Induction of Polarized Cell-Cell Association and Retardation of Growth by Activation of the E-Cadherin-Catenin Adhesion System in a Dispersed Carcinoma Line

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Abstract. PC9 lung carcinoma cells cannot tightly associate with one another, and therefore grow singly, despite their expression of E-cadherin, because of their lack of α-catenin, a cadherin-associated protein. However, when the E-cadherin is activated by transfection with α-catenin cDNA, they form spherical aggregates, each consisting of an enclosed monolayer cell sheet. In the present work, we examined whether the α-catenin-transfected cell layers expressed epithelial phenotypes, by determining the distribution of various cell adhesion molecules on their surfaces, including E-cadherin, ZO-1, desmoplakin, integrins, and laminin. In untransfected PC9 cells, all these molecules were randomly distributed on their cell surface. In the transfected cells, however, each of them was redistributed into a characteristic polarized pattern without a change in the amount of expression. Electron microscopic study demonstrated that the α-catenin-transfected cell layers acquired apical-basal polarity typical of simple epithelia; they formed microvilli only on the outer surface of the aggregates, and a junctional complex composed of tight junction, adherens junction, and desmosome arranged in this order. These results indicate that the activation of E-cadherin triggered the formation of the junctional complex and the polarized distribution of cell surface proteins and structures. We also found that, in untransfected PC9 cells, ZO-1 formed condensed clusters and colocalized with E-cadherin, but that other adhesion molecules rarely showed such colocalization with E-cadherin, suggesting that there is some specific interaction between ZO-1 and E-cadherin even in the absence of cell-cell contacts. In addition, we found that the activation of E-cadherin caused a retardation of PC9 cell growth. Thus, we concluded that the E-cadherin-catenin adhesion system is essential not only for structural organization of epithelial cells but also for the control of their growth.

CELLS of simple epithelia have apical-basal polarity, which condition is essential for their structure and function (Rodriguez-Boulan and Nelson, 1989). Most of simple epithelial cells are linked together by a junctional complex comprised of tight junction, adherens junction (zonula adherens or intermediate junction) and desmosome, which are arranged in this order from the apical portion of the cell-cell contact (Farquhar and Palade, 1963). The basal plasma membrane of the cells attaches to the basement membrane, while the apical-free surface develops microvilli for responding to the extracellular environment. Many carcinoma cells, however, lose such polarized phenotypes during tumor progression. Little is known about the mechanism of how the polarized epithelial cell-cell contacts are established, or of how they are disrupted during carcinogenesis.

It has been suggested that the cadherin cell-cell adhesion system plays a role in initiation of polarized cell-cell association (Gumbiner and Simons, 1986; Gumbiner et al., 1988). Cadherins are a family of cell-cell adhesion molecules (Takeichi, 1991). A subfamily of them, the so called “classic” cadherins including E-cadherin, are localized in the adherens junctions (AJ) (Kemler, 1993), and another subfamily, composed of desmogleins and desmocollins, are restricted to desmosomes (Buxton et al., 1993). Without cadherins, cells are unable to tightly associate with each other, and do not show polarity. Introducing a cadherin into cadherin-deficient cells induces a polarized distribution of surface proteins such as Na⁺, K⁺-ATPase (McNeill et al., 1990) and enhances gap junction formation (Mege et al., 1988; Matsuzaki et al., 1990). On the other hand, blocking cadherin function in epithelial cells with antibodies results in disruption of the junctional complexes (Gumbiner and Si-

1. Abbreviation used in this paper: AJ, adherens junctions.
junctional complex, which is characteristic of simple epithelial cell-cell contacts regulate not only the structural organization but also their growth property. These results indicate that not only is α-catenin indispensable for cadherin function but also that the cadherin–catenin system provides some signals for cells to arrange themselves into an epithelioid pattern.

In the present study, we performed detailed analyses of the structure of the α-catenin–transfected PC9 cells and also of their growth property. The results show that these cells acquire apical–basal polarity including the formation of a junctional complex, which is characteristic of simple epithelia. Thus, the dispersed carcinoma cells regained an almost complete set of epithelial–specific adhesive properties as the result of α-catenin transfection. We also show that the growth of PC9 cells is significantly suppressed following α-catenin transfection. These results suggest that the cadherin–mediated cell–cell contacts regulate not only the structural organization of cell layers but also their growth property.

Materials and Methods

Cell Cultures and α-Catenin cDNA Transfection

Human lung carcinoma PC9 (Kinjo et al., 1979) and PC9-αN, a line of PC9 transfected with chicken αN-catenin cDNA (Hirano et al., 1992), were cultured in a 1:1 mixture of DMEM and Ham's F12 supplemented with 10% FCS (DH10).

The expression vector of mouse αE-catenin cDNA, pBAT-α, was made by replacing the βGlobin-XbaI fragment of pBAT-EM2 (Nose et al., 1988) with the BamHI-XbaI fragment of pSK102B (Nagafuchi and Tsukita, 1994). Transfection of PC9 cells with pBAT-α was performed as described previously (Hirano et al., 1992). Cells were cotransfected with pPGKneo bpA (Soriano et al., 1991) and selected in G418-containing medium. Isolated cells were transfected. These results suggest that the E-cadherin–mediated adhesion might activate a PKC signaling system to induce the formation of tight junction (Balda et al., 1993).

Our recent studies presented another example showing the importance of cadherin function in epithelial organization (Hirano et al., 1992). Cadherin in the AI are associated with a group of cytoplasmic proteins, α, β-catenins (McCrea et al., 1991) and plakoglobin (Knudsen and Wheelock, 1992; Piepenhagen and Nelson, 1993). α-Catenin is divided into two subtypes, αE-catenin, which is expressed in many kinds of tissues (Nagafuchi et al., 1991; Herrenknecht et al., 1991; Nagafuchi and Tsukita, 1994), and αN-catenin, expressed mainly in the nervous system (Hirano et al., 1992; Uchida et al., 1994). Cells of the lung carcinoma line PC9 express neither of these α-catenin subtypes but do express E-cadherin and α-catenin, and they cannot aggregate and thus grow singly in suspension (Hirano et al., 1992; Shimoyama et al., 1992). Transfection of PC9 cells with αN-catenin cDNA activates the E-cadherin, leading them to acquire cadherin-mediated cell–cell associations (Hirano et al., 1992). Moreover, these transfected cells display an epithelioid arrangement in the aggregates. These results indicate that not only is α-catenin indispensable for cadherin function but also that the cadherin–catenin system provides some signals for cells to arrange themselves into an epithelioid pattern.

Antibodies

The following antibodies were used: mouse mAbs HECDC-1 (Shimoyama et al., 1989) and SHE7R-7 (Takara Shuzo Co., Ltd., Kyoto, Japan) to E-cadherin; rat mAb ECD-2 to E-cadherin (Shirayoshi et al., 1986); rat mAb 809 to αE-catenin (Nagafuchi and Tsukita, 1984); rabbit polyclonal antibody (pAb) to β-catenin (Shibamoto et al., 1994); mouse mAb PG5.1 to plakoglobin (61005; Progen Biotechnik GmbH, Heidelberg, Germany); mouse mAb 24-25 to ZO-1 (Itoh et al., 1991); mouse mAb 11-5F to desmoplakin (Parrish et al., 1987); rabbit pAb 4316 to βI integrin (a gift of Kenneth Yamada); rat mAb 13 to βII integrin (Akiyama et al., 1989); and rabbit pAb to laminin (AT-2404, EY Laboratories, Inc., San Mateo, CA).

For detection of primary antibodies, we used sheep biotinylated species-specific antibody to mouse Ig (RPN1001; Amersham International, Amersham, UK) and to rat Ig (RP7101; Amersham International); donkey biotinylated species-specific antibody to rabbit Ig (RPN1004; Amersham International); FITC-labeled streptavidin (RPN1232; Amersham International); Texas red–labeled streptavidin (RPN1233; Amersham International); goat Cy3-labeled species-specific antibody to rat IgG (AP-183C; Chemicon International, Inc., Temecula, CA); sheep FITC-labeled antibody to mouse Ig (N903; Amersham International); goat rhodamine-labeled antibody to rat IgG (55763; Organon Technika N. V. Cappel Products, West Chester, PA); sheep HRP-linked species-specific antibody to mouse Ig (NA9310; Amersham International) and to rat Ig (NA932; Amersham International); goat HRP-linked antibody to rabbit IgG (3212-0081; Cappel Products); and Sepharose 4B-linked goat antibody to mouse IgG (62-6541; Zymed Laboratories, Inc., San Francisco, CA), and to rat IgG (62-9541; Zymed Laboratories).

Immunofluorescence Staining

Cells suspended in culture medium were collected by centrifugation, resuspended in Hepes-buffered (pH 7.4) HBSS, and placed on coverslips that had been pre-coated with 1 mg/ml polyethylenimine by incubation overnight at room temperature. After 15 min at room temperature, the cells were fixed as follows. For immunofluorescence staining for desmoplakin, cells were fixed and permeabilized by treatment with methanol on ice for 5 min. For staining for other molecules, cells were treated as for the desmoplakin staining, or they were fixed with 3.5% paraformaldehyde in HBSS at 4°C for 20 min, and permeabilized by treatment with methanol at −20°C for 20 min. For avoidance of nonspecific binding of antibodies, the fixed cells were pre-treated with 5% skim milk for more than 15 min. They were subsequently incubated with a primary antibody, a biotinylated secondary antibody, and FITC or Texas red–labeled streptavidin. For double-immunostaining, fixed cells were first treated with a mixture of two primary antibodies, followed by incubation with a biotinylated antibody to one primary antibody, and then a mixture of FITC or Texas red–labeled streptavidin and Texas red– or FITC-labeled secondary antibody to the other primary antibody. In some experiments, Cy3-labeled secondary antibody was substituted for Texas red–labeled antibody. Cross-reactivity of each secondary antibody sample was carefully checked to avoid false double-staining prior to experiments. The immunostained samples were examined under Zeiss Axiophot and confocal microscopes. Confocal pictures were taken to show single optical x-y sections.

Immunoblotting

Cells were scraped out of dishes with a rubber policeman, dissolved in Laemmlli's SDS sample buffer, and boiled with 5% β-mercaptoethanol for 10 min. Proteins were separated by 7.5, 5, or 3.5% polyacrylamide gel electrophoresis, followed by transfer to nitrocellulose filters. The filters were incubated with primary antibodies and then with HRP-conjugated secondary antibodies. Bound HRP was visualized by use of the enhanced chemiluminescence system (RPN2106, Amersham International).

Immunoprecipitation

All steps in the following immunoprecipitation protocol were carried out either on ice or at 4°C. Cells attached to dishes were scraped off with a rubber policeman, and the resulting suspension of cells was transferred to a test tube. The cell suspensions were centrifuged at 150 g for 5 min, and the pellets were resuspended in HBSS and transferred to Eppendorf tubes. Cells were centrifuged, and then 1 ml of an ice-cold detergent extraction buffer (1% NP-40), 1 Triton X-100, 1 mM CaCl2, and 1 mM PMSE in 50 mM TBS, pH 7.6) was added to each pellet. After incubation for 30 min, the crude extracts were centrifuged at 100,000 g for 10 min. The supernatants were transferred to Eppendorf tubes. The extracts were preclariﬁed by incubation with Sepharose 4B for 30 min. These samples were then centrifuged at 1,200 g for 10 s, and the resulting supernatants were transferred to new tubes. The primary antibody was added to the cleared extracts, which were incubated for 1 h. Then the extracts were incubated with Sepharose 4B-
Adhesive Properties of PC9 Cells Transfected with \textit{αE}- and \textit{αN}-Catenin cDNA

In our previous work, PC9 cells were transfected with chicken \textit{αN}-catenin cDNA, and a transfected line, PC9-\textit{αNA}, was isolated (Hirano et al., 1992). Since the PC9 line was derived from lung carcinoma (Kinjo et al., 1979), the original \textit{α}-catenin of the cells was supposedly \textit{αE}-catenin. To restore \textit{αE}-catenin expression to this cell line, we transfected PC9 cells with mouse \textit{αE}-catenin cDNA and obtained transfected lines, one of which was designated as PC9-\textit{αE1}. Cells of the \textit{αE}-catenin-transfected lines formed aggregates (Fig. 1 \textit{b}), each consisting of an enclosed epithelial cell monolayer (Fig. 1 \textit{c}), in contrast to the dispersed nature of the parent PC9 cells (Fig. 1 \textit{a}). When PC9-\textit{αE1} cells were cultured with anti-E-cadherin antibodies, they did not form such epithelialoid aggregates, being indistinguishable from the untransfected PC9 cells in morphology (Fig. 1 \textit{d}), as previously found for PC9-\textit{αNA}. Thus, both subtypes of \textit{α}-catenin showed a similar effect on E-cadherin-dependent aggregation of PC9 cells. In the following experiments, PC9-\textit{αE1} was generally used, and PC9-\textit{αNA} was occasionally used to confirm the generality of the phenotypes of \textit{α}-catenin-transfected PC9 cell lines. Both cell lines gave essentially the same results in all the following experiments. The term \textit{α}-catenin was used as a collective name of \textit{αE}- and \textit{αN}-catenin.

We noticed that the cultures of the above transfected lines contained not only spherical aggregates but also a cell population adherent to the dish (Fig. 1 \textit{b}). The latter formed monolayer cell sheets. The proportion of the attached and nonattached cells varied from culture to culture. To determine the relationship between the two cell populations, we separated and cultured them independently. Each of them, however, always produced both nonattached and attached cells in a culture, suggesting that these two forms are convertible into each other. Probably, the aggregated cells occasionally attach to the substratum and spread on it, but some of them again detach and grow in the form of aggregates. On the other hand, the majority of the untransfected PC9 cells remained in suspension except for a certain population of attached cells. In the following experiments, the two cell populations were not separated for biochemical and growth analyses, but the suspended form was mainly used for morphological analyses. In addition, \textit{α}-catenin expression in the transfected lines was not extremely stable, and nonadhesive single cells were constantly produced in cultures of the transfectants. Thus, a certain level of contamination of those cells was unavoidable in the assay of the transfectants, but their presence did not largely affect the results.

Formation of Polarized Cell–Cell Association after \textit{α}-Catenin cDNA Transfection

Simple epithelia are characterized by polarized distributions of cell adhesion structures and molecules. To determine whether the epithelioid PC9-\textit{αE1} cell layers indeed express epithelial phenotypes, we studied the distribution of several adhesion-related proteins, ZO-1 (tight junction protein), E-cadherin, β1, α2, α3, and α6-integrins, desmoplakin (desmosomal plaque protein), and laminin (basement membrane protein) by conventional or confocal immunofluorescence microscopy. In untransfected PC9 cells, all these molecules were randomly distributed on the cell surface or in the cytoplasm. ZO-1 was detected as a limited number of small clusters with an irregular shape on the surface of the cells (Fig. 2 \textit{a}). E-cadherin (Fig. 2 \textit{b}), β1-integrin (Fig. 2 \textit{c}), and lami-
n (data not shown) were scattered on the cell surface or in the cytoplasm as diffused clusters. The distributions of \( \alpha \)-integrins were similar to the distribution of the \( \beta \)-chain (data not shown). Desmoplakin was detected mostly in the cytoplasm as small clusters (Fig. 2 d), as found in low Ca\(^{2+} \) media (Mattey and Garrod, 1986b; Green et al., 1987; Pasdar and Nelson, 1988).

Interestingly, when ZO-1 and E-cadherin were double-stained, some ZO-1 clusters, but not all of them, were colocalized with E-cadherin staining (compare Fig. 2, a and b); this was observed under two different fixation conditions, methanol treatment alone, and paraformaldehyde fixation followed by methanol treatment. The ZO-1 clustering itself occurred without E-cadherin, since a similar ZO-1 distribution was observed in a population of PC9 cells not expressing E-cadherin, which cells were often observed in the PC9 cultures. On the other hand, the majority of E-cadherin sites were free of ZO-1 (\( \alpha \))-integrin and desmoplakin did not show such an obvious colocalization with E-cadherin on the cell surface, although their colocalization was sometimes observed in the cytoplasm.

In aggregates of PC9-\( \alpha \)-E1 cells, all the above adhesion molecules exhibited polarized distributions. The outer surface of the aggregates was devoid of any of these molecules. ZO-1 was detected in a pinpoint immunofluorescence at the outermost portion of cell–cell contacts (Fig. 3 a), as found in normal simple epithelial cells. The ZO-1 staining was contiguous to E-cadherin staining, as observed in double-immunostained samples (Fig. 3, a and b). E-cadherin was localized to lateral cell–cell contacts (Fig. 3 b), but it tended to be concentrated more in cell–cell contact in the outer regions of many aggregates (Fig. 3 d). On the other hand, \( \beta \)-integrin was detected evenly at cell–cell contacts as well as at the inner free surface of the aggregates; obvious differences between the \( \beta \)-integrin and E-cadherin distributions were observed in double-immunostained samples (compare Fig. 5, c and d). All \( \alpha \)-chains of the integrin studied here colocalized with \( \beta \)-chain (data not shown). Desmoplakin was detected as characteristic punctuated lines at cell–cell boundaries (Fig. 3 e), and laminin was localized in both cell–cell contacts and the inner surfaces of the aggregates, whose pattern was similar to that of integrins (Fig. 3 f). The cellular distributions of all these proteins are quite similar to those in normal simple epithelia, except for laminin; this protein is not found in cell–cell contacts in normal tissues. These results suggest that the \( \alpha \)-E-catenin–transfected PC9 cell layers acquired apical–basal polarity, in which the apical pole was oriented to the outer surface of the aggregates. PC9-\( \alpha \)-E1 cells showed a similar phenotype with regard to the distribution of these cell adhesion molecules. The polarized distribution of cell adhesion molecules in these \( \alpha \)-catenin–transfected PC9 cells was abolished when the cells were cultured in the presence of anti-E–cadherin antibodies (data not shown).

To confirm the above observations, we used electron microscopy. Untransfected PC9 cells did not exhibit any polarized and specialized structures on their surface (data not shown). In contrast, PC9-\( \alpha \)-E1 aggregates showed various polarized structures: in low-power views, we could observe microvilli only on the outer surface of the aggregates (Fig. 4 a), which was reminiscent of the apical surface of normal simple epithelia. Closer examinations, then, demonstrated that tight junction, adherens junction and desmosome were present in this order from the outside in many aggregates (Fig. 4 b). This set of junctional structures was indistinguishable from the typical junctional complex observed in normal simple epithelia (Farquhar and Palade, 1963). However, we could not detect the basement membrane in PC9-\( \alpha \)-E1 aggregates.

**Expression of Cell Adhesion Molecules before and after \( \alpha \)-Catenin Transfection**

We tested whether the expression level of the various cell adhesion molecules examined was changed or not after \( \alpha \)-catenin transfection. Immunoblot and immunoprecipitation analyses showed that there was little difference in the level of ZO-1, laminin, desmoplakin, \( \beta \)-integrin (Fig. 5), or E-cadherin (Fig. 6, lanes 5 and 6) before and after the \( \alpha \)-catenin transfection. These results suggest that cell–cell junctions formed in the \( \alpha \)-catenin transfected cells were organized without additional synthesis of necessary components.

We then examined by immunoprecipitation experiments whether the expression of \( \alpha \)-catenin affected the association of E-cadherin with other catenins. In detergent extracts of PC9 cells, E-cadherin coinmunoprecipitated with \( \beta \)-catenin and plakoglobin (Fig. 6, lanes 1 and 3). In PC9-\( \alpha \)-E1 cells, similar amounts of \( \beta \)-catenin or plakoglobin were coprecipitated with E-cadherin (Fig. 6, lanes 2 and 4). This suggests that the binding of these two proteins to E-cadherin is not affected by \( \alpha \)-catenin. We also tested the possibility that ZO-1 and E-cadherin may be co-purified upon immunoprecipitation, because of their colocalization in PC9 cells, but the results were negative (data not shown).
Further, we tested the detergent solubility of E-cadherin before and after α-catenin transfection. Most of E-cadherin molecules were solubilized with the detergent extraction buffer containing 1% NP-40 and 1% Triton-X in the absence of α-catenin (Fig. 7, A and B, lanes 1 and 2). In PC9-αE1 cells, however, an insoluble fraction of E-cadherin was detected after extraction with the same detergents for 5 to 10 min (Fig. 7 A, lane 4), although this fraction disappeared after a 1-h extraction of the cells (Fig. 7, lane 4). Thus, E-cadherin was more resistant to detergent extraction after α-catenin transfection.

Retarded Growth of the α-Catenin-transfected PC9 Cells

Finally, we studied whether or not proliferation of PC9 cells was affected by α-catenin transfection. As shown in Fig. 8, PC9-αE1 cells grew much more slowly than the parent PC9 cells. To check whether this growth retardation of PC9-αE1 cells is directly associated with the activation of E-cadherin, the effect of blocking antibodies to E-cadherin was examined. By the antibody treatments, the growth of PC9-αE1 cells was considerably enhanced (Fig. 8); two blocking mAbs HECD-1 and SHE78-7 showed a similar effect, but other antibodies, that could not disrupt cell–cell contacts, had no effect. The growth rate of the transfectants in the presence of anti-E-cadherin antibodies was a little lower than that of the parent PC9 cells even in the presence of saturated concentrations of the antibodies. This is probably due to either a possible failure in the complete blocking of E-cadherin by the antibodies or a clonal variation in growth rate between the parent and transfectant lines.

Discussion

Two important findings were made in this work. First, the activation of E-cadherin by means of α-catenin transfection...
of the originally dispersed PC9 carcinoma cells induced polarized cell–cell associations characteristic of simple epithelia. Second, the restoration of E-cadherin–mediated cell–cell contacts in this cell line caused growth retardation. These effects of α-catenin transfection were abolished by treatment with antibodies specific for E-cadherin, indicating that E-cadherin and α-catenin are not separable in their functions. The phenomena observed here should thus be interpreted to be based on a cooperative action of these two molecules, not on the sole action of α-catenin which was exogenously introduced.

The role of α-catenin in cadherin activity is not fully understood. The present study demonstrated that α-catenin transfection of PC9 cells slightly reduced the detergent solubility of E-cadherin. This finding supports the idea that α-catenin may play a role in mediating the interactions of cadherins with the actin-based cytoskeleton (Ozawa et al., 1990; Kemler, 1993). In addition, we found that binding of β-catenin or plakoglobin to cadherins was independent of the presence or absence of α-catenin, as consistent with previous observations (McCrea and Gumbiner, 1991).

Previous studies have suggested that the classic cadherin-mediated cell–cell contact is a prerequisite for the formation of tight junction and desmosome. This idea is supported by indirect evidence such as that tight junction formation is Ca^{2+} dependent (see reviews by Citi, 1993; Anderson et al., 1993) and that the desmosome is sensitive to Ca^{2+} as well (Volk and Geiger, 1986; Pasdar and Nelson, 1986; Mattey and Garrod, 1986a, b; Green et al., 1987; Gumbiner et al., 1988). More direct evidence was obtained by the observation that antibodies to E-cadherin (uvomorulin) inhibited the formation of these junctions (Gumbiner and Simons, 1986; Gumbiner et al., 1988; Fleming et al., 1989; Balda et al., 1993). However, such evidence is not conclusive for the role...
of cadherins in junctional complex formation, because the effect of anti-cadherin antibodies was always only partial in inhibition. Most anti-cadherin antibodies cannot completely block cadherin activity in a given cell; one reason for this inability is that the majority of cells express multiple types of cadherin including unidentified ones, and another reason is that there is a limit in the action of antibodies in blocking cadherin activity, especially when monoclonal antibodies are used. In the present approach, we could overcome these problems by use of a cell line in which normal cadherin activity was genetically blocked (Shimoyama et al., 1992).

Thus, the present study provided the most clear-cut evidence for the requirement of the classic cadherin adhesion system for the formation of the junctional complex.

How can E-cadherin initiate the formation of other junctions? Untransfected PC9 cells expressed all the adhesion molecules studied, and their expression level was not significantly altered following α-catenin transfection. E-cadherin-mediated cell-cell contacts, therefore, seem to have

![Figure 5](image1)

**Figure 5.** Immunoblot analysis of the expression of adhesion proteins in PC9 (lane 1) and PC9-αE1 (lane 2) cells. (A) ZO-1, (B) Laminin, B1- and B2-chain. (C) Two isoforms of desmoplakin. Other bands are perhaps degradation products. (D) β1-integrin. The specific protein bands detected are shown by arrowheads. Ig represents immunoglobulin chains. Whole cell lysates (A and B) or immunoprecipitants obtained with anti-desmoplakin antibody (C) or anti-β1-integrin antibody (D) were subjected to immunoblotting. Polyacrylamide gels of 3.75, 5, and 7.5% were used for A and B, C, and D, respectively. Positions of molecular weight markers are 200, 116, and 97 x 10^3 for A and B; 200, 116, 97, and 66 x 10^3 for C; and 116, 97, 66, and 45 x 10^3 for D.

![Figure 6](image2)

**Figure 6.** Immunoblot analysis of E-cadherin-associated proteins.

An equal number of PC9 cells (lanes 1, 3, 5, and 7) and PC9-αE1 cells (lanes 2, 4, 6, and 8) were detergent-extracted and -soluble proteins were subjected to immunoprecipitation with HECD-1. Immunoprecipitants were detected by immunoblotting with antibodies to β-catenin (lanes 1 and 2), to plakoglobin (lanes 3 and 4), to E-cadherin (lanes 5 and 6), and to α-catenin (lanes 7 and 8). Note that almost equal amounts of β-catenin and plakoglobin relative to E-cadherin were coprecipitated with the E-cadherin, regardless of the binding of α-catenin. Positions of molecular weight markers are 200, 116, 97, 66, and 45 x 10^3.

![Figure 7](image3)

**Figure 7.** Detergent extractability of E-cadherin. PC9 cells (lanes 1 and 2) and PC9-αE1 cells (lanes 3 and 4) were extracted with a mixture of NP-40 and Triton X-100 for 10 min (A) or 1 h (B), and soluble (lanes 1 and 3) and insoluble fractions (lanes 2 and 4) were subjected to immunoblotting with HECD-1. With the 10-min treatment, all E-cadherin molecules in PC9 cells were extracted, while a small fraction of insoluble E-cadherin molecules were detected in PC9-αE1 cells. Positions of molecular weight markers are 200, 116, 97, 66, and 45 x 10^3.

![Figure 8](image4)

**Figure 8.** Effect of α-catenin transfection and anti-E-cadherin antibody treatment on cell growth.

Equal numbers of PC9 cells (PC9) and PC9-αE1 cells (α) were seeded in wells, and the cell numbers were counted 6 and 12 d after incubation without or with 0.1 µg/ml (+ A) or 1 µg/ml (+ 10A) HECD-1. The former concentration was sufficient to block E-cadherin-mediated aggregation of PC9-αE1 cells. The horizontal axis depicts the days after seeding, and the vertical axis common logarithms of cell numbers. Vertical error bars represent standard deviations.
merely induced their structural reorganization. The simplest model for the role of E-cadherin would be that this molecule plays no specific roles in the formation of other junctions, except providing initial cell–cell contacts. Tight junctions and desmosomes could be formed regardless of cadherin function, if two cell membranes were brought into close contacts with each other. On the other hand, it is equally possible that E-cadherin plays a signaling role in the formation of other junctions. A recent study suggested that PKC activation is involved in tight junction formation, and this process may be initiated by E-cadherin–mediated cell contacts (Balda et al., 1993).

We should also consider the possibility that there may be structural links between the E-cadherin–catenin complex and other junctions, and the former may control the assembly of the latter through this system, including their polarized arrangement. In this context, it should be particularly noted that E-cadherin was found to colocalize with ZO-1 on the free surface of PC9 cells, as observed in certain types of cell–cell junctions (Itoh et al., 1993). An important aspect of this finding is that this colocalization occurred between free molecules in the absence of cell–cell contacts. This suggests that these two molecules can interact with one another, directly or indirectly, at the prejunctional stage. Tight junctions and the cadherin-based AJs are formed side by side in most epithelial cells. This may not be coincidental, and the interaction between cadherin and ZO-1 may serve to maintain their intimate spatial relationship. Since we could not copurify ZO-1 and E-cadherin by immunoprecipitation, their interaction, if present, may be not so stable. On the other hand, the formation of desmosomes could be more or less independent of classic cadherin function, as antibodies to classic cadherins do not always disrupt desmosomes once formed (Wheelock and Jensen, 1992; Watabe et al., 1993; Hodivala and Watt, 1994). The present observation that PC9 cells do not form desmosomes without active E-cadherin, however, suggests that desmosomal formation requires at least preceding cell–cell contacts.

We found in the present study that activation of E-cadherin was sufficient to reconstruct an almost complete epithelium-specific architecture in cells which otherwise would have remained disperse. The restored phenotypes included the formation of the junctional complex, the basolateral localization of integrins, and the apical development of microvilli, all of which are characteristic of normal simple epithelia. Previous studies showed that introduction of cadherin cDNA into cells that have no endogenous cadherins was capable of redistributing cell surface proteins into a polarized pattern. In those studies, however, only partial epithelium-specific phenotypes were restored in the transfected cells; for example, L cells transfected with E-cadherin cDNA did not form tight junctions (McNeill et al., 1990). Differences between the present and previous results may be attributed to differences in the cell lines used. PC9 cells are supposed to be of epithelial origin, and must genetically inherit most of their original phenotypes; reactivation of the cadherin system must have triggered the re-expression of these phenotypes. Cell lines like L, which cannot reorganize epithelium-specific structures, may not be of epithelial origin or may have lost genes necessary for epithelial phenotypes. The apical–basal polarity formed in the α-catenin–transfected PC9 cells was similar to that in mouse blastocysts (Fleming et al., 1992), but opposite to that in normal epithelia in which the apical surface faces the lumen enclosed by a cell sheet. A similar inverted polarity is often observed with aggregates of other epithelial cells in vitro (Rodriguez-Boulan and Nelson, 1989), and the orientation of cell polarity seems to depend on cellular environments or intrinsic phenotypes. The only abnormal features in the α-catenin–transfected PC9 cells were the lack of the basement membrane and the unusual distribution of laminin, which was localized all over the basolateral surfaces of these cells. The reason for this phenomenon remains to be studied; the formation of the basement membrane perhaps requires some factors that PC9 cells fail to express.

Concerning the inhibitory effect on cell growth of the activation of E-cadherin, a similar phenomenon was reported based on studies using certain carcinoma lines (Navarro et al., 1991), but such inhibition is not widely seen in other systems. Probably, sensitivity of cell growth to cadherin-mediated cell–cell contacts varies with the cell types examined. Actually, many tumor cells are highly proliferative regardless of their normal cadherin activity and tight cell–cell associations, suggesting their inability to respond to cell contacts. However, the growth of normal cells is known to be contact dependent. From this point of view, it is likely that PC9 cells might have responded to the E-cadherin–mediated cell contacts in a way of normal cells. As to the mechanism of how cadherin-mediated cell–cell contacts affect cell growth, at least four possibilities can be considered. First, cadherin–cadherin interactions at their extracellular domain may generate some signals to regulate cell growth, in a manner as found in a variety of receptor–ligand interactions. The AJ contains molecules that could serve for signaling processes, e.g., members of the Src tyrosine kinase family are localized in this cell junction (Tsukita et al., 1991; Tsukita et al., 1993). Second, cell–cell association mediated by cadherins may trigger interactions of other cell surface receptors with their ligands, leading to growth control. If the ligands are membrane-bound, cadherin-mediated cell–cell contact may be a prerequisite for their interactions with the receptors. It is known that some receptors such as EGF receptors are localized around the AJ (Fukuyama and Shimizu, 1991). Third, cadherins may control cell growth via the induction of tight junction formation. ZO-1, a component of the tight junction, has homology to the product of the Drosophila lethal(l)discs-large (dlg) gene (Woods and Bryant, 1991; Itoh et al., 1993; Willott et al., 1993), which mutation causes abnormal overgrowth of tissues (Woods and Bryant, 1991). Tight junction formation thus could affect cell growth. Fourth, the layers of cells connected with the junctional complex could be less accessible to growth factors in culture medium than dispersed cells. This possibility cannot be ignored when essential growth factor receptors are localized to the basolateral surfaces, especially for closed cystic aggregates in which tight junctions could form a perfect permeability barrier to extracellular factors. Whatever the mechanism is, our present findings indicate that the cadherin-catenin adhesion system may serve as a direct or indirect regulator of cell growth.

In conjunction with the above discussion, an intriguing observation was recently reported, that the tumor suppressor gene APC product binds to β- and α-catenins (Rubinfeld et al., 1993; Su et al., 1993), implying that catenins might be
involved in control of tumorigenic growth through their association with APC. It should also be noted that β-catenin is involved in axis determination in Xenopus embryos (McCrea et al., 1993) and that its Drosophila homologue, Armadillo, is essential for establishment of segment polarity (Riggleman et al., 1990; Peifer and Wieschaus, 1990). Moreover, β-catenin is sensitive to extracellular and intracellular signals; for example, tyrosine phosphorylation of this molecule is induced by treatment of cells with hepatocyte or epidermal growth factor (Shibamoto et al., 1994) as well as by v-src transformation (Matsuyoshi et al., 1992; Hamaguchi et al., 1993; Behrens et al., 1993). These findings suggest that β-catenin itself may play a signaling role in cell growth and differentiation. Recent work suggests that cadherin-dependent cell contacts also regulate cell differentiation (Burdal et al., 1993) and integrin expression (Hodivala and Watt, 1994). These findings support the possibility that cadherins and catenins contribute to signaling processes involved in cell–cell interactions.

In conclusion, the cadherin–catenin system plays a central role in structural and functional organization of epithelial cell–cell contacts. The loss of cadherin activity impairs other adhesion systems, and affects cell growth. Such effects may be implicated in various abnormal behaviors of tumor cells, in which cadherin activity is often down-regulated (Takeichi, 1993). Finally, it should be emphasized that the molecular basis for the classical “contact inhibition” is not yet fully understood. Cadherin-mediated growth suppression is likely related to this very important cellular behavior.

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