out to investigate changes in the N-cadherin adhesion complexes that might accompany transendothelial migration. When cocultures were stained for the catenins, both N-cadherin and \( \beta \)-catenin were found to concentrate in the heterotypic contact upon adherence of melanoma cells on the endothelium (Figure 4A, a and d). Surprisingly, when melanoma cells began to transmigrate across the endothelial junction, most of the heterotypic contacts became devoid of \( \beta \)-catenin (Figure 4Ae). In contrast, p120\(^{ctn}\) remained clearly detectable in the heterotypic contact (Figure 4Ag). When homotypic contacts between melanoma cells were examined, both \( \beta \)-catenin and p120\(^{ctn}\) were observed (Figure 4A, f and h). On the other hand, \( \gamma \)-catenin staining was absent from both
heterotypic and homotypic contacts (Figure 4A, i and j), although it was expressed in WM239 cells. These results indicate that both N-cadherin and β-catenin were concentrated in the heterotypic contact during the initial stages of cell attachment, but signals accompanying the initiation of transendothelial migration somehow led to the dissociation of β-catenin from the N-cadherin adhesion complex.

Further examination of the micrographs showed that β-catenin became detectable in the nucleus of melanoma cells during transendothelial migration and displayed a punctate pattern of staining (Figure 4Ae). To provide biochemical evidence for β-catenin redistribution, immunoprecipitation studies were carried out (Figure 4B). The 5-h coculture was compared with the 0-h time point when the two cell types were simply mixed in order to provide the reference value before cell–cell interactions during transendothelial migration. Quantitative analysis showed a 50% reduction in β-catenin coprecipitated with N-cadherin at 5 h of coculture, although the total cellular level of β-catenin did not change over time. Subcellular fractionation studies showed a significant reduction of β-catenin associated with the membrane fraction at 5 h of coculture, which was accompanied by a corresponding increase in the cytosolic level of β-catenin (Figure 4C). An analysis of the nuclear fraction obtained from cocultures showed a 5–6-fold increase in the nuclear level of β-catenin (Figure 4D). As a control, melanoma cells and endothelial cells were cultured separately on Matrigel and the results showed that Matrigel did not induce the nuclear translocation of β-catenin. Together, these results suggest the presence of an active mechanism involved in the translocation of β-catenin from the N-cadherin adhesion complex to the nucleus.

β-catenin Signaling Is Activated during Transendothelial Migration of Melanoma Cells

What then is the role of β-catenin in the nucleus during melanoma cell transmigration? It is well known that β-catenin interacts with the LEF/TCF family of transcription factors upon activation of Wnt signaling pathway. To determine whether β-catenin had a role in gene regulation during transendothelial migration, WM239 cells were transiently transfected with the TOPflash vector that contained wild-type TCF/LEF binding sites before a luciferase reporter gene. Changes in β-catenin-mediated transcription were examined during transendothelial migration. A time-dependent increase in luciferase activity was observed, probably reflecting a steady increase in the nuclear accumulation of β-catenin (Figure 5A). In contrast, luciferase activity remained at the background level in cells transfected with the FOPflash vector, which contained mutated TCF/LEF binding sites. After background subtraction, there was a sixfold increase in luciferase activity at 5 h of coculture, correlating well with the 5–6-fold increase in nuclear β-catenin (see Figure 4D).

Northern blot analysis was carried out using total RNA isolated from cocultures of WM239 cells and endothelial cells at 0 and 5 h. The RNA blots were probed for transcripts of N-cadherin, and the known β-catenin target genes CD44 and MT1-MMP. Quantitative analysis showed that N-cadherin had a 2.5-fold increase and CD44 displayed a threefold increase at 5 h of coculture. However, only a 1.2-fold increase was observed for MT1-MMP (Figure 5B). To confirm that the increase was due to β-catenin signaling in transmigrating melanoma cells, A21 cells which expressed a dominant-negative form of β-catenin (see next section) was used in coculture. As expected, there was little increase in the transcript level of the β-catenin target genes in these cocultures (Figure 5B).

To assess the effects of N-cadherin-mediated adhesion on β-catenin-dependent gene activation, N-cadherin present on the surface of TOPflash-transfected WM239 cells was blocked using the anti-N-cadherin mAb GC4. Luciferase activity was reduced by 50% at 5 h of coculture (Figure 5A). Furthermore, A2 and A35 cells were transfected with the TOPflash vector. A35 cells, which expressed only 10% of the normal level of N-cadherin, were unable to sequester β-catenin in the cell membrane. When luciferase activity was assayed, they showed a higher level of background activation that remained relatively constant during the 5-h period of coculture. On the other hand, the luciferase activity of A2 cells, which expressed the normal level of N-cadherin, was comparable to that of the parental WM239 cells (Figure 5C). Together, these results suggest a role for N-cadherin-mediated cell adhesion in the activation of the β-catenin signaling pathway.

Activation of β-catenin Signaling Facilitates Transendothelial Migration of Melanoma Cells

To provide direct evidence that β-catenin signaling was a key component of the transendothelial migration process, we transfected WM239 cells with either a dominant-negative form or a dominant-active form of β-catenin (DasGupta et al., 2002). The N-terminal domain of β-catenin contains GSK phosphorylation sites that will target β-catenin to the ubiquitin-proteasome degradation pathway when phosphorylated. The central armadillo repeats of β-catenin are respon-
sible for binding with cadherin and the TCF/LEF transcription factors, whereas the C-terminal domain is the major transactivation domain. In this study, a β-catenin construct lacking both N- and C-terminal domains served as a dominant-negative form, whereas β-catenin lacking the N-terminal domain served as a dominant-active form (Figure 6A). The N-terminus of these mutant forms was fused to a TAP-tag, so that their expression in stably transfected cell lines could be probed with mAb against protein A (Figure 6B). The distribution of the dominant-negative and dominant-active forms of β-catenin in transfecteds was examined by double immunofluorescence staining with anti-β-catenin pAb and anti-β-catenin polyclonal antibody. Although both mutant forms of β-catenin showed cytoplasmic and nuclear localization, a higher level was observed in the nucleus (Figure 6C). Interestingly, these two mutant forms of β-catenin did not colocalize with the endogenous β-catenin at the cell-cell contact regions, suggesting that they might not compete with the endogenous β-catenin for N-cadherin binding.
Clones F13 (ΔN) and A21 (ΔN+ΔC) expressed high levels of their respective mutant β-catenin and were chosen for luciferase assays (Figure 6D) and transendothelial migration assays (Figure 6E). At 5 h of coculture, F13 cells showed 25% and 35% increase in transendothelial migration and β-catenin-mediated transcription, respectively. In contrast, A21 cells showed 50% reduction in β-catenin-induced luciferase activity and 40% reduction in transendothelial migration. For comparison, control transfectants with the TAP vector were assayed and they showed levels of luciferase activity and transmigration efficiency similar to those of WM239 cells. The data indicate a close correlation between the level of β-catenin-mediated transcription and the transmigration process.

**Tyrosine Phosphorylation of N-cadherin Regulates the Binding of β-catenin during Transendothelial Migration**

It remains to be determined what regulates the interactions between β-catenin and N-cadherin during transendothelial migration. Tyrosine phosphorylation has been implicated in regulation of cadherin-β-catenin interaction (Gumbiner, 2000; Nelson and Nusse, 2004). When immunofluorescence studies were carried out to localize phospho-tyrosine, relatively weak signals were detected in the heterotypic contacts between transmigrating melanoma cells and endothelial cells (Figure 7A). To maximally visualize the phospho-tyrosine staining, cocultures were treated with the tyrosine phosphatase inhibitor pervanadate for 5 min before fixation. In the presence of pervanadate, the phospho-tyrosine staining became clearly visible in the heterotypic contact regions of transmigrating cells as well as the homotypic contact regions between endothelial cells. Notably, phospho-tyrosine staining was absent in the heterotypic contact before transmigration took place even in the presence of pervanadate (Figure 7A). As a control, WM239 cells alone were stained for phospho-tyrosine. Punctate staining was observed in the cytoplasm but absent from the cell-cell contact regions (Figure 7B).

To further assess the role of protein phosphorylation during transendothelial migration, cocultures were carried out in the presence of different kinase inhibitors. The protein tyrosine kinase inhibitor genistein eliminated the phospho-tyrosine signal and inhibited the dissociation of β-catenin from the N-cadherin complex at the heterotypic contact (Figure 7C). When the number of transmigrated cells was scored at 5 h of coculture, addition of genistein and calphos-tein C (protein kinase C [PKC] inhibitor) resulted in 60% and 35% inhibition, respectively (Figure 7D). However, the protein kinase A inhibitor H8 had no effect.

To determine which protein was phosphorylated, the N-cadherin complex was isolated by immunoprecipitation and the protein blot was probed with anti-phospho-tyrosine mAb. A tyrosine-phosphorylated band of ~130 kDa, consistent with the size of N-cadherin, was detected at 5 h of coculture, but not at 0 h (Figure 8A). Although similar levels of N-cadherin were detected in the precipitates of the 0- and 5-h coculture samples, the amount of coprecipitated β-catenin decreased by ~50% at 5 h. To confirm the identity of the 130-kDa band, immunoprecipitation was performed using the anti-phospho-tyrosine mAb, and the blot was probed with mAb against N-cadherin and β-catenin. The antibody pulled down N-cadherin but not β-catenin at 5 h of coculture (Figure 8A), confirming that N-cadherin contained phosphorylated tyrosine but β-catenin did not.

To assess whether there were other tyrosine-phosphorylated proteins in the heterotypic contact, A35 cells, which expressed <10% of the cellular level of N-cadherin than the parental WM239 cells, were examined. When A35 cells were examined using the transendothelial migration assay, neither N-cadherin nor phospho-tyrosine staining was detected in the heterotypic contact (Figure 8B). The data indicate that N-cadherin was the major component that became tyrosine-
phosphorylated in the heterotypic contact regions between melanoma cells and endothelial cells.

DISCUSSION

Extravasation of cancer cells is a complicated event that involves both adhesive interactions and chemotaxis (Voura et al., 2001; Ramjeesingh et al., 2003). In this report, we show that N-cadherin plays a key role in the transendothelial migration of melanoma cells. It is concentrated in the heterotypic contact between melanoma cells and endothelial cells. However, N-cadherin forms a novel type of adhesion complex that lacks β-catenin, which allows the activation of β-catenin signaling in transmigrating melanoma cells. Transendothelial migration of melanoma cells is impaired by blocking N-cadherin synthesis via antisense transfection or by inhibiting β-catenin signaling via the expression of a dominant-negative β-catenin in melanoma cells. It becomes apparent that both N-cadherin-mediated adhesion and β-catenin signaling are involved in transendothelial migration of melanoma cells.

Endothelial cells express two major cadherins, VE- and N-cadherin. Despite the relative abundance of N- and VE-cadherin, only VE-cadherin is concentrated in the endothelial junction. VE-cadherin is known to exclude N-cadherin from the endothelial junction via the juxtamembrane region of its cytoplasmic domain (Navarro et al., 1998). Our previous work localized dissolution of VE-cadherin from the endothelial junction upon the attachment of melanoma cells (Sandig et al., 1997b; Voura et al., 2000). As VE-cadherin moves out, N-cadherin can now move in and becomes concentrated in the heterotypic contact during melanoma cell transmigration. N-cadherin is also enriched on filopodia, pseudopodia, and the migrating front of melanoma cells, consistent with the observation that cadherin is clustered in the tip of membrane processes where it acts as traction points for spreading and migration (Sandig et al., 1997a; Adams et al., 1998; Vasioukhin et al., 2000).

In many tumors, down-regulation of E-cadherin is accompanied by up-regulation of N-cadherin (Islam et al., 1996; Nieman et al., 1999; Li et al., 2001). This cadherin switch has been suggested to represent an invasive phenotype of tumors as an enhanced level of N-cadherin has been found to promote cell motility, invasion, and survival (Nieman et al., 1999; Hazan et al., 2000; Li et al., 2001; Suyama et al., 2002). Our data show that interactions between endothelial cells and melanoma cells stimulate a twofold increase in N-cadherin in the melanoma cells. Because transmigrating melanoma cells are surrounded on all sides by endothelial cells, the increase in N-cadherin may facilitate the formation of
heterotypic contacts with the endothelial cells. N-cadherin is apparently up-regulated at the transcription level by \( \beta \)-catenin signaling in transmigrating melanoma cells. In contrast, the level of N-cadherin in the endothelial cells remains relatively constant. This may be due to the lack of \( \beta \)-catenin signaling in endothelial cells because only a small area of the endothelial membrane is engaged in heterotypic contact formation and, therefore, most of the \( \beta \)-catenin would remain sequestered in the homotypic endothelial junction via interaction with VE-cadherin.

It is well known that \( \beta \)-catenin maintains the stability of adhesion complexes mediated by classical cadherins by linking them to the actin cytoskeleton. Mutations in \( \beta \)-catenin or \( \alpha \)-catenin that disrupt this linkage eliminate adhesiveness mediated by cadherin (Kawanishi et al., 1995; Bullions et al., 1997). Also, cadherin loses its binding activity after truncation or deletion of its cytoplasmic domain (Nagafuchi and Takeichi, 1988; Ozawa et al., 1990). In our studies, coimmunolocalization and coimmunoprecipitation studies have been used to confirm the association of \( \beta \)-catenin with N-cadherin at the initial stage of cell attachment. However, in subsequent stages of transmigration, the level of \( \beta \)-catenin associated with the heterotypic contact or the N-cadherin complex is reduced significantly. Cadherins without linkage to the actin cytoskeleton may undergo weaker interactions. Because tight adhesion is believed to be detrimental to cell migration (Nakagawa and Takeichi, 1998), weaker adhesive interactions may promote the transmigration of melanoma cell.

In the N-cadherin immunoprecipitates, a substantial amount of p120ctn was also present. p120ctn binds to the juxtamembrane region of cadherins and has been shown to facilitate the clustering of C-cadherin even in the absence of \( \beta \)-catenin binding (Yap et al., 1998) and enhance E-cadherin-mediated adhesion (Thoreson et al., 2000). It is possible that p120ctn may contribute to the stability of the N-cadherin complex and prevent its dissolution in the heterotypic contact during melanoma cell transmigration.

In contrast to reports showing that elevated levels of either N- or E-cadherin can inhibit Wnt signaling by sequestering \( \beta \)-catenin (Sadot et al., 1998; Nelson and Nusse, 2004), we have found that \( \beta \)-catenin dissociates from the N-cadherin

![Figure 7. Tyrosine phosphorylation in the heterotypic contact and \( \beta \)-catenin redistribution. (A) Tyrosine phosphorylation of proteins in the heterotypic contact during transendothelial migration of WM239 cells (labeled red). Cocultures were fixed at 1 or 5 h with or without the prior addition of 1 mM pervanadate (+ PV or −PV) for 5 min and then stained for phospho-tyrosine (green). Arrows indicate heterotypic contacts between melanoma cells and endothelial cells. (B) Lack of phospho-tyrosine staining in homotypic contacts of melanoma cells. WM239 cells were cultured on Matrigel, treated with 1 mM pervanadate for 5 min before fixation, and then double stained for \( \beta \)-catenin (red) and phospho-tyrosine (P-Tyr; green). (C) Inhibition of \( \beta \)-catenin dissociation from the heterotypic contact by genistein. WM239 cells (red) were cultured on top of a HMVEC monolayer in the presence of genistein (10 μM). Coverslips was fixed and stained with mAb against phospho-tyrosine, N-cadherin, or \( \beta \)-catenin. Arrows indicate heterotypic contacts which showed N-cadherin (green) and \( \beta \)-catenin (green) localization but lacked phospho-tyrosine staining. Similar experiments were carried out with calphostin C and H8, but they had no effect (unpublished data). (D) Effects of protein kinase inhibitors on melanoma cell transmigration. Transendothelial migration assays were carried out in the presence of genistein, calphostin C or H8, and transmigrated cells were scored at 5 h. Data represent the mean ± SD (n = 9). Bars, 10 μm.](image-url)
herin adhesion complex and activates the $\beta$-catenin signaling pathway in transmigrating melanoma cells despite an up-regulation of N-cadherin. The $\beta$-catenin signaling pathway has been implicated in cancer metastasis (Smalley and Dale, 1999; Peifer and Polakis, 2000). More than 80% of colorectal carcinomas contain mutations in either APC or $\beta$-catenin, leading to constitutive activation of the $\beta$-catenin signaling pathway (Bienz and Clevers, 2000). Nuclear $\beta$-catenin is frequently observed in colon cancer cells at the invasion front (Brabletz et al., 2001; Hiendlmeyer et al., 2004), suggesting the possible involvement of $\beta$-catenin signaling in invasion. It is of interest to note that among the known $\beta$-catenin target genes are those that code for proteins involved in the invasive process of cancer cells, such as CD44 (Wielenga et al., 1999), uPA (Hiendlmeyer et al., 2004), uPAR (Mann et al., 1999), MMP-7 (Brabletz et al., 1999), and MT1-MMP (Takahashi et al., 2002), and we have confirmed the up-regulation of CD44. Up-regulation of invasion-related genes should augment the transmigration process by promoting invasion of the basement membrane. Indeed, blocking $\beta$-catenin signaling by expressing a dominant-negative $\beta$-catenin results in significant inhibition of transendothelial migration, whereas the expression of a dominant-active $\beta$-catenin promotes transmigration.

Normally, excess $\beta$-catenin will be targeted for degradation in the absence of the Wnt signal (Jamora and Fuchs, 2002). However, the level of $\beta$-catenin does not change in our coculture system, suggesting that the melanoma cells might utilize a different mechanism to stabilize $\beta$-catenin. In addition, both dominant-active and dominant-negative mutants of $\beta$-catenin show preferential nuclear localization. These two mutant forms lack the N-terminal degradation signal, thus allowing them to escape degradation by the ubiquitin-proteasome pathway, whereas their armadillo repeats might facilitate their nuclear translocation (Fagotto et al., 1998; Yokoya et al., 1999). Sequestration of the mutant $\beta$-catenins in the nucleus would lower their cytoplasmic level, rendering them unable to compete with the endogenous $\beta$-catenin for binding to the membrane-associated N-cadherin. However, their high concentration in the nucleus should allow them to compete efficiently with the endogenous $\beta$-catenin for binding to the target genes.

The dissociation of $\beta$-catenin is a postattachment event, concomitant with the transmigration process. We speculate that heterotypic cell-cell interactions generate a signal that is capable of triggering a cascade of events, which in turn leads to $\beta$-catenin dissociation. Although the nature of this signal is not known, our results demonstrate a correlation between tyrosine phosphorylation of N-cadherin and the dissociation of $\beta$-catenin from the adhesion complex. In contrast to E-, N-, or P-cadherins which are either not phosphorylated or poorly phosphorylated, VE-cadherin can be heavily phosphorylated on its tyrosine residues under physiological conditions (Lampugnani et al., 1997). PECAM-1 is also known to contain phospho-tyrosine (Osawa et al., 2002). However, both VE-cadherin and PECAM-1 are absent from heterotypic contacts (Sandig et al., 1997b; Voura et al., 2000), suggesting that the phospho-tyrosine staining in the heterotypic contact is not due to either of these adhesion proteins. Notably, the phospho-tyrosine staining disappears from the heterotypic contacts when N-cadherin is knocked down in melanoma cells, indicating that the phospho-tyrosine residues are associated with N-cadherin.

It is likely that heterotypic cell-cell interactions between melanoma cells and endothelial cells stimulate the activity of a protein tyrosine kinase, which phosphorylates the cytoplasmic domain of N-cadherin, thereby destabilizing the binding of $\beta$-catenin to N-cadherin. In coculture studies, addition of genistein inhibits tyrosine phosphorylation of N-cadherin and prevents $\beta$-catenin dissociation. Genistein also inhibits transendothelial migration by 60%.

Although tyrosine phosphorylation of N-cadherin is observed, we have failed to detect tyrosine-phosphorylated $\beta$-catenin. Our results, therefore, stand in striking contrast to what is commonly held regarding the regulation of cadherin-$\beta$ catenin interaction. Tyrosine phosphorylation of $\beta$-catenin has long been believed to perturb cadherin-mediated adhesion by disrupting cadherin-$\beta$-catenin interaction.
β-catenin becomes phosphorylated, resulting in the disruption of cell-cell adhesion, when cells are transfected with v-Src, treated with tyrosine phosphate inhibitor or stimulated with growth factors (Volberg et al., 1992; Behrens et al., 1993; Shibamoto et al., 1994; Kinch et al., 1995; Balsamo et al., 1996; Nelson and Nusse, 2004). Tyrosine-phosphorylated β-catenin shows reduced binding to E-cadherin in vitro, and crystallography studies suggest that tyrosine phosphorylation of β-catenin may disrupt its interaction with E-cadherin through steric effects (Roura et al., 1999; Huber and Weis, 2001). However, little is known about the effect of tyrosine phosphorylation of N-cadherin on β-catenin binding because N-cadherin is poorly phosphorylated on tyrosine residues.

Recently, an active c-Src mutant has been reported to induce tyrosine phosphorylation of cadherin but not β-catenin (Irby and Yeatman, 2002). N-cadherin possesses several tyrosine residues that are potential substrates of Src and several of them reside within the β-catenin binding region. It would be of interest to determine whether Src activation is responsible for the tyrosine phosphorylation of N-cadherin, leading to the subsequent dissociation of β-catenin during melanoma cell transmigration.

Using other kinase inhibitors, we have found that H8 does not inhibit transendothelial migration, suggesting that protein kinase A may not play a role here. On the other hand, PKCβ has been implicated in cancer cell chemotaxis, migration, and invasion (Masur et al., 2001; Koivunen et al., 2004; Sun et al., 2005). Indeed, calphostin C inhibits transendothelial migration by almost 40%, suggesting that multiple signaling pathways are activated during extravasation. Future studies involving the identification of the kinases and phosphatases that regulate the phosphorylation state of N-cadherin and other signaling components should help define the signaling pathways involved in cancer cell transendothelial migration.

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