P-Cadherin Is Up-Regulated by the Antiestrogen ICI 182,780 and Promotes Invasion of Human Breast Cancer Cells

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ABSTRACT

P-cadherin expression in breast carcinomas has been associated with tumors of high histologic grade and lacking estrogen receptor-α, suggesting a link between these proteins. In the MCF-7/AZ breast cancer cell line, blocking estrogen receptor-α signaling with the antiestrogen ICI 182,780 induced an increase of P-cadherin, which coincided with induction of in vitro invasion. Retroviral transduction of MCF-7/AZ cells, as well as HEK 293T cells, showed the proinvasive activity of P-cadherin, which requires the juxtamembrane domain of its cytoplasmic tail. This study establishes a direct link between P-cadherin expression and the lack of estrogen receptor-α signaling in breast cancer cells and suggests a role for P-cadherin in invasion, through its interaction with proteins bound to the juxtamembrane domain.

INTRODUCTION

Classical cadherins are a superfamily of transmembrane glycoproteins responsible for calcium-dependent cell-cell adhesion, mediating homophilic protein interactions (1). These are modulated by their conserved cytoplasmic juxtamembrane domain and catenin-binding domain, linking them to the actin cytoskeleton. β-γ, p120-γ, and α-Catenins are the best-documented interaction partners (2). β-Catenin (and perhaps also γ-catenin) is a signaling molecule, implicated in tissue patterning, of which the functions are regulated by binding to the catenin-binding domain of cadherins and by interactions with receptor tyrosine kinases and transcription factors of the lymphocyte enhancer factor/T-cell factor family (2). P120-catenin was identified as a substrate for Src and several receptor tyrosine kinases and interacts directly with the juxtamembrane domain of cadherins, modulating cadherin clustering and cell motility in a cell-type and phosphorylation state-dependent way (3). The cadherin/catenin functional complex is linked to the actin cytoskeleton via α-catenin, thus strengthening its adhesive force (1).

Reduced expression of E-cadherin is associated with tumor progression in many different cancers, including breast cancer (4), and may result from mutations, loss of heterozygosity, promoter hypermethylation, or up-regulation of transcriptional repressors, as SIP1, Snail, Slug, or Twist (1). Moreover, the invasion suppressor function of normally expressed E-cadherin may be overcome by the aberrant expression of N-cadherin (5) or cadherin-11 (6), which have been associated with progression of breast carcinoma through interference with E-cadherin function (7).

P-cadherin, another classical cadherin, is expressed in ectodermal tissues, more specifically in the basal layers of stratified epithelia, and in myoepithelial cells of the breast (10). P-cadherin is implicated in growth and differentiation, as evidenced by knockout mice displaying precocious differentiation of the mammary gland (11), and is aberrantly expressed in mammary carcinomas of high histologic grade and with a poor prognosis (12–16), as well as in other types of carcinomas and proliferative inflammatory lesions (17–19). It has been suggested that suppression of the P-cadherin gene is lost during carcinogenesis (9), but the nature of this mechanism and the biological role of the newly acquired P-cadherin remain to be investigated.

Because aberrant expression of P-cadherin identified a subgroup of estrogen receptor-α-negative breast carcinomas (16), we raised the hypothesis that the expression of P-cadherin in mammary epithelial cells is hormonally regulated, as described for E-cadherin (20), N-cadherin (21), and cadherin-11 (22).

In mammary epithelial cells, estrogen receptor-α is a key regulator of proliferation and differentiation and a crucial prognostic indicator and therapeutic target in breast cancer. Estrogen receptor-α is a ligand-dependent transcription factor acting through direct transcriptional target activation (23). Estradiol acts as a potent mitogen for many breast cancer cell lines, and ~70% of breast carcinomas are estrogen receptor-α positive. This mitogenic effect is blocked by estrogen antagonists. Pure antiestrogens (like ICI 182,780) and selective estrogen receptor modulators (like tamoxifen; ref. 24) are used for the treatment of osteoporosis, breast cancer, and other diseases. Continuous exposure of steroid–hormone-responsive breast cancer cell lines to ICI 182,780 leads to resistant sublines, with signaling pathways alternative to estrogen receptor-α (25). Similarly, in breast cancer, a high number of patients eventually develop antiestrogen resistance for unknown reasons.

Using the antiestrogen ICI 182,780, we investigated a putative molecular and functional link between the absence of estrogen receptor-α signaling and P-cadherin expression in breast cancer cells. To understand the relationship between P-cadherin and the aggressive breast cancer phenotype, we studied the effect of wild-type P-cadherin and several mutants on cell aggregation and invasion. We report that aberrant expression of P-cadherin may result from a lack of estrogen receptor-α signaling and may induce cell invasion in a juxtamembrane domain-dependent manner.

MATERIALS AND METHODS

Plasmids and cDNA Constructs. The hP-cad/pBR322–23-b expression vector, containing the 3.2kb cDNA encoding full-length human P-cadherin (8), was kindly provided by Prof. Keith R. Johnson (Department of Oral Biology, College of Dentistry and the Eppley Cancer Center, Nebraska Medical Center, Omaha, NE), with the permission from Prof. Yukata Shimoyama (Department of Surgery, International Catholic Hospital, Nakaohiai, Shinjuku, Tokyo, Japan). The cDNA encoding full-length mouse E-cadherin was kindly provided by Jolanda van Hengel (Department of Molecular Biomedical Research, VIB-Ghent University, Ghent, Belgium). Both cDNAs (pc-WT and mcEC-WT) were transferred to the expression vector pIRE2-EGFP (Clontech, Palo Alto, CA), allowing easy evaluation of transfection efficiencies due to co-tranfection of a green fluorescent protein (GFP) expression vector and an EGFP expression vector with a Renilla luciferase reporter gene. The efficiency of transfection was evaluated by co-transfection of the pIRE2-EGFP expression vector with a Renilla luciferase reporter gene, which allows easy evaluation of transfection efficiencies.
expression of enhanced green fluorescent protein (EGFP). To generate P-cadherin deletion mutants, P-cadherin was EcoRI subcloned into pBluescript (Promega, Madison, WI) and NdeI/SalI digested to remove the region encoding its COOH-terminal tail. PCR fragments corresponding to different lengths of the removed tail, flanked by NdeI/SalI restriction enzyme digest sites at the 5' and 3' ends, respectively, were obtained always using the same sense primer (5'-AGACAGGATCATGATGAGC-3') and different antisense primers for the following constructions: PC-CT682: 5'-CTCGGTGGCGCATCCTGCTG-3'; PC-CT702: 5'-CTCGTGGCGCATCACTGACTGG-3'; PC-CT711: 5'-CTCGTGGCGCATCCTGACTCG-3'; PC-CT719: 5'-CTCGTGCGCATCCTGACTGAGCTG-3'; PC-CT727: 5'-CTCCTGCGTGACACTCCTGGCGTC-3'; and PC-CT762: 5'-CTCGTGCGTCGACCTCAGGTTCTCTAAT-3'. After NdeI/SalI digestion, these products were ligated into NdeI/SalI digested pCD-Bluescript, and the resulting construct was EcoRI/SalI transferred to pRES2-EGFP. Additionally, a mutant with a small deletion in the P20-catenin-binding sequence (lacking the nucleotides coding EGFP) and retaining the intact catenin-binding domain was created (PC-Δ703–707). Therefore, pcd-RES2-EGFP was XhoI/SmaI digested, and the removed fragment was cut with EarlI. After removal of the small fragment between the two EarlI restriction sites (encoding EGFP), the two remaining fragments (XhoI/EarlI and EarlI/SmaI) were ligated into XhoI/SmaI digested pC-RES2-EGFP. To create the P-cadherin point mutant (PC-R503H), a PCR product, encompassing the point mutation, was used, using the following primers: a sense primer (5'-GGACACCTCCTGACATGAGGATGTAG-3') with the TaqI restriction site in italics and the mutation point in bold; and the antisense primer used for generating PC-CT762. This product was TaqI/NdeI digested and used in a three-point ligation with a BamHI/TaqI and a BamHI/NdeI fragment of pCD-Bluescript. Followed by EcoRI/HindIII digestion of the construct to pRES2-EGFP. Direct sequencing (ABI, Perkin-Elmer, Foster City, CA) was performed for all of the constructs to confirm their integrity.

Restriction Enzymes, Antibodies, and Chemical Reagents. All of the restriction enzymes were purchased from New England BioLabs (Beverly, MA). Antihuman primary mouse monoclonal antibodies used were against P-cadherin (clone 56) and P120-catenin (clone 98; BD Transduction Laboratories, Lexington, KY), N-cadherin (CH-19 and GC-4), α-tubulin (B-5; 1-2; Sigma-Aldrich, Bornem, Belgium), e-cadherin (HECD-1; Takara Biochemicals, Kyoto, Japan), and estrogen receptor-α (NCL-α-ER-6F1; Novocastra, Newcastle, United Kingdom). 17β-Estradiol was purchased from Sigma-Aldrich Química (Sintra, Portugal) and ICI 182,780 was kindly provided by AstraZeneca (Barcarena, Portugal). Both drugs were dissolved in 100% EtOH and added to the culture media. The concentrations used were 10 nmol/L for 17β-estradiol and 100 nmol/L for ICI 182,780, unless mentioned otherwise. Cycloheximide was obtained from Sigma and used at 25 μg/mL. For the control situations, cells were treated only with 100% EtOH.

Cells and Transient Transfection. Human cancer cell lines were obtained as described: BT-20 from Peter Coopman (Laboratory of Molecular Biology, Ghent University, Belgium), MF-7 (MCF-7) from Per Briand (The Fibiger Institute, Copenhagen, Denmark), ZR-75.1 and T47D from American Type Culture Collection (Manassas, VA), and HEK 293T (HEK) cells from Veerle De Corte (Department of Biochemistry, Faculty of Medicine and Health Sciences, VIB-Ghent University, Belgium). Cell lines were routinely maintained at 37°C, 10% CO2, in the following media (Invitrogen): 50% DMEM/50% HamF12 (MCF7), DMEM (BT-20, T47D, HEK), or RPMI 1640 (ZR-75.1). All of the media for the routine culture contained 10% heat-inactivated fetal bovine serum (Greiner bio--one, Wemmel, Belgium), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B (Invitrogen). To obtain transient transfectants, appropriate expression vectors digested from pIRES2-EGFP and cals, Kyoto, Japan), and estrogen receptor-heat-inactivated fetal bovine serum (Greiner bio-one, Wemmel, Belgium), 100 μg/mL amphotericin B, and 50% DMEM/50% HamF12 (MCF7), DMEM (BT-20, T47D, HEK), or RPMI 1640 (ZR-75.1). Cell lines were routinely maintained at 37°C, 10% CO2, in the following media (Invitrogen): 50% DMEM/50% HamF12 (MCF7), DMEM (BT-20, T47D, HEK), or RPMI 1640 (ZR-75.1). 17β-Estradiol and 100 nmol/L for ICI 182,780, unless mentioned otherwise. 150 mm dishes were seeded with 100,000 cells per dish. The next day, cells were transfected with 3 μg of pCD-Bluescript, and the resulting construct was EcoRI/SalI transferred to pRES2-EGFP. Additionally, a mutant with a small deletion in the P20-catenin-binding sequence (lacking the nucleotides coding EGFP) and retaining the intact catenin-binding domain was created (PC-Δ703–707). Therefore, pcd-RES2-EGFP was XhoI/SmaI digested, and the removed fragment was cut with EarlI. After removal of the small fragment between the two EarlI restriction sites (encoding EGFP), the two remaining fragments (XhoI/EarlI and EarlI/SmaI) were ligated into XhoI/SmaI digested pC-RES2-EGFP. To create the P-cadherin point mutant (PC-R503H), a PCR product, encompassing the point mutation, was used, using the following primers: a sense primer (5'-GGACACCTCCTGACATGAGGATGTAG-3') with the TaqI restriction site in italics and the mutation point in bold; and the antisense primer used for generating PC-CT762. This product was TaqI/NdeI digested and used in a three-point ligation with a BamHI/TaqI and a BamHI/NdeI fragment of pCD-Bluescript. Followed by EcoRI/HindIII digestion of the construct to pRES2-EGFP. Direct sequencing (ABI, Perkin-Elmer, Foster City, CA) was performed for all of the constructs to confirm their integrity.

Aggregation Assays. For semi-solid substratum, 2 × 105 cells in 200 μL medium were seeded on solidified agar in a 96-well plate (27). Aggregate formation was evaluated under an inverted microscope after 24, 48, and 72 hours. In suspension, 6 × 105 cells were added to 50 mL Erlenmeyer flasks in 6 mL of medium. The flasks were incubated on a Gyrotory shaker (New Brunswick Scientific Co., New Brunswick, NJ) at 72 rpm and continuously gassed with humidified 10% CO2 in air. The particle size distribution of the aggregates was measured with a Coulter Particle Size Counter (LS2000, Coulter Company, Miami, FL). The diameter of the particles can be considered as a measure for aggregate formation. Statistical analysis of differences between the particle size distribution curves was done with the Kolmogorov-Smirnov method.
RESULTS

The Antiestrogen ICI 182,780 Up-Regulates P-Cadherin in Estrogen Receptor-α-Positive Breast Cancer Cell Lines. To test the hypothesis that estrogen receptor-α negatively regulates P-cadherin, we examined the expression of estrogen receptor-α and cadherins in breast cancer cell lines by Western blot (Fig. 1A). Interestingly, higher levels of P-cadherin were found in estrogen receptor-α-negative BT-20 cells.

A 24-hour treatment with the antiestrogen ICI 182,780 (10^{-7} mol/L) increased P-cadherin protein levels in MCF7 and ZR-75.1 cells but not in BT-20 cells (Fig. 1B). There were no significant changes in P-cadherin levels observed in T47D cells, bearing already higher pretreatment levels of P-cadherin and lower levels of estrogen receptor-α than the responsive cell types. ICI 182,780-induced increase of P-cadherin was associated with a decline of estrogen receptor-α levels (Fig. 1B).

For additional investigation, we chose the MCF7 cell line, because it is estrogen receptor-α positive, highly responsive to estrogen, and extensively investigated as a model of breast cancer. In these cells, ICI 182,780 induced, respectively, up- and down-regulation of P-cadherin and estrogen receptor-α in a time- and dose-dependent way (Fig. 2, A and B). A decrease of estrogen receptor-α levels was already observed after 6 hours of treatment, whereas P-cadherin levels nearly doubled after 12 hours. After 24 hours of exposure to ICI 182,780, higher P-cadherin and lower estrogen receptor-α levels persisted for several days, with normalization 96 hours after ICI 182,780 withdrawal (Fig. 2C). To examine whether or not the effect of ICI 182,780 on P-cadherin expression was mediated via estrogen receptor-α, we did a competition experiment. As already described (30), 17β-estradiol readily decreased estrogen receptor-α levels, although to a lesser extent than ICI 182,780 (Fig. 2D). Importantly, 17β-estradiol counteracted the ICI 182,780-induced up-regulation of P-cadherin (Fig. 2D) and accelerated normalization of P-cadherin levels in cells treated for 24 hours with ICI 182,780 (Fig. 2E).

Together, these results suggest that not the decrease in estrogen receptor-α, but the lack of estrogen receptor-α signaling is responsible for the increase of P-cadherin by ICI 182,780.

RT-PCR revealed an increase of P-cadherin mRNA after ICI 182,780 treatment, suggesting that the higher P-cadherin protein expression results from an up-regulation of P-cadherin transcripts (Fig. 2F). This was confirmed by a micro-array study performed on 17β-estradiol– or ICI 182,780-treated MCF7 cells, in which 17β-estradiol did not alter P-cadherin mRNA levels, whereas ICI 182,780 induced an 8-fold increase. Finally, it remained to be determined whether induction of the P-cadherin gene (CDH3) was a direct effect of ICI 182,780 or required prior induction of other genes. We addressed this question by blocking protein synthesis in cells, because the induction of primary target proteins or immediate early genes should not be sensitive, whereas secondary targets should be blocked. The treatment of MCF7 cells with cycloheximide, a de novo protein synthesis inhibitor, largely blocked P-cadherin up-regulation by ICI 182,780 (Fig. 2G), which is consistent with a requirement for newly synthesized proteins, probably induced by ICI 182,780, before CDH3 activation. In contrast, as expected, this drug did not block estrogen receptor-α down-regulation mediated by ICI 182,780 (Fig. 2G).

ICI 182,780 Decreases Cell–Cell Adhesion and Increases Invasiveness of MCF-7/AZ Cells. MCF7 cells formed compact aggregates on top of soft agar or when incubated in Erlemeyer flasks under continuous shaking (Fig. 3A, panel i, and Fig. 3B). In presence of ICI 182,780, this effect was counteracted (Fig. 3A, panel ii, and 3B). Even a 24-hour pretreatment with ICI 182,780, followed by testing these cells in the absence of ICI 182,780, was sufficient to prevent the formation of large aggregates (Fig. 3A, panel iii). On plastic substratum, no changes in morphology or migrating behavior (as measured by a wound healing assay) could be observed upon treatment with ICI 182,780 (data not shown).

Whereas MCF7 cells failed to invade in collagen type I and Matrigel invasion assays, a 24-hour pretreatment with ICI 182,780 was sufficient to induce invasion of these cells in both assays (Fig. 3, C and D). These proinvasive effects of ICI 182,780 were counteracted by 17β-estradiol (Fig. 3, C and D), indicating that they are mediated by interference with estrogen receptor-α signaling.

Aggregation and invasion of MCF7 cells, in the presence of ICI 182,780, mimics the behavior of the poorly aggregating and invasive estrogen receptor-α-negative and P-cadherin-positive BT-20 cells (Fig. 1A), which remained unchanged upon treatment with ICI 182,780 (Fig. 3, E and F).

P-Cadherin Expression Increases Invasiveness but Does Not Alter Cell–Cell Adhesion of MCF-7/AZ cells. Cells, retrovirally transduced to encode only EGFP (MCF7.LIE) or both P-cadherin and EGFP (MCF7.P-cad), were sorted to >90% EGFP positivity (Fig. 4A). P-cadherin levels were higher at the cell surface in P-cadherin–transduced cells (Fig. 4B), the levels of cell-surface E-cadherin were the same in P-cadherin–transduced cells, as in vector-transduced cells (Fig. 4, A and B), excluding an effect of the exogenous cadherin on the levels of the major endogenous cadherin.

On plastic substratum, P-cadherin–transduced MCF7 cells, like their parental or vector-transduced cells, formed epithelioid islands, showing no morphotype differences (data not shown). Transduction with P-cadherin did not interfere with E-cadherin–mediated cell–cell adhesion (Fig. 4, C and D). However, in a wound healing migration assay, P-cadherin–transduced cells migrated faster (data not shown) and, in contrast to parental or vector-transduced (LZRS-RES-EGFP) controls, invaded into collagen type I and Matrigel (Fig. 4, E and F).

P-Cadherin-Induced Invasion Is Not Breast Cancer Cell or Endogenous Cadherin-Specific. P-cadherin retroviral transduction was also done on HEK cells, expressing at their surface low and
Fig. 2. Regulation of P-cad expression by an ERα-dependent signaling pathway in MCF7 breast cancer cells. Immunoblotting, for P-cad and ERα analysis, of cell lysates from MCF7 cells that had been treated with the indicated concentrations of ICI for the indicated time points. Band quantification was done relative to the expression levels in untreated cells. Immunostaining for anti-α-tubulin was done to control for equal loading. A, ICI induces up-regulation of P-cad and down-regulation of ERα levels in a time-dependent manner, being maximal after 12 hours of treatment. B, a 24-hour treatment with ICI induces up-regulation of P-cad and down-regulation of ERα levels in a dose-dependent way, the higher concentrations leading to a more pronounced effect. C, MCF7 cells were grown in the presence of ICI for 24 hours. At time 0 hours, ICI was withdrawn, and cell lysates were prepared at the indicated time points. Immunoblotting was performed to analyze P-cad (□) and ERα (●) expression. The levels of both proteins start to normalize again 96 hours after ICI withdrawal, showing the reversibility of the effect. D, Cells were treated with ICI, E2, or a combination of both, for 24 hours. Although both ICI and E2 decreased ERα levels, the ICI-induced up-regulation of P-cad was counteracted by estradiol. E, MCF7 cells were grown in the presence of solvent control (lanes 1 and 2) or ICI (lanes 3 and 4) for 24 hours. After that, ICI was withdrawn, all media were refreshed, and cells were treated (lanes 2 and 4) or not (lanes 1 and 3) with E2 for additional 24 hours. E2 accelerated the reversion of P-cad expression to control levels in cells that had been treated with ICI. F, RT-PCR analysis of P-cad mRNA levels after ICI treatment of MCF7 cells for 24 hours. The analysis was done after the indicated number of cycles of PCR amplification. P-cad mRNA increased in the presence of ICI (more evident at the 30-cycle point, in the exponential phase). Band quantification is presented in the graph shown. G, Immunoblotting, using anti-P-cad and anti-ERα antibodies, of lysates from cells treated with CHX during 24 hours, alone or in combination with ICI. Band quantification was done relative to the expression levels in untreated cells. CHX blocked P-cad up-regulation induced by ICI, suggesting the involvement of de novo protein synthesis. (cad, cadherin; ER, estrogen receptor; ICI, ICI 182,780; E2, 17β-estradiol; CHX, cycloheximide)

high levels of E- and N-cadherin, respectively (Fig. 1A and Fig. 5A), and being invasive neither into collagen type I nor into Matrigel. Sorting of vector- or P-cadherin–transduced cells resulted in populations having either moderate or high EGFP expression (HEK.LIE.Med, HEK.LIE.High, HEK.P-cad.Med, and HEK.P-cad.High; Fig. 5, A and B). As for MCF7 cells, no differences in morphotype or aggregation were observed between parental and transduced cells (Fig. 5, C and D). Although there was a down-regulation of superficial N-cadherin in the highest P-cadherin–expressing cells (Fig. 5B), this did not result in a significant decrease in total levels of N-cadherin (Fig. 5A). P-cadherin–transduced cells were significantly more invasive into collagen type I or Matrigel than vector-transduced cells, with higher invasiveness of the cells expressing more P-cadherin (Fig. 5, E and F). In both assays, the control cells with higher LZRS-IRES-EGFP expression levels showed an increased invasion index when compared with the ones with moderate levels of expression. This may be due to the insertion of viral promoters into the host genome, leading to the aberrant activation of host genes. However, although this observation highlights the care that should be taken when using these systems, it does not influence the interpretation of our results as such: the values of the P-cadherin–transduced cells remain significantly different from those of the respective vector-transduced cells.

P-Cadherin Mediates Invasion of HEK 293T Cells via Its Juxtamembrane Domain. To identify the P-cadherin domain(s) necessary for its proinvasive effects, we used several P-cadherin constructs (Fig. 6A) for transient transfection of the HEK cell line. Biotinylation and immunoblotting confirmed expression of all of the constructs at the plasma membrane (Fig. 6B). Transient transfection with P-cadherin induced invasion into collagen type I, as observed with stably transduced HEK cells (Fig. 6C). The P-cadherin point mutant, PC-R503H (Fig. 6A), representing the missense mutation in CDH3, found in hypotrichosis with juvenile macular dystrophy (31), failed to support strong cell–cell adhesion...
Unlike wild-type P-cadherin. Most likely, the reason for this failure is the disruption of the strongly conserved LDRE Ca\(^{2+}\)-binding motif in the fourth extracellular domain of P-cadherin. Nevertheless, PC-R503H still induced invasion (Fig. 6C).

Mutants of the P-cadherin cytoplasmic tail were also generated (Fig. 6A). Transfection into HEK cells showed that PC-CT762, retaining the intact P-cadherin juxtamembrane domain, induced invasion-like wild-type P-cadherin (Fig. 6C). Because this mutant is truncated just before the catenin-binding domain, we assume that β-catenin, γ-catenin, or any other protein that binds to this region are not needed for P-cadherin-mediated invasion.

With mutants within the juxtamembrane domain (Fig. 6A), statistically significant invasion into collagen was seen only with the truncation mutants that still retained the intact juxtamembrane domain (PC-CT719 and PC-CT727; Fig. 6C). The somewhat decreased ability of PC-CT719 to induce invasion (Fig. 6C) might be due to its lower expression levels (Fig. 6B). In line with the results obtained with the truncation mutants and confirming that the catenin-binding domain is not involved in the proinvasive effects, the PC-Δ703–707 mutant (lacking EEgG in the P120-catenin binding site), with impaired P120-catenin binding (Fig. 6D), was not able to induce invasion of HEK cells into collagen type I (Fig. 6C). In conclusion, P-cadherin needs its intact juxtamembrane domain to induce invasion of HEK cells into collagen type I.

To exclude that the gain of any exogenous cadherin, retaining its juxtamembrane domain, would be sufficient for a proinvasive effect, we demonstrated that HEK cells transfected with mouse wild-type E-cadherin cDNA (Fig. 6A) failed to invade into collagen type I (Fig. 6C). In conclusion, the juxtamembrane domain of P-cadherin confers to this molecule the specific ability to induce invasion of HEK cells, in the presence of the endogenously expressed cadherin.

**DISCUSSION**

We demonstrated that the antiestrogen ICI 182,780 increased time- and dose-dependently P-cadherin expression in estrogen receptor-α-positive breast cancer cells. This increase could be completely reverted by 17β-estradiol, categorizing CDH3 as an estrogen-repressed gene and pointing to 17β-estradiol as a key regulator of this cadherin. In addition to competing for binding to estrogen receptor-α, ICI 182,780 also increases its breakdown (24). As a result, ICI 182,780 abrogates estrogen receptor-α signaling and the subsequent regulation of 17β-estradiol responsive genes. Because the human P-cadherin promoter (GI: 2950171) does not contain the consensus sequence 5’-GGTCAnnnTGACC-3’ of the estrogen-responsive elements (32), 17β-estradiol is unlikely to have a direct inhibitory effect on transcription of the CDH3 gene. Instead, the increase of P-cadherin by ICI 182,780, some hours after the decrease of estrogen receptor-α, and its
inhibition by cycloheximide, pleads for the existence of a CDH3-regulating transcription factor. In the absence of estrogen receptor-α signaling (as in estrogen receptor-α-positive cells treated with ICI 182,780 or in estrogen receptor-α-negative cells), this 17β-estradiol-regulated factor might account for the high P-cadherin levels in some breast cancer cell lines and for the inverse correlation between estrogen receptor-α and P-cadherin expression in mammary tumors.

In MCF7 breast cancer cells, ICI 182,780 treatment led to a decreased cell–cell adhesion and promotion of invasion in vitro. This is in line with the finding that 17β-estradiol (33) and even the unliganded receptor (34) may decrease in vitro invasiveness and motility of breast cancer cells, suggesting that some estrogen-regulated genes negatively control invasion. Because this control is lost in cells treated with high concentrations of ICI 182,780, which up-regulate P-cadherin, the effect of the latter was additionally investigated on in vitro aggregation and invasion of cells retrovirally transduced with P-cadherin. Surprisingly, retroviral transduction of MCF7 and HEK cells with P-cadherin had no detectable influence on cell–cell adhesion. This result suggests that P-cadherin does not shift the aggregation balance established by the other cadherins in these systems. By contrast, such balance may well be changed for invasion, as demonstrated with P-cadherin–transduced cells. It should be noted that this

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**Fig. 4.** Cell aggregation and in vitro invasion of stably transduced MCF7 cells with P-cad cDNA. A. Flow cytometric evaluation of EGFP expression (X axis) and E-cad expression (Y axis) in MCF7.LIE and MCF7.P-cad cells. P-cad expression did not induce alterations in E-cad expression levels (right plot). The percentage of EGFP positivity is indicated for each cell line. B. Immunoblotting, using anti-E-cad and anti-P-cad antibodies, of lysates from biotinylated MCF7.LIE and MCF7.P-cad cells. C. In the slow aggregation assay in suspension, both MCF7.LIE and MCF7.P-cad cells form similar compact aggregates after 48 hours. The arrow indicates the measurement of a single cell suspension at the beginning of the experiment. D. Pictures, after 48 hours, of the slow aggregation assay on semi-solid substratum. MCF7.LIE (panel i) and MCF7.P-cad (panel ii) cells form round and compact aggregates, with no differences observed. E. In the collagen type I invasion assay, MCF7.P-cad cells invade significantly more (P = 0.0024). F. Representative experiment of Matrigel invasion assay, where MCF7.P-cad cells invade significantly more than empty-vector transduced cells (P < 0.001). (cad, cadherin)

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**Fig. 5.** Cell aggregation and in vitro invasion of stably transduced HEK cells with P-cad cDNA. A. Immunoblotting, using anti-N-cad and anti-P-cad antibodies, of lysates from biotinylated HEK, HEK.LIE.Med, HEK.P-cad.Med, HEK.LIE.High, and HEK.P-cad.High cells. Band quantification was done relatively to the expression levels in control cells. To control for equal loading, immunostaining with anti-α-tubulin was done. B. Flow cytometric analysis of EGFP expression (X axis) and N-cad expression (Y axis) in the indicated cell lines. High levels of P-cad in the HEK.P-cad.High cell line induced down-regulation of endogenous N-cad. The percentage of EGFP positivity is indicated for each cell line. C. In the slow aggregation assay in suspension, HEK.LIE.Med, HEK.P-cad.Med, HEK.LIE.High, and HEK.P-cad.High cell line forms similar compact aggregates after 48 hours. The arrow indicates the measurement of a single cell suspension at the beginning of the experiment. D. Pictures, after 48 hours, of the slow aggregation assay on semi-solid substratum. HEK.LIE.Med (panel i), HEK.LIE.High (panel ii), HEK.P-cad.Med (panel iii), and HEK.P-cad.High (panel iv) cells form round and compact aggregates, with no differences observed. E. A single cell suspension of these cell lines was seeded on top of collagen type I gels. After 24 hours of incubation, HEK.P-cad.Med and HEK.P-cad.High cells invaded significantly more than empty-vector transduced cells (P < 0.001). F. Representative experiment of Matrigel invasion assay. HEK.P-cad.Med and HEK.P-cad.High cells invaded significantly more than empty-vector-transduced cells (P = 0.0024). Also here, although HEK.LIE.High differed significantly from control, the comparison with HEK.P-cad.High still shows the significant effect of P-cad on invasion (P < 0.001). (cad, cadherin)
does not allow us to draw conclusions about the necessity of P-cadherin up-regulation for ICI 182,780-induced invasion of MCF7 cells. In contrast to P-cadherin–transduced cells, which migrated faster than controls in a wound healing migration assay, ICI 182,780-treated cells did not. This might be due to the fact that the extent by which P-cadherin is up-regulated by ICI 182,780 may not be sufficient to promote motility as such or, alternatively, the growth-inhibitory effect of ICI 182,780 nullified the promigratory effect of P-cadherin in this assay. Furthermore, ICI 182,780 up-regulated additional proinvasive genes in MCF7 cells, such as MMP-2 and -9, of which the expression was not influenced by P-cadherin (data not shown). Hence, whereas high levels of P-cadherin may be sufficient for induction of invasion, ICI 182,780-induced invasion might require the synergistic action of multiple genes. This hypothesis, in which a critical level of P-cadherin seems to be needed for its proinvasive activity, is supported by the comparison between the invasive and highly P-cadherin–positive BT-20 and T47D cells and the noninvasive and weakly P-cadherin–positive MCF7 and ZR-75.1 cells (Fig. 1A). In contrast to its proinvasive activity in our cells, transfection of other cell lines with P-cadherin inhibited invasion (35, 36), suggesting that P-cadherin may act both as an invasion promoter and suppressor, depending on the cell type and its invasive status. Transgenic mice expressing high levels of P-cadherin in the normal mammary epithelium (37) contributed little to this issue, because they did not produce tumors, and because neu oncogene-induced mammary tumors in P-cadherin transgenic mice were always P-cadherin negative.

In the present study, the proinvasive action of P-cadherin is unlikely to be the result of alterations in cell–cell adhesion, because the assays score invasion of single cells into or through a matrix, the retroviral transduction of MCF7 and HEK cells with P-cadherin did not change aggregation, and the point mutant PC-R503H, incapable of supporting strong P-cadherin mediated adhesion, still induced invasion. We presume that the proinvasive activity of P-cadherin is due to changes in signaling pathways.

Recently, Wong and Gumbiner (38) attributed the anti-invasive activity of wild-type E-cadherin to its interaction with β-catenin. An E-cadherin mutant, retaining the catenin-binding domain but with a point mutation that abolishes P120-catenin binding, was still able to suppress invasion. By contrast, in P-cadherin, maintenance of the juxtamembrane domain is crucial for the induction of invasion, irrespective of the catenin-binding domain. Although the juxtamembrane domain is highly conserved between cadherins, its function is very context-dependent, being implicated in both positive and negative regulation of cadherin activity. Cells expressing mutated E-cadherin juxtamembrane domain are weakly adherent (39), more motile, but still epithelioid. Upon formation of adhesive contacts, the juxtamembrane domain recruits and activates Rac, regulating the actin cytoskeleton (40). In another context, the juxtamembrane domain may inhibit aggregation mediated by classical cadherins and induce cell motility (41, 42) or, alternatively, exclude another cadherin from junctions and regulate cell proliferation (43). Via its binding to P120-catenin, this domain has been implicated recently in maintenance of the stability of endogenous cadherins (44, 45). Thus, a possible mechanism for the induction of invasion by P-cadherin might be its competition with the endogenous cadherin for the available P120-catenin, leading to the destabilization of pre-existing anti-invasive cadherin/catenin complexes. Yet, we consider this possibility less likely. Although the down-regulation of N-cadherin in HEK cells by high levels of the several P-cadherin constructs coincided with stimulation of invasion (Supplementary Data), moderate P-cadherin expression levels, leaving the endogenous cadherin unchanged, were sufficient to induce invasion. Furthermore, transfection of HEK cells with E-cadherin did not induce invasion (Fig. 6C), despite decreased endogenous cadherin in highly expressing cells (Supplementary Data) and expected competition for cadherin-binding proteins.

Alternatively, P-cadherin may generate a specific proinvasive signal via its juxtamembrane domain. In this hypothesis, the binding of proteins to the P-cadherin juxtamembrane domain may differ from their binding to E- or N-cadherin by strength, conformation, or recruitment of other members of the complex. This, in turn, may result in the activation of pathways that overcome the suppressive signals mediated by the endogenous cadherins.
Although binding of proteins to the juxtamembrane domain of P-cadherin has just been documented for P120-catenin (46), other molecules, like Hakai and presenilin-1 (PS-1), have been reported to bind to the juxtamembrane domain of classical cadherins as well, to a sequence adjacent to or overlapping the P120-catenin–binding domain, thereby competing with P120-catenin for binding (47, 48). Although the significance of these interactions is not well known, we cannot exclude the possibility that disruption of the P120-catenin–binding sequence introduces conformational changes and/or uncoupled the interactions of these or other proteins, which could be responsible for our observations. Striking examples of this were shown for E-cadherin, where functional differences have been noted between larger and minimal deletions of the juxtamembrane domain, with even the minimal changes disrupting binding of multiple molecules (47).

Data about the role of P120-catenin in normal and cancer cells are conflicting. Positive and negative regulation of cell–cell adhesion and motility possibly reflect differences in cell type, cadherins, P120-catenin isoforms, and shutting between cadherin-bound and cytoplasmic pools (3). When overexpressed in the cytoplasm, P120-catenin may regulate the actin cytoskeleton and cell motility, through Rho GTPases (49). Similar to the differences seen between E- and N-cadherin in terms of strength (3) and preference (50) of binding to GTPases (49). Similar to the differences seen between E- and N-cadherin, this unique interaction may influence its impact on the activity of the Rho GTPases, possibly making the cells more prone to invade. Alternatively, the panel of molecules recruited by P120-catenin may differ depending on the isoform or on the cadherin it is bound to.

In conclusion, our study establishes an as yet unknown role for P-cadherin in cancer: (1) P-cadherin expression is regulated through estrogen receptor-α signaling, suggesting that the inverse in vivo correlation between these molecules stems from a causal relationship; (2) P-cadherin induces invasion, in the context of endogenous E- or N-cadherin expression; because P-cadherin expression in breast cancer is far more frequent than aberrant expression of N-cadherin, its physiologic relevance is more likely to be higher (15); (3) except from the presently demonstrated induction of invasion, no regulatory functions have been described for the P-cadherin juxtamembrane domain.

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