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Distribution of the Cadherin-Catenin Complex in Normal Human Thyroid Epithelium and a Thyroid Carcinoma Cell Line

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Abstract E-cadherin is the major cell-cell adhesion molecule expressed by epithelial cells. Cadherins form a complex with three cytoplasmic proteins, α-, β-, and γ-catenin, and the interaction between them is crucial for anchoring the actin cytoskeleton to the intercellular adherens junctions. The invasive behavior of cancer cells has been attributed to a dysfunction of these molecules. In this study, we examined the distribution of the cadherin-catenin complex in a Chinese human thyroid cancer cell line, CGTH W-2, compared with that in normal human thyroid epithelial cells. In the normal cells, using immunofluorescence staining, E-cadherin and α-, β-, and γ-catenin were found to be localized at the intercellular junction and appeared as 135, 102, 90, and 80 kD proteins on Western blots. In CGTH W-2 cells, no E-cadherin and γ-catenin immunoreactivity was detected by immunofluorescence or Western blotting; α- and β-catenin were detected as 102 and 90 kD proteins on blots but gave a diffuse cytoplasmic immunofluorescence staining pattern in most cells, while β-catenin was also distributed throughout the cytoplasm in most cells but was found at the cell junction in some, where it colocalized with α-actinin. The present data indicate that the loss of cell adhesiveness in these cancer cells may be due to incomplete assembly of the cadherin-catenin complex at the cell junction. However, this defect did not affect the linkage of actin bundles to vinculin-enriched intercellular junctions. J. Cell. Biochem. 70:330–337, 1998.

Key words: cadherin; catenins; thyroid carcinoma cell; epithelial cell; cell-cell adhesion

The epithelial cell-cell junction is a specialized region of the plasma membrane in which cadherins act as the transmembrane adhesion molecules and actin filaments are densely associated with the plasma membrane through a well-developed plasmalemmal undercoat [Geiger and Ginsberg, 1991]. Epithelial (E)-cadherin, which is identical to uvomorulin and homologous to L-CAM, cell-CAM 120/80, and Arc-1 [Nagafuchi et al., 1987], is expressed as early as the fertilized egg and is necessary for the development of the blastocyst, the first-formed epithelium of the embryo [Ekilobaseslom et al., 1986]. It consists of a 723–748 amino acid transmembrane polypeptide, the extracellular portion of which binds Ca^{2+}, and E-cadherin molecules of adjacent cells [Takeichi, 1988, 1991]. A recent study has demonstrated that the cytoplasmic portion of the cadherin molecule is associated with three cytosolic proteins, the catenins (α, β, and γ), which play an important role in linking the microfilament network to the cadherins [Ozawa et al., 1989]. In addition to contributing to the mechanical properties of the adherens junction, the cadherin-catenin complex, it is now widely believed, may also play crucial roles in the control of cell growth and tissue morphogenesis by means of signal transduction pathways [Takeichi, 1991].

Studies of the expression of E-cadherin in a number of epithelial cells suggest an important role for this protein in the regulation of cell polarity and in development [Ekilobaseslom et

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al., 1986; Wollner et al., 1992]. Thyrocytes are polarized epithelial cells with a highly restricted distribution of plasma membrane proteins that are segregated into two structurally and functionally distinct domains (apical and basolateral). E-cadherin is located in adherens junctions of the basolateral domain and is responsible for cell-cell adhesion [Yap et al., 1995]. Northern blot analysis and immunofluorescence staining studies on the expression of E-cadherin in thyroid cancers have shown that the immunoreactivity of E-cadherin is variably reduced or even lost when compared with normal thyroid tissue [Brabant et al., 1993; Naitoh et al., 1995; Scheumman et al., 1995]. Gene analysis has shown that allele mutation or loss of the E-cadherin gene are infrequent in thyroid cancers, suggesting that the reduced expression of E-cadherin may be due to an impairment on transcriptional regulation or posttranscriptional modulation [Soares et al., 1997]. Although the loss of E-cadherin expression has been suggested to play a pathogenetic role in thyroid tumor invasion [Reinhard et al., 1997], the distribution of α-, β-, and γ-catenins in thyroid cancer tissue has not been studied.

In the present study, we used immunofluorescence microscopy and Western blot analysis to examine the expression of E-cadherin and α-, β-, and γ-catenin in normal thyroid tissue and in a cancer cell line (CGTH W-2) [Lin et al., 1996], derived from the metastatic area of a Chinese patient with thyroid follicular cancer, to obtain better insight into the intercellular adhesive property of thyroid cancer cells.

MATERIALS AND METHODS

Cell Culture

The CGTH W-2 cell line, derived from the metastatic thyroid follicular carcinoma of a patient and the first Chinese human thyroid cancer line to be established, was a generous gift from Dr. Lin Jen-Der [Lin et al., 1996]. The cells have a doubling time of approximately 18 h and lose virtually all their iodine-concentrating ability and the capacity for thyroglobulin (Tg) synthesis after the sixth passage in culture [Lin et al., 1996]. The culture medium was RPMI 1640 containing 10% fetal calf serum, 1 μM sodium pyruvate, 1% penicillin, and 1% streptomycin (pH 7.2–7.4), and the cells were grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% O_2.

Immunofluorescence Microscopy

Antibody reactions were at 37°C and all other manipulations at room temperature. Cells grown on coverslips were briefly washed in phosphate buffered saline (PBS), fixed in 10% formalin in PBS for 10 min, and permeabilized using 0.1% Triton X-100 in PBS for another 10 min. After a PBS wash, nonspecific binding was blocked by treatment with 5% nonfat milk in PBS for 15 min. Mouse monoclonal antibodies against E-cadherin, β-catenin, or γ-catenin were purchased from Transduction Laboratories (Lexington, KY); mouse monoclonal antibody against vinculin and rabbit antiserum against α-catenin were purchased from Sigma (St. Louis, MO). After a PBS wash, cells were incubated with primary antibodies for 1 h; then bound primary antibodies were visualized by incubation with an appropriate secondary antibody (FITC-conjugated goat antimouse IgG [1:50] or FITC-conjugated goat antirabbit IgG [1:50]) for 1 h. Double-labeling immunofluorescence microscopy was performed using a mixture of rabbit anti–β-catenin antiserum and rat anti–α-actinin antiserum [manuscript in preparation], followed by a mixture of FITC-conjugated goat antirabbit IgG (1:50) and Texas red–conjugated goat antirat IgG (1:50). Double-labeling for β-catenin and F-actin or vinculin and F-actin was performed by incubation with anti–β-catenin or antivinculin antibodies, followed by incubation with Texas red–conjugated goat antimouse IgG (1:50) for 1 h at 37°C and FITC-conjugated phalloidin (Sigma) for another 30 min at room temperature. After extensive washes with PBS, the cells were mounted on the slide using PBS containing 2% n-propyl gallate and 60% glycerol, pH 8.0, and sealed with nail polish. Smear preparations of normal human thyroid tissue obtained from patients undergoing surgery for thyroid problems were also processed for immunofluorescence microscopy. Specimens were examined using a Reichert Polyvar 2 microscope (Leica, Vienna, Austria), equipped for epifluorescence, and were photographed using Kodak T-Max 400 film.

Western Blotting

CGTH W-2 cells were hypotonically lysed, and thyroid tissue was homogenized in lysis buffer O [O'Farrell, 1975] containing 5 mM phenylmethylsulfonylfluoride. Gel electrophore-

sis and immunoblotting were performed as described by Fritz et al. [1989] and Towbin et al. [1979]. Proteins were electrophoretically transferred to nitrocellulose membrane at 200 V for 1 h and the nitrocellulose membrane sheets blocked using 5% nonfat milk in PBS containing 0.1% Tween-20 for 1 h at room temperature. After washing in Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 8.2) containing 0.1% Tween-20 (TBS-Tween), the blots were incubated overnight at 4°C with a 1:500 dilution of primary antibodies consisting of mouse monoclonal antibodies specific for E-cadherin, β-catenin, or γ-catenin (Transduction Laboratories) or a rabbit polyclonal anti–α-catenin antiserum (Sigma). After an extensive wash with TBS-Tween, the blots were incubated for 2 h at 37°C with a 1:7,500 dilution of alkaline phosphatase (AP)–conjugated goat antimouse IgG or AP-conjugated goat antirabbit IgG (both from Promega, Madison, WI), as appropriate. Reactive bands were visualized by substrate development using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in Tris buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris, pH 9.5).

RESULTS

Immunostaining of smear preparation of normal thyroid tissue showed E-cadherin to be expressed only by the thyroid follicular epithelium and to be confined to the interthyrocyte intercellular junction (Fig. 1A). Staining with antibodies specific for the E-cadherin–associated proteins, α-, β-, or γ-catenin, was also localized to the cell junction between thyrocytes (Fig. 1B–D). The expression of E-cadherin and the three associated proteins in normal thyroid tissue was further examined using Western blots. Analyses using anti–E-cadherin antibody

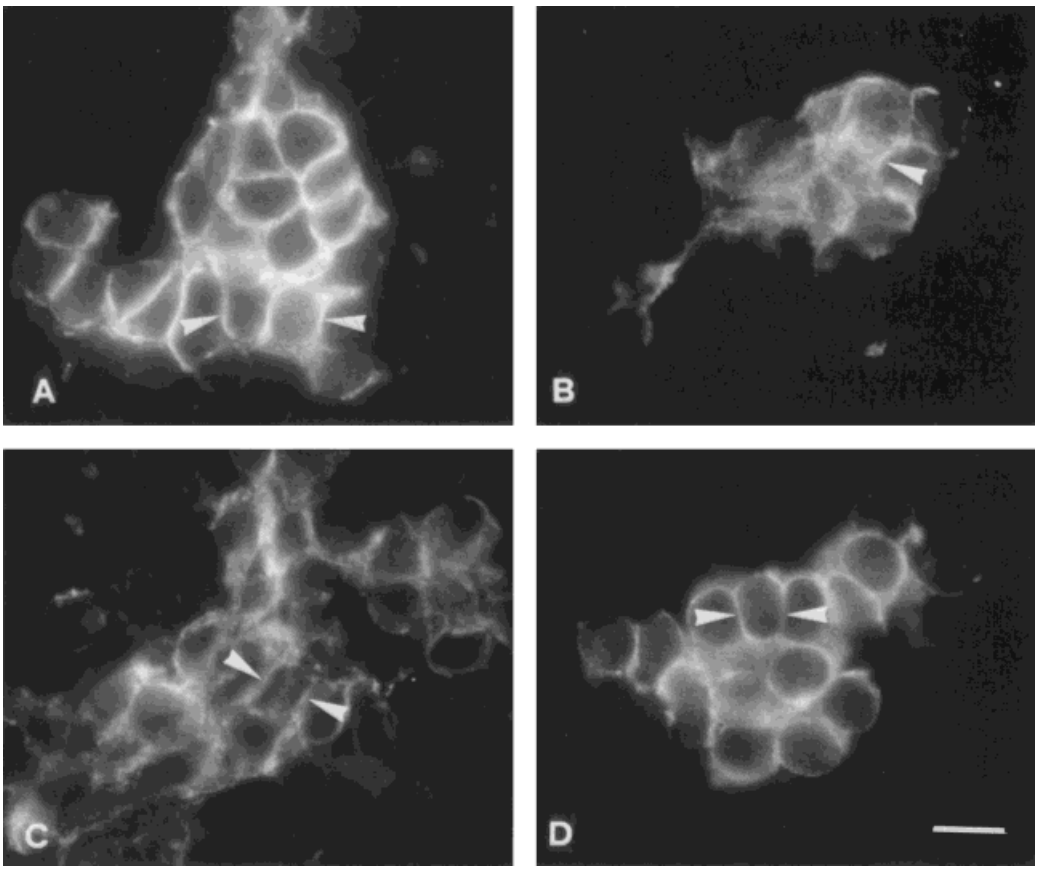

Fig. 1. Immunofluorescence staining for E-cadherin and α-, β-, and γ-catenin in a smear preparation of normal human thyroid tissue. The contact surfaces (arrowheads) of the thyroid epithelial cells are positively stained for E-cadherin (**A**), α- (**B**), β- (**C**), and γ-catenin (**D**). Scale bar = 10 μm.

showed that a normal amount of E-cadherin was present in the thyroid tissue (Fig. 2 lane b) and that α-, β-, and γ-catenin were all visualized on the blots using specific antibodies (Fig. 2, lanes c–e).

In contrast, immunofluorescence studies on the follicular thyroid cancer cell line (CGTH W-2) showed no E-cadherin staining and a diffuse cytoplasmic staining pattern for α-catenin (Fig. 3C,D). A rabbit polyclonal antipan cadherin antibody, which recognizes members of cadherin family, also failed to detect any cadherin in these cells by immunoblotting and immunostaining (data not shown). Most of the cells exhibited cytoplasmic staining for β-catenin; however, about 10% of the cell population showed a normal β-catenin distribution at cell-cell junctions (Fig. 3E,F). γ-catenin was not detected in these cells by immunostaining (Fig. 3G,H). On Western blot analysis, E-cadherin was again undetectable (Fig. 4, lane a), but anti–α-catenin antibody reacted with a band of 102 kD molecular weight and with another band of lower molecular weight, probably a degradation product of α-catenin (Fig. 4, lane b); anti–β-catenin antibody reacted with 90 kD β-catenin (Fig. 4, lane c), whereas no band was detectable using anti–γ-catenin antibody (Fig. 4, lane d).

On the basis of immunofluorescence and biochemical analyses of the cadherin-catenin complex–related proteins, only β-catenin and α-catenin were detectable in CGTH W-2 cells; in addition, the distribution of the latter was unusual. Since α-catenin is responsible for the binding of β-catenin to vinculin, we further examined the interactions between β-catenin and F-actin, β-catenin and α-actinin, and vinculin and F-actin in the absence of α-catenin by double-labeling immunofluorescence (double-labeling for vinculin and β-catenin was not possible, as both primary antibodies were of the same mouse IgG subclass). Small actin filament bundles were found to be vertically inserted onto β-catenin–positive junctions (Fig. 5A,B), indicating that the binding of the actin cytoskeleton to the cell membrane occurred normally. α-actinin colocalized with β-catenin at the cell junction (Fig. 5C,D) and probably contributed to the direct binding of the actin cytoskeleton to the vinculin. Vinculin, seen as oval patches at all cell junctions and at focal adhesion plaques, was the insertion site for the actin filament bundles (Fig. 5E,F).

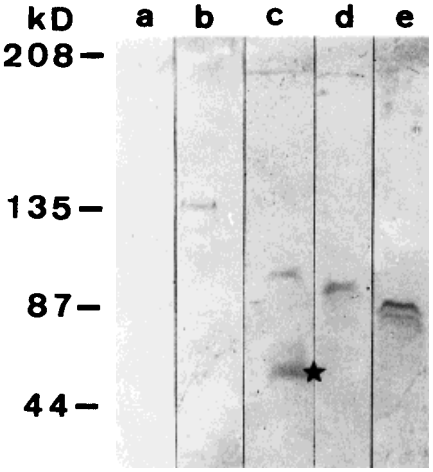

Fig. 2. Immunoblot analysis for E-cadherin and α-, β-, and γ-catenin in normal thyroid tissue. Anti–E-cadherin antibody reacts with a protein band with an apparent molecular weight of 135 kD (**lane b**). Anti–α-catenin antibody reacts with 102 kD α-catenin and a low molecular weight protein (*star*), probably a degradation product of α-catenin (**lane c**). Anti–β-catenin antibody reacts with 90 kD β-catenin (**lane d**). Anti–γ-catenin antibody reacts with 80 kD γ-catenin (**lane, e**). **Lane a:** Blank control without primary antibody showing a clear background. The positions of the molecular weight markers are indicated on the left of the blot.

DISCUSSION

In this study, we examined the distribution of the cadherin-catenin complex in normal human thyroid epithelial cells and in a Chinese thyroid cancer cell line, CGTH W-2. In the normal cells, a typical distribution of E-cadherin and α-, β-, and γ-catenin in the lateral cell membrane was seen. In contrast, in thyroid cancer cells, incomplete assembly of the cadherin-catenin complex at the cell junction was seen, as shown by the lack of E-cadherin and γ-catenin immunoreactivity; α-catenin was present in the cytosol, while β-catenin was also found in the cytosol and occasionally at the cell junction. The present observation correlates well with the loss of contact inhibition and invasive behavior seen with this cancer cell line, since subcutaneous transplantation of CGTH cells into severe combined immunodeficient mice generated invasive and metastatic tumors in the infrahepatic area and the omentum [Lin et al., 1996].

An absence of E-cadherin and γ-catenin has been reported in gastric carcinoma [Ochiai et

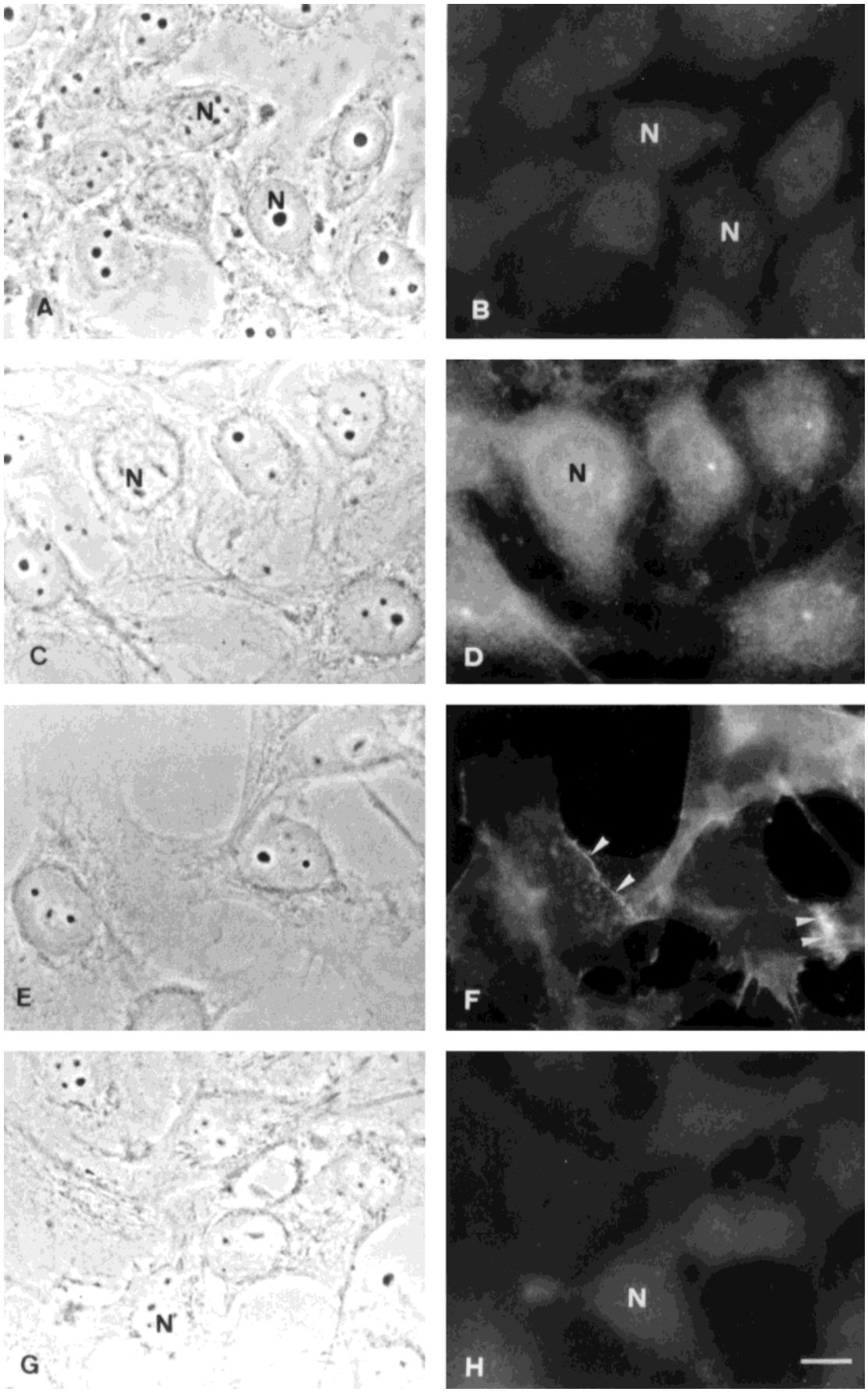

Figure 3.

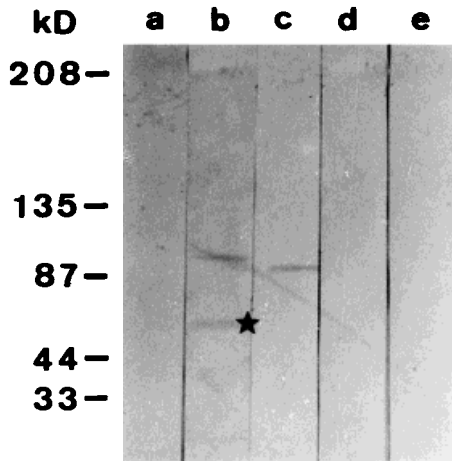

Fig. 4. Immunoblot analysis for E-cadherin and α-, β-, and γ-catenin in CGTH W-2 cells. No immunoreactivity is seen with antibody against E-cadherin (**lane a**). Anti–α-catenin antibody reacts with 102 kD α-catenin and a low molecular weight protein (*star*), probably a degradation product of α-catenin (**lane b**). Anti–β-catenin antibody reacts with 90 kD β-catenin (**lane c**). Anti–γ-catenin antibody fails to detect γ-catenin (**lane d**). **Lane e:** Blank control without primary antibody showing a clear background. The positions of the molecular weight markers are indicated on the left of the blot.

al., 1994]. Currently, we cannot determine whether the lack of E-cadherin and γ-catenin immunoreactivity is due to an epitope change resulting from gene mutation of these molecules, preventing their recognition by the monoclonal antibodies used, or the result of the defective transcription or translation of these proteins. An oncogene, Wnt-1, is able to regulate the expression of both E-cadherin and γ-catenin [Bradley et al., 1993]. Whether the present observation of an apparent lack of expression of E-cadherin and γ-catenin in this cell line is Wnt-1 gene–related requires further investigation.

In 10% of CG cancer cells, β-catenin was present at the cell junction. However, in the majority of cells, diffuse cytosolic staining, of variable intensity, was noted, indicating an increase in the free form of β-catenin. β-catenin is involved in both cell adhesion and intracellular signaling [Ilyas et al., 1997], since it forms a complex with transcription factor Tcf-4, to inhibit apoptosis and stimulate cell proliferation, and another transcription factor, LEF-1, to regulate E-cadherin gene expression via binding to the promotor region of the E-cadherin gene in vitro [Behrens et al., 1996; Huber et al., 1996; Peifer, 1997]. The gene product of a tumor suppressor gene, adenomatous polyposis coli (APC), is able to bind β-catenin but not E-cadherin and thus regulate cell adhesion and cell signaling [Rubinfeld et al., 1993]. An increase in the free form of β-catenin has been demonstrated in several cancer cell types, in which the APC gene was mutated, the Wnt-1 gene highly expressed [Ilyas et al., 1997], or β-catenin mutated [Ilyas et al., 1997]. Since no E-cadherin expression was detected in CGTH W-2 cells, a protein equivalent to E-cadherin in terms of β-catenin binding to the cell membrane may be present in these cancer cells.

α-Catenin is also an APC-binding protein [Su et al., 1993] and thus may regulate the activity of the APC product in a similar way to β-catenin. α-catenin appeared in a diffuse cytosolic form in CGTH W-2 cells, suggesting a decreased affinity for β-catenin, probably due to a structural change in either β- or α-catenin themselves, affecting their interaction. Reduced expression of α-catenin has been reported in gastric carcinomas with invasive tendencies [Ochiai et al., 1994], and deletion or mutation of the α-catenin gene has been seen in a human lung cell line [Shimoyama et al., 1992]. Thus, a lack of this protein has been implicated in tumorogenesis [Hirano et al., 1992].

Since α-catenin is important in mediating the binding between β-catenin and the vinculin–α-actinin complex [Tsukita and Nagafuchi, 1992], the cadherin complex cannot associate with the actin cytoskeleton if α-catenin is inactivated or absent; however, as shown by double-labeling, the actin cytoskeleton was still associated with β-catenin in these cells, and it is possible that a linker protein may be present in the cell membrane and replace the function of α-catenin. Vinculin appeared normally distributed as small oval plaques at focal adhesions and near the lateral cell membrane and was associated with one end of the actin bundles. Thus, the interactions between vinculin and actin and between vinculin and the cell membrane can occur in the absence of effective cell adhesion.

Fig. 3. Immunofluorescence staining for E-cadherin and α-, β-, and γ-catenin in CGTH W-2 cells. Panels **A, C, E,** and **G** are the phase-contrast pairs of panels **B, D, F,** and **H,** respectively. B: No E-cadherin immunoreactivity is seen. D: α-catenin immunoreactivity is detected as diffuse cytoplasmic staining. F: β-catenin immunoreactivity is localized at the cell surface with an increased intensity at cell-cell contact sites (arrowheads). H: γ-catenin immunoreactivity is seen in these cells. N, nucleus. Scale bar = 10 μm.

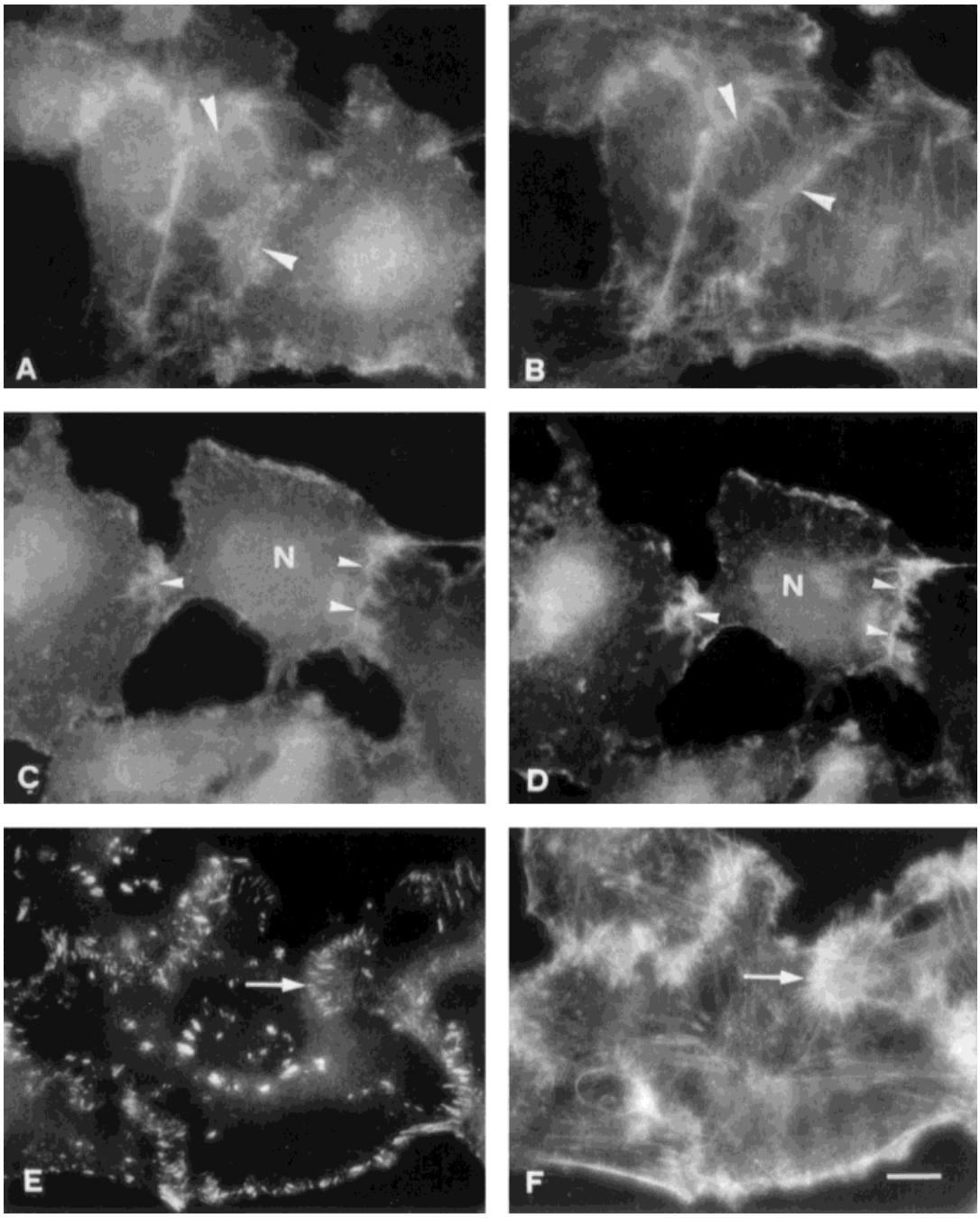

Fig. 5. Staining of catenin-associated proteins in CGTH W-2 cells. **A,B:** Double-labeling for β-catenin and actin filaments (FITC-phallotoxin). **C,D:** Double-labeling for β-catenin and α-actinin. **E,F:** Double-labeling for vinculin and actin filaments. Arrowheads and arrows indicate the location of cell-cell function. N, nucleus. Scale bar = 10 μm.

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