P-Cadherin Is a Basal Cell-specific Epithelial Marker That Is Not Expressed in Prostate Cancer

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ABSTRACT

P-Cadherin is a member of the cadherin family of cell surface glycoproteins that mediate Ca\(^{2+}\)-dependent cell-cell adhesion and is expressed in a differential fashion in normal epithelial tissues. The expression of P-cadherin in human prostate cancer development has not been investigated previously. By immunohistochemistry, we show that P-cadherin expression is restricted to the cell-cell border of basal epithelial cells in 30 normal prostate samples. This staining is down-regulated in prostatic intraepithelial neoplasia and is absent in all 25 of the well to poorly differentiated prostate cancer specimens analyzed. To examine potential P-cadherin-regulatory elements, we sequenced the 5'-flanking region of this gene. Similar to the mouse gene, the human P-cadherin promoter is TATA-less, contains an Sp-1 binding site and, analogous to the human E-cadherin sequence, demonstrates a GC-rich region characteristic of a CpG island. Cytosine methylation of this region occurs in prostate cancer specimens. To demonstrate that the expression of the basal cell marker P-cadherin is lost in prostate cancer development and that in vivo mechanisms other than cytosine methylation regulate this consistent loss of expression.

INTRODUCTION

Cadherins are cell-cell adhesion molecules that form important Ca\(^{2+}\)-dependent intercellular junctional structures and play an essential morphoregulatory role in the development and maintenance of multicellular organs (1, 2). During embryogenesis, cellular expression of specific cadherins results in homophilic interactions that are critical in the processes of cell sorting and tissue stratification (3-5). Alterations in these cellular attachments play a permissive role in the disassociation of cells and may modify the carefully regulated differentiation processes in epithelial structures (2, 6-8). For this reason, the functional loss of cadherin expression and the molecular mechanisms underlying the control of these genes have been implicated in malignant progression (9).

The cadherin family is subdivided into various types, including E-, P-, and N-cadherin, with each cadherin class demonstrating a unique tissue distribution (2). Although E-cadherin is expressed in virtually all epithelial tissues, the expression of P-cadherin is restricted to the basal or lower layers of stratified epithelia in selected organs, including breast and skin (7, 10). In a preliminary screening study performed on human tissues, a basal expression pattern for P-cadherin was noted by Shimoyama et al. (10) in the prostate. This organization of cadherin expression suggests that, in addition to maintaining cellular adhesion, P-cadherin may also have other undefined functions important in differentiation and cell growth (11). A disruption of the normal epithelial architecture, with a loss of the basal epithelial cell layer, is histologically diagnostic for prostate cancer, and has been demonstrated to be frequently associated with a down-regulation in the expression of E-cadherin (12). This differential pattern of E-cadherin expression in prostate cancer development, coupled with the frequent loss of the basal epithelial layer, prompted us to investigate P-cadherin expression in prostate cancers.

A comparison of the primary structure of human E- and P-cadherin demonstrates a 58% homology in their amino acid sequences and colinear organization with unique extracellular domains (13). The 5'-flanking sequence of the E-cadherin gene has recently been sequenced and found to be extremely GC enriched, meeting the criteria for a “CpG island” (14, 15). Aberrant methylation across the CpG island located in the promoter region of E-cadherin and other tumor suppressor genes may result in a selective inactivation of transcription (16, 17). Methylation of E-cadherin has recently been demonstrated in prostate and other cancer cell lines, as well as in breast cancer and an undifferentiated prostate cancer specimen (17). This functional block of E-cadherin expression is removed with exposure of cell lines to demethylating agents in vitro. Additional factors within the E-cadherin gene that appear important to transcriptional regulation include a palindromic sequence...
PROBE A new series of prostate tissues and test the hypothesis that P-cadherin expression is down-regulated during the development of prostate cancer. To investigate the loss of P-cadherin expression in cancer specimens, we explore possible mechanisms for the transcriptional control of this gene by sequencing the P-cadherin 5'-flanking region and characterize the methylation status of a CpG island within this promoter region.

MATERIALS AND METHODS

Prostate Samples and Establishment of Cultures. Prostatectomy samples were obtained at surgery from men ages 54–68 who had been diagnosed with cancer. Portions of each tumor, surrounding normal peripheral prostate tissue, and peri-urethral benign prostate hyperplasia specimens were frozen immediately at −70°C for DNA and RNA analysis. Frozen sections were stained with H&E for histological evaluation of each tissue specimen. In the case of tumor tissue, tissue blocks were trimmed to yield samples containing ≥70% tumor nuclei. Cultured prostate cancer cell lines LNCaP, Du145, PC3, and PPC1 were obtained from the American Type Culture Collection. DuPRO and TSU-PR1 were obtained from other sources (19).

Immunohistochemistry and Immunofluorescence Staining. Frozen prostate specimens were sectioned, mounted, and fixed in 100% ethanol for 5 min at room temperature. After rinsing (three times for 2 min each) in TNC buffer (10 mM TRIS, 150 mM NaCl, and 2 mM CuCl2 (pH 7.4)), the sections were incubated for 1 h (25°C) with mouse monoclonal antibody against P-cadherin (20). Anti-P-cadherin was diluted 1:10 in 3.75% BSA/TNC buffer. After washes with TNC buffer (three times for 2 min each), sections were incubated with a biotinylated secondary antibody (goat antimouse IgG; Zymed Laboratories, Inc., San Francisco, CA) for 30 min. Slides were rinsed with TNC buffer (three times for 2 min each), and conjugated with a streptavidin-peroxidase complex (Zymed) for 15 min. After washing in TNC (three times for 1 min each), staining was developed with an aminothiol carbazole chromagen substrate (Zymed) in 0.006% peroxide-containing buffer for 30 min, followed by rinsing with tap water (three times). As a negative control, we used mouse serum at a dilution of 1:100 as a primary antibody. The sections were then counterstained with hematoxylin, dehydrated, and mounted.

To analyze immunofluorescence staining of cell lines, we generated semi-confluent cultures on two-chamber microscopic slides (Nunc Inc., Naperville, IL). The medium was removed, and cells were fixed in 100% ethanol for 5 min at room temperature. Rinsing with TNC buffer (three times for 2 min), blocking, and incubation with the primary P-cadherin antibody at a dilution of 1:10 (at 4°C) were performed as above. After rinsing, the secondary antibody, a rhodamine-conjugated (tetramethylrhodamine isothiocyanate) donkey antimouse IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA) was applied and incubated at 4°C for 1 h. After rinsing with TNC, slides were treated with SlowFade antifade kit (Molecular Probes, Inc., Eugene, OR) and mounted.

Isolation of the 5'-Flanking Sequences of the Human P-Cadherin Gene. Using pPCad-MV1-HPI (containing the most 5' 172 bp of the human P-cadherin cDNA; Ref. 21) as a probe, a human fetal brain cosmide library (Stratagene) was screened according to Bussamakers et al. (14) and Sambrook et al. (22). Positive clones were selected, and their DNA was digested with EcoRI. DNA sequences were determined and edited using IntelliGenetics computer software. Computer comparison studies were performed using sequences obtained from the European Molecular Biology Laboratory and GenBank nucleotide sequence databases using CAMMSA computer software (23).

Southern Analysis of P-Cadherin Methylation. Tumor and normal prostate DNAs (8 μg) were digested sequentially with the methylation-sensitive enzymes Smal, HaelII, or Thal (10 units/μg) and the methylation-insensitive enzyme BamHI (10 units/μg; New England Biolabs, Beverly, MA) overnight. The digested DNA was then electrophoresed on a 1.2% vertical agarose gel and transferred to a Hybond N+ nylon membrane (Amersham Corp.) for Southern blot analysis. After UV crosslinking (Stratagene), filters were hybridized overnight at 62°C in 10 ml of 1% SDS, 1 mM NaCl, 10 mM dextran sulfate, and 0.5 μg alkali-sheared salmon sperm with 0.5–1.0 × 106 cpm/ml [32P]dCTP random-primed (Amersham) 604-bp DNA probe generated from the 5'-P-cadherin sequence (63% GC rich) designated PCad5'. Primers to generate this probe were chosen from the 5'-flanking sequence and included 5'-ACG GGA GGT GGA GAA AGA G-3' (sense) and 5'-ACG CCG AGG CTG TGG AGT A-3' (antisense). Conditions for this P-cadherin amplification included 35 cycles of 95°C for 1 min, 61°C for 30 s, and 72°C for 30 s, followed by incubation at 72°C for 5 min. An additional smaller probe, PCadHAE (320 bp), was generated by digesting the full-length probe with HaelII. After hybridization, the blots were washed at 65°C with 0.1X SSC-0.5% saline-sodium phosphate-EDTA for approximately 10–20 min. Autoradiographs were generated after exposure to radiographic film overnight (Kodak). Experiments were repeated in duplicate, and additional enzyme did not alter the methylated signal, and blots were reprobed with additional genes known to be unmethylated to confirm complete enzyme digestion.

RT-PCR for P-Cadherin Expression. To evaluate P-cadherin reexpression, the demethylating agent 5'-deoxycytidine (0.5–10 μM) was added to ~50% confluent cell cultures, redosed at days 2 and 4, and harvested at day 5 for RNA as described previously (24). Longer-term LNCaP cultures (6 months) at a dose of 2 μM were established and also analyzed. Primers used for cDNA amplification of P-cadherin included 5'-TCT CGC TGC TCT CCT CCT TCT-3' (sense) and 5'-GCC TGT GCT CCG GTC TCT CCT-3' (antisense) at an annealing temperature of 58°C for 30 cycles. β-Actin primers (Stratagene) were used as a control.

RESULTS

Expression of P-Cadherin Protein in Normal Prostate and Tumor Samples. To characterize P-cadherin expression in the prostate, immunohistochemistry was performed on 30 histolog-
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demonstrated normal basal epithelial staining as described above. Specimens containing high-grade PIN, considered a precursor to prostate carcinoma (25), demonstrated a discontinuous pattern of basal layer staining with scattered positive-staining cells (Fig. 1f).

Using immunofluorescence, an analysis of a series of prostate cancer cell lines derived from metastatic lesions was performed. No protein expression of P-cadherin by immunofluorescence was noted in cell lines LNCaP, TSU-PR1, and DuPRO (Fig. 2 and data not shown). However, cell lines PC3, Du145, and PPC1 (Fig. 2) revealed a continuous staining of the cell membrane with an accentuation of immunostaining at points of cell-cell contact. Minimal staining heterogeneity was noted in these cultures. RT-PCR on these prostate cancer cell lines confirmed these findings (data not shown). We have demonstrated previously by Western blotting that normal prostate epithelial cells in culture express P-cadherin at moderate levels similar to the level observed in PC-3 (19). When stained in culture, normal prostate epithelial cells display a positive staining pattern for P-cadherin expression similar to the pattern observed for DU145 cells.

**Sequence of the 5' Human P-Cadherin Gene.** To examine possible mechanisms for the transcriptional regulation of P-cadherin expression in the prostate, we sequenced the 5'-flanking region of the human P-cadherin gene. A human fetal brain cosmid library was screened with pPCad-MV1-HPl, and a cosmid clone cHPC8 was isolated. A 2.5-kb EcoRI fragment of clone cHPC8 was found to contain the 5'-end of the previously characterized human P-cadherin cDNA (21). Comparison of the immediate 5'-flanking sequences of the human P-cadherin gene (GenBank accession number X95824) with those reported for the mouse P-cadherin gene promoter (26, 27) reveals a 73% homology. There is conservation of a CCAAT box at -64, with no TATA box. In the human P-cadherin promoter, three E boxes are present (helix-loop-helix binding motif; Ref. 18), compared to two found in the mouse. A putative Sp-1 binding site (−88) is also conserved. The probable initiation site for transcription is 70 nucleotides upstream of the translation start site based on a comparison with the mouse sequence (27). An Alu repeat is present approximately 700 bp upstream from the translation start site.

**Methylation and Expression of P-Cadherin in Normal Prostate, Primary Prostate Cancers, and Cell Lines.** The P-cadherin gene contains a CpG island 5' and extending into the first exon on the basis of several criteria, including a G and C content of 73%, a CG:GC ratio of greater than 0.6, and extension of this island over more than 500 bp (Ref. 28; Fig. 3A). Restriction with the methylation-insensitive restriction enzyme BamHI provides a flanking cut for this region. Digestion with the methylation-sensitive enzymes Thal and HaelII revealed methylation in the LNCaP, TSU-PR1, and DuPRO prostate cancer cell lines at sites located around the CpG island promoter elements in the 5'-flanking region, using both probes PCad5' and the truncated version, PCadHaelII (Fig. 3B). Sites upstream of the putative transcription start site were completely methylated in LNCaP and partially in TSU-PR1 and DuPRO (see Table 1). However, in these three cell lines, the SmaI as well as the HaelII methyl-sensitive sites in close proximity to the transcription start site (within 50 bp on either side) were never methylated. Indeed, complete methylation across this region was never seen. No methylation of any site is demonstrated in Du145, PC3, or PPC1 cell lines.

Aberrant methylation of GC-rich promoter regions has been demonstrated to correlate with transcriptional inactivation for a number of specific genes (16). The detection of any methylation within the P-cadherin promoter region Cpg island (e.g., TSU-PR1, DuPRO, or LNCaP) is always associated with transcriptional inactivation by both RT-PCR (data not shown) and immunohistochemistry (Fig. 2). Cell lines expressing detectable P-cadherin message or protein, including Du145, PC3, and PPC1, are unmethylated at all restriction sites analyzed. Treatment of the cell lines TSU-PR1 and LNCaP with 5-aza-2-deoxycytidine and 5-azacytidine, both demethylating agents, fail to reexpress P-cadherin message by RT-PCR (data not shown). Both short-term exposure (3 days) and longer dosing for 6 months did not reactivate P-cadherin, although glutathione S-transferase-π, which is methylated extensively in LNCaP (29), was reactivated in this long-term culture.

The methylation status of this Cpg island was examined in a series of 12 normal and matched primary prostate cancers using the methylation-sensitive enzymes Thal, HaelII (Fig. 3C), and SmaI (data not shown). All of these restriction sites were

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5 W. G. Nelson, personal communication.
A

PCad5' (604bp)
- BamHI
- HaeII
- HaeII
- BamHI

PCadHaeII (320bp)
- Thal (four sites)
- HaeII (three sites)
- Smal (one site)

Exon 1

500
1000
1400

Fig. 3  Restriction map and Southern blot methylation analysis of P-cadherin in prostate cancer cell lines and tumor samples. A, the restriction enzyme map and CG dinucleotide density of the 5' promoter region of P-cadherin. Exon 1 is encompassed by a 1.9-kb BamHI-flanking restriction fragment, which includes the ~1-kb promoter CpG island. The density of CG dinucleotides in the 930-bp flanking sequence and 3' downstream gene is shown below the sequence. Note the increase in CpG density across exon 1 and the promoter region. Methylation analysis is performed by digestion with the methyl-sensitive enzymes Thal (four sites), HaeII (three sites), or Smal (one site). The sequence spanned by the probes PCad5' and PCixdHaeII (used for Southern analysis) are shown.

B, methylation analysis of the 5'-CpG island in cultured prostate cancer cell lines. All restrictions were performed with 10 units/1μg DNA. *, methylation bands. A BamiI "flanking" cut (Lane 1) and blood DNA (Lane 2) are controls. Cell lines Du145 (Lane 3), PC3 (Lane 5), and PPC-1 (Lane 6) are unmethylated (464-bp band) and express the P-cadherin gene. In contrast, LNCaP (Lane 4), TSU-PR1 (Lane 6), and DuPRO (Lane 7) are methylated (see Table 1) and do not express the P-cadherin gene. Complete methylation to 1.9 kb across this CpG island was not observed. Southern blots generated using Thal and HaeII. Paired prostate tumor samples (T) and normal prostate tissues (A0) from three patients demonstrate no methylation. The left panel was probed with PCad5' and the right with probe PCadHaeII (see map shown in A).

Table 1  Mapping of the methylation-sensitive restriction sites in the P-cadherin 5' CpG Island

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<th>Smal (830)</th>
<th>Thal (423)</th>
<th>Thal (886)</th>
<th>Thal (942)</th>
<th>Thal (1193)</th>
<th>HaeII (470)</th>
<th>HaeII (777)</th>
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<tr>
<td>Du145</td>
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<td>LnCaP</td>
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<td>M</td>
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<td>PC3</td>
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<td>TSU-PR1</td>
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<td>DuPRO</td>
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* U, unmethylated alleles; P, partial methylation; M, complete methylation.

found to be unmethylated in normal tissues, including blood and normal prostate. Although these prostate cancers were negative for P-cadherin protein by immunohistochemical staining (see above), no detectable methylation was demonstrated in any tumor sample. No deletions of this region were detected using this 5' P-cadherin-specific probe.

DISCUSSION

The expression of the cell adhesion molecules, the cadherins, appears to play an essential role in the stabilization and maintenance of nonmalignant differentiated tissues. In the present study, we demonstrate that P-cadherin is expressed in the...
basal epithelial layer of the acinar ducts in all 30 normal prostate tissues examined. No stromal expression is noted. Benign prostatic hyperplasia specimens reveal a similar immunohistochemical staining pattern. This immunostaining accumulates in regions of cell-cell apposition in a manner similar to the expression of other classical cadherins, including E-cadherin (10). However, E-cadherin is expressed in all epithelial layers (12), a fact that suggests profoundly different roles for these two closely related cadherins. The prostate epithelium is multilayered, with a subset of prostate basal cells functioning most likely as stem cells that give rise to ductular secretory epithelial cells (30–32). During breast development, P-cadherin appears to play a fundamental morphoregulatory role as evidenced by its selective expression in the cap cells of budding ductules (11). Thus, segregation of P-cadherin in the basal layer of the prostate and other tissues (7, 10) suggests a potential role for this gene in the regulation of epithelial intercellular junctional adhesion, as well as in the critical process of secretory cell differentiation.

Progression to histological prostate cancer is characterized by profound alterations in intercellular and cell-substratum interactions. These include a disruption of the normal glandular epithelial hierarchy and the development of dysplastic ductules lined by a single layer of pleomorphic cells. P-cadherin protein expression is clearly absent in all 25 well to poorly differentiated (Gleason 4–9) primary prostatic adenocarcinomas assessed. Furthermore, an incremental decrease in P-cadherin staining is noted during the transition from a normal acinar gland to PIN, believed to be a preneoplastic lesion (25). This striking absence of P-cadherin in histological prostate cancer occurs more frequently and is less heterogeneous than alterations in staining seen with the panepithelial marker E-cadherin (12).

The loss of P-cadherin immunostaining is not a universal finding in other tumor systems and does not occur to the complete extent that we demonstrate in prostate specimens. Only subsets of breast, lung, and gastric cancers have been found to be negative (10, 33–35). No clear correlation is seen with histology, grade, or prognosis in these studies, although several have suggested that a reduction in expression occurs with progression to a less-differentiated histology (33, 36). These data suggest that the regulation of P-cadherin in these tumor systems may involve several tissue-specific transcriptional mechanisms. Cytokines appear to up-regulate P-cadherin in PC3 prostate cancer cells (37). With regard to deletional events at 16q22, a region that encompasses both the E- and P-cadherin genes, a loss of heterozygosity is seen in ~30% of primary prostate cancers (38). No mutational analyses of P-cadherin have been published to date.

Human E-cadherin transcriptional regulation has been widely investigated (14, 39); however, less is known of the factors involved in P-cadherin expression. Mouse promoter studies have suggested that highly complex combinations of elements in the promoter and second intron regulate transcription in a cell type-specific fashion (27). Our sequence data reveal that the P-cadherin and E-cadherin promoters contain similar putative regulatory elements, as well as other related structural features, such as the presence of Alu repeats and B2/E2 repetitive elements. E2 elements are believed to be important in tissue-specific expression (40). Similar to the E-cadherin promoter (14), the putative transcription start site for P-cadherin (based on a comparison with the mouse promoter; Ref. 27) lies within a GC-rich region (>70%) that fulfills all of the criteria for a CpG island (28). Additional 5' sequences and studies using promoter constructs should confirm this transcription start site and the role these promoter elements play in P-cadherin transcription.

The finding of a CpG island in the promoter region prompted an investigation of DNA methylation in prostate cancer cell lines and tissues. Methylation appears to be a mechanism important in the transcriptional control of the E-cadherin gene in vivo and in vitro (17). Prostate cancer cell lines expressing P-cadherin are found to be completely unmethylated at all CG sites within this island. We have demonstrated a complete ablation of transcriptional activity when hypermethylation is present to any extent within this GC-rich promoter region. Several interesting observations were made: (a) the presence of incomplete methylation within this 5'-CG-rich region was also associated with transcriptional loss. Whether this represents a heterogeneous cellular population or monoallelic methylation is unknown; (b) we note that methylation appeared to involve CG dinucleotide sites located peripherally in this island more frequently than sites directly adjacent to the putative transcription start site (see Table 1). It has recently been hypothesized by Graff et al. (41) that the aberrant neoplasia-associated methylation of CpG islands spreads from outside CG-rich regions inward, possibly from Alu sites that often contain methylation;

(c) extensive culture with several demethylating agents was not capable of selectively reactivating P-cadherin transcription in vitro. This observation suggests that hypermethylation is not involved directly in the inactivation of P-cadherin expression in these prostate cancer cell lines; and (d) the examination of 12 P-cadherin protein-negative prostate tumors reveal no evidence of methylation in vivo. This would suggest that these hypermethylation events represent an in vitro phenomenon. Immortalized cell lines demonstrate frequent CpG island methylation (~50–70% CpG islands methylated; 42), and this may reflect altered transcriptional mechanisms for protecting inactive genes from methylation (43).

The absence of P-cadherin expression may reflect not a transcriptional alteration but the loss of this basal cell population during cancer formation. Our observation that intermittent, scattered P-cadherin-positive basal cells remain in specimens containing PIN, considered a premalignant lesion (25), might suggest that these cells are gradually lost during tumor formation. In vivo, other basal cell markers, notably the Ca2+-independent cell-cell adhesion molecule C-CAM, demonstrates this pattern of loss of expression in the progression to PIN and frank prostate cancer (44). Other basal cell epithelial genes that are not expressed in the histological prostate cancer includes the high molecular weight cytokeratins 5, 10, and 11 (45). This body of evidence would support the hypothesis that prostate cancers originate from a morphologically intermediate epithelial cell rather than a basal cell (30, 32).

P-cadherin represents a basal epithelial marker in normal prostate tissues that is inherently lost during the formation of prostate cancer. All 25 of the prostate cancers examined were immunohistochemically negative for P-cadherin, and therefore P-cadherin provides a useful marker for the histological diag-
nosis of prostate cancer. The molecular mechanisms underlying the regulation of the E-cadherin gene are complex, and similarly, our promoter sequence data for P-cadherin suggest that many putative regulatory mechanisms may be important. The role that P-cadherin plays in the transition from basal cell to secretory epithelial cell and alterations in this process in prostate carcinogenesis remain important questions for further investigation.

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REFERENCES


