P-Cadherin Promotes Cell-Cell Adhesion and Counteracts Invasion in Human Melanoma

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Abstract

Malignant transformation of melanocytes frequently coincides with alterations in epithelial cadherin (E-cadherin) expression, switching on of neural cadherin (N-cadherin), and, when progressed to a metastatic stage, loss of membranous placental cadherin (P-cadherin). In vitro studies of melanoma cell lines have shown invasion suppressor and promoter roles for E-cadherin and N-cadherin, respectively. In the present study, we investigated the effect of P-cadherin on aggregation and invasion using melanoma cells retrovirally transduced with human P-cadherin. De novo expression of P-cadherin in P-cadherin–negative cell lines (BLM and HMB2) promoted cell-cell contacts and Ca2+-dependent cell-cell aggregation in two- and three-dimensional cultures, whereas it counteracted invasion. These effects were not observed following P-cadherin transduction of endogenously P-cadherin–positive MeWo cells. In addition, P-cadherin–transduced BLM cells coaggregated with keratinocytes and showed markedly reduced invasion in a reconstructed skin model. The proadhesive and anti-invasive effects of P-cadherin were abolished on targeted mutation of its intracellular juxtamembrane domain or its extracellular domain. For the latter mutation, we mimicked a known missense mutation in P-cadherin (R503H), which is associated with congenital hypotrichosis with juvenile macular dystrophy. (Cancer Res 2005;65(19):8774-83)

Introduction

Malignant melanoma incidence in Western countries has increased substantially during the last decades (1). A powerful prognostic determinant of malignant melanoma is the invasion depth of the primary tumors, as clinically represented by the Breslow index (2) or Clark’s level of invasion (3), which correlate with the metastasis rate. Invasion is indeed one of the first steps of the metastasis cascade and is closely linked with the adhesive properties of carcinoma but also of melanoma cells.

Altered expression of cell adhesion molecules, such as integrins and molecules belonging to the immunoglobulin or cadherin superfamily, frequently coincides with melanoma progression (4). Cadherins (Ca2+-dependent adherent proteins) are transmembrane glycoproteins establishing homophilic adhesion between neighboring cells through their extracellular cadherin repeats (5). The adhesive and anti-invasive functions of cadherins are modulated by cytoplasmic binding partners, such as the catenins. Whereas β-catenin binds to the cadherin’s COOH-terminal tail, providing a link to the actin cytoskeleton through α-catenin (6), p120 catenin is linked to the juxtamembrane domain, modulating cadherin trafficking and cadherin-dependent cell-cell adhesion and migration (7).

In the normal skin, melanocytes predominantly express the classic epithelial cadherin (E-cadherin) and placental cadherin (P-cadherin)/catenin complexes, as do the surrounding basal-type keratinocytes. E-cadherin has been suggested to be a prime mediator for the heterotypic adhesion between keratinocytes and melanocytes (8), providing growth control of the latter (9). Studies on melanoma cell lines showed decreased or absent E- and P-cadherin (10, 11), together with an up-regulation of neural cadherin (N-cadherin; ref. 10), in comparison with normal melanocytes. Using those cadherin switches, melanoma cells may escape from the control by the surrounding keratinocytes in the epidermis, allowing them to infiltrate and to interact, by means of their newly acquired N-cadherin, with constituents of the dermis, such as fibroblasts and blood vessel endothelium (12–15). Immunohistochemical studies have produced conflicting data regarding the expression of E-, P- and N-cadherin in malignant melanoma. The down-regulation of E-cadherin observed in vitro seems an early and reversible event in melanoma development, because the higher E-cadherin expression was found in deeply infiltrating melanomas and/or in metastases (16, 17). Krengel et al. (18), however, suggested that rather the microenvironment of melanocytic tumor cells determined their cadherin expression pattern, as E-cadherin and/or P-cadherin expression gradually diminished with tumor depth either in malignant melanoma or in benign nevi. Sanders et al. (19) mainly observed significant derangement of P-cadherin, its expression switching from membranous to cytoplasmic in metastasizing melanomas. These data suggested that in malignant melanoma P-cadherin might act as an invasion suppressor, like E-cadherin. However, there are no in vitro studies that explore this hypothesis.

In this study, we investigated the effect of P-cadherin on melanoma cell-cell adhesion and invasion using a panel of invasive melanoma cell lines that were retrovirally transduced with full-length human P-cadherin cDNA. The parental cell lines chosen could serve as models for aggressive, late-stage melanoma, as they were derived from lymph node (HMB2 and MeWo; refs. 20, 21) or experimentally induced lung metastases (BLM; ref. 22).

To explore the possible mechanisms of action, we also studied two P-cadherin mutants, targeting regions that are highly...
conserved in classic cadherins: the juxtamembrane p120 catenin-binding domain of which the corresponding site in E- and N-cadherin has been implicated in cell-cell adhesion and motility (23, 24) and the LDLRE motif in the fourth extracellular cadherin (EC4) repeat, which is likely involved in Ca2+-dependent cell-cell adhesion, as has been shown for E-cadherin (25). For the latter P-cadherin mutant, we mimicked the R503H mutation found in congenital hypotrichosis with juvenile macular dystrophy (HJMD) patients (26, 27).

Materials and Methods

Plasmids and cDNA constructs, retroviral transduction, and cell sorting. Generation of constructs, production of retroviral supernatant, transduction, flow cytometric evaluation of enhanced green fluorescent protein (EGFP) expression, and cell sorting were done as described earlier (28, 29).

Cells, antibodies, and chemical reagents. The human melanoma cell lines BLM, HMB2, and MeWo were obtained and cultured as described before (30). Primary keratinocytes and fibroblasts used for skin reconstruction were derived from skin specimens of normal human adults who underwent plastic surgery (abdominoplasty or breast reconstruction). Primary human keratinocytes were cultured on irradiated 3T3 feeder cells and stored in liquid nitrogen (31). On thawing, they were grown in defined serum-free, low-calcium keratinocyte medium (K-SEMs, Invitrogen, Merelbeke, Belgium) and used at their first or second passage. Primary human fibroblasts were cultivated in Opti-MEM I (Invitrogen) supplemented with 5% FCS, 2% UltraSer G, and 1% glutamine. In the reconstructed skin model, the primary keratinocyte/melanoma cell mixture was first incubated in a 1:1 mixture of K-SFM/DMEM supplemented with 10% FCS. During final submersion and during cultivation at an air-liquid interface, we used serum-free DMEM/Ham’s F-12 (3:1) supplemented with 10 ng/mL epidermal growth factor, 0.4 μg/mL hydrocortisone, 5 μg/mL insulin, and 2 × 10−5 M L-glutamine. Antibodies used were monoclonal anti–P-cadherin (clone 56) and anti–p120 catenin (clone 98; BD Transduction Laboratories, Lexington, KY), monoclonal anti–E-cadherin (Heccd-1, Takara, San Diego, CA), monoclonal anti–N-cadherin (CH19) and anti–α-tubulin (B-5-1-2) and polyclonal anti–α-catenin and anti–β-catenin (Sigma-Aldrich, Bornem, Belgium), and anti-GFP (Abcam, Cambridge, United Kingdom). The cocktail of monoclonal antibodies, clone AE1/AE3, was used as a pan-cytokeratin antibody (DAKO, Glostrup, Denmark). The pan-cadherin antibody we used was a polyclonal rabbit antiseraum generated against a 15-mer peptide corresponding to the COOH terminus of E-cadherin, which is identical in P- and N-cadherin.

Biotinylation, immunoprecipitation, and immunoblotting. Immunoprecipitation and immunoblotting experiments were done as published before, except that for preparation of cell lysates used for immunoprecipitation 0.7% NP40 in PBS was used as lysis buffer (30). Preparation of cells for biotinylation experiments was done as described earlier (28). Lysates containing 500 μg protein were precipitated with streptavidin beads (3 × 25 μL, Sigma-Aldrich). To establish the ratio between the levels of cytoplasmic P-cadherin (spontaneous after precipitation with streptavidin beads) and total P-cadherin (total cell lysate) for the different cell lines, the volume of supernatant to be loaded on gel was kept equal to that of the corresponding total lysate, which on its turn was calculated to contain equal amounts of total protein. The latter, as for any immunoblot, was controlled by immunostainings with anti–α-tubulin. Quantification of the band intensities was done with the Quantity One software (Bio-Rad, Hercules, CA). All experiments were done at least in duplo.

Slow aggregation assays. On semisolid substratum, 2 × 104 cells in 200 μL medium were seeded on solidified agar in a 96-well-plate (32). Aggregation formation was evaluated under an inverted microscope at the indicated time points.

In suspension, 6 mL containing 6 × 103 melanoma cells or primary keratinocytes/melanoma cells (1:1 in their proper medium) were incubated for 24 or 48 hours in continuously gassed (10% humidified CO2) 50 mL Erlenmeyer flasks on a Gyrotory shaker at 72 rpm (New Brunswick Scientific Co., New Brunswick, NJ). After fixation in 10% phosphate-buffered formaldehyde, the particle size of the aggregates, representing the aggregation capacity of the cells, was measured with a Coulter particle size counter (LS20000, Coulter Co., Miami, FL) or by phase-contrast microscopy. For the latter, from aggregates in 10 randomly selected × fields the shortest (a) and largest (b) diameters were assessed using Axiovision software (Carl Zeiss, Göttingen, Germany) and the volume was estimated with the equation: 0.4 a2 × b (33). Statistical analysis of the particle size distribution curves was done with the Kolmogorov-Smirnov method (Coulter counter) or the Student’s t test; differences in aggregate diameters were considered statistically significant if P < 0.05.

Chick heart invasion assay. The chick heart invasion assay was done as described earlier (34). Briefly, precultured heart fragments (PHF) of 9-day-old chick embryos were brought into contact with monolayer fragments of melanoma cells on semisolid agar for 24 hours and then kept in suspension under continuous Gyrotory shaking for 4 or 8 days. The confrontations were fixed in Bouin-Holland’s solution, embedded in paraffin, serially sectioned, and stained with H&E or processed for immunohistochemistry. Invasion was scored as follows: 0 if only PHF is found and no confronting cells can be observed; 1 if the confronting cells are attached to the PHF and do not occupy the heart tissue; 2 if occupation of the PHF is limited to the outer fibroblast-like and myoblast cell layers; 3 if the confronting cells have occupied the PHF but have left more than half of the original amount of heart tissue intact; and 4 if the confronting cells have occupied more than half of the original volume of the PHF.

Invasion assay in reconstructed human skin. Skin reconstructions were generated using published techniques (35, 36) with modifications. First, dead de-epidermized dermis (DED) was produced from human skin fragments derived from plastic surgery (abdominoplasty or breast reconstructions). The skin fragments were cut into 2 × 2 cm pieces and incubated in PBS at 37°C for 10 days or until the epidermis was separable from the dermis. DED was then retrieved by gently scraping off the epidermis and was stored in PBS at −20°C. On processing, the DED was thawed, washed with PBS, and reduced in thickness (to 3-4 mm).Suspensions (500 μL) of 3 × 105 primary fibroblasts and 3 × 105 primary keratinocytes mixed with melanoma cells at a ratio of 1:10 or 1:100 (melanoma cells/keratinocytes) were seeded onto the reticular and papillary surface of the composite, respectively, according to Eves et al. Incubation periods of the cells in the rings were limited to 24 hours, after which the composites were submerged in the proper medium for an additional 24 hours (fibroblasts) and 72 hours (melanoma cells/keratinocytes) before continuing with the next step. After 10 to 14 days of incubation at an air-liquid interface, the composites were fixed overnight in 10% phosphate-buffered formaldehyde. Two representative sections through the seeded area were embedded in paraffin, processed for histology using a vacuum tissue processor (APS 300, Leica, Germany), serially sectioned, and stained with H&E at three levels or immunohistochemically with a pan-cytokeratin antibody (the alternating sections). For quantification, each BLM P-cadherin (BLM P-cad) slide was randomly coupled to a BLM pLZRS/ internal ribosomal entry site (ires)/EGFP (LIE) slide (with the same ratio of melanoma cells/keratinocytes), and for each composite, one representative H&E-stained section was scored blind-coded. The alternating anti-cytokeratin–stained section was first screened to exclude all cytokeratin-positive cells present in the dermis. For each representative H&E section, the total number of cytokeratin-negative cells present in the dermis was counted and the distance to the nearest basement membrane, representing the minimal invasion depth, was assessed using the Axiovision software (Carl Zeiss, Göttingen, Germany). Statistical evaluation of both variables (number of invasive cells and depth of invasion) was done with the Wilcoxon signed-rank test (37). To exclude irrelevant outliers regarding the invasion depth, the 90th percentile of invasion depth was taken into account.

Immunohistochemistry, cytochemistry, and cell labeling. Tissue sections were deparaffinized, rehydrated, and stained according to the modified avidin-biotin-peroxidase method. Briefly, sections were incubated with the primary anti-GFP or anti–P-cadherin antibody followed by horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch) and developed with 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO).
by, respectively, biotinylated donkey anti-rabbit or biotinylated sheep anti-mouse secondary antibody (Amersham Biosciences Europe, Brecht, Belgium) and by streptavidin–Texas red–conjugated streptavidin (Amersham Biosciences Europe). Detection was done by incubation with 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Sections were counterstained with hematoxylin. Slides for anti–P-cadherin staining were first boiled for 20 minutes in 1 mmol/L EDTA buffer (pH 8.0; antigen retrieval). Confluent cultures on glass coverslips were fixed for 10 minutes in methanol at –20°C. The secondary biotinylated antibody was detected with Texas red–conjugated streptavidin (Amersham Biosciences Europe). Nuclei were stained with 4',6-diamidino-2-phénylindole. Photomicrographs were taken with the Axiosvert 20 microscope (Carl Zeiss).

BLM and keratinocyte cultures were incubated for 30 minutes with 1.5 mL of 5 μL/mL Vibrant DIO and DiI cell labeling solution, respectively (Molecular Probes, Eugene, Leiden, the Netherlands). After washing with PBS, cultures were trypsinized and suspended for further experiments.

Results

P-cadherin transduction of melanoma cell lines. BLM, HMB2, and MeWo were selected from a panel of melanoma cell lines tested by Western blotting for its expression of the classic cadherins, E-, N- and P-cadherin. Whereas BLM showed no immunoreactivity for anti–pan-cadherin or for any of the E-, P- or N-cadherin–specific antibodies, HMB2 and MeWo cells displayed strong expression of N-cadherin. In addition, moderate endogenous P-cadherin expression was noted in the MeWo cell line (Fig. 1A).

To explore the functional effects of P-cadherin expression, the selected cell lines were infected with the retroviral vector pLZRS/P-cadherin/IRESC/EGFP, further coined P-cad, or with the empty vector LIE. Transduction was followed by cell sorting, yielding a population of >96% EGFP-positive cells (data not shown). Expression of E-, N- and P-cadherin in the transduced cell lines was verified by Western blotting. Unlike in the total lysates of parental BLM and HMB2 cells, a 118-kDa band reactive with a monoclonal anti–P-cadherin antibody was revealed in the P-cadherin–transduced BLM cells (BLM P-cad) and HMB2 cells (HMB2 P-cad), whereas an increased P-cadherin level was found in MeWo P-cad. No major changes in the expression levels of N-cadherin were observed on (over)expression of P-cadherin in the HMB2 and MeWo cell lines (Fig. 1B). Biotinylation experiments and immunocytochemistry confirmed the presence of N-cadherin and/or P-cadherin at the surface of all transduced cell lines (Fig. 1C and D; Supplementary Fig. S1; see also Fig. 6). These transduced melanoma cell lines enabled us to investigate the function of P-cadherin in relation to a different endogenous cadherin background.

De novo expressed P-cadherin imposes an epithelioid morphology on melanoma cells. On plastic culture dishes or glass coverslips, the de novo P-cadherin–expressing melanoma cells adopted an altered, epithelioid morphotype. BLM P-cad cells were arranged in cell rows or clusters, whereas the polygonal or spindle-shaped parental or vector-transduced cells displayed only minimal cell-cell contacts. These alterations were particularly evident at subconfluence (Fig. 1E, left). HMB2 P-cad cells, at high cell density, also displayed an epithelioid morphotype, although the formation of cell rows or clusters was less pronounced in subconfluent cells (Fig. 1E, middle). No changes could be observed in MeWo P-cad cells, which were elongated or spindle-shaped like the nontransduced or vector-transduced controls (Fig. 1E, right).

In summary, a P-cadherin–induced epithelioid morphotype is present in melanoma cells that do not express this protein natively, irrespective of the coexpression of N-cadherin.

De novo expressed P-cadherin stimulates homotypic and heterotypic cell-cell aggregation of melanoma cells. Because the appearance of epithelioid clusters in BLM P-cad cultures suggested that P-cadherin plays a role in melanoma cell adhesion, we tested our panel of cell lines for their ability to form cell-cell aggregates. On a semisolid agar substratum, BLM and BLM LIE cells did not aggregate after a short period (up to 24 hours) and formed only loose and irregular aggregates after 72 hours. BLM P-cad cells, however, formed compact aggregates that were already noticeable after 24 hours (Fig. 2A, top). Similarly, P-cadherin conferred increased aggregation to the HMB2 cell line, of which both parental and vector-transduced cells readily organized into small clusters, whereas HMB2 P-cad cells progressively formed more compact aggregates after prolonged incubation periods (Fig. 2A, middle). In contrast to these effects of de novo P-cadherin expression, the introduction of additional P-cadherin in the low P-cadherin–expressing MeWo cells did not result in any changes in cell-cell aggregation neither at early (data not shown) nor at later stages (Fig. 2A, bottom).

Another approach, in which cell-cell aggregation was quantified after incubating the respective cell lines in suspension under continuous shaking, gave similar results. As evidenced by a shift of the particle size distribution curve in Fig. 2B, significantly larger aggregates were formed by HMB2 P-cad cells after 48 hours compared with the control cell lines. Aggregation of the BLM P-cad cells was increased to such an extent that the volumes largely exceeded the detection limits of the particle size counter even after 24 hours of incubation. Consequently, the aggregate diameters of the BLM cell lines were measured by phase-contrast microscopy to calculate the aggregate volumes (Fig. 2C; see also Fig. 5B). Consistent with the aggregation assay on agar, transduction of MeWo cells with P-cadherin did not enhance their aggregating behavior in suspension (Fig. 2B).

Immunocytochemistry of HMB2 and MeWo cell aggregates, showing the presence of N-cadherin (endogenous) and/or P-cadherin (endogenous or overexpressed) at sites of cell-cell contacts suggests the involvement of these cadherins in the formation of these aggregates (Supplementary Fig. S1).

Because the surrounding normal skin has an important regulatory effect on melanoma development (12), we also studied aggregation of melanoma cells and keratinocytes in suspension cocultures. Dil-labeled keratinocytes (red) and DiO-labeled BLM LIE or P-cad cells (green) sorted out after 24 hours (data not shown) or 48 hours of incubation (Fig. 2D). Also here, BLM P-cad cells formed large aggregates, unlike BLM LIE cells. These aggregates showed cross-adherence, attaching to and partially enveloping the keratinocyte clusters (see Discussion). The absence of such heterotypic adhesion in BLM LIE cocultures suggests the involvement of P-cadherin in this process.

In conclusion, P-cadherin stimulates homotypic and heterotypic cell aggregation when de novo expressed in melanoma cells.

De novo expressed P-cadherin counteracts invasion of melanoma cells. To test the putative role of P-cadherin as an invasion suppressor in malignant melanoma, we subjected all cell lines of our panel to the chick heart invasion assay (34). The parental and vector-transduced BLM cells strongly invaded into the chick heart fragments after 4 days (Fig. 3A, top left and
In contrast, invasion was largely suppressed in BLM P-cad cells, which enveloped the chick heart fragments instead, as highlighted by the anti-GFP immunohistochemical stainings (Fig. 3A, top right; see also Fig. 5C and D). In the strongly invasive HMB2 cell line, the invasive capacity was also attenuated by introduction of P-cadherin (Fig. 3A, middle). The P-cadherin–positive MeWo cell line, also strongly invasive in this assay, was not affected in its invasive behavior by P-cadherin transduction (Fig. 3A, bottom). These differences could be appreciated in eight of nine “couples” (i.e., blinded and randomly coupled histologic sections of independent experiments). We quantified the extent of invasion by counting the invading melanoma cells and measuring their invasion depth as described in Materials and Methods. Quantification of a representative couple is depicted in Fig. 3C, which ranks all invading BLM LIE or P-cad cells by increasing invasion depth. Figure 3D represents the proportion of invasive BLM LIE and P-cad cells relative to the total number of invasive cells within each couple. According to the nonparametric Wilcoxon signed-rank test (37), both the number of invasive cells and the invasion depth were significantly different in the BLM LIE and BLM P-cad samples (Ps = 0.0039 and 0.027, respectively).

In summary, we showed that, paralleling its stimulating effect on aggregation, de novo expressed P-cad counteracts invasion of melanoma cells irrespective of the coexpression of N-cadherin.
Generation of BLM P-cad mutant cell lines. To identify which domains of P-cadherin were important for its proadhesive and anti-invasive properties, two P-cadherin mutants were generated, targeting regions that can be expected to alter P-cadherin function: P-cadΔp120, lacking 5 amino acids (EEGGG) in the p120 catenin–binding site of the juxtamembrane domain of P-cadherin, and P-cadR503H, in which an arginine residue in the LDRE motif of the EC4 repeat was substituted by a histidine.

BLM cells were transduced with these P-cadherin mutants and sorted for EGFP expression to >95% purity as had been done for wild-type (wt) P-cadherin– and vector-transduced cells. Integrity of these constructs was confirmed by Western blotting of BLM P-cadΔp120 and BLM P-cadR503H total cell lysates. P-cadΔp120 migrated slightly faster and its levels were consistently lower than the wt P-cadherin or P-cadR503H levels (Fig. 1B) as could be expected from literature data (38). To check localization at the plasma membrane, lysates of biotinylated cells were subjected to precipitation with streptavidin beads followed by immunoblotting of the different fractions for P-cadherin. The ratio between P-cadherin levels in the nonbiotinylated fraction and the total lysate was similar in the three transduced cell lines (Fig. 1D, bottom). As revealed by the precipitated (biotinylated) fraction, both P-cadherin mutants are expressed at the cell surface, although their levels vary when compared with wt P-cadherin (Fig. 1C versus D, middle; see also Discussion). This particularly holds true for the P-cadΔp120 cell line, although also in these cells levels of cell surface expression can approach those of BLM P-cad (Fig. 1C).

Western blotting was also done to verify whether introduction of P-cadherin or P-cadherin mutants into BLM cells affected the levels of β-catenin and p120 catenin. Whereas all cell lines showed similar levels of p120 catenin, β-catenin levels were increased in all P-cadherin–transduced cell lines, suggesting its stabilization by recruitment to the P-cadherin constructs (Fig. 4A). The smaller increase in BLM P-cadΔp120 cells likely reflects the lower levels of P-cadherin in this cell line. These findings fit with the known metabolic stability of p120 catenin and the stabilizing role of cadherins toward β-catenin (23). Subsequently, communoprecipitation studies revealed that α-catenin and β-catenin coimmunoprecipitated with all P-cadherin constructs (Fig. 4B, top and middle). p120 catenin, on the other hand, associated only poorly with P-cadΔp120, in contrast to wt P-cadherin and P-cadR503H, confirming the selective deficiency of p120 catenin in binding P-cadΔp120 (Fig. 4B, bottom).

Mutation of the p120 catenin–binding domain or the extracellular domain of P-cadherin interferes with its proadhesive and anti-invasive effects. Subconfluent cultures of BLM P-cadΔp120 and BLM P-cadR503H revealed a spindle-cell morphotype indistinguishable from parental and vector control BLM cells.

Figure 2. De novo expressed P-cadherin stimulates cell-cell adhesion. A, slow aggregation assay on soft agar with the indicated cell lines transduced with the indicated constructs. Phase-contrast micrographs were taken at the indicated time points. Bar, 500 μm. B, particle size distribution of HMB2 or MeWo cell aggregates in a slow aggregation assay after 48 hours in suspension. n, parental cell lines; E, LIE-transduced cell lines; P-cad, P-cadherin–transduced cell lines. C, mean calculated aggregate volumes of parental (-) and transduced BLM cells after 24 hours in a slow aggregation assay in suspension. Aggregates formed by P-cadherin–transduced BLM cells are significantly (asterisk) larger than nontransduced or vector-transduced BLM cells. D, fluorographs of DiO-labeled BLM LIE or BLM P-cad cells (green) cocultured with DiI-labeled primary keratinocytes (red) for 48 hours in a slow aggregation assay in suspension. BLM P-cad shows homotypic and heterotypic cell-cell adhesion in contrast to BLM LIE. Bar, 500 μm.
could be ascribed to a technical failure. With BLM LIE cells, except for one outlier (included in the statistics), which markedly lower number of invading melanoma cells compared with composites one coupled experiment. Composites seeded with BLM P-cad cells show a reduced in BLM P-cad (n = 32) compared with BLM LIE (n = 762). Combination of invading melanoma cells into the dermal part of the reconstructed skin composites (blue, BLM P-cad; red, BLM LIE) relative to the total number of invading cells of the respective couple. Each column refers to one coupled experiment. Composites seeded with BLM P-cad cells show a markedly lower number of invading melanoma cells compared with composites with BLM LIE cells, except for one outlier (included in the statistics), which could be ascribed to a technical failure.

In summary, we showed that disruption of the extracellular or the p120 catenin–binding domain of P-cadherin interferes with its ability to establish stable cell-cell contacts and with its anti-invasive function in BLM melanoma cells.

**Mutation of the p120 catenin–binding domain or the extracellular domain of P-cadherin interferes with the localization of P-cadherin at the cell-cell junctions.** We did immunocytochemistry to check the subcellular localization of the P-cadherin mutants and of β-catenin and p120 catenin, which are known to form complexes with classic cadherins at sites of intercellular contact. Cell cultures were stained at (near) confluence, as the BLM control cells and the P-cadherin mutant cells tended to form cell-cell contacts.

Pan-cadherin, β-catenin, and p120 catenin stainings of BLM P-cad cells revealed a membrane-associated honeycomb texture, indicating that P-cadherin/catenin complexes accumulated at the intercellular junctions on the plasma membrane (Fig. 6). This staining pattern contrasted with the absent (pan-cadherin) and the diffuse cytoplasmic (β-catenin and p120 catenin) staining of parental BLM (data not shown) and BLM LIE cells. The P-cadherin mutant cells also displayed striking differences with wt BLM P-cad. In the P-cadherinR503H cells, on the other hand, formed a single aggregate resembling the BLM P-cadherin aggregates at any time point. However, whereas the BLM P-cad cells formed a very tight, compact aggregate characterized by a smooth perimeter, the BLM P-cadherin association remained loose (Fig. 5A, right). In the slow aggregation assay in suspension, both mutations clearly interfered with the intercellular aggregation induced by P-cadherin (Fig. 5D). When analyzed by the particle size counter, these BLM P-cadherin mutant cells (6 of 6) were not significantly different from the control aggregates (data not shown). Together, these data indicated that P-cadherin mutated in its p120 catenin–binding domain has a strongly impaired ability to mediate cell-cell aggregation, whereas P-cadherinR503H, at most, mediates weak intercellular contacts.

To determine the involvement of the p120 catenin–binding domain and the EC4 repeat in the anti-invasive function of P-cadherin, the BLM P-cad mutants were examined in the chick heart invasion assay. As shown in Fig. 5C for 4-day-old confronting cultures, BLM P-cadherinΔp120 and BLM P-cadherinR503H cells extensively invaded the chick heart tissue. Figure 5D depicts the invasion scores (see Materials and Methods): all BLM P-cad confronting cultures exhibited only minimal invasion (score 2; 6 of 6), whereas all BLM P-cadherin mutant cell lines (6 of 6 and 9 of 9) invaded the chick heart fragments similarly to parental BLM (5 of 5) and BLM LIE (6 of 6) cells (score 3 or 4).

De novo expressed P-cadherin counteracts invasion. A, light micrographs of histologic sections of 4-day-old chick heart confrontation cultures with the indicated cell lines. The parental BLM cell line (·) and the HMB2 (·, LIE, and P-cad) and MeWo (·, LIE, and P-cad) cell lines were stained with H&E, whereas BLM LIE and BLM P-cad confrontation cultures were immunostained with an anti-GFP antibody. Whereas all parental and LIE-transduced cells as well as MeWo P-cad cells exhibit massive infiltration of the chick heart fragment (C), P-cadherin–transduced BLM and HMB2 cells rather surround the chick heart fragments with only minimal infiltration of the outermost layers of the chick heart fragment. Bar, 100 μm. B, light micrographs of H&E-stained histologic sections through human skin reconstructions. BLM LIE cells markedly infiltrate the dermal collagen (D), whereas BLM P-cad cells remain clustered in the lower part of the epidermis, extending downward without penetrating the basement membrane. The reconstructed epidermis can be recognized, consisting of keratinocytes (K), which show differentiation toward a cornified layer at the surface. Arrowheads, positions of the basement membrane. Bar, 100 μm. C, cumulative distribution curve of invasion depth measurements on representative sections of a couple of reconstructed skin composites, representing all BLM LIE or P-cad cells invading into the dermis, ranked by their invasion depth. Both the number of invasive cells and the depth of invasion are strongly reduced in BLM P-cad (n = 32) compared with BLM LIE (n = 762).
catenin (P-cadR503H) and/or β-catenin (both mutants) but fails to localize in organized and continuous cell-cell junctions. This could explain the decreased adhesive capacity and, as a consequence, the abrogated anti-invasive function observed in the mutant P-cadherin–transduced cell lines.

**Discussion**

Down-regulation of P-cadherin has been observed during melanoma progression, particularly in the deeply infiltrating part of primary tumors or in metastasizing melanoma (18, 19, 11). In the present *in vitro* study, using a selected panel of P-cadherin–transduced cell lines, we examined the function of P-cadherin in late-stage malignant melanoma, focusing on intercellular adhesion and invasion.

We showed that P-cadherin, when de novo expressed, has a strong anti-invasive activity in BLM and HMB2 melanoma cells. MeWo (P-cad) cells, however, were readily invasive despite their endogenous or de novo P-cadherin expression, which suggests that in this cell line other mechanisms have evolved that bypass or render the cells resistant to the anti-invasive P-cadherin effect. Our results agree with an earlier *in vitro* study of P-cadherin function in Lewis lung carcinoma cells (39). Interestingly, in parallel with the *in vivo* data on melanomas (18, 19), loss of P-cadherin correlating with decreased differentiation was also described in a small series of non–small cell lung carcinomas (68% of 28 cases; ref. 40). This is opposed to, for example, breast cancer, where P-cadherin is aberrantly expressed in a subset of tumors and strongly correlates with a poor 5-year patient survival (41). In line with this, we recently found that P-cadherin is regulated by estrogen receptor signaling and has a proinvasive function in breast cancer cell lines (28). Our studies therefore support a model in which the ultimate function of P-cadherin reflects the alterations in expression throughout tumor progression. P-cadherin could act as an invasion suppressor in a cancer cell type in which it is down-regulated during progression but as an invasion promoter in a cancer cell type in which aberrant expression occurs throughout progression. Importantly, our results with HMB2 P-cad suggest that in this cell line P-cadherin has a dominant effect over the endogenous N-cadherin. This is remarkable in view of the N-cadherin dominance over E-cadherin described in breast cancer cells. E-cadherin did not suppress invasion of N-cadherin–expressing MDA-MB-435 cells (42), whereas N-cadherin conferred more invasiveness to E-cadherin–expressing MCF-7 cells (43).

The invasion suppressor function of P-cadherin was evident in a collagen type I invasion assay, which showed a 2-fold decreased invasion index of BLM P-cad and HMB2 P-cad cells compared with vector-transduced cells (data not shown). We further showed the anti-invasive effect of P-cadherin by two additional invasion assays, the chick heart invasion assay and a reconstructed human skin model. In the former, tumor cells were confronted with myocardium of embryonic chick heart fragments, whereas the latter model, consisting of human keratinocytes, basement membrane, dermal fibroblasts, and dermal collagen, more closely recapitulates the microenvironment of a melanocytic tumor *in vivo*. Whereas others have mainly applied this model in a qualitative or semiquantitative way (36, 44), we quantified the invasion of our tested cells. We counted the cells that had invaded into the dermal collagen and measured the minimal invasion depth of each cell relative to the basement membrane as an *in vitro* correlate of the Breslow index (tumor invasion depth), a prognostic factor routinely assessed in the clinical setting (2).

P-cadherin–mediated suppression of invasion is most likely linked to its concurrent proadhesive effect, which we showed in calcium-dependent aggregation assays. In the coaggregation assay in suspension, melanoma cells and keratinocytes sorted out, whereas BLM P-cad aggregates also showed cross-adherence, incompletely enveloping the keratinocyte clusters. This corresponds to earlier reports on coaggregation assays with E- or P-cadherin–positive L-cells, in which both homophilic and heterophilic (E-cadherin/P-cadherin) adhesion occurred with equal...
strength and was determined by the total cadherin levels rather than by the cadherin types involved (39, 45). On BLM P-cad cells, these levels are most probably lower than on the double P-cadherin/E-cadherin−positive keratinocytes, explaining the particular pattern of cross-adherence. Similarly, sorting out of keratinocytes and melanoma cells occurred in the reconstructed epidermis, the melanoma cells did not intermingle with the keratinocytes. The differences in invasive capacity between BLM LIE and P-cad suggest that P-cadherin−mediated contacts determine and could be sufficient to control melanoma invasion. Similarly, previous studies on the function of E-cadherin have shown that forced E-cadherin expression in melanoma cells restored keratinocyte-mediated growth control and inhibited invasion (9, 46). Therefore, we can speculate on the redundancy of E- and P-cadherin in melanocytes and melanoma cells. This would parallel the redundant roles of these cadherins in basal keratinocytes as shown in vitro (47) and in conditional E-cadherin knockout mice where P-cadherin partially compensated for loss of E-cadherin (48).

To gain more insight into the molecular mechanisms of P-cadherin action, we created two P-cadherin mutants. First, P-cadΔp120 was created to study the juxtamembrane domain. In E- and N-cadherin, this domain is involved in tight cell-cell adhesion and cell motility through direct binding of p120 catenin (23, 24). Here, BLM P-cadΔp120 cells displayed severely impaired cell-cell adhesion and abrogation of P-cadherin−mediated invasion suppression, indicating the involvement of the juxtamembrane domain in P-cadherin's effect on adhesion and invasion. Several studies have shown a critical role for p120 catenin in maintaining the steady-state cadherin levels and stabilizing them at the cell membrane (38, 49, 50). We also observed less overall P-cad Δp120 than wt P-cadherin in the respective BLM cell lines and, at subconfluency, a lower ratio of cell surface versus overall P-cad Δp120 levels compared with wt P-cadherin (data not shown). In confluent cultures, however, the relative P-cad Δp120 levels at the cell surface could approximate those of wt P-cadherin (see Fig. 1C). This suggests that with increasing cell density the cadherin/catenin complex is retained at the cell surface in a p120 catenin−independent way. This is in line with studies on E-cadherin

Figure 5. Strong cell-cell adhesion and the capacity to counteract invasion are lost in BLM cells transduced with mutant P-cadherin. A, slow aggregation assay on soft agar with BLM-derived cell lines as indicated. Phase-contrast micrographs were taken at the indicated time points. Right, magnification of bottom middle: the perimeter of the BLM P-cad aggregates is smooth (arrowheads) compared with the more loosely associated BLM P-cad R503H aggregates, indicating less strong adhesive capacity of the latter. Bar, 500 μm (left) and 100 μm (right). B, slow aggregation assay in suspension with the indicated BLM-derived cell lines. Phase-contrast micrographs were taken after 48 hours of incubation. Bar, 100 μm. C, light micrographs of H&E-stained histologic sections of 4-day-old chick heart confrontation cultures with the indicated BLM-derived cell lines. c, chick heart tissue. Bar, 100 μm. D, table depicting the invasion scores for all BLM (-derived) cell lines after 4 days of confrontation in the chick heart invasion assay. Each circle represents one independent experiment. In contrast to BLM P-cad cells, which are clearly less invasive than nontransduced or vector-transduced cells, BLM P-cad R503H and BLM P-cadΔp120 strongly invade the chick heart fragment.
mutated in its p120 cadherin–binding domain, which showed membranous localization, albeit with a decreased efficiency in generating continuous cell-cell contacts (23, 51). Our immunocytochemical analyses, however, could not show membranous P-cadherin, whereas membranous β-catenin staining was only weak. Given the fact that immunocytochemical detection of a molecule strongly relies on its concentration at a particular subcellular localization, we hypothesize that in BLM cells, unlike the wt P-cadherin/β-catenin complex, the P-cadΔp120/β-catenin complex is not concentrated at cell-cell junctions but diffusely present at the cell membrane. Interestingly, the similar cytoplasmic staining patterns of pan-cadherin and β-catenin in BLM P-cad and BLM P-cadΔp120 cells, suggesting an intact P-cadherin/β-catenin complex, whereas uncoupling of p120 cadherin but retained β-catenin immunocolocalization is seen in BLM P-cadΔp120. Bar, 50 μm.

The second mutant, P-cadR503H, mimicked a homozygous missense mutation detected in patients with congenital HJMD. This is an autosomal recessive disorder mainly characterized by hair loss and progressive macular degeneration leading to early blindness (52). The R503H mutation results in a single histidine-for-arginine substitution at position 503 (26, 27), which is part of the highly conserved Ca2+-binding LDRE motif in the EC4 repeat of P-cadherin. As shown in cadherins related to P-cadherin, the LDRE motif is crucial for the proper folding of the extracellular domain of the cadherin molecule, enabling stable cis and trans cadherin interactions (25, 53). In our study of BLM melanoma cells, we showed that the extracellular domain of P-cadherin is implicated in its anti-invasive effect. As expected, the targeted domain was necessary for strong cell-cell adhesion, as compaction of BLM P-cadΔR503H aggregates was impaired in the aggregation assay on agar and only minor aggregation occurred when the cells were kept in suspension. Our immunocytochemical data suggested inefficient recruitment of the P-cadR503H/β-catenin complex to cell-cell junctions. This was, however, less severe than in BLM P-cadΔp120 cells, which might in part explain the less drastic effect of the R503H mutation on aggregation (see Fig. 5A). As coimmunoprecipitation and immunocytochemical colocalization of β-catenin (and p120 cadherin) with P-cadR503H were preserved, we consider it unlikely that signaling via the catenins is of major importance in the P-cadR503H phenotype.

In conclusion, we showed that P-cadherin may function as an anti-invasive factor in human melanoma irrespective of concurrent N-cadherin expression. Based on the functional consequences of transduction with (mutant) P-cadherin, we propose that this anti-invasive property is attributed to its strong proadhesive activity, ensuring both homotypic and heterotypic cell-cell contacts. Our in vitro results suggest an active role for P-cadherin in melanoma progression and support the functional relevance of the down-regulation of P-cadherin in biopsies of melanoma patients described in literature.

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References


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