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E-, P-, and N-Cadherin Are Co-expressed in the Nasopharyngeal Carcinoma Cell Line TW-039

Pei-Jen Lou,[1,3] Wen-Pin Chen,[1] Chin-Tarng Lin,[2] Robert M. DePhilip,[4] and Jiahn-Chun Wu[1]*

[1]Department of Anatomy, College of Medicine, National Taiwan University, Taipei 10018, Taiwan
[2]Department of Pathology, College of Medicine, National Taiwan University, Taipei 10018, Taiwan
[3]Department of Otolaryngology, College of Medicine, National Taiwan University, Taipei 10018, Taiwan
[4]Department of Anatomy and Medical Education, College of Medicine and Public Health, Ohio State University, Columbus, Ohio 43210

Abstract The cadherin/catenin complex plays a key role in the initiation of cell-cell recognition, and adhesion, and the elaboration of structural and functional organization in multicellular tissues and organs. It is associated with tumor metastasis and also acts as an ''invasion suppressor'' of cancer cells. Nasopharyngeal carcinoma (NPC) is notorious for its highly metastatic nature. The expression of the E-cadherin/catenin complex is down-regulated in NPC tumor specimens. To obtain better insight into the intercellular adhesive property of NPC cells, we used immunofluorescence microscopy, immunoprecipitation, and immunoblot analysis to examine the expression of the classical cadherins and β-catenin in a NPC cell line, TW-039. The results demonstrate a change in the distribution of E-cadherin from cytosolic flakes to cell-cell contacts with increasing time in culture. Between days 1 and 5 after plating, the detergent-insoluble fraction of E-cadherin increased from 20% to 37% of total E-cadherin, and that for P-cadherin increased from 33% to 40%. By contrast, the values for β-catenin remained unchanged (26% and 25%). Both immunofluorescence and immunoblot studies suggested that P-cadherin may be involved in pioneer contact adhesion of TW-039 cells. Interestingly, E-, P-, and N-cadherin are co-expressed in this cell line. Immunoprecipitation studies also showed that other members of the cadherin family may be involved in the contact adhesion of TW-039 cells. J. Cell. Biochem. 76:161–172, 1999.

Key words: cadherin; β-catenin; nasopharyngeal carcinoma; immunocytochemistry; immunoprecipitation; immunoblot

Cadherins constitute a gene family of Ca^{2+}-dependent cell-cell adhesion molecules that are important in tissue morphogenesis, cell polarity, and tumor invasiveness [Takeichi, 1991; Mareel et al., 1997]. Most members of the cadherins contain a large extracellular domain, a single-pass transmembrane segment, and a cytoplasmic portion. The cytoplasmic portion is associated with a cytoplasmic pool of proteins, the catenins [Ozawa et al., 1989; Tsukita et al., 1992]. Among the catenins, β- and γ-catenin (plakoglobin) associate directly with the cadherins and α-catenin, which, in turn, associates with the actin-based cytoskeleton [Nathke et al., 1994]. This linkage between the cadherin/catenin complex and actin filaments is required for the functional integrity of the cadherins.

Of the members of the cadherin family, E-cadherin is distributed widely and is the most important intercellular adhesion molecule in epithelial cells [Takeichi, 1990]. In cancer cells, early studies showed that inhibiting E-cadherin activity with function-perturbing antibodies or cadherin-specific antisense mRNA transformed noninvasive cells into invasive cells [Behrens et al., 1989; Vlemincks et al., 1991]. In addition, invasive carcinoma cells can converted into a noninvasive phenotype by transfection with a cDNA encoding E-cadherin [Frixen et al., 1991]. Over the past decade, numerous studies have addressed the association between cadherin expression and tumor invasiveness and metastasis [reviewed by

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*Correspondence to: Jiahn-Chun Wu, Department of Anatomy, College of Medicine, National Taiwan University, 1–1 Jen-Ai Road, Taipei 10018, Taiwan. E-mail: jcwu@ha.mc.ntu.edu.tw

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Shiozaki et al., 1996]. Taken together, their results support an association between down-regulation of E-cadherin expression and tumor dedifferentiation, increased invasiveness, and a high incidence of lymph node metastasis in various human carcinomas.

P-cadherin was first purified from a mouse visceral endodermal cell line, PSA5-E, and is expressed most abundantly in the mouse placenta and uterine decidua [Nose and Takeichi, 1986]. P-cadherin expression is transient in many tissues, whereas its permanent expression is restricted to certain tissues, such as the mesothelium, the corneal endothelium, and the basal layers of the epidermis [Nose and Takeichi, 1986]. Whereas both P- and E-cadherin have been shown to be associated with catenins in human epidermoid carcinoma A-431 cells, they appear to be present in separate cadherin/catenin complexes [Johnson et al., 1993]. Based on the fact that monoclonal antibodies directed against E- or P-cadherin disturb the normal organization of the mouse skin [Hirai et al., 1989] and the stratification of cultured keratinocytes [Wheelock and Jensen, 1992], these two cadherins are thought to play a pivotal role in the maintenance of epidermal structure. Although E-cadherin has been thought to play a role as an invasion suppressor of cancer cells, the expression and functional roles of P-cadherin in cancer cells have not been clearly defined. Previous studies have demonstrated altered E- and P-cadherin expression in murine p53-null papillomas [Cano et al., 1996], human skin cancers [Shirahama et al., 1996], and breast carcinomas [Palacios et al., 1995], in which E-cadherin expression is generally reduced, whereas P-cadherin expression is increased. This finding suggests a relationship between reduced expression of E-cadherin and the invasive and metastatic features of carcinomas and between increased expression and abnormal distribution of P-cadherin and the proliferative nature of cancer cells.

N-cadherin is a 127-kDa protein expressed in neuroectodermal and mesodermal derived tissues [Hatta and Takeichi, 1986]. Previous studies have demonstrated involvement of N-cadherin in developmental morphogenesis [reviewed by Takeichi, 1991] and adherens junction assembly of cardiomyocytes [Volt and Geiger, 1986]. In cancer cells, N-cadherin is found in tumors of mesodermal and neuroectodermal origin, such as pleural mesotheliomas [Han et al., 1997], astrocytomas, and glioblastomas [Shinoura et al., 1995]. Anomalous expression of N-cadherin in carcinoma cells is a rare phenomenon and is thought to be associated with an invasive nature and scattered fibroblastic phenotypes [Islam et al., 1996]. However, the expression and functional roles of N-cadherin in carcinoma cells have not been satisfactorily studied.

Nasopharyngeal carcinoma (NPC) is a rare tumor in most parts of the world, but it occurs at high rates in southeast Asia. Unlike other head and neck malignancies, NPC is notorious for its highly metastatic nature [Hsu and Tu, 1983]. In a previous study, we studied the expression of the E-cadherin/catenin complex in biopsy specimens from a series of untreated NPC patients and showed that down-regulated expression of this complex is associated with the occurrence of tumor intracranial invasion [Lou et al., 1999]. To obtain better insight into the intercellular adhesive property of NPC cells, we have now used immunofluorescence microscopy, immunoprecipitation, and immunoblot analysis to examine expression of the classical cadherins and β-catenin in a NPC cell line, TW-039 [Lin et al., 1990]. The results demonstrate a change in the distribution of E-cadherin with increasing time in culture. Detergent extractability of E-, P-cadherin and β-catenin was also checked. Interestingly, all three members of the classical cadherins were co-expressed in this cell line. Immunoprecipitation studies also revealed that other members of the cadherin family may be involved in contact adhesion of TW-039 cells.

MATERIALS AND METHODS

Cell Culture

The nasopharyngeal carcinoma cell line, NPC TW-039 [Lin et al., 1990], was grown in Dulbecco's modified Eagle's medium (DMEM) containing 1.8 mM Ca^{2+} and 10% fetal calf serum (FCS) in a 5% CO_2 atmosphere at 37°C. Cells at confluence (5×10^5 cells/cm^2) were plated either on coverslips in 35-mm Petri dishes or in 25-cm^2 flasks (Costar, Cambridge, MA) and maintained for 1 or 5 days. The cells grown on coverslips were processed for immunofluorescence, whereas those in the flasks were processed for immunoprecipitation and immunoblotting. All data were obtained from at least three independent experiments.

Antibodies

Mouse monoclonal antibodies against E-cadherin (C20820), P-cadherin (C24120), or β-catenin (C19220) were purchased from Transduction Laboratories (Lexington, KY). Rabbit anti-β-catenin antiserum (C-2206), anti-pan-cadherin antiserum (C-3678), and TRITC-conjugated phalloidin were purchased from Sigma Chemical Co. (St. Louis, MO). Mouse monoclonal antibody (13A9) recognizing N-cadherin was generated against a fusion protein containing a cytoplasmic fragment of human N-cadherin [Knudsen et al., 1995].

Detergent Extraction of Cells

Detergent extraction of cells was performed as described previously, with slight modification [Nagafuchi and Takeichi, 1988]. Cultured cells were washed three times with ice-cold Hepes-buffered magnesium-free saline (HMF: 10 mM Hepes and 1 mM $CaCl_2$), collected into an Eppendorf microtube by scraping with a rubber policeman, and then centrifuged at 14,000*g* for 5 min. After removal of the supernatant, 100 µl of 0.5% Nonidet P-40 (NP-40) in HMF was added to the pellet. The samples were then incubated for 5 min with mild pipetting and centrifuged at 14,000*g* for 30 min; 2× Laemmli sample buffer was added to the supernatant, for a total volume of 250 µl; this was then used as the detergent-soluble fraction, whereas the pellet fraction was dissolved in 250 µl of 1× Laemmli sample buffer and used as the detergent-insoluble fraction. Both fractions were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and processed for immunoblotting.

Immunocytochemistry

All antibody reactions were at 37°C, whereas all other manipulations were at room temperature. Cells grown on coverslips were washed briefly 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% skimmed milk in PBS for 30 min; the cells were then incubated for 2 h with mouse monoclonal antibody against E-cadherin and rabbit anti-β-catenin antiserum or mouse monoclonal antibody against P-cadherin and rabbit anti-β-catenin antiserum. After thorough washing, bound primary antibodies were visualized by incubation for 1 h at 37°C with a mixture of FITC-conjugated goat anti-mouse IgG (Sigma) and Texas red-conjugated goat anti-rabbit IgG (Sigma). Double-labeling for β-catenin and F-actin was performed by incubation with mouse monoclonal antibody against β-catenin, followed by incubation with FITC-conjugated goat anti-mouse IgG for 1 h at 37°C and TRITC-conjugated phalloidin for an additional 30 min at room temperature. After extensive washes with PBS, the cells were mounted on a slide, using PBS containing 2% n-propyl gallate and 60% glycerol, pH 8.0, sealed with nail polish, viewed using a Zeiss Axiophot microscope equipped with epifluorescence, and photographed using Kodak T-Max 400 film. Cells treated with detergent, with prior fixation, were stained as follows. Cultures were incubated with 0.5% NP-40 (NP-40; Sigma) in HMF for 5 min at 4°C. After thorough washing with ice-cold HMF to remove NP-40, the cells were then fixed with 10% formalin, blocked with 5% skimmed milk, and processed for antibody incubation as described above.

Immunoprecipitation

Monolayers of cells were washed 3 times with PBS at 4°C, and each 25-cm^2 flask was extracted at 0°C for 30 min with 1 ml of detergent solution (10 mM Tris-HCl, pH 7.5, 0.5% NP-40, 1 mM EDTA, and 1 mM phenylmethylsulfonylfluoride [PMSF]). Insoluble materials were removed by centrifugation at 12,000*g* for 10 min. The supernatant was transferred to a new microtube and mixed with 1 µg of monoclonal anti-β-catenin at 4°C. After 1 h, Protein G-Sepharose (Pharmacia, Uppsala, Sweden) was added and mixing continued for another 30 min. The Sepharose-bound immune complexes were collected by centrifugation and washed 5 times with detergent solution. The pellets were boiled in Laemmli sample buffer and the bound proteins separated by SDS-PAGE.

Immunoblotting

Gel electrophoresis and immunoblotting were performed as previously described [Fritz et al., 1989; Towbin et al., 1979] using a 10% resolving gel and a 3.5% stacking gel. SDS-PAGE-separated proteins from cell extracts or immunoprecipitates were transferred electrophoretically to a nitrocellulose membrane and the membrane blocked with 5% skimmed milk. The vari-

ous proteins of interest were detected using specific monoclonal or polyclonal antibodies as indicated, followed by species-specific alkaline phosphatase-conjugated antibodies (Promega, Madison, WI). The immunoreactive bands were visualized by substrate development using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as previously described [Wu et al., 1993]. Quantification of the resultant bands was performed by densitometer analysis (IS-1000 Digital Imaging System).

RESULTS

Immunocytochemical Localization of E- and P-Cadherin and β-Catenin in TW-039 Cells

Confluent monolayers of NPC TW-039 cells displayed the typical cobblestone appearance of squamous epithelial cells. One day after plating, E-cadherin staining exhibited a unique flake pattern and was diffusely distributed in the cytosol (Fig. 1A,C). β-Catenin immunoreactivity was mainly located at cell-cell contacts (Fig. 1B,D). Colocalization of E-cadherin and β-catenin could be seen at cell-cell contacts in confluent areas (Fig. 1A,B). Detection of β-catenin immunoreactivity without colocalization of E-cadherin was occasionally seen in some cells at the leading edges of subconfluent areas (Fig. 1C,D). Five days after plating, all the cells showed colocalization of E-cadherin and β-catenin at cell-cell contact sites (Fig. 2A–F). Cytosolic flake staining for E-cadherin was not observed at compact areas of cell aggregates (Fig. 2A) but was apparent at areas of lower condensation (Fig. 2E) and was perijunctionally distributed (Fig. 2C) at areas of intermediate condensation. P-cadherin staining in cell-cell contacts colocalized well with β-catenin both at 1 day (Fig. 3A,B) and at 5 days after plating (Fig. 3C,D). In addition, 5 days after plating, the immunoreactivity of P-cadherin was as strong as that of E-cadherin (cf. Figs. 2A,C, 3C).

Immunofluorescence Study of E-Cadherin and β-Catenin in TW-039 Cells Extracted With NP-40

Cross-linking of the cadherin/catenin complex by the actin cytoskeleton is of prime importance in mediating cell-cell adhesion. The cytoskeleton-bound cadherin/catenin complex is thought to be resistant to detergent extraction and this detergent-insoluble pool of proteins is important in maintaining adhesion. We therefore performed nonionic detergent extraction (NP-40) of TW-039 cells 1 day (Fig. 4A,B,E–G) or 5 days (Fig. 4C,D,H) after plating. Immunocytochemical staining demonstrated colocalization of E-cadherin and β-catenin at cell-cell contacts (Fig. 4A–D); the diffuse flake staining of E-cadherin was reduced in detergent-extracted cells (Fig. 4A,C). However, the actin cytoskeleton was quite resistant to detergent extraction. A double-labeling study of detergent-extracted cells showed colocalization of F-actin and β-catenin at cell-cell contacts (Fig. 4E,F). After detergent extraction, P-cadherin staining was also noted at cell-cell contacts either 1 day (Fig. 4G) or 5 days (Fig. 4H) after plating.

Immunoblot Analysis of Detergent Extractability of E- and P-Cadherin and β-Catenin in TW-039 Cells

The immunofluorescence study demonstrated the presence of detergent-soluble and detergent-insoluble pools of E-cadherin in TW-039 cells. Because the detergent-insoluble pool of proteins is important in maintaining adhesion, biochemical studies were warranted to examine the detergent extractability of E- and P-cadherin and β-catenin in TW-039 cells with special reference to adhesion functions, so immunoblot analysis was performed. One day after plating, the detergent-insoluble pools represented 20%, 33%, or 26%, respectively, of the total E- or P-cadherin or β-catenin (Fig. 5A); 5 days after plating, these values were 37%, 40%, and 25%, respectively (Fig. 5B). Quantitative analysis (Fig. 5C) demonstrated a significant increase in the detergent-insoluble pool of E-cadherin 5 days after plating (Student's *t*-test, $P < 0.01$). The detergent-insoluble pool of P-cadherin also increased after prolonged culture, but the increase was not statistically significant. By contrast, the detergent-insoluble fraction of β-catenin remained unchanged over the 5-day period.

E-, P-, and Other Cadherins Could Be Involved in Contact Adhesion of TW-039 Cells

The cadherin family consists of a group of proteins with similar biochemical properties. Of this family, E- and P-cadherin are the two classical cadherins found in epithelial cells. A previous study showed that both E- and P-cadherin have the ability to promote tumor cell cohesion [Foty and Steinberg, 1997]. In TW-039 cells, only 37% of the E-cadherin molecules were NP-40-insoluble after prolonged culture.

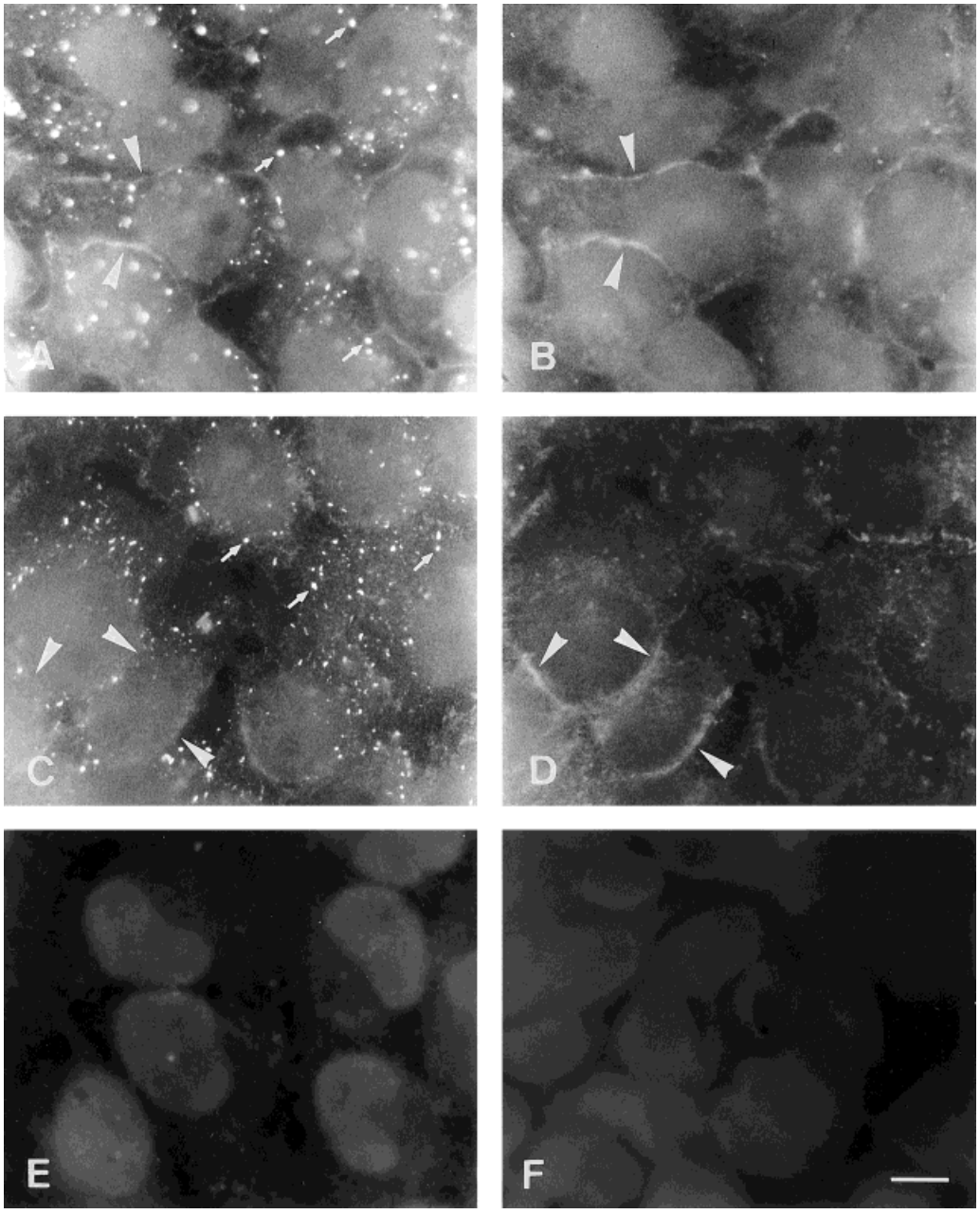

Fig. 1. Immunofluorescence localization of E-cadherin and β-catenin in TW-039 cells 1 day after plating. TW-039 cells double-labeled for E-cadherin **(A,C)** and β-catenin **(B,D)**. At confluent areas **(A,B)**, E-cadherin **(A)** colocalizes with β-catenin **(B)** at cell-cell contacts (arrowheads, **A,B**). The leading edges of subconfluent areas **(C,D)** show contact staining of β-catenin (arrowheads, **D**) without colocalization of E-cadherin (arrowheads, **C**). Most cells exhibit a cytosolic flake pattern of E-cadherin staining (arrows, **A,C**). Negative controls were performed by omission of the primary antibodies and subsequent labeling with FITC- **(E)** or Texas red- **(F)** conjugated secondary antibodies. Scale bar = 10 μm.

Such a low percentage suggests that other members of the cadherin family might be present in TW-039 cells. Because anchorage of the cadherins in a cell is known to occur by means of β-catenin, we immunoprecipitated β-catenin and probed the precipitate with pan-cadherin antiserum to see whether other members of the cadherin family were involved in the contact adhesion of TW-039 cells. Immunoblot study demonstrated that both E- and P-cadherin coimmunoprecipitated with β-catenin (Fig. 6, lanes b,c). Large amounts of proteins of the cadherin family, with a molecular mass of about 130 kDa, were detected by probing with anti-pan-

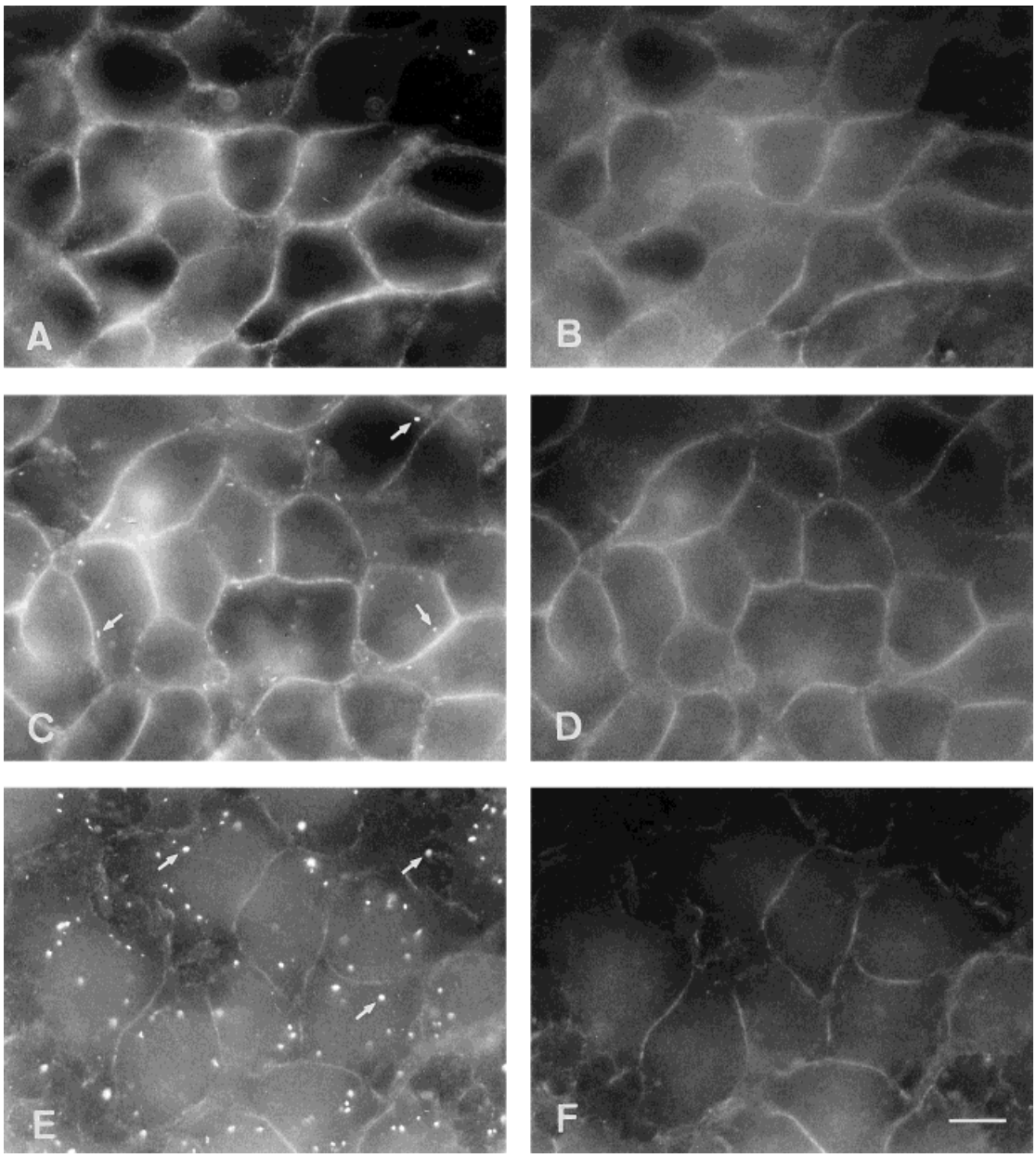

Fig. 2. Immunofluorescence localization of E-cadherin and β-catenin in TW-039 cells 5 days after plating. TW-039 cells double-labeled for E-cadherin **(A,C,E)** and β-catenin **(B,D,F)**. Strong E-cadherin **(A,C)** immunoreactivity colocalizes with β-catenin (B, D) at cell-cell contacts of compact **(A,B)** and intermediately condensed areas **(C,D)**. A weaker E-cadherin **(E)** and β-catenin **(F)** immunoreactivity colocalizes in cell-cell contacts at areas of less condensation **(E,F)**. Arrows **(C)** indicate the perijunctional staining of E-cadherin. Arrows **(E)** indicate the cytosolic flake pattern of E-cadherin. Scale bar = 10 μm.

cadherin antisera (Fig. 6, lane a). This result shows that other members of the cadherin family could be involved in the contact adhesion of NPC TW-039 cells.

N-Cadherin Is Also Expressed in TW-039 Cells

E-, P-, and N-cadherin are the classical cadherins. The molecular mass of N-cadherin is about 127 kDa [Hatta et al., 1985], similar to that of the cadherin detected in NPC TW-039 cells using pan-cadherin antisera (Fig. 6, lane a). Immunoblot analysis was thus employed to check classical cadherin expression in TW-039 cells and in two other commonly-used epithelial carcinoma cell lines: A-431, a vulvar epidermoid carcinoma cell line, and HeLa, a cervical

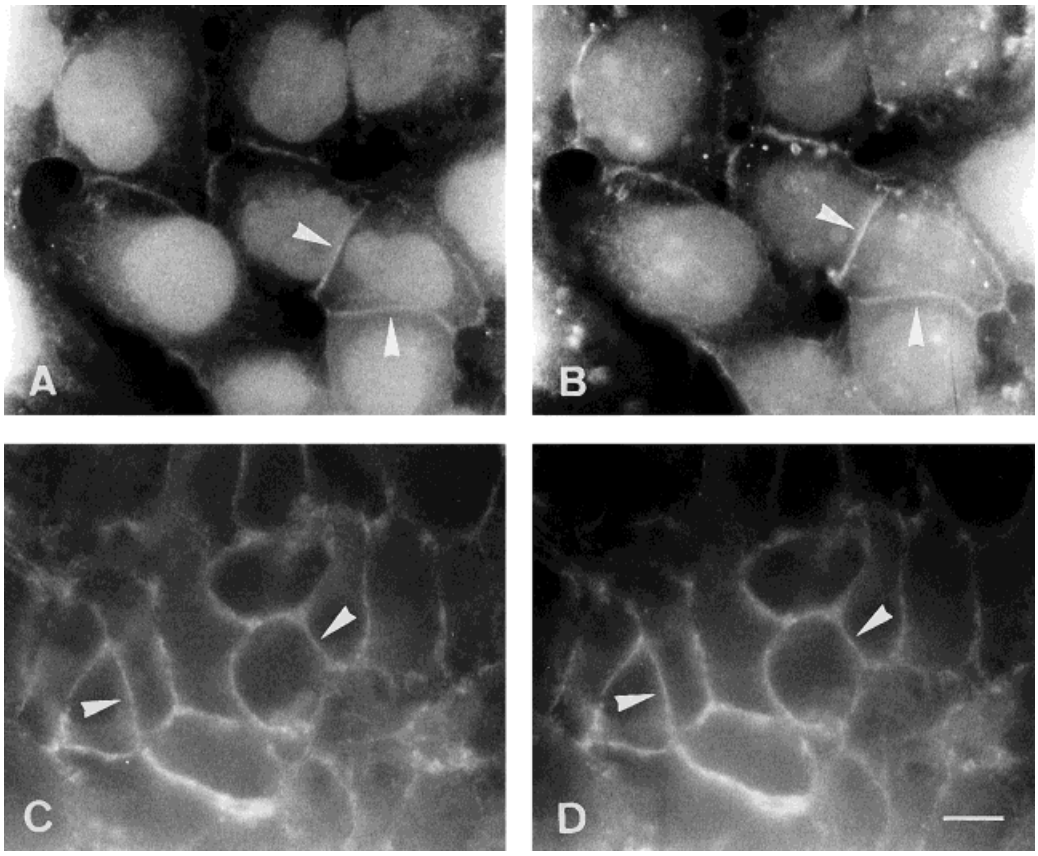

Fig. 3. Immunofluorescence localization of P-cadherin and β-catenin in TW-039 cells 1 and 5 days after plating. TW-039 cells double-labeled for P-cadherin **(A,C)** and β-catenin **(B,D)** 1 day **(A,B)** or 5 days **(C,D)** after plating. P-cadherin (arrowheads, **A,C**) colocalizes with β-catenin (arrowheads, **B,D**) at cell-cell contacts. Scale bar = 10 μm.

cancer cell line. The results showed that, although A-431 cells did not express N-cadherin and HeLa cells did not express either E- or P-cadherin, NPC TW-039 cells contained all three classical cadherins (Fig. 7). An immunofluorescence study demonstrated localization of N-cadherin at cell-cell contacts either 1 day (Fig. 8A) or 5 days (Fig. 8B) after plating.

DISCUSSION

The distribution of the E-cadherin/catenin complex has been studied in many cancers [reviewed by Shiozaki et al., 1996]. Although most reports demonstrate that its expression is down-regulated, much remains unclear about the involvement of this complex in the process of carcinogenesis and metastasis. In addition to the quantitative down-regulation, problems in assembly of the E-cadherin/catenin complex may also be involved in the process of tumor invasion and metastasis. In the present study, only weak contact labeling of E-cadherin was noted in TW-039 cells 1 day after plating. Most cells showed diffuse cytosolic flakes of E-cadherin that were considered to be nonfunctional in maintaining adhesion, as they were extracted by NP-40. A previous study using the same antibody, which recognizes the E-cadherin cytoplasmic domain, showed similar flake staining in cells without completely assembled E-cadherin [Fischer and Quinlan, 1998]. This pattern of E-cadherin staining can also be seen in A-431 cells during the first few hours of Ca^{2+}-induced cell-cell contact (data not shown). When A-431 cells become condensed, however, the flake staining is no longer observed. Thus, the cytosolic flake pattern of E-cadherin staining is not specific for TW-039 cells, but may be attributed to delayed assembly of E-cadherin into cell-cell contacts. Whether this delayed assembly is responsible for the highly metastatic and invasive nature of NPC cells requires further investigation.

E-cadherin is the most important intercellular adhesion molecule in epithelial cells. As long as the cadherins are functional, inactiva-

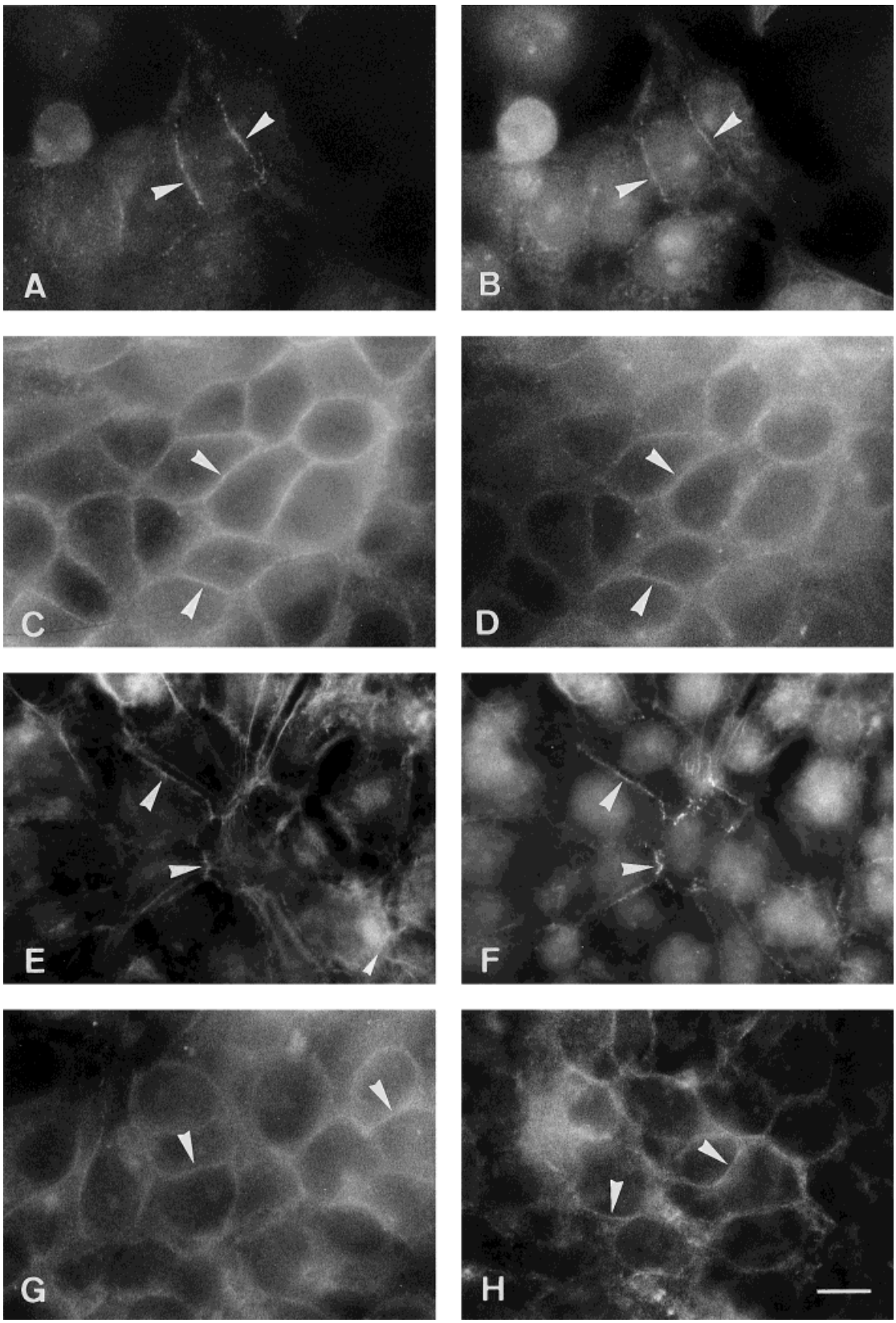

Fig. 4. Effects of detergent treatment on E-, P-cadherin, β-catenin, and F-actin immunofluorescence localization in TW-039 cells. TW-039 cells were extracted with 0.5% NP-40, fixed with formalin, and double-labeled for E-cadherin **(A,C)** and β-catenin **(B,D)**, double-labeled for F-actin **(E)** and β-catenin (F), or immunolabeled with anti-P-cadherin antibody **(G,H)** 1 day **(A,B,E–G)** or 5 days **(C,D,H)** after plating. Arrowheads **(A,B)** indicate colocalization of E-cadherin **(A)** and β-catenin **(B)** at cell-cell contacts. The cytosolic flakes of E-cadherin staining cannot be seen after extraction **(A,C)**. Arrowheads **(E,F)** indicate colocalization of F-actin **(E)** and β-catenin **(F)** at cell-cell contacts. Arrowheads **(G,H)** indicate contact staining of P-cadherin. Scale bar = 10 μm.

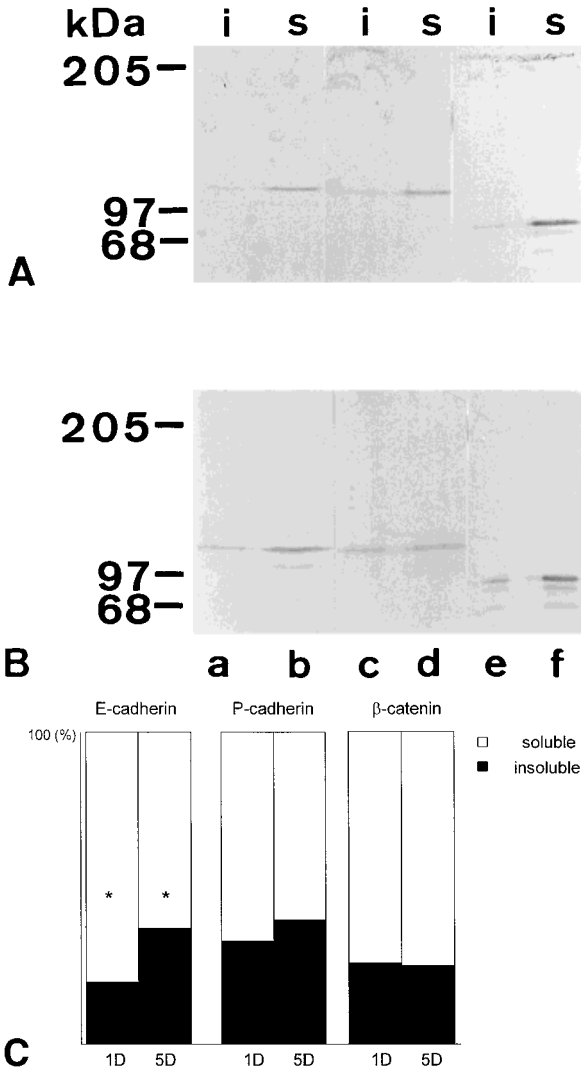

Fig. 5. Immunoblot analysis of detergent-soluble and detergent-insoluble pools of E-cadherin, P-cadherin, and β-catenin in TW-039 cells. TW-039 cells grown in culture for 1 day **(A)** or 5 days **(B)** were subjected to 0.5% NP-40 extraction. The detergent-soluble (s) and detergent-insoluble (i) pools of proteins were separated on 10% polyacrylamide gels, transblotted, and probed with antibodies specific for E-cadherin **(lanes a,b)**, P-cadherin **(lanes c,d)**, or β-catenin **(lanes e,f)**. Molecular mass markers are indicated on the left of the blots. Bands with smaller molecular mass detected in some lanes are probably degradation products. **C:** Each band in **A** and **B** was quantified. The total expressed protein (s + i) was expressed as 100% and the expression level of (i) or (s) presented as a fraction. $*P < .01$.

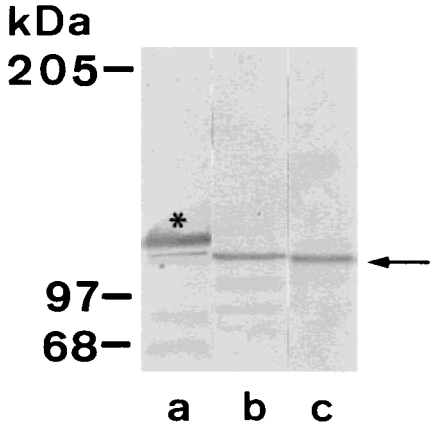

Fig. 6. E-, P-, and other cadherins coimmunoprecipitate with β-catenin in TW-039 cells. Immunoprecipitates of β-catenin were separated on 10% polyacrylamide gels, transblotted, then probed with anti-pan-cadherin **(lane a)**, anti-E-cadherin **(lane b)**, or anti-P-cadherin **(lane c)** . An unknown member of the cadherin family (asterisk) with a molecular mass greater than E- or P-cadherin (arrow) is seen in β-catenin immunoprecipitates. Molecular mass markers are indicated on the left of the blots. Bands with smaller molecular mass detected in some lanes are probably degradation products.

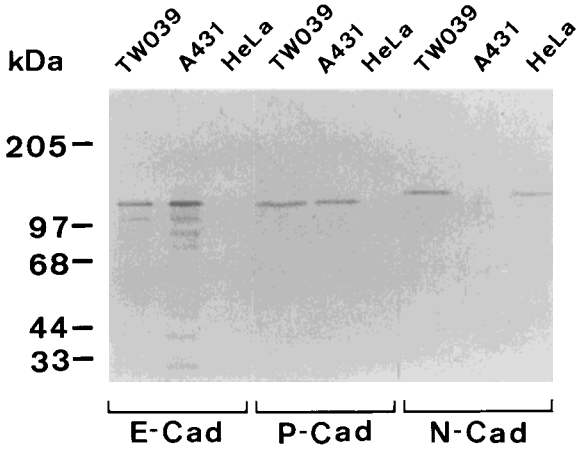

Fig. 7. E-, P-, and N-cadherin are co-expressed in TW-039 cells. Immunoblot analysis shows co-expression of E-, P-, and N-cadherin in TW-039 cells. Equal amount of protein lysates (5 μg) from TW-039 cells, A-431 cells, and HeLa cells were electrophoresed, transblotted, and probed with E-, P-, or N-cadherin antibodies. Molecular mass markers are indicated on the left of the blots. Bands with smaller molecular mass detected in some lanes are probably degradation products.

tion of other adhesion systems has little effect on cell-cell adhesion [Takeichi, 1990]. It has been demonstrated that the cytoplasmic portion of cadherin is directly associated with β-catenin [Ozawa et al., 1989; Tsukita et al., 1992; Nathke et al., 1994]. In the present study, although most cells exhibited colocalization of E-cadherin and β-catenin at contact sites of cell aggregates, some cells of the leading edge displayed contact staining for β-catenin, but not E-cadherin. This finding implies that other cadherins may be responsible for the pioneer contact adhesion of TW-039 cells. Of the members of the cadherin family, P-cadherin is known to be expressed in epithelial cells, but its actual functional role is not clearly understood.

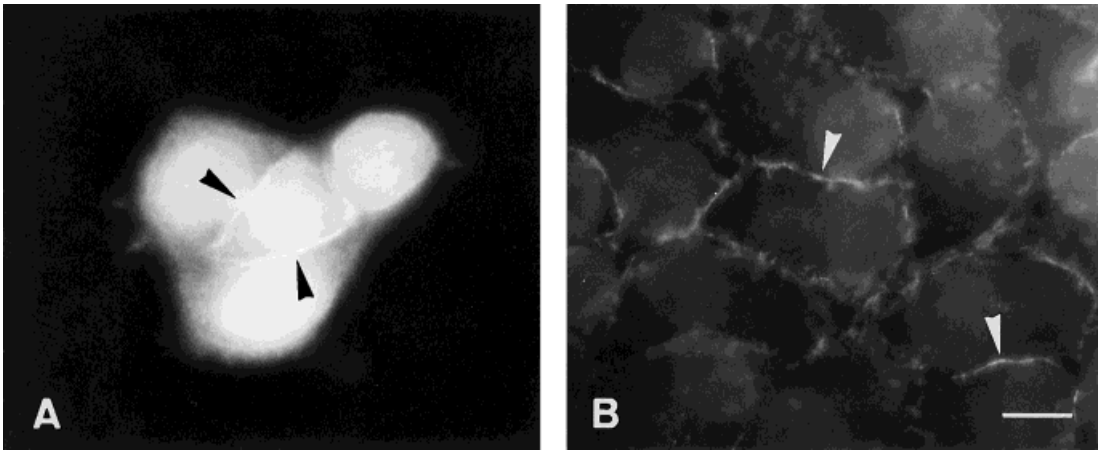

Fig. 8. Immunofluorescence localization of N-cadherin in TW-039 cells 1 or 5 days after plating. TW-039 cells show positive contact staining (arrowheads) of N-cadherin 1 day **(A)** and 5 days **(B)** after plating. Scale bar = 10 μm.

Previous studies have demonstrated altered E- and P-cadherin expression in various cancers [Cano et al., 1996; Shirahama et al., 1996; Palacios et al., 1995; Shimoyama and Hirohashi, 1991], with P-cadherin expression generally increased, especially at areas of reduced E-cadherin expression. These findings suggest either that P-cadherin acts as a compensatory adhesion molecule for E-cadherin in maintaining the baseline adhesion of cancer cells, or that it may belong to the oncofetal protein family which is associated with the proliferative nature of cancer cells [Shimoyama and Hirohashi, 1991]. In the present study, at 1 day after plating, P-cadherin colocalized with β-catenin at cell-cell contacts in some cells at the leading edges of subconfluent areas, in the absence of E-cadherin contact staining. In addition, immunoblot analysis showed that, at the same time, 33% of the P-cadherin was NP-40-insoluble (compare this with 20% of E-cadherin). These findings suggest that P-cadherin may compensate for E-cadherin and be involved in the pioneer contact adhesion of TW-039 cells.

The formation of a detergent-insoluble cadherin/catenin complex is crucial to the formation of stable cell-cell contacts [McNeill et al., 1993]. The NP-40-insoluble pool of the cadherin/catenin complex is important in maintaining adhesion and is cytoskeleton-bound [Nagafuchi and Takeichi, 1988]. In the present study, we showed the presence of NP-40-soluble and NP-40-insoluble pools of cadherin/catenin complex in TW-039 cells. However, TW-039 cells are intolerant of detergent extraction before fixation, using prolonged detergent incubation (10 min) as performed by Nagafuchi and Takeichi [1988], and more than 50% of the cells float, a phenomenon that we attribute to a weak cadherin/catenin-cytoskeleleton association. Even using our modified protocol (5-min extraction), immunoblot analysis demonstrates that about 60% of E- and P-cadherin remains in the NP-40-soluble pools 5 days after plating. These findings suggest a relatively weak association of the cadherin/catenin complex with the actin cytoskeleton, and also suggest the possibility that other cadherins may be involved in the contact adhesion of TW-039 cells.

Immunoprecipitation and a subsequent immunoblot study confirmed the presence of other cadherins, in addition to E- and P-cadherin, in TW-039 cells. Immunofluorescence and immunoblot studies demonstrated the expression of N-cadherin in TW-039 cells. N-cadherin is primarily expressed by cells of mesodermal and neuroectodermal origin, and plays an essential role in the formation of the strong cell-cell contacts of cardiomyocytes [Soler and Knudsen, 1994]. However, fibroblasts, which express approximately the same amounts of N-cadherin and N-cadherin/catenin complex as cardiac muscle cells, do not display close cell-cell contacts, but act as solitary cells most of the time [Wheelock and Knudsen, 1991; Knudsen et al., 1995]. Although E-cadherin is well documented in maintaining intimate cell-cell contacts and suppressing invasion by cancer cells, the role of anomalously expressed N-cadherin in epithelial cells is not completely understood. Squamous carcinoma cell lines that express N-cadherin display a scattered fibroblastic

phenotype, together with decreased expression of E- and P-cadherin and increased invasiveness of the cancer cells [Islam et al., 1996]. Transfection of L-CAM cDNA into cells expressing N-cadherin, on the other hand, induces fibroblast-epidermoid transition and downregulates the endogenous N-cadherin [Li et al., 1998]. These studies suggest a reciprocal regulatory relationship between N-cadherin and E-cadherin and that cells anomalously expressing N-cadherin will transform into a fibroblastic appearance. Our results show that TW-039 cells express E-, P-, and N-cadherin at approximately the same levels found in A-431 cells (E- and P-cadherin) or HeLa cells (N-cadherin). Although showing high expression of N-cadherin, TW-039 cells exhibited a cobblestone appearance characteristic of epidermoid cells. When Parker et al. [Parker et al., 1998] introduced plakoglobin cDNA into a plakoglobin- and E-cadherin-deficient, but N-cadherin-expressing, epidermoid carcinoma cell line, desmosome formation and fibroblastic-epidermoid transition occurred. As TW-039 cells also contain desmosomes [Lin et al., 1990], these structures may possibly be responsible for the formation of epidermoid phenotypes.

The finding that E-, P-, and N-cadherin are co-expressed at high levels in TW-039 cells is also interesting. As already mentioned, previous studies have shown that, when N-cadherin expression is increased, E-cadherin expression is decreased, and vice versa [Islam et al., 1996; Li et al., 1998]. A similar reciprocal expression can also be seen in morphogenetic events, with cells that segregate from the ectoderm gradually ceasing to express E-cadherin and beginning to express N-cadherin [Takeichi, 1987]. On the basis of these observations, it is postulated that cells containing multiple cadherin genes do not express all types of cadherin but may selectively express certain cadherins in response to different biophysical events. Co-expression of three classical cadherins in TW-039 cells may thus be a result of a simultaneous turn-on of the classical cadherins. In the present study in TW-039 cells, we have demonstrated a time-related change in E-cadherin distribution that might be caused by delayed assembly of E-cadherin.

Immunofluorescence and extraction studies both suggest that P-cadherin may play a role in the pioneer contact adhesion of TW-039 cells. Interestingly, all three members of the classical cadherins are co-expressed at high levels in TW-039 cells. Further studies are needed to clarify whether the expression of N-cadherin correlates with the invasive and metastatic nature of NPC cells.

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