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Cadherin Cell-Adhesion Molecules in Human Epithelial Tissues and Carcinomas[1]

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ABSTRACT

Two distinct calcium-sensitive cell-cell adhesion molecules were identified in human epithelial tissues and carcinomas using two monoclonal antibodies raised against vulvar epidermoid carcinoma A-431 and human mammary carcinoma MCF-7 and selected on the basis of their activities to disrupt cell-cell adhesion. In immunoblot analysis, these antibodies, designated NCC-CAD-299 and HECD-1, detected main bands of M_r 118,000 and 124,000, respectively. Purified tryptic fragments of the antigen recognized by NCC-CAD-299 showed cross-reactivity with a rabbit antiserum against mouse P-cadherin, indicating that this molecule was the human homologue of P-cadherin. On the other hand, the antigen recognized by HECD-1 showed essentially the same tissue distribution pattern as E-cadherin in the mouse, suggesting that this molecule is the human homologue of E-cadherin. Availability of these monoclonal antibodies to human P- and E-cadherin allowed us to examine their distributions in human tissues immunohistochemically. Both antigens were detected in epithelial tissues, but they showed distributions that were distinct from each other. The antigen recognized by HECD-1 was expressed in almost all epithelial tissues, while distribution of the other one recognized by NCC-CAD-299 was restricted to the basal or lower layers of stratified epithelia in which both antigens were coexpressed. Moreover, immunohistochemical examination of 44 lung carcinomas showed that both molecules were coexpressed in all of them, and suggested that expression of P-cadherin was closely related to the differentiation of carcinoma cells.

INTRODUCTION

Cell-cell adhesion plays essential roles in organogenesis, physical transport, signal transmission, and immunological function in multicellular organisms. Calcium-dependent cell-cell adhesion molecules, termed "cadherins" (1), form one class of molecules responsible for such mechanisms, and have been defined in several laboratories, mainly in experimental animals. One of us has so far reported three subclasses of mouse cadherins, which possess tissue distributions and binding specificities distinct from each other: E-cadherin (1), N-cadherin (2), and P-cadherin (3). E-cadherin and its homologues in other animals are also known as uvomorulin (4), L-CAM in chicken (5), Arc-1 in dog (6), and cell-CAM 120/80 in human (7). A-CAM, originally found in chicken cardiac muscle (8), has similar properties to N-cadherin (9). Recent studies on the cloning of cDNA encoding cadherins have revealed that these substances constitute a gene family (10–12). In embryogenesis, E-cadherin is considered to be significantly involved in the process of compaction, because it was inhibited by mAb[4] ECCD-1 raised against mouse E-cadherin (13). In addition, the assembly, segregation, and rearrangement of cells during development appear to be regulated by changes in cadherin type (3, 14, 15). These findings suggest that cadherins play a significant part in morphogenetic events, and it is highly probable that cancer cells employ these molecules for the formation and maintenance of cancer tissues. It is therefore of interest to study how cadherins are involved in growth, differentiation and metastasis of cancer cells.

Human E-cadherin was first identified by Damsky *et al.* as cell-CAM 120/80 using a polyclonal antibody (7). Here we report for the first time isolation and characterization of mAbs which identify human P- and E-cadherin. Two mAbs were raised against two human carcinoma cell lines, A-431 and MCF-7, and selected on the basis of their activities to disrupt cell-cell adhesion. The expression of both cadherins in human tissues and carcinomas was immunohistochemically examined in detail using the two mAbs.

MATERIALS AND METHODS

Human Carcinoma Cell Lines. Human carcinoma cell lines used in this study were vulvar epidermoid carcinoma A-431 (16), mammary adenocarcinoma MCF-7 (17), gastric adenocarcinoma MKN-7 (18), and cervical epidermoid carcinomas SKG-1 (19), SKG-II (20), SKG-IIIa, and SKG-IIIb (21). A-431 and MCF-7 cells and the other five strains were maintained in Dulbecco's modified Eagle medium plus 10% FCS and RPMI 1640 plus 10% FCS, respectively. All cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2/95% air.

Antibodies. A hybridoma clone producing mAbs against a cell-cell adhesion molecule expressed in A-431 cells was obtained as follows. BALB/c, *nu/nu* mice were immunized with xenotransplanted A-431 cells using the "rejection method" as previously reported (22). Three days after an injection of A-431 cells as a booster, spleen cells were removed and fused with mouse myeloma P3-X63-Ag8-U1 cells according to the method of Köhler and Milstein (23). Hybridomas were then screened for the production of antibodies capable of disrupting the cell-cell adhesion of cultured A-431 cells, and one clone, designated NCC-CAD-299, was selected.

BALB/c, +/+ mice were immunized by several i.p. injections of MCF-7 cells homogenized with Freund's adjuvant. Three days after the last injection, cell fusions were performed as mentioned above. One hybridoma clone producing mAbs capable of inducing the disruption of cell-cell adhesion in monolayer cultures of MCF-7 cells, designated HECD-1, was isolated. These hybridomas and P3-X63-Ag8-U1 cells were cultured in RPMI 1640 supplemented with 10% FCS under the same conditions as those used for human carcinoma cells.

Rabbit antisera against mouse P- and E-cadherin were prepared as previously described (10, 11).

Immunoblot Analysis. Cultured cells were lysed by brief sonication in Laemmli's sample buffer (24) containing 1 mM $CaCl_2$. Cell lysates were denatured at 100°C for 5 min, and loaded on 7.5% polyacrylamide gel. Proteins were fractionated by SDS-PAGE, and transferred onto Durapore membranes (Nihon Millipore Kogyo, Yonezawa, Japan) in a transfer buffer consisting of 25 mM Tris, 192 mM glycine, 20% methanol, and 0.1% SDS. The membranes were then processed successively as follows: (*a*) incubation with a blocking buffer consisting of 5% skim milk (Difco, Detroit, MI), 1% bovine serum albumin (fraction V; Sigma, St. Louis, MO), and 0.1% Antifoam A (Sigma) in Pi/NaCl-Ca for 3 h,

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[4] The abbreviations used are: mAb, monoclonal antibody; FCS, fetal calf serum; Pi/NaCl-Ca, phosphate-buffered saline containing 1 mM $CaCl_2$; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

(*b*) incubation with primary antibodies overnight at 4°C, (*c*) washing in 0.1% Tween-20 in Pi/NaCl-Ca and Pi/NaCl-Ca only, three times respectively, (*d*) brief rinsing with the blocking buffer, (*e*) incubation for 30 min with peroxidase-conjugated anti-mouse or anti-rabbit IgG (Cappel, West Chester, PA) diluted 1/50 with the blocking buffer, (*f*) washing as in step *c*, and (*g*) staining with diaminobenzidine. Primary antibodies used were NCC-CAD-299 hybridoma supernatant, HECD-1 hybridoma supernatant, and rabbit antisera against mouse P- and E-cadherin diluted 1/100 with the blocking buffer.

Purification of Tryptic Fragments of the Cadherin Recognized by NCC-CAD-299. Tryptic fragments of the cadherin reacting with NCC-CAD-299 were purified from A-431 tumors grown in nude mice as previously described (25). Briefly, membrane fractions of A-431 tumors were treated with 0.01% trypsin (type I; Sigma) in the presence of Ca^{2+} for 1 h at 37°C, and loaded onto an affinity column (CNBr-activated Sepharose 4B; Pharmacia, Uppsala, Sweden) conjugated with NCC-CAD-299 antibody. The eluates were then concentrated using Amicon YM10 membrane filters and fractionated on a Superose 12 gel filtration column (Pharmacia) employing a Pharmacia FPLC system. The peak fractions detected by NCC-CAD-299 upon immunoblotting were collected and used for examination of reactivity with other antibodies.

Immunohistochemistry. Human tissues obtained at surgery or autopsy were fixed in 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 1 h followed by infiltration with a graded series of sucrose (10–20%) in phosphate buffer at 4°C. They were then embedded in OCT compound (Miles, Elkhart, IN) and frozen on a sheet of copper at −70°C. Frozen sections were cut on a cryostat at a thickness of 6 μm, mounted on albumin-coated slides, and air dried. The sections were then stained by the immunoperoxidase method using biotinylated anti-mouse IgG and avidin-biotin-peroxidase complex (Vector, Burlingame, CA) as previously reported (22), except that all the buffers contained 1 mM $CaCl_2$. NCC-CAD-299 and HECD-1 antibodies were produced in ascitic fluids of mice inoculated intraperitoneally with each hybridoma cell, diluted 1/1000, and used as primary antibodies.

RESULTS

Monoclonal Antibodies Disrupting Cell-Cell Adhesion of Human Carcinoma Cells. Two mAbs capable of inducing the disruption of cell-cell adhesion were obtained separately. NCC-CAD-299 (IgG1) and HECD-1 (IgG1) were selected on the basis of their abilities to disrupt the cell-cell adhesion of A-431 (Fig. 1) and MCF-7 cells, respectively. The disruptions were observed within 30 min after addition of the antibodies, and were reversible as described previously (1).

Trypsin and Ca^{2+} Sensitivities of the Antigens. A-431 and MCF-7 cells were incubated at 37°C for 1 h with the following four treatments: 1 mM EGTA (E), 0.0001% trypsin plus 1 mM EGTA (LTE), 0.01% trypsin plus 1 mM EGTA (TE), or 0.01% trypsin plus 1 mM $CaCl_2$ (TC) in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered Ca^{2+}- and Mg^{2+}-free saline (26). Cells were then harvested, washed twice with Dulbecco's phosphate-buffered saline containing Ca^{2+} and Mg^{2+}, and lysed by brief sonication. For LTE- or TE- and TC-treated cells, the cells were suspended in 0.0005% or 0.05% soybean trypsin inhibitor (type I; Sigma) in the above 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered saline prior to washing. Immunoblots of A-431 cell lysates using NCC-CAD-299 are shown in Fig. 2. NCC-CAD-299 detected a band of M_r 118,000 in E- and TC-treated cells (*lanes 1* and *4*), but not in LTE- and TE-treated cells (*lanes 2* and *3*). These results indicate that the antigen recognized by NCC-CAD-299 is a calcium-sensitive cell-cell adhesion molecule (27), which has been defined as "cadherin" (1). Similar results were obtained in immunoblottings of MCF-7 cells using HECD-1, and the main band was detected at M_r 124,000 (data not shown).

Distribution of the Antigens in Human Carcinoma Lines. Seven human carcinoma cell lines were analyzed by immunoblotting. Antigens transferred onto filters were detected by NCC-CAD-299, HECD-1, and rabbit antiserum against mouse P- or E-cadherin. Both NCC-CAD-299 and antiserum against mouse P-cadherin detected a main band of M_r 118,000 strongly in A-431 and SKG-IIIb, moderately in SKG-II and SKG-IIIa, faintly in SKG-I and MKN-7, but not in MCF-7 (Fig. 3, *A* and *C*). Both HECD-1 and antiserum against mouse E-cadherin detected a main band of M_r 124,000 and some lower bands, which probably represented degradation productions of the M_r 124,000 antigen, with the same pattern in all the carcinomas (Fig. 3, *B* and *D*). These results suggested that the antigens detected by NCC-CAD-299 and HECD-1 are homologues of mouse P- and E-cadherin, respectively, and indicated that these two cadherins are coexpressed in the same cell in certain cell types. The difference of migration on SDS-PAGE and the coexpression of the two cadherins was visualized clearly using a mixture of NCC-CAD-299 and HECD-1 for detection of the cadherin (Fig. 4).

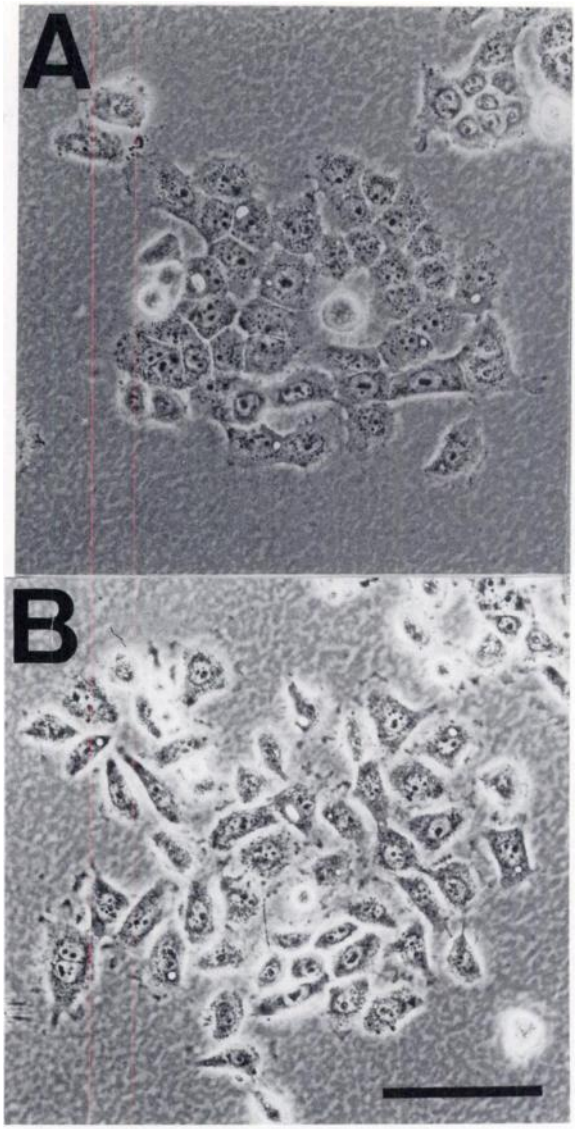

Fig. 1. Effect of NCC-CAD-299 on A-431 cells. *A*, a colony of A-431 cells before addition of NCC-CAD-299; *B*, the same colony as in *A* 4 hr after the addition of NCC-CAD-299 at a final concentration of 100 μg/ml. *Scale bar*, 120 μm.

Immunoblot Analysis of Purified Tryptic Fragments of the Antigen Recognized by NCC-CAD-299. M_r 72,000 tryptic fragments of the M_r 118,000 NCC-CAD-299 antigen on SDS-

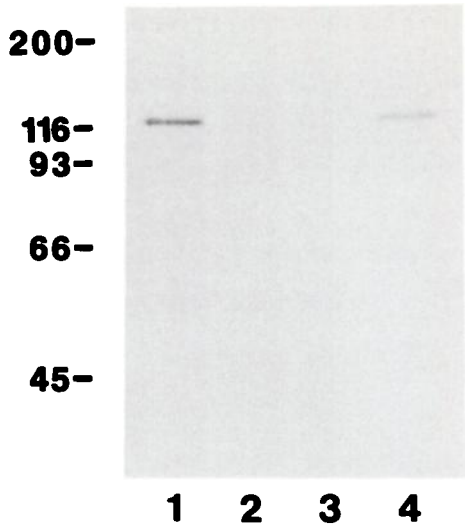

Fig. 2. Immunoblot analysis of A-431 cell lysates treated under four different conditions. A-431 cells were incubated with *lane 1*, E; *lane 2*, LTE; *lane 3*, TE; or *lane 4*, TC before harvesting. Cell lysates were loaded on 7.5% polyacrylamide gel at 50 μg·protein/lane determined by Protein Assay (Bio-Rad), transferred onto a Durapore membrane, and detected by NCC-CAD-299. *Bars* show positions of molecular weight markers in $M_r \times 10^3$.

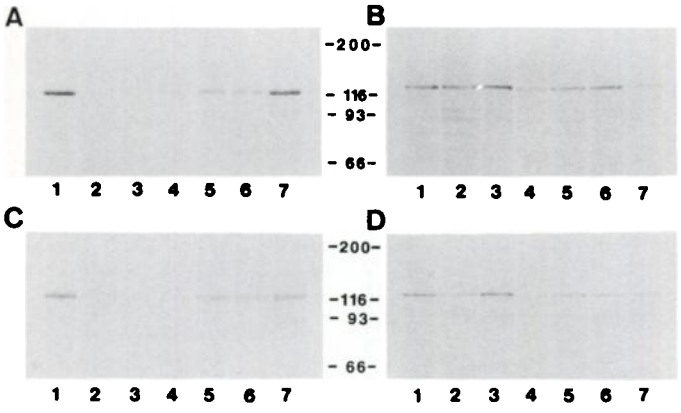

Fig. 3. Immunoblot analysis of human carcinoma cells. Cell lysates: *lane 1*, A-431; *lane 2*, MKN-7; *lane 3*, MCF-7; *lane 4*, SKG-I; *lane 5*, SKG-II; *lane 6*, SKG-IIIa; and *lane 7*, SKG-IIIb, were loaded on 7.5% polyacrylamide gel at 50 μg·protein/lane, transferred onto Durapore membranes, and detected by NCC-CAD-299 (*A*), HECD-1 (*B*), and rabbit antiserum against mouse P- (*C*), or E-cadherin (*D*).

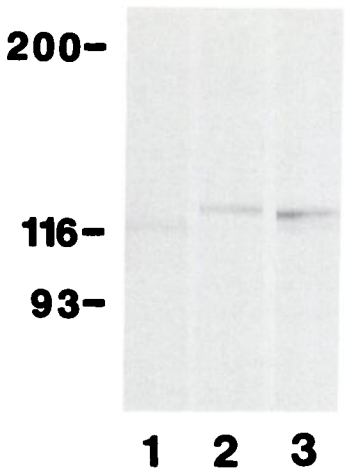

Fig. 4. Immunoblot analysis of SKG-IIIa cells using NCC-CAD-299 and HECD-1. A cell lysate of SKG-IIIa cells was loaded on 7.5% polyacrylamide gel at 50 μg·protein/lane, transferred onto a Durpore membrane, and detected by *lane 1*, NCC-CAD-299; *lane 2*, HECD-1; or *lane 3*, a mixture of NCC-CAD-299 and HECD-1.

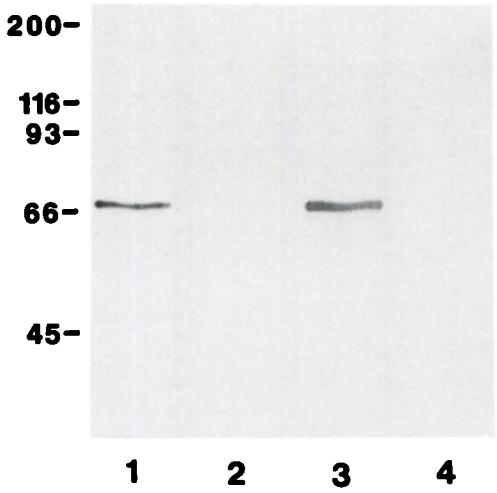

Fig. 5. Immunoblot analysis of purified tryptic fragments of the cadherin recognized by NCC-CAD-299. Tryptic fragments of the cadherin purified from A-431 tumors in Laemmli's sample buffer were loaded on 7.5% polyacrylamide gel, transferred onto a Durapore membrane, and detected by *lane 1*, NCC-CAD-299; *lane 2*, HECD-1; and rabbit antiserum against mouse *lane 3*, P-; or *lane 4*, E-cadherin.

PAGE were purified from A-431 tumors. Fig. 5 shows reactivities of antibodies with the M_r 72,000 fragments upon immunoblotting. Antiserum against mouse P-cadherin, as well as NCC-CAD-299, reacted with the fragments. However, neither HECD-1 nor the antiserum against mouse E-cadherin detected them.

Distribution of the Two Cadherins in Normal Human Tissues. Serial frozen sections were immunohistochemically examined using NCC-CAD-299 and HECD-1, and the results are summarized in Table 1. In adult tissues, both cadherins were detected only in epithelial tissues and localized at the cell to cell borders. The cadherin recognized by NCC-CAD-299 was stained in stratified or pseudostratified epithelia such as squamous epithelium (Fig. 6*A*), bronchial epithelium (Fig. 6*C*), transitional epithelium, and excretory duct epithelium of glands, but not stained in simple or unstratified epithelia such as gastrointestinal epithelium and hepatocytes (Fig. 6*E*). The other cadherin recognized by HECD-1 was, however, detected in almost all epithelial tissues (Fig. 6, *B*, *D*, and *F*). In stratified or pseudostratified epithelia, the cadherin recognized by NCC-CAD-299 was detected only in basal or lower layers (Fig. 6, *A* and *C*). The cadherin recognized by HECD-1 was, however, distributed over almost all layers, although its staining was somewhat strong in lower layers (Fig. 6*B*). In placental tissues, which were examined at between 6 weeks of gestation and delivery, NCC-CAD-299 did not react with any cells, while HECD-1 continuously reacted with cytotrophoblasts and decidua (Fig. 6, *G* and *H*).

Expression of the Two Cadherins in Human Lung Carcinomas. 44 lung carcinomas which consisted of 21 adenocarcinomas, 16 squamous cell carcinomas, four large cell carcinomas, two small cell carcinomas, and one carcinoid tumor were examined immunohistochemically using NCC-CAD-299 and HECD-1 mAbs. Although staining intensity of each cadherin varied considerably from case to case and one or both cadherins were only partially detected in some cases, both cadherins were detected in all the cases at the cell to cell borders (Fig. 7). Highly keratinized cancer pearls in squamous cell carcinomas, resembling upper layers of stratified squamous epithelium, did not express P-cadherin (Fig. 7, *A* and *B*). In addition, it was

Table 1 *Distribution of cadherins detected by NCC-CAD-299 and HECD-1 in immunohistochemistry*

Organ	NCC-CAD-299[a]	HECD-1[b]
Brain	ND[c]	ND
Cardiac muscle	ND	ND
Skeletal muscle	ND	ND
Skin	Epidermis, duct of sweat gland	Epidermis, duct of sweat gland
Mammary gland	Mammary duct	Mammary duct
Bronchus	Bronchial epithelium, duct of bronchial gland	Bronchial epithelium, duct and acinus of bronchial gland
Lung	ND	Alveolar lining
Tongue	SE[d]	SE
Esophagus	SE	SE
Stomach	ND	SE[e], fundic and pyloric gland
Intestine	ND	SE
Liver	ND	Hepatocyte
Bile duct	ND	SE
Pancreas	ND	Acinus, pancreatic duct
Kidney	ND	Distal and collecting tubule
Ureter	SE	SE
Bladder	SE	SE
Prostate gland	Glandular epithelium	Glandular epithelium
Testis	ND	ND
Ovary	ND	ND
Vagina	SE	SE
Thyroid gland	ND	Follicle epithelium
Adrenal gland	ND	ND
Placenta	ND	Cytotrophoblast, decidua

[a] In all epithelial tissues which showed positive staining with NCC-CAD-299, the antigen was detected in the basal or lower layers of the epithelium.
[b] In all epithelial tissues which showed positive staining with HECD-1, the antigen was detected in almost all layers of the epithelium.
[c] ND, not detectable.
[d] SE, surface epithelium.
[e] Intestinal metaplasia was also stained positively.

remarkable that expression of P-cadherin was very weak in comparison with that of E-cadherin in all of six well-differentiated adenocarcinomas (Fig. 7, *C* and *D*), resembling simple glandular epithelia. We also examined cadherin expression in lymph node metastases of five cases in comparison with each primary tumor. Cadherins were detected not only in primary tumors but also in metastases at the almost same level (Fig. 7, *E–H*). Mesenchymal cells in the cancer stroma were negative for both cadherins.

DISCUSSION

Two cell-cell adhesion molecules were detected in human normal tissues and carcinomas by two functional mAbs. The effect of several trypsin treatments on these molecules suggested that they were of the calcium-dependent type, named cadherins. The cadherin recognized by NCC-CAD-299 is ascertained to be the human homologue of mouse P-cadherin because of the cross-reactivity of its M_r 72,000 fragments with antiserum against mouse P-cadherin. The other cadherin recognized by HECD-1 is considered to be human E-cadherin, because it exhibited a distribution in adult tissues consistent with that of mouse E-cadherin (2) and its homologues (6, 28), and rabbit antiserum against mouse E-cadherin detected identical bands to this cadherin upon immunoblotting. This molecule is also considered to be identical to cell-CAM 120/80, because it was originally identified in MCF-7 cells (7).

P-Cadherin showed unique tissue distribution in normal human tissues. It was detected only in the basal or lower layers of stratified or pseudostratified epithelia in normal human tissues, which possess a high proliferative potential, but not in simple epithelia. This expression pattern of P-cadherin was similar to that of epidermal growth factor receptor, the expression level of which indicates proliferative ability in certain carcinomas

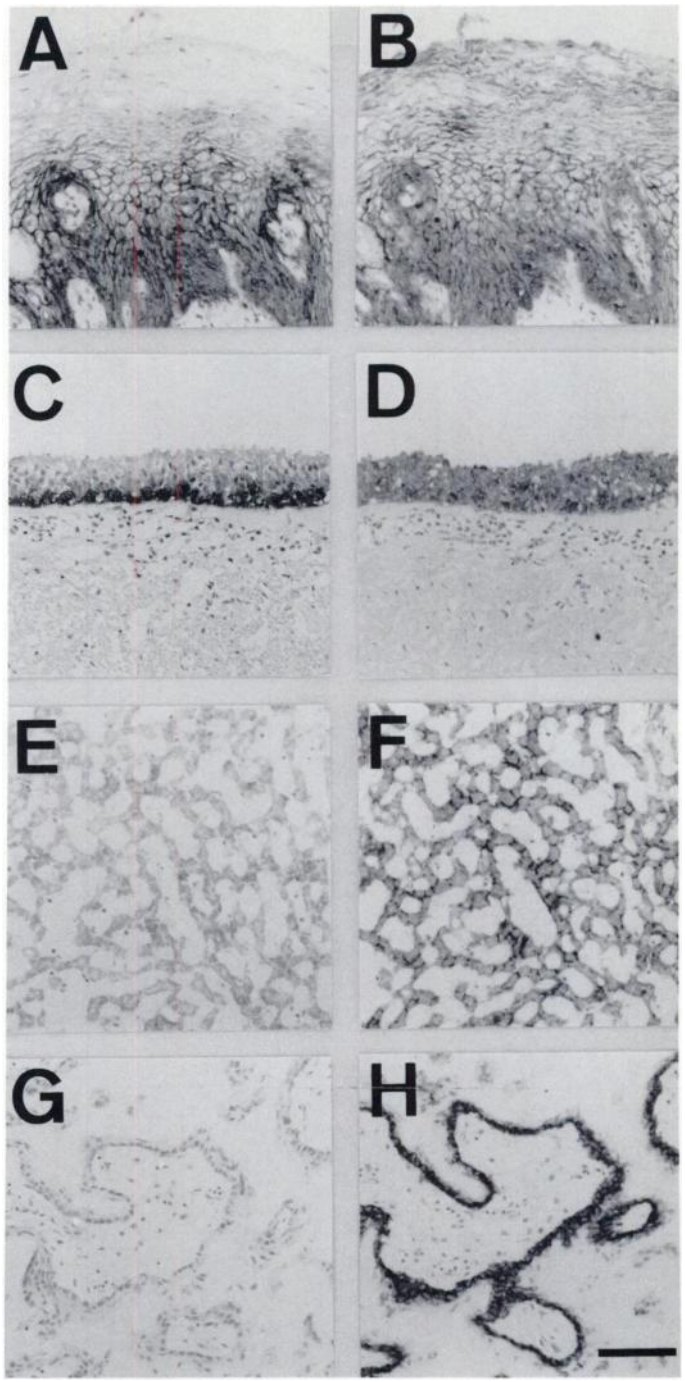

Fig. 6. Immunohistochemical localization of cadherin detected by NCC-CAD-299 and HECD-1. Cadherins in (*A* and *B*) esophageal epithelium, (*C* and *D*) bronchial epithelium, (*E* and *F*) liver, and (*G* and *H*) chorionic villi of placenta at 7 weeks of gestation were stained using avidin-biotin-peroxidase complex. (*A*, *C*, *E*, and *G*) and (*B*, *D*, *F*, and *H*) were stained by NCC-CAD-299 and HECD-1, respectively. *Scale bar*, 20 μm.

(29). Moreover, human P-cadherin was not detected in human placenta despite the strong expression of mouse P-cadherin in mouse placenta (3). Instead, human E-cadherin showed consistently strong staining in human placenta. This discrepancy in the tissue distribution of cadherin between species is an unexpected finding. However, human placental tissues before 6 weeks of gestation were not examined, and further study is required to infer the role of each cadherin subclass in human embryogenesis.

E-Cadherin was stained in almost all epithelial tissues except for a few epithelia such as that of proximal tubules, in which

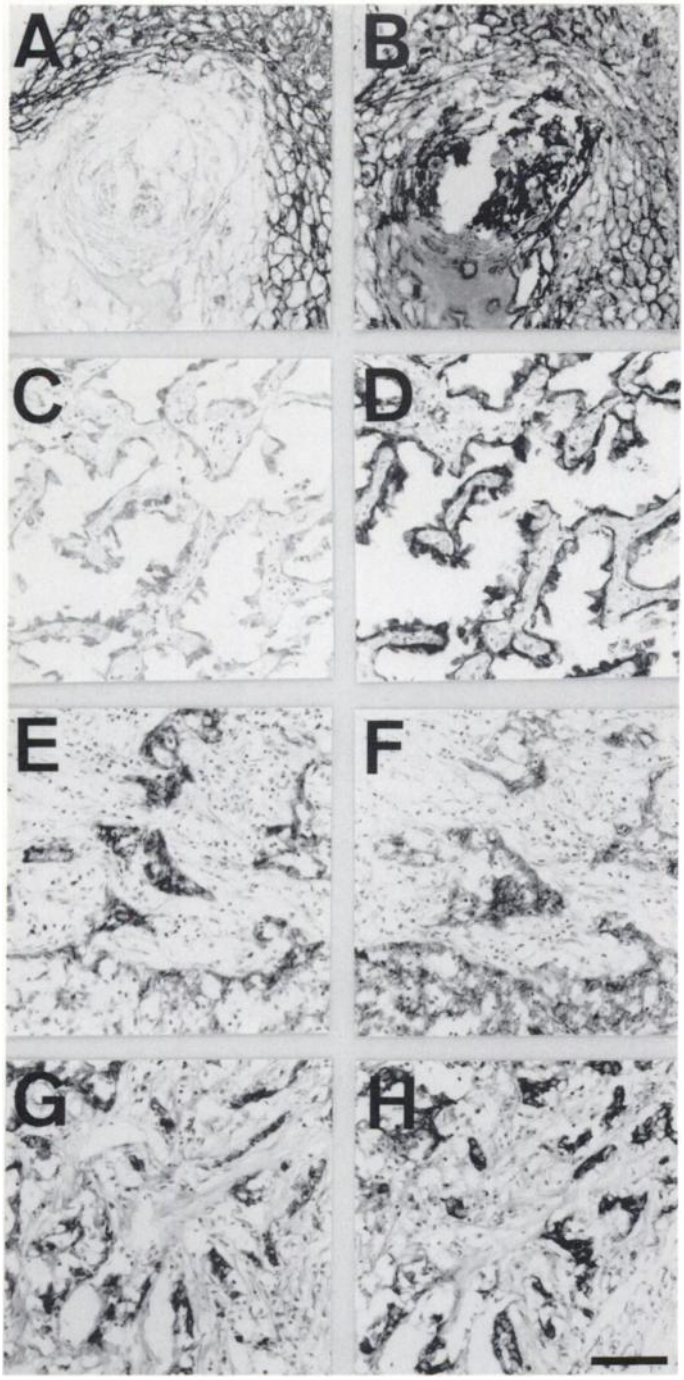

Fig. 7. Cadherins detected by NCC-CAD-299 and HECD-1 in lung carcinomas. Cadherins in (*A* and *B*) squamous cell carcinoma with cancer pearl formation, (*C* and *D*) well-differentiated adenocarcinoma, (*E* and *F*) poorly differentiated adenocarcinoma, and (*G* and *H*) metastasis to a regional lymph node of the same patient as *E* and *F* were stained using avidin-biotin-peroxidase complex. (*A*, *C*, *E*, and *G*) and (*B*, *D*, *F*, and *H*) were stained by NCC-CAD-299 and HECD-1, respectively. *Scale bar*, 20 μm.

clear boundaries between cells cannot be seen at the light microscopic level. The same finding has previously been reported in canine kidney (6). This cadherin could not be detected either in placental syncytiotrophoblasts, a multicellular complex, despite the staining shown in cytotrophoblasts. However, the results obtained here suggest that E-cadherin is a common cell-cell adhesion molecule in epithelial tissues.

Although human P- and E-cadherin showed tissue distribution distinct from each other, the basal or lower layers of stratified or pseudostratified epithelia expressed both molecules immunohistochemically. The coexpression of both cadherins was also demonstrated in cultured carcinoma cells using immunoblot analysis and in lung carcinomas immunohistochemically. However, P-cadherin was not or very weakly expressed in cancer pearls of squamous cell carcinomas and in well differentiated adenocarcinomas of the lung tested. These findings correspond well to the absence of P-cadherin in upper layers of stratified squamous epithelium and in normal glandular epithelia, and show that the amount of P-cadherin is reduced as the cells differentiate.

It has been reported that calcium-dependent cell-cell adhesion molecules play an essential role in embryogenesis. Even in adult tissues, however, these molecules are thought to be indispensable for the maintenance of orderly tissue structures. The results obtained in this study suggest that two cadherins probably contribute to human epithelial structures. One of them, P-cadherin, may be correlated with the maintenance of the proliferative compartment of certain epithelia in view of its restricted distribution, while the other one, E-cadherin, perhaps plays a main role in the formation and maintenance of epithelial tissues because of its broad distribution. In addition, both cadherins were found to be conserved and coexpressed in carcinoma cells. There is no doubt cancer cells employ cadherins for the formation and maintenance of cancer cell nests. Although the amounts of P- and E-cadherin in the metastases were almost the same as those in their primary tumors in the present study, further study is required for possible involvement of cadherin in the process of metastasis. Availability of mAbs to human P- and E-cadherin provide us an opportunity to further investigate the roles of cadherin in growth, differentiation, and metastasis.

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