Advances in Brief

Chromosome 5 Suppresses Tumorigenicity of PC3 Prostate Cancer Cells: Correlation with Re-Expression of α-Catenin and Restoration of E-Cadherin Function


Department of Urology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21287 (C. M. E., Ning Ru, Ronald A. Morton, John C. Robinson, Margaret J. Wheelock, Keith R. Johnson); Department of Biology, University of Toledo, Toledo, Ohio 43606 (J. Carl Barrett, William B. Isaacs); and Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 (J. C. B.)

Abstract

Considerable evidence now exists to support an important role for the E-cadherin-mediated cell-cell adhesion pathway as a suppressor of the invasive phenotype in adenocarcinoma cells. Previous studies have found that this pathway is frequently aberrant in prostate cancers, particularly those that are likely to metastasize. In this study, we report on the effects of re-establishment of this pathway in a prostate cancer cell line, PC-3, in which this adhesion system is dysfunctional by virtue of a deletion of the gene that codes for α-catenin, an E-cadherin-associated protein necessary for normal E-cadherin function. Re-expression of α-catenin was accomplished either by transfection of PC-3 cells with a copy of the α-catenin cDNA under the control of a heterologous promoter or by microcell-mediated transfer of chromosome 5, which contains the α-catenin gene and its normal regulatory elements. In both cases, re-expression of α-catenin is associated with a similar, dramatic alteration in cell morphology, whereby extensive cell-cell contact is observed. In the case of transfection of the cDNA, this expression is only transient, because the transfected cells either cease to proliferate or, more commonly, revert to the parental phenotype with concomitant cessation of α-catenin expression. In contrast, cells containing one or more copies of microcell-transferred chromosome 5 express α-catenin in a stable manner and continue to proliferate. Upon injection into nude mice, these latter cells are no longer tumorigenic, or form only slowly growing tumors with greatly diminished invasive potential that accompanies tumor progression (7-9).

We have demonstrated previously that down-regulation of E-cadherin levels is commonly found in prostate cancers, particularly in those which are likely to metastasize (18, 19). Furthermore, we found that the E-cadherin cell-cell adhesion pathway could be rendered nonfunctional by a means other that decreased levels of E-cadherin through loss of α-catenin expression (20). This lack of expression of α-catenin in the PC-3 prostate cancer cell line is due to a homozygous deletion of the gene, which has been mapped recently to chromosome 5q (21, 22).

To directly assess the contribution of altered cell-cell adhesion to the tumorigenic phenotype in prostate cancer cells, we describe here the effect on cell morphology, growth, and tumorigenicity of re-establishment of E-cadherin-mediated cell-cell adhesion in the PC-3 prostate cancer cell line by DNA transfection and chromosome microcell transfer techniques.

Materials and Methods

Cell Lines. Prostate cancer cell lines were obtained from either American Type Culture Collection (PC-3; Ref. 23) or from Dr. John Isaacs, Johns Hopkins University (TSU-Pr1; Ref. 24). Both lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Cells were grown at 37°C in humidified 5% CO2 atmosphere.

Plasmids and Transfection. pCMV-Neo-Bam4 (25), a gift of Dr. Bert Vogelstein (Johns Hopkins) contains a cytomegalovirus promoter-enhancer upstream of the insertion site (BamHI) for CDNA sequences to be expressed, along with a second transcription unit containing a neomycin resistance gene driven by an SV40 promoter-enhancer to allow for selection of colonies in geneicin. A full-length mouse α-catenin cDNA (a gift from Drs. S. Tsukita and A. Nagafuchi, Kyoto University, Kyoto, Japan; Ref. 17) was modified by the addition of EcoRI-XmnI-BamHI linkers (New England BioLabs). The modified cDNA was then cloned into the BamHI site of the pCMV-Neo-Bam vector. After appropriate screening, a sense-oriented clone was obtained and investigators to propose that dysfunction of this adhesion pathway is an important causal event in the loss of differentiated function and acquisition of invasive potential that accompanies tumor progression (7-9).

The function of E-cadherin, and indeed each of the classical cadherins, as mediators of cell-cell interactions is dependent upon their interaction with a family of cytoplasmic proteins termed the catenins (10-12). β-Catenin binds directly to the COOH-terminal cytoplasmic tail of E-cadherin (13), and α-catenin is thought to link this cadherin-catenin complex to the microfilament cytoskeleton (3, 14, 15). Such a function for α-catenin is further suggested by its sequence homology with the adhesion plaque protein, vinculin, which plays a critical role in mediating the interaction of integrins and the actin-based cytoskeleton (16, 17). Deletions of the cytoplasmic portion of E-cadherin, blocking interaction with the catenins, abolish the cell-cell aggregating ability of the cadherin in experimental settings (14).

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1 Supported by NHLBI grant CAS8236.
2 Present address: Department of Urology, Baylor College of Medicine, Houston, TX 77030.

3 To whom requests for reprints should be addressed, at Department of Urology, Marburg 130b, Johns Hopkins Hospital, 600 North Wolfe Street, Baltimore, MD 21287.

The abbreviation used is: CMV, cytomegalovirus.
used for the transfection of prostate cancer cell lines as described previously (26). Transfected cells were selected by treatment with 500 µg/ml of geneticin (G418; Gibco) in standard growth medium.

**Microcell Transfer.** Mouse A9 cells containing a copy of human chromosome 5 into which a neomycin-resistance gene had integrated were derived as described previously (27) and maintained in DMEM supplemented with 20% FBS and 500 µg/ml of geneticin (G418; Gibco). Microcell transfer was performed as described previously (28). The absence of transferred mouse DNA in the recipient clones was confirmed by *in situ* hybridization with total mouse genomic DNA.

**Tumor Growth Studies.** Male athymic nude mice (nu/nu; Harlan-Sprague-Dawley), 4–6 weeks old, were inoculated s.c. in the flank with 1 × 10⁶ wild-type or microcell-derived clones. Tumor measurements were made weekly, and tumor volumes were calculated according to I s aacs and C offey (29). Animals were sacrificed at 110 days after inoculation or when the tumor volumes reached greater than 0.5 ml. To measure *in vitro* growth, 5 × 10⁶ cells were inoculated into 60-mm dishes in growth media (RPMI 1640 + 10% fetal calf serum). Cells were harvested by trypsinization 24 h after plating and every 2 days thereafter. Cell number was determined by Coulter counter.

**Antibodies.** A mouse mAb against E-cadherin (HECD-1; Ref. 30) was obtained from Zymed Laboratories, Inc. The mouse monoclonal, 1G5, against α-catenin was prepared against a fusion protein as described (31). Monoclonal anti-WAF1/Cip1/p21 antibody was obtained from Santa Cruz Biotechnology. 4-6 weeks old, were inoculated s.c. in the flank with 1 × 10⁶ wild-type or microcell-derived clones. Tumor measurements were made weekly, and tumor volumes were calculated according to Isaacs and Coffey (29). Animals were sacrificed at 110 days after inoculation or when the tumor volumes reached greater than 0.5 ml. To measure *in vitro* growth, 5 × 10⁶ cells were inoculated into 60-mm dishes in growth media (RPMI 1640 + 10% fetal calf serum). Cells were harvested by trypsinization 24 h after plating and every 2 days thereafter. Cell number was determined by Coulter counter.

**Results**

**PC-3 Cells Express E-Cadherin but not α-Catenin.** Previous studies have found that prostate cancer cells frequently display aberrant calcium-dependent, cell-cell adhesion due to altered expression of either E-cadherin or α-catenin, or both. In one prostate cancer cell line, PC-3, derived from a bone metastasis, dysfunction of this adhesion pathway can be traced to the complete lack of α-catenin expression due to a homozygous deletion of the α-catenin gene (20). This cell line grows as a monolayer as loosely adherent clusters of cells, with pleomorphic appearance (Fig. 1A) and minimal formation of intercellular junctions (23). The cells are keratin positive and form rapidly growing tumors when injected into nude mice. Interestingly, this cell line continues to synthesize both E-cadherin and β-catenin, which form α-catenin-deficient, nonfunctional complexes (20).

**Transfection of α-Catenin cDNA into PC-3 Cells.** To assess the effects of re-expression of α-catenin in PC-3 cells, an expression vector was constructed by cloning the murine α-catenin cDNA into the pCMV-Neo-Bam plasmid. This vector, which places the cloned cDNA under the control of the CMV promoter (25), has been shown previously to be active in this cell line (26). When transfections are carried out with the α-catenin cDNA-containing plasmid, colonies are obtained that have an altered, tightly packed, epithelioid morphology and now express α-catenin that is localized at areas of cell-cell contact (Fig. 1C). This expression is only transient, however, because these colonies either cease to proliferate after accumulating between 50 and 100 cells, or cells begin to appear within these colonies that have the parental phenotype. This pattern of transient α-catenin expression was observed in each of three separate transfection experiments performed; and in each case, attempts to isolate clones that expressed α-catenin in a stable fashion failed. G418-resistant clones, which have the morphology of untransfected PC-3 cells and which continue to proliferate, are readily obtained, however. Southern analysis of five such randomly selected clones is shown in Fig. 2. Although each of these clones integrated the neomycin-resistance portion of the expres-
Techniques, we successfully introduced one or more copies of chromosome 5 into PC-3 cells. The presence of the introduced chromosome 5 was verified by cytogenetic analysis, as seen in Fig. 3, where examples of clones containing either one or two copies of transferred chromosome 5 are shown. In each of four independent microcell transfer experiments, each G418-resistant colony obtained had a similar morphology dramatically different from the parental cells (Fig. 1). Each colony arising after microcell transfer has a tightly packed epithelioid morphology indistinguishable from that obtained following transfection of the α-catenin cDNA as described above (Fig. 1). In contrast to the colonies obtained after cDNA transfection, however, clones containing transferred chromosome 5 continue to proliferate, maintaining their altered morphology. In addition, in each case, the continued expression of α-catenin is readily detectable by either immunofluorescence, Western blot, or immunoprecipitation (Figs. 1 and 4). The formation of protein complexes containing both E-cadherin and α-catenin is demonstrated by immunoprecipitation with E-cadherin antibodies, followed by Western blotting with α-catenin antibodies (Fig. 4C). Functionality of these complexes is revealed by the re-acquisition of calcium-dependent, cell-cell adhesion in cell aggregation assays (data not shown).

Reversion of PC-3(5) Cells to the Parental Phenotype. In the course of passage of PC-3 cells containing transferred copies of chromosome 5 [PC-3(5) cells], cells with the parental phenotype begin to appear (Fig. 1F), particularly in the clones of PC-3(5) that contain only a single transferred copy of chromosome 5. When assayed for α-catenin production, it is apparent that this morphological reversion to the parental phenotype is associated with a cessation of α-catenin synthesis (Fig. 1G). We isolated clones from distinct revertant colonies for further analysis. Southern analysis reveals that in each case of reversion to the parental phenotype, the portion of chromosome 5 containing the α-catenin gene has been deleted (Fig. 4A). As would be predicted, since the cells are grown continually in the presence of G418, the portion of the transferred chromosome containing the neomycin resistance gene is retained in each revertant, as is the region of the introduced chromosome containing the APC gene, located at 5q21 (data not shown). Thus, as with the transfection experiments described above, there is a selection against continued re-expression of α-catenin, and deletion (or lack of integration, in the case of transfection) of the gene appears to be the primary mechanism by which expression of this gene is terminated.

In Vitro and in Vivo Growth Rates. To assess the effect of re-establishment of the E-cadherin pathway on the growth of PC-3 cells, both in vitro and in vivo growth rates were examined Fig. 5. In
culture, in cells containing transferred chromosome 5, there is a marked decrease in the initial plating efficiency compared to the parental cells. However, after several days in culture, PC-3(5) cells attain a growth rate that is essentially equivalent to the wild-type cells (Fig. 5A). Thus, whereas the number of cells which are capable of forming proliferative colonies is greatly reduced by introduction of chromosome 5, once colonies are established, growth rates are largely unchanged. This effect is markedly cell density dependent, since plating PC-3(5) cells at low densities (i.e., requiring clonal growth) results essentially in no detectable increase in cell number, with the plated cells remaining viable but assuming a highly flattened mor-

Discussion

Previous studies have implicated the E-cadherin cell-cell adhesion system as a suppressor of metastatic behavior through its ability to block invasiveness. Indeed, the original intent of the studies described herein was to assess the effect of a-catenin reexpression and concomitant restoration of E-cadherin function on the metastatic behavior of the PC-3 prostate cancer cell line, a line derived from a bone metastasis. Somewhat surprisingly, re-expression of a-catenin in these cells, either by transfection or microcell-mediated chromosomal transfer, was strongly associated with in vitro and in vivo growth suppression, respectively. These results suggest that the cadherin/catenin cell-cell adhesion system, in addition to its demonstrated ability to suppress invasiveness, may also play a role in regulating cellular growth potential and overall tumorigenicity. (In vitro invasiveness is also suppressed by introduction of chromosome 5, and these findings will be presented elsewhere). The mechanism by which growth and/or tumorigenicity is suppressed by a-catenin re-expression is unknown. However, it is clear from a variety of studies that this pathway is critical to the establishment and maintenance of a number of characteristics of normal epithelial cells including cell polarity and cell-to-cell communication (1). In relation to this, this re-expression in PC-3 cells is associated with the formation of tight junctions and dramatic alterations in the microfilament cytoskeleton. It is possible that restoration of a more normal cell-cell interaction mechanism as provided by an intact cadherin system may affect the function of a variety of genes involved in growth regulatory pathways. Studies are underway using differential display techniques to examine the possible downstream effects on gene expression that may be induced by restoration of cadherin-based adhesion in this system.

As discussed, a strong positive correlation is found between expression of a-catenin and suppression of tumorigenicity. The possibility exists, however, that other genes on chromosome 5 are responsible for the observed suppression of tumorigenicity or that other such genes work in concert with a-catenin to mediate the observed suppression. One such candidate is the product of the APC gene, localized proximal to a-catenin on chromosome 5q at 5q21. The APC protein may be particularly relevant in this respect because recent studies have demonstrated the specific interaction of APC with ß-catenin (34, 35). Interestingly, the binding of APC to ß-catenin appears to preclude binding to E-cadherin, such that APC may in fact act as a negative regulator of cadherin-mediated adhesion (13). Thus, the contribution of APC to the increase in adhesive function observed here is not immediately clear. In related studies, Smith et al. (36) examined the expression of the APC gene in the PC-3 cell line, finding that full-length protein was readily detectable in these cells. We have

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C. M. Ewing and W. B. Isaacs, unpublished observations.
confirmed these findings in the PC-3 cells used in this study. As the majority of APC gene mutations characterized to date result in truncation of the protein product, the finding of full-length APC protein suggests that the APC gene is intact in PC-3 cells. Taken together, these data suggest that complementation of APC function is not related to the suppression of tumorigenicity observed in this study.

Perhaps the strongest evidence for the specific role of α-catenin in the phenotypic alterations observed here is from the transfection studies in which re-expression of α-catenin alone results in an adhesive phenotype concomitant with cessation of growth and that each clone that continues to proliferate does so in the absence of α-catenin cDNA integration and expression. Similarly, after microcell transfer of chromosome 5, each tumorigenic revertant that develops with passage in culture is characterized by loss of α-catenin expression. Further supporting evidence for a direct role for α-catenin in mediating the phenotypic alterations observed here is provided by recent studies of Watabe et al. (37). Similar to the findings presented here, these workers demonstrated that re-expression of α-catenin in an α-catenin-deficient lung cancer cell line resulted in profound morphological alterations and a marked suppression in 
in vitro growth, whereas in vivo tumorigenicity was not examined.

These experiments suggest that deletion of the α-catenin gene is a relatively common mechanism leading to the loss of E-cadherin function, at least in the case of PC-3 cells. The genetic mechanisms responsible for this preselection to fragmentation of chromosome 5 in these cells are unknown, although a fragile site localized to the 5q31 region may be related to the chromosomal instability observed in this process (38). Originally, we reported mapping the α-catenin gene to chromosome 5q21-22 (22), but subsequently Nollé et al. determined (40) that this is the location of an α-catenin pseudogene, with the gene being located more distally at 5q31, as had been previously reported by Furukawa et al. (21). We have confirmed these mapping assignments. Interestingly, allelic loss of this region in prostate cancer is quite high, particularly in advanced prostate cancers (39). Whether the α-catenin gene is a target of these allelic loss events remains to be determined. In summary, the results presented here provide evidence for a novel, growth-regulatory role for the cadherin pathway and the role of α-catenin in mediating this activity.

References


6 C. M. Ewing and R. A. Morton, unpublished observation.

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