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Cadherin Dysfunction in a Human Cancer Cell Line: Possible Involvement of Loss of α-Catenin Expression in Reduced Cell-Cell Adhesiveness[1]

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ABSTRACT

A human lung cancer cell line, PC 9, was analyzed to elucidate the molecular mechanisms of dysfunction of cadherin-mediated cell-cell adhesion in cancer. Although PC 9 cells strongly expressed E-cadherin at the cell membrane, which was indistinguishable immunochemically from functional E-cadherin, they did not show tight cell-cell adhesion and had reduced E-cadherin-mediated aggregation activity. Immunoprecipitation with E-cadherin and Western blot analysis revealed that PC 9 cells did not express α-catenin, a cadherin-associated protein, suggesting that this was the cause of the cadherin dysfunction in the cell line. In addition, Northern and Southern blot analyses disclosed homozygous deletion of part of the α-catenin gene, which might have resulted in the loss of α-catenin expression in PC 9 cells.

INTRODUCTION

Disruption of cell-cell adhesion must be indispensable for the growth profile specific to cancer. If cancer cells proliferated while maintaining tight cell-cell adhesion, they would show expansive growth similar to that of benign tumors, and they would neither metastasize to distant organs nor invade the surrounding tissues. In this respect, analysis of the molecular basis of cancer cell detachment seems to be a very important aspect of cancer research.

A few years ago, we started studying cadherin cell-cell adhesion molecules in human cancer, which are responsible for tight and strong cell-cell adhesion (1, 2). Initially, we identified two cadherin molecules, E- and P-cadherin, in human epithelial tissues and carcinomas by establishing respective specific mAbs[3] (3). We then examined in detail the expression of these cadherins in gastric and hepatocellular carcinomas. We found that some carcinomas completely lacked cadherin expression and also exhibited a "scattered" histopathological appearance, suggesting that loss of cadherin expression freed cells from tight cell-cell association and provided them with some cancer-specific properties (4, 5). However, we also found that a considerable number of "scattered" gastric carcinomas strongly expressed E-cadherin which was indistinguishable immunochemically from functional E-cadherin (4). The question then arose as to why these cancer cells could not form tight cell-cell associations.

It is known that some cytoplasmic proteins are coimmunoprecipitated with E-cadherin (6, 7). Recent studies have demonstrated that E-cadherin becomes associated with these proteins, termed "catenins," through its cytoplasmic domain (8), suggesting that molecular interactions among these proteins are necessary for the cell-cell binding function of E-cadherin (9, 10). These findings, and the successful molecular cloning of catenins (CAP 102/α-catenin, β-catenin) (11–13), have prompted us to investigate whether any alterations in catenins actually cause the dysfunction of cadherins in cancers. To this aim, we looked for cancer cell lines expressing cadherins but lacking tight cell-cell adhesion. We then discovered a cancer line that expressed E-cadherin but not α-catenin and showed insufficient cadherin function. In this paper, we describe the properties of this cell line and discuss the possible relationship between cadherin dysfunction and loss of α-catenin expression.

MATERIALS AND METHODS

Cells. Human lung carcinoma PC 9 (14) and colon carcinoma HCT-15 cells (15) were used. Both cell lines were cultured in RPMI 1640 medium supplemented with 10% FCS in a humidified atmosphere containing 5% CO_2/95% air. HCT-15 cells were used in this study as representative cells expressing functional E-cadherin molecules.

Antibodies. Two mAbs, HECD-1 (3) and α-18, which recognize E-cadherin and α-catenin, respectively, were used. The establishment of mAb α-18 will be reported elsewhere.[4]

Immunocytochemistry. PC 9 cells were smeared on a poly-D-lysine-coated slide, and HCT-15 cells were cultured on a plastic dish, for E-cadherin staining with HECD-1. These cells were fixed with 3.5% paraformaldehyde in HNC at 4°C for 15 min and then with methanol at −20°C for 15 min. After rinsing with HNC, they were treated with 2% swine serum in HNC for 30 min followed by incubation with HECD-1 for 1 h. They were then incubated with biotinylated anti-mouse IgG for 30 min, followed by another 30-min incubation with fluorescein isothiocyanate-conjugated streptavidin. After each period of incubation, the samples were rinsed with HNC and treated briefly with 2% swine serum in HNC. Finally, after extensive rinsing with HNC, they were mounted and examined with a MRC-500 confocal imaging system (Bio-Rad Microscience Division, Watford, United Kingdom).

Detergent Extraction of Cells. Cells were washed 3 times with HNC. After removal of the supernatant, 100 μl of 1% Nonidet P-40 in HCMF containing 5 mM $CaCl_2$, 2 mM phenylmethylsulfonyl fluoride, and 20 μg/ml of leupeptin were added to 40 μl of the pellet, and the cells were incubated for 10 min with mild pipetting. They were then centrifugated at 100,000 × *g* for 30 min at 4°C. To the supernatant, 2 × SDS-PAGE sample buffer was added to give a total volume of 250 μl and used as the detergent-soluble fraction. The residual pellet was then dissolved in 250 μl of 1 × SDS-PAGE sample buffer with brief sonication on ice and used as the detergent-insoluble fraction.

SDS-PAGE and Western Blot Analysis. SDS-PAGE and Western blot analysis were carried out as described previously (4), except that an ECL Western blotting detection system (Amersham International plc., Amersham, United Kingdom) was used according to the manufacturer's instructions.

Cell Aggregation. A cell aggregation experiment was performed as described previously (16) with some minor modifications. Briefly, to

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[3] The abbreviations used are: mAb, monoclonal antibody; FCS, fetal calf serum; HNC, a buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 150 mM NaCl, and 2 mM $CaCl_2$; HCMF, 10 mM 4-(2-hydroxyethyl)--1-piperazineethanesulfonic acid-buffered Ca^{2+}, Mg^{2+}-free Hanks' solution; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IgG, immunoglobulin G; poly(A)$^+$ RNA, polyadenylate-containing RNA.

[4] A. Nagafuchi and S. Tsukita, manuscript in preparation.

obtain dispersed suspensions of cells with intact cadherin molecules, the cells were treated with 0.01% trypsin and 5 mM $CaCl_2$ in HCMF at 37°C for 15 min on a gyratory shaker at 75 rpm. Fifty thousand cells suspended in 0.5 ml of HCMF containing 1% bovine serum albumin were placed in each well of a 24-well plastic plate and allowed to aggregate at 37°C for 30 min at 75 rpm. The extent of cell aggregation was represented by the index $(N_0 - N_t)/N_0$, where N_t is the total particle number after incubation time t and N_0 is the total particle number at the start of incubation.

Immunoprecipitation. [^{35}S]Methionine-labeled cells were lysed with 1% Nonidet P-40 and 1% Triton X-100 in HNC containing 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml of leupeptin, and 100 kallikrein inhibitor units/ml of aprotinin. The cell lysates were clarified by centrifugation at 100,000 × g for 30 min at 4°C and incubated with CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) conjugated with normal mouse IgG overnight at 4°C to adsorb materials which might bind nonspecifically to the Sepharose and IgG. Then, the cadherin-catenin complex was precipitated from the supernatants by incubation for 2 h at 4°C with CNBr-activated Sepharose 4B conjugated with HECD-1 and brief centrifugation, released from the Sepharose by heating in 1 × SDS-PAGE sample buffer (3), and analyzed by SDS-PAGE using a 7.5% polyacrylamide gel. The gel was fixed with 10% acetic acid/30% methanol for 1 h, immersed in EN^{3}HANCE (E.I. du Pont de Nemous & Co., Boston, MA) for 1 h, dried, and then exposed to a XAR film (Eastman Kodak Co., Rochester, NY).

Northern and Southern Blot Analyses. Northern and Southern blottings were performed as described previously (17, 18). A 2.9-kilobase pair *Eco*RI-*Hin*dIII fragment of a mouse α-catenin complementary DNA (11), which covers the entire open reading frame, was used as a probe.

RESULTS

Characterization of E-Cadherin Expressed in PC 9 Cells. PC 9 cells grew in suspension as very loosely adherent aggregates as shown in Fig. 1 and were easily dispersed by pipetting. Immunofluorescence cytochemistry of PC 9 cells revealed E-cadherin all along the cell membrane (Fig. 1). By contrast, the HCT-15 cells used as a control expressing functional E-cadherin molecules formed tight cell-cell aggregates attached to the plastic dishes and expressed E-cadherin at cell-cell boundaries but little at the free cell border (Fig. 1). Western blot analysis showed that the E-cadherin expressed on PC 9 cells was the same molecular size as that on HCT-15 cells, and it also showed the same Ca^{2+} sensitivity as the functional E-cadherin which had been reported previously (19) (Fig. 2); that is, E-cadherin on PC 9 cells was resistant to trypsin treatment in the presence of Ca^{2+}. Also, PC 9 E-cadherin showed a detergent solubility similar to that of the functional molecule; a considerable amount of E-cadherin could not be extracted with Nonidet P-40, as found before (19). No alteration in the E-cadherin gene of PC 9 cells could be found by Southern blotting (data not shown). P-cadherin was not detected in PC 9 cells by either immunocytochemistry or Western blotting (data not shown).

In cell aggregation experiments, PC 9 cells showed only a low rate of E-cadherin-mediated aggregation, which was completely blocked by addition of anti-E-cadherin mAb (Fig. 3). In contrast, HCT-15 cells showed strong aggregation (Fig. 3). Moreover, PC 9 cells formed more fragile aggregates than HCT-15 cells. A considerable proportion of them were disrupted by shaking at 150 times/min (Fig. 3).

Analysis of α-Catenin in PC 9 Cells. We next examined whether PC 9 cells had any abnormalities of cadherin-associated proteins, known as catenins. Catenins on PC 9 and HCT-15 cells were examined by coimmunoprecipitation with E-cadherin using HECD-1. As shown in Fig. 4, the precipitate from PC 9 cells contained a protein considered to be β-catenin but no molecule at the position for α-catenin, whereas HECD-1 precipitated two major catenins, α- and β-catenins, with E-cadherin from HCT-15 cells. The immunoprecipitate from PC 9 also contained a few bands of proteins with lower molecular weights than that of α-catenin. The precipitate from PC 9 or HCT-15 was then analyzed by Western blotting using a newly established mAb against α-catenin, α-18, and this confirmed that α-catenin was missing from the PC 9 cells (Fig. 4). The

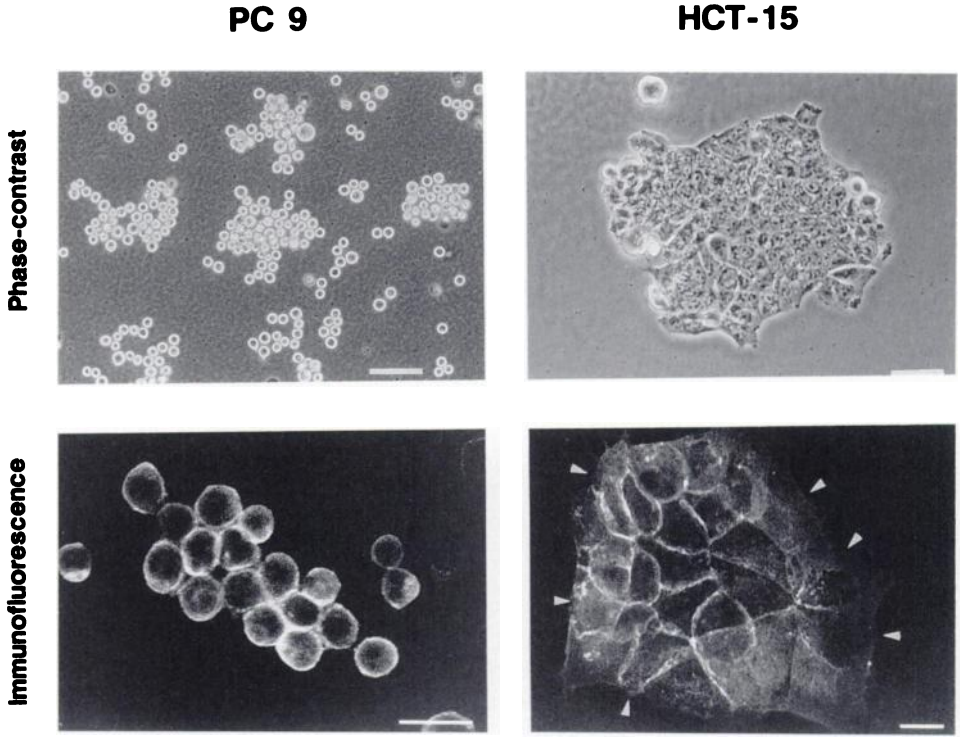

Fig. 1. Phase-contrast micrograph and immunofluorescence staining of E-cadherin in PC 9 and HCT-15 cells. *Arrowheads*, free cell border of HCT-15 cells. *Scale bar*, 100 μm (*left upper*), 25 μm (*left lower*), 50 μm (*right upper*), and 10 μm (*right lower*).

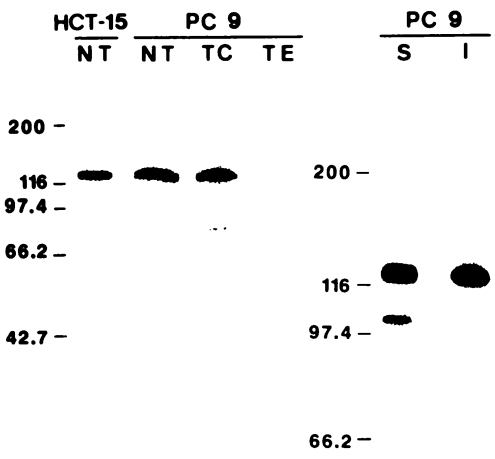

Fig. 2. Western blot analysis of E-cadherin. Samples were obtained from non-trypsinized cells (*NT*), from cells treated with trypsin in the presence of Ca^{2+} (*TC*), and from cells treated with trypsin and ethyleneglycol-bis(β-aminoethyl-ether)-*N*,*N*-tetraacetic acid in the absence of Ca^{2+} (*TE*), as described previously (19). Detergent-soluble (*S*) and detergent-insoluble (*I*) fractions of PC 9 cells were also analyzed. Samples in each lane were derived from 1 μl of the pellet. *Bars* show positions of molecular weight markers in $M_r \times 10^3$.

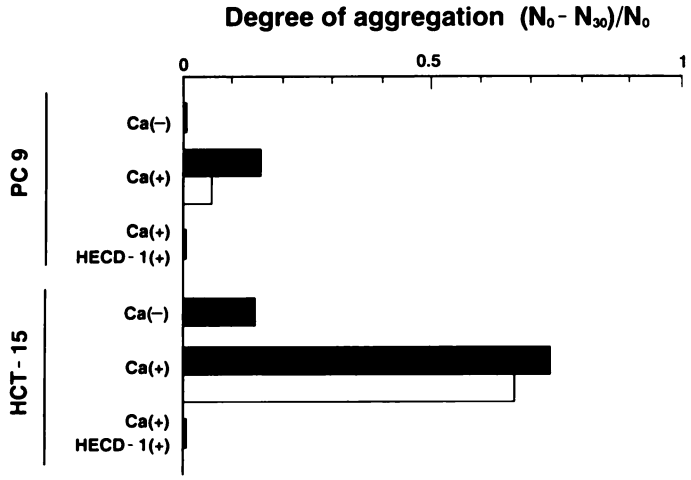

Fig. 3. Aggregation of cells under different conditions. Cells were allowed to aggregate in the absence of Ca^{2+}, in the presence of Ca^{2+} (5 mM $CaCl_2$), or in the presence of Ca^{2+} and HECD-1 (20 μg/ml), and each degree of aggregation is indicated by *closed bars*. The aggregates formed in the presence of Ca^{2+} (cadherin-mediated aggregates) were shaken reciprocally at 150 times/min for 30 min to examine the strength of cell-cell adhesion in the aggregates. *Open bars* show the extent of cell aggregation after this treatment.

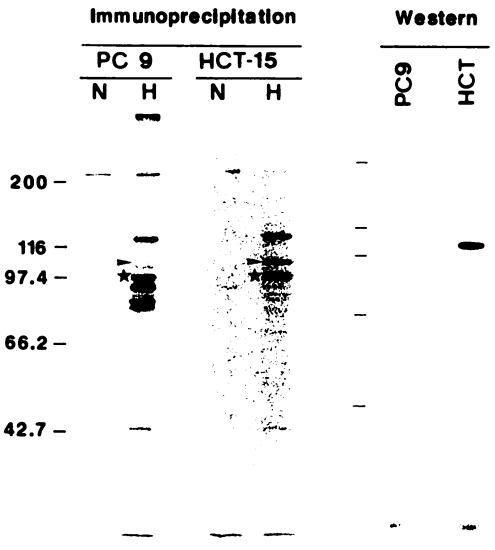

Fig. 4. Immunoprecipitation with E-cadherin and Western blot analysis of α-catenin. Lysates of PC 9 and HCT-15 cells were precipitated with normal mouse IgG (*N*) or anti-E-cadherin mAb, HECD-1 (*H*). The position of α-catenin is indicated by *arrowheads*. Bands corresponding to the mobility of β-catenin are indicated by *closed stars*. Other bands detected in PC 9 cells are presently unknown. Part of the sample precipitated with HECD-1 was also subjected to Western blotting using anti-α-catenin mAb, α-18.

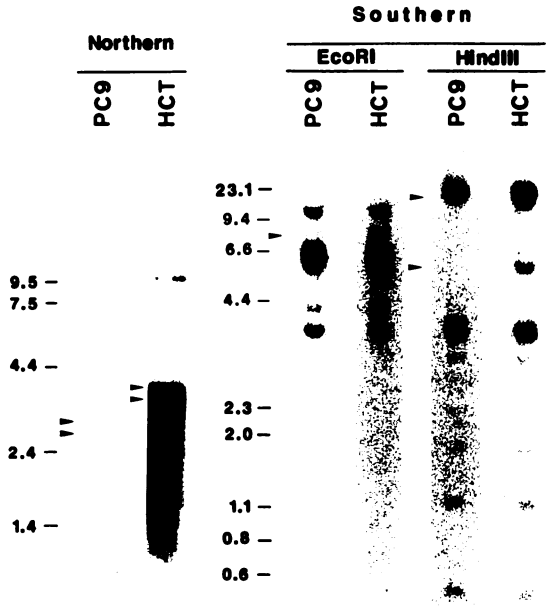

Fig. 5. Northern and Southern blot analyses of α-catenin. For Northern blotting, 3 μg of poly(A)$^+$ RNA were loaded in each lane. The positions of α-catenin mRNAs are indicated by *arrowheads*. *Bars* show positions of molecular size marker mRNAs in kilobases. For Southern blotting, 5 μg of high-molecular-weight DNA were digested with *Eco*RI or *Hin*dIII and loaded on each lane. The bands lacking in PC 9 samples are indicated by *arrowheads*. The 15-kilobase band in the *Hin*dIII digest of HCT-15 DNA is fused with the band just above it. *Bars* show positions of molecular size marker DNAs in kilobases.

lower-molecular-weight bands in the precipitate from PC 9 cells (Fig. 4) did not react with α-18, although these could have been abnormal α-catenin peptides. α-Catenin was also not detected by Western blotting in a whole cell lysate of PC 9 (data not shown).

Northern blotting using an α-catenin complementary DNA probe covering the entire open reading frame detected very faint 3.0- and 2.7-kilobase bands in poly(A)$^+$ RNA of PC 9 cells, whereas it detected intense bands of 3.8- and 3.4-kilobase transcripts in HCT-15 cells (Fig. 5). We then examined the α-catenin gene of PC 9 cells by Southern blotting using the same probe as above. As shown in Fig. 5, a 7.5-kilobase fragment in the *Eco*RI digest and also 15- and 5.4-kilobase fragments in the *Hin*dIII digest which were present in HCT-15 were not detected in PC 9. We also examined normal tissues in which cadherins were assumed to be functional and confirmed that the restriction pattern of their α-catenin gene was the same as that of HCT-15 (data not shown).

DISCUSSION

It has been demonstrated that suppression of cadherin-mediated cell-cell adhesion leads to enhanced invasiveness (20–22). It has also been reported that a highly metastatic ovarian tumor line showed characteristic unstable cadherin expression (23),

and loss of cadherins has been implicated in the final stage of mouse epidermal carcinogenesis (24). These experimental findings strongly suggested a causal relationship between suppression of cadherin function and progression of cancers. In fact, loss or reduction of cadherin expression, possibly linked with a "scattered" histopathological appearance and increased invasiveness, has been found by immunohistochemistry in resected human gastric, hepatocellular, and other carcinomas (4, 5, 25). However, in the previous study, we encountered many gastric carcinomas which had no tight cell-cell adhesion despite strong expression of E-cadherin (4), suggesting that these cancer cells might have some mechanism which suppressed cadherin function.

In the present study, we found that PC 9, a lung carcinoma line, strongly expressed E-cadherin molecules which were indistinguishable from functional ones with regard to various properties, but showed very weak cell-cell adhesiveness. It is well known that cadherins are concentrated at cell-cell contact sites and that free cell borders lack, or have less, cadherins (17, 26, 27). In general, cells with functional cadherins adhere tightly to one another, leading to deformation of their shape in order to maximize the area of cell-cell contact (16). All of these features were observed in cell-cell adhesion of HCT-15 cells but not of PC 9 cells. PC 9 cells adhered to one another retaining a spherical shape, as if they were aggregated due to Ca^{2+}-independent cell-cell adhesion (16). However, the cell aggregation experiment showed that E-cadherin of PC 9 cells functioned very weakly, and it was blocked by anti-E-cadherin mAb. These observations suggest that E-cadherin molecules expressed on PC 9 cells are normal and can bind cells together via small areas of contact, but some mechanism for eliciting the full function of E-cadherin, for example one which concentrates the molecule at cell-cell contact sites, is deficient in PC 9 cells.

Immunoprecipitation and Western blot analyses revealed that PC 9 cells did not express normal α-catenin, although we could not exclude the possibility that they expressed some fragments of α-catenin. Functions of catenins in cadherin-mediated cell-cell adhesion are still unclear. However, since E-cadherin molecules with deletions in the cytoplasmic domain lose the ability to bind to one another and cannot associate with catenins (8, 10), it is considered that cadherin-catenin complex formation is indispensable for the expression of cadherin function. Therefore, it is conceivable that loss of α-catenin results in insufficient E-cadherin function in PC 9 cells. Recently, we attempted to transfect PC 9 cells with a complementary DNA for a subtype of α-catenin and found that the transfected cells formed tight cell-cell adhesions mediated by E-cadherin (28). This result confirms our speculations that PC 9 cells express normal E-cadherin molecules and that loss of α-catenin results in cadherin dysfunction.

Northern and Southern blot analyses performed in this study revealed a possible explanation for the mechanism of loss of PC 9 α-catenin expression. Northern blotting demonstrated that PC 9 cells faintly expressed smaller α-catenin mRNAs in comparison with HCT-15 cells showing normal cadherin function. Some fragments of the α-catenin gene were not detected in Southern blots hybridized with an α-catenin complementary DNA probe which covered the entire open reading frame. These results suggest that part of the α-catenin gene containing some exon(s) is homozygously deleted in PC 9, resulting in weak expression of abnormal mRNAs, so that finally PC 9 cells cannot produce normal α-catenin molecules.

Although the present work focused on only one cancer line lacking α-catenin, the same mechanism could be involved in cell detachment of many cancer types. Following the present study, we have examined expression of α-catenin as well as E-cadherin in surgically resected gastric carcinomas by immunohistochemistry and immunoblotting, and we have found a considerable number of "scattered" carcinomas expressing E-cadherin but not α-catenin.[5] Actually, 11 of 24 E-cadherin-positive "scattered" gastric carcinomas, 45.8%, lacked α-catenin expression. It is likely that in those cases the loss of α-catenin caused the "scattered" phenotype and enhanced invasiveness. PC 9 cells would be a useful model material for further analysis of cell detachment mechanisms and α-catenin functions.

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