Molecular Cloning of a Human Ca²⁺-dependent Cell–Cell Adhesion Molecule Homologous to Mouse Placental Cadherin: Its Low Expression in Human Placental Tissues

Yutaka Shimoyama,*‡ Teruhiko Yoshida,* Masaaki Terada,* Yukio Shimosato,* Osahiko Abe,‡ and Setsuo Hirohashi*

*Pathology and Genetics Divisions, National Cancer Center Research Institute, Tokyo 104, Japan; and ‡Department of Surgery, School of Medicine, Keio University, Tokyo 160, Japan

Abstract. P-cadherin is a subclass of Ca²⁺-dependent cell–cell adhesion molecules present in mouse placenta, where its localization suggests a function of connecting the embryo to the uterus (Nose, A., and M. Takeichi. 1986. *J. Cell Biol.* 103:2649–2658). We recently identified a human cadherin detected by an mAb capable of disrupting cell–cell adhesion of A-431 cells, and found that it was closely related immunochemically to mouse P-cadherin. Curiously, this cadherin was undetectable in human placenta by immunohistochemical examination (Shimoyama, Y., S. Hirohashi, S. Hirano, M. Noguchi, Y. Shimosato, M. Takeichi, and O. Abe. 1989. *Cancer Res.* 49:2128–2133). We here report the cloning and sequencing of a cDNA clone encoding the human homologue of mouse P-cadherin. The deduced amino acid sequence of the human P-cadherin consists of 829 amino acids and shows striking homology with mouse P-cadherin. On Northern blot analysis, human P-cadherin was scarcely expressed in human placenta in contrast to mouse P-cadherin, which was abundantly expressed in mouse placenta throughout pregnancy, and it was shown that E-cadherin, but not P-cadherin, was the major cadherin molecule in human placenta. Moreover, NIH3T3 cells transfected with human P-cadherin cDNA expressed the functional cadherin molecule, which was identical to the cadherin we had previously identified using the mAb, showing that this molecule really does mediate cell–cell adhesion and that the cadherin we detected immunochemically is undoubtedly human P-cadherin. The results obtained in this study support the idea that P-cadherin plays little role, if any, in Ca²⁺-dependent cell–cell binding in human placental tissue at least after several weeks of pregnancy.

CADHERINS are integral membrane glycoproteins responsible for Ca²⁺-dependent cell–cell adhesion. At present it is known that they constitute a gene family consisting of at least three subclasses, E-, N-, and P-cadherin. In developing embryos, each subclass shows a unique spatiotemporal pattern of expression that coincides with the movement and rearrangement of cell collectives, suggesting that cadherins play a key role in morphogenetic events (for review, see reference 22). Recent studies using cells transfected with cadherin cDNAs have demonstrated experimentally that cadherin molecules are directly involved in cell–cell binding (2, 15), and that they can control cell sorting in vitro (18). Their cell–cell binding function is mediated not by a ligand-receptor complex but occurs in a homophilic manner (2) in cooperation with certain cytoskeletal components (15).

We recently reported the establishment of two mAbs recognizing two human cadherins, which are distinct from each other in terms of immunological specificity, molecular weight, and tissue distribution (20). One of them showed a broad spectrum of expression in epithelial tissues with few exceptions, and was subsequently identified as human E-cadherin, which is possibly identical to cell–CAM 120/80 (1). In contrast, the tissue distribution of the other cadherin was very unique, being detected immunohistochemically in stratified epithelia only, and not in simple epithelia. Even in stratified epithelia, this cadherin showed a characteristic expression pattern: it was detected in the basal or parabasal layers but not in the upper layers, suggesting a close relationship with differentiation. Since the purified extracellular fragments of this molecule showed specific cross-reactivity with anti-mouse P-cadherin sera raised in rabbit, this molecule was defined as human P-cadherin. P-cadherin was originally identified in mouse placenta (16), and no data are available on its homologues in other species. Unexpectedly, however, the molecule defined as human P-cadherin by the mAb was immunohistochemically undetectable in human placental tissues despite the strong expression of E-cadherin. Therefore, the possibility remained that this molecule was P-related cadherin and distinct from authentic human P-cad-

herin. To settle this issue, we cloned and sequenced human P-cadherin cDNA to examine the expression of human P-cadherin in human placenta and to elucidate whether the molecule identified by the mAb was, in fact, human P-cadherin.

Materials and Methods

Cell Cultures

Vulvar epidermoid carcinoma A-431 cells, which coexpress P- and E-cadherin (20), were cultured in DME supplemented with 10% FCS at 37°C in a humidified atmosphere containing 5% CO_2/95% air. NIH3T3 cells were maintained in DME supplemented with 10% calf serum under the same conditions as those above.

Construction and Screening of cDNA Library

Poly(A)$^+$RNA was isolated from A-431 cells as described by Maniatis et al. (12). Double-stranded cDNA was synthesized using a cDNA synthesis system (Bethesda Research Laboratories, Gaithersburg, MD) according to the manufacturer's instructions followed by methylation of the Eco RI site and ligation to the arms of bacteriophage λgt10 using Eco RI linkers (8). The recombinant phages were packaged in vitro using a λ in vitro packaging kit (Amersham, UK), and then plated onto Escherichia coli strain NM514. The phage DNAs were transferred onto nylon filters. The filters were hybridized at 42°C in a buffer containing 50% formamide, 0.65 M NaCl and a 5′ Eco RV fragment of mouse P-cadherin cDNA (17) labeled with [α-^{32}P]dCTP by random priming, washed at 65°C in 0.1 × SSC/0.1% SDS, and exposed to film (XAR; Eastman Kodak Co., Rochester, NY) at −70°C with intensifying screens. Positive plaques were picked up and purified under the same conditions as those above.

DNA Sequence Analysis

A human P-cadherin cDNA was cloned into the Eco RI site of phage M13mpl8, and overlapping subclones were prepared by the stepwise deletion method (25). The nucleotide sequence was determined by the dideoxy chain-termination method (19) using a 370A DNA sequencer (Applied Biosystems, Foster City, CA) as reported previously (5). Deoxy-7-deazaguanosine triphosphate and sequenase (United States Biochemical Corp., Cleveland, OH) were used in place of dGTP and Klenow fragment, respectively. DNA sequencing was performed in full for both strands. Nucleotide and amino acid sequences were analyzed using GENETYX programs (Software Development Co. Ltd., Tokyo, Japan) for a microcomputer and the IDEAS programs (10) for a VAX/VMS computer.

Plasmid Construction

For the expression of human P-cadherin in NIH3T3 cells, an expression vector, pβact-hP, which contains the chicken β actin promoter upstream from the human P-cadherin cDNA fragment, was constructed from pβact-CAT9 (3). The human P-cadherin cDNA fragment, which contains the entire open reading frame and has the Hind III–Eco RI sequence of the multicloning site of M13mpl8 and an Eco RI linker at its 5′ end, was obtained from M13mpl8 containing the cDNA in its Eco RI site by double digestion with Hind III and Stu I. To generate pβact-hP, the Hind III–Hpa I fragment encoding the CAT gene of pβact-CAT9 was replaced with this Hind III–Stu I fragment of human P-cadherin cDNA.

Transfection

Transfection of human P-cadherin cDNA into NIH3T3 cells was performed by calcium-phosphate coprecipitation (24) using 30 μg of salmon testis DNA, 2 μg of pβact-hP, and 0.2 μg of pSTneoB (11) per 1 × 10⁶ cells in a 100-mm plastic dish. At 8 h after the addition of DNAs, the culture medium was replaced with fresh medium. The cells were cultured for another 12 h, and then transferred to other dishes in DME plus 10% calf serum with G418 at 0.4 mg/ml. After ∼2 wk of culture, G418-resistant colonies were isolated, and examined for their reactivities with mAb NCC-CAD-299. Positive cells were then recloned. Transfectants were maintained in the above medium with G418 under the same conditions as those for NIH3T3 cells.

Antibody

NCC-CAD-299 mAb, which recognizes a human cadherin closely related to mouse P-cadherin, was produced in ascitic fluid of mice inoculated intraperitoneally with its hybridomas (20). The ascitic fluid was diluted 1:1,000, and used as a primary antibody for immunocytochemistry and Western blot analysis. The antibody was purified by an Affi-Gel Protein A MAPS II kit (Bio-Rad Laboratories, Richmond, CA), and used for functional testing against transfectants of NIH3T3 cells expressing human P-cadherin.

Immunocytochemistry

Cells cultured on chamber slides (Nunc, Roskilde, Denmark) were rinsed with a buffer containing 10 mM Hepes (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$ (HNC), fixed with isopropanol for 10 min at 4°C, and air dried. After rinsing with HNC, the samples were treated with 2% swine serum in HNC (SS-HNC) for 30 min at room temperature, and then incubated with mAb NCC-CAD-299 overnight at 4°C. After washing with HNC and brief rinsing with SS-HNC, the samples were incubated with biotinylated anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA) diluted 1/200 with SS-HNC for 30 min at room temperature. After washing with HNC, the samples were incubated with avidin-biotin-peroxidase complex (ABC; Vector Laboratories, Inc.) diluted 1:100 with HNC for 30 min at room temperature. After extensive washing with HNC, they were then stained with diaminobenzidine, and counterstained with hematoxylin.

Western and Northern Blot Analyses

Western blot analysis was performed as described previously (20) except that diluted ascitic fluid was used as the primary antibody. Extraction of poly(A)$^+$RNAs and Northern blot analysis were performed essentially as described by Maniatis et al. (12). Poly(A)$^+$RNAs (2 μg/lane) were separated in 0.9% agarose/formaldehyde gels, transferred onto nitrocellulose filters, and hybridized at 42°C in a buffer containing 50% formamide and 0.65 M NaCl with probes labeled with [α-^{32}P]dCTP by random priming. Probes used in this study were a 0.6-kb Sac I fragment of λhp23 cDNA for the detection of human P-cadherin mRNA, a 0.8-kb Hinc II fragment of mouse P-cadherin cDNA (17) for mouse P-cadherin, and a 0.6-kb Eco RI fragment of mouse E-cadherin cDNA (15) for human and mouse E-cadherin. Each probe mainly consisted of the 5′ noncoding exon of each cDNA to avoid crosshybridization with mRNAs of different subclasses. The filters were washed at 65°C in 0.1 × SSC/0.1% SDS for human P-, mouse P- and E-cadherin, or at 52°C in 2 × SSC/20 mM sodium phosphate buffer/0.06% sodium pyrophosphate/0.05% SDS for human E-cadherin. The RNA blot filters were then exposed to film (XAR; Eastman Kodak Co.) at −70°C with intensifying screens.

Results

cDNA Cloning

The λgt10 cDNA library constructed from A-431 poly(A)$^+$ RNA was screened with the mouse P-cadherin probe. Approximately 10⁵ recombinants were screened, and 41 positive plaques were identified. These were then purified and examined for their cDNA inserts. One clone, designated λhP23, contained a cDNA insert of ∼3.2 kb, which was almost identical in size to human P-cadherin mRNA predicted from preliminary Northern blot analysis using a mouse P-cadherin probe (data not shown). Moreover, this insert appeared to hybridize with the same band as the mouse P-cadherin probe on Northern blot analysis (data not shown). This cDNA was cloned into the Eco RI site of M13mpl8 in both directions for the following sequence analysis.

cDNA and Deduced Amino Acid Sequences

The nucleotide sequence and restriction endonuclease map of

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GCGGAACACC GGCCCGCCGT CGCGGCAGCT GCTTCACCCC TCTCTCTGCA GCCATGGGGC TCCCTCGTGG ACCTCTCGCG TCTCTCCTCC TTCTCCAGGT   100
TTGCTGGCTG CAGTGCGCGG CCTCCGAGCC GTGCCGGGCG GTCTTCAGGG AGGCTGAAGT GACCTTGGAG GCGGGAGGCG CGGAGCAGGA GCCCGGCCAG   200
GCGCTGGGGA AAGTATTCAT GGGCTGCCCT GGGCAAGAGC CAGCTCTGTT TAGCACTGAT AATGATGACT TCACTGGCAG GAATGGCGAG ACAGTCCAGG   300
AAAGAAGGTC ACTGAAGGAA AGGAATCCAT TGAAGATCTT CCCATCCAAA CGTATCTTAC GAAGACACAA GAGAGATTGG GTGGTTGCTC CAATATCTGT   400
CCCTGAAAAT GGCAAGGGTC CCTTCCCCCA GAGACTGAAT CAGCTCAAGT CTAATAAAGA TAGAGACACC AAGATTTTCT ACAGCATCAC GGGGCCGGGG   500
GCAGACAGCC CCCCTGAGGG TGTCTTCGCT GTAGAGAAGG AGACAGGCTG GTTGTTGTTG AATAAGCCAC TGGACCGGGA GGAGATTGCC AAGTATGAGC   600
TCTTTGGCCA CGCTGTGTCA GAGAATGGTG CCTCAGTGGA GGACCCCATG AACATCTCCA TCATCGTGAC CGACCAGAAT GACCACAAGC CCAAGTTTAC   700
CCAGGACACC TTCCGAGGGA GTGTCTTAGA GGGAGTCCTA CCAGGTACTT CTGTGATGCA GGTGACAGCC ACAGATGAGG ATGATGCCAT CTACACCTAC   800
AATGGGGTGG TTGCTTACTC CATCCATAGC CAAGAACCAA AGGACCCACA CGACCTCATG TTCACAATTC ACCGGAGCAC AGGCACCATC AGCGTCATCT   900
CCAGTGGCCT GGACCGGGAA AAAGTCCCTG AGTACACACT GACCATCCAG GCCACAGACA TGGATGGGGA CGGCTCCACC ACCACGGCAG TGGCAGTAGT   1000
GGAGATCCTT GATGCCAATG ACAATGCTCC CATGTTTGAC CCCCAGAAGT ACGAGGCCCA TGTGCCTGAG AATGCAGTGG GCCATGAGGT GCAGAGGCTG   1100
ACGGTCACTG ATCTGGACGC CCCCAACTCA CCAGCGTGGC GTGCCACCTA CCTTATCATG GGCGGTGACG ACGGGGGACCA TTTTACCATC ACCACCCACC   1200
CTGAGAGCAA CCAGGGCATC CTGACAACCA GGAAGGGTTT GGATTTTGAG GCCAAAAACC AGCACACCCT GTACGTTGAA GTGACCAACG AGGCCCCTTT   1300
TGTGCTGAAG CTCCCAACCT CCACAGCCAC CATAGTGGTC CACGTGGAGG ATGTGAATGA GGCACCTGTG TTTGTCCCAC CCTCCAAAGT CGTTGAGGTC   1400
CAGGAGGGCA TCCCCACTGG GGAGCCTGTG TGTGTCTACA CTGCAGAAGA CCCTGACAAG GAGAATCAAA AGATCAGCTA CCGCATCCTG AGAGACCCAG   1500
CAGGGTGGCT AGCCATGGAC CCAGACAGTG GGCAGGTCAC AGCTGTGGGC ACCCTGCACC GTGAGGATGA GCAGTTTGTG AGGAACAACA TCTATGAAGT   1600
CATGGTCTTG GCCATGGACA ATGGAAGCCC TCCCACCACT GGCACGGGAA CCCTTCTGCT AACACTGATT GATGTCAACG ACCATGGCCC AGTCCCTGAG   1700
CCCCGTCAGA TCACCATCTG CAACCAAAGC CCTGTGCGCC ACGTGCTGAA CATCACGGAC AAGGACCTGT CTCCCCACAC CTCCCCTTTC CAGGCCCAGC   1800
TCACAGATGA CTCAGACATC TACTGGACGG CAGAGGTCAA CGAGGAAGGT GACACAGTGG TCTTGTCCCT GAAGAAGTTC CTGAAGCAGG ATACATATGA   1900
CGTGCACCTT TCTCTGTCTG ACCATGGCAA CAAAGAGCAG CTGACCGGTGA TCAGGGCCAC TGTGTGCGAC TGCCATGGCC ATGTCGAAAC CTGCCCTGGA   2000
CCCTGGAAAG GAGGTTTCAT CCTCCCTGTG CTGGGGGCTG TCCTGGCTCT GCTGTTCCTC CTGCTGGTGC TGCTTTTGTT GGTGAGAAAG AAGCGGAAGA   2100
TCAAGGAGCC CCTCCTACTC CCAGAAGATG ACACCCGTGA CAACGTCTTC TACTATGGCG AAGAGGGGGG TGGCGAAGAG GACCAGGACT ATGACATCAC   2200
CCAGCTCCAC CGAGGTCTGG AGGCCAGGCC GGAGGTGGTT CTCCGCAATG ACGTGGCCACC AACCATCATC CCGACACCCA TGTACCGTCC TAGGCCAGCC   2300
AACCCAGATG AAATCGGCAA CTTTATAATT GAGAACCTGA AGGCGGCTAA CACAGACCCC ACAGCCCCGC CCTACGACAC CCTCTTGGTG TTCGACTATG   2400
AGGGCAGCGG CTCCGACGCC GCGTCCCTGA GCTCCCTCAC CTCCTCCGCC TCCGACCAAG ACCAAGATTA CGATTATCTG AACGAGTGGG GCAGCCGCTT   2500
CAAGAAGCTG GCAGACATGT ACGGTGGCGG GGAGGACGAC TAGGCGGCCT GCCTGCAGGG CTGGGGACCA AACGTCAGGC CACAGAGCAT CTCCAAGGGG   2600
TCTCAGTTCC CCCTTCAGCT GAGGACTTCG GAGCTTGTCA GGAAGTGGCC GTAGCAACTT GGCGGAGACA GGCTATGAGT CTGACGTTAG AGTGGTTGCT   2700
TCCTTAGCCT TTCAGGATGG AGGAATGTGG GCAGTTTGAC TTCAGCACTG AAAACCTCTC CACCTGGGCC AGGGTTGCCT CAGAGGCCAA GTTTCCAGAA   2800
GCCTCTTACC TGCCGTAAAA TGCTCAACCC TGTGTCCTGG GCCTGGGCCT GCTGTGACTG ACCTACAGTG GACTTTCTCT CTGGAATGGA ACCTTCTTAG   2900
GCCTCCTGGT GCAACTTAAT TTTTTTTTTT AATGCTATCT TCAAAACGTT AGAGAAAGTT CTTCAAAAGT GCAGCCCAGA GCTGCTGGGC CCACTGGCCG   3000
TCCTGCATTT CTGGTTTCCA GACCCCAATG CCTCCCATTC GGATGGATCT CTGCGTTTTT ATACTGAGTG TGCCTAGGTT GCCCCTTATT TTTTATTTTC   3100
CCTGTTGCGT TGCTATAGAT GAAGGGTGAG GACAATCGTG TATATGTACT AGAACTTTTT TATTAAAGAA A                                   3171
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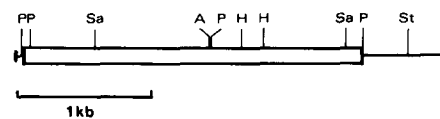λhP23 cDNA are shown in Figs. 1 and 2, respectively. λhP23 contains an insert consisting of 3,171 nucleotides, which show 77.0% homology with the nucleotide sequence of mouse P-cadherin cDNA (17). The open reading frame begins with an ATG codon at positions 54–56, terminates at a TAG codon at positions 2541–2543, and contains 2,487 nucleotides that correspond to 829 amino acids. Although no poly(A) tail is present in λhP23, a polyadenylation signal (ATTAAA), which is common to other cadherins reported so far (6, 15, 17), is identified at positions 3162–3167.

The deduced amino acid sequence of the polypeptide encoded by λhP23 cDNA as compared with that of mouse P-cadherin (17) is shown in Fig. 3. This protein, like mouse P-cadherin, has two hydrophobic regions that are considered to be signal and transmembrane sequences, and four cysteine residues in the extracellular domain that are conserved among the cadherin family. A major internal repeat found in mouse P-cadherin (17) is also observed, and three out of four possible NH₂-linked glycosylation sites of mouse P-cadherin are conserved in human P-cadherin. Homologies between the protein encoded by λhP23 cDNA (λhP23 protein) and cadherins that have been sequenced so far are shown in Fig. 4. The λhP23 protein is strikingly homologous with mouse P-cadherin; homology between the two proteins is 82% for the whole sequence and 87% for the putative mature protein. Homologies for the putative mature form between the λhP23 protein and mouse E-cadherin (15), chicken L-CAM (4, 21), which is considered to be chicken E-cadherin, and chicken N-cadherin (6) are lower, 58%, 58%, and 47%, respectively. Moreover, the precursor region of the λhP23 protein is far diversified from that of mouse E-cadherin, chicken L-CAM or chicken N-cadherin (Fig. 4). Human uvomorulin or cell-

CAM 120/80 is considered to be human E-cadherin, and is the only human cadherin molecule for which sequences have been reported (13, 23). In the 155 amino acid sequences of human uvomorulin that have been determined (23), there is only 67% homology with the λhP23 protein, whereas in the corresponding sequences homology between the λhP23 protein and mouse P-cadherin is 88%. These data strongly suggest that λhP23 cDNA encodes human P-cadherin.

mRNA Expression of P- and E-cadherin in Placenta

Although P-cadherin has been shown to be involved in the function of mouse placenta (16), its probable human homologue was not detected immunohistochemically in human placenta in contrast to the strong expression of E-cadherin (20). In this study, we examined the mRNA expression of human P- and E-cadherin in placenta as compared with those of mouse. Fig. 5 represents the mRNA expression of human P- (Fig. 5 a) and E-cadherin (Fig. 5 b). Human P-cadherin mRNA was detected as a single band, with an approximate molecular size of 3.2 kb, in poly(A)⁺RNA isolated from A-431 cells but not in placental poly(A)⁺RNAs after exposure for 12 h (Fig. 5 a, lanes 1–5). When the filter was exposed

Figure 2. Restriction endonuclease map of λhP23 cDNA. The open reading frame is shown as a clear box. P, Pst I; Sa, Sac I; A, Acc I; H, Hinc II; St, Stu I.

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MGLPRGPLASLLLL-QVCWLQCAASEPCRAVFR-EAEVTLEAGGAEQEPGQALGKVFMGCPGQEPALFSTDNDDFTVRNG    78
*  *  **  *  ****  *****     ***  **  *  **  ****  *   **  *  ****        **       **  *
MELLSGPHAFLLLLLQVCWLRSVVSEPYRAGFIGEAGVTLEVEGTDLEPSQVLGKVALA--GQ--GMHHADNGDIIMLTR    76


ETVQERRSLKERNPLKIFPSKRILRRHKRDWVVAPISVPENGKGPFPQRLNQLKSNKDRDTKIFYSITGPGADSPPEGVF   158
***     *      *  *****  **   **  ****************************  ********************
GTVQG--G-KD--AMHS-PPTRILRRRKREWVMPPIFVPENGKGPFPQRLNQLKSNKDRGTKIFYSITGPGADSPPEGVF   150
                                    ▼
AVEKETGWLLLNKPLDREEIAKYELFGHAVSENGASVEDPMNISIIVTDQNDHKPKFTQDTFRGSVLEGVLPGTSVMQVT   238
   ***  *****  *****  *  ****  ************  *************  ***  **************   *********
TIEKESGWLLLHMPLDREKIVKYELYGHAVSENGASVEEPMNISIIVTDQNDNKPKFTQDTFRGSVLEGVMPGTSVMQVT   230
                                                     ▲

ATDEDDAIYTYNGVVAYSIHSQEPKDPHDLMFTIHRSTGTISVISSGLDREKVPEYTLTIQATDMDGDGSTTTAVAVVEI   318
*******  ****************************  ********  ***  *************  ******  **  *******  ******  *** *
ATDEDDAVNTYNGVVAYSIHSQEPKEPHDLMFTIHKSTGTISVISSGLDGEKVPEYRLTVQATDMDGEGSTTTAEAVVQI   310


LDANDNAPMFDPQKYEAHVPENAVGHEVQRLTVTDLDAPNSPAWRATYLIMGGDDGDHFTITTHPESNQGILTTRKGLDF   398
********  *  ******  ****  **************  **  *******  *  **************  ***  ***  *****
LDANDNAPEFEPQKYEAWVPENEVGHEVQRLTVTDLDVPNWPAWRATYHIVGGDDGDHFTITTHPETNQGVLTTKKGLDF   390


EAKNQHTLYVEVTNEAPFVLKLPTSTATIVVHVEDVNEAPVFVPPSKVVEVQEGIPTGEPVCVYTAEDPDKENQKISYRI   478
**  **************  ****  ***  ****  *************  *  ****   **  **  ***  *****  *****  *
EAQDQHTLYVEVTNEAPFAVKLPTATATVVVHVKDVNEAPVFVPPSKVIEAQEGISIGELVCIYTAQDPDKEDQKISYTI   470
                                    ▼                                        ▲▼
LRDPAGWLAMDPDSGQVTAVGTLDREDEQFVRNNIYEVMVLAMDNGSPPTTGTGTLLLTLIDVNDHGPVPEPRQITICNQ   558
****  ***  ******  **  *  *********  **  *******  *  *  *************  *  *****  ******  ****
SRDPANWLAVDPDSGQITAAGILDREDEQFVKNNVYEVMVLATDSGNPPTTGTGTLLLTLTDINDHGPIPEPRQIIICNQ   550
                                                                           ▲▲
▼
SPVRHVLNITDKDLSPHTSPFQAQLTDDSDIYWTAEVNEEGDTVVLSLKKFLKQDTYDVHLSLSDHGNKEQLTVIRATVC   638
***  ***********  ********  ******  ***  *  ****  *************  *********  ****  ******
SPVPQVLNITDKDLSPNSSPFQAQLTHDSDIYWMAEVSEKGDTVALSLKKFLKQDTYDLHLSLSDHGNREQLTMIRATVC   630
◆     ▲◆        ▲                                                                                  ◆
DCHGHV-ETCPGPWKGGFILPVLGAVLALLFLLLVLLLLVRKKRKIKEPLLLPEDDTRDNVFYYGEEGGGEEDQDYDITQ   717
****  *    **  *********  ********  ***  **********  *****************************************
DCHGQVFNDCPRPWKGGFILPILGAVLALLTLLLALLLLVRKKRKVKEPLLLPEDDTRDNVFYYGEEGGGEEDQDYDITQ   710
◆      ◆                                                                                ◆
LHRGLEARPEVVLRNDVAPTIIPTPMYRPRPANPDEIGNFIIENLKAANTDPTAPPYDTLLVFDYEGSGSDAASLSSLTS   797
***************  **  ***********************************************  *  ******************
LHRGLEARPEVVLRNDVVPTFIPTPMYRPRPANPDEIGNFIIENLKAANTDPTAPPYDSLMVFDYEGSGSDAASLSSLTT   790


SASDQDQDYDYLNEWGSRFKKLADMYGGGEDD   829
********  *********************
SASDQDQDYNYLNEWGSRFKKLADMYGGGEDD   822
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Figure 3. Comparison of amino acid sequences of the protein encoded by λhP23 cDNA (*upper*) and mouse P-cadherin (*lower*) (17). Conserved residues are marked by asterisks. The putative signal peptide and transmembrane regions are indicated by bold lines. The NH₂ terminus of the mature protein predicted from data on other cadherins is indicated by a vertical line. The major internal repeats and possible NH₂-linked glycosylation sites are shown by arrows and arrowheads, respectively. The four cysteine residues conserved among the cadherin family are marked by rhombi.

for 5 d, a faint band appeared at a position of ∼3.2 kb even in poly(A)⁺RNAs of early-stage placenta (Fig. 5 *a*, lanes 6-8). However, human E-cadherin mRNA, ∼4.5 kb in size, was clearly detected in poly(A)⁺RNAs isolated from both

A-431 cells and placental tissues at 8 to 11 wk of pregnancy (Fig. 5 *b*). These results are consistent with the immunohistochemical data previously reported (20). In mouse placental tissues, both P- and E-cadherin mRNAs were clearly

Figure 4. Homologies between the protein encoded by λhP23cDNA and other cadherins in separate regions, whole protein and putative mature protein. The extracellular domain was divided into 5 regions according to Hatta et al. (6). *mP* (●), *hU* (□), *mE* (○), *cL* (△), and *cN* (▲) represent percentage homology between the protein encoded by λhP23cDNA and mouse P-cadherin, human uvomorulin (cell–CAM 120/80), mouse E-cadherin, chicken L-CAM, and chicken N-cadherin, respectively. The sequence data for human uvomorulin (cell–CAM 120/80) are only partially available at present (13, 23). *S*, signal peptide; *P*, precursor region; *EC*, extracellular domain; *TM*, transmembrane domain; *IC*, intracellular domain; *WP*, whole protein; *MP*, mature protein.

Figure 5. Northern blot analysis of poly(A)⁺RNAs isolated from human carcinoma cells and placenta. (*a*) Poly(A)⁺ RNAs from A-431 cells (lane *1*), and human placenta at 8 (lanes *2* and *6*), 10 (lanes *3* and *7*), 11 wk (lanes *4* and *8*), and 38 wk (lanes *5* and *9*) of pregnancy were hybridized with a human P-cadherin probe. Lanes *6–9* show lanes *2–5* after exposure periods ten times longer than those for lanes *1–5*. (*b*) The same filter as that in (*a*) was rehybridized with a mouse E-cadherin probe. Positions of 28S and 18S ribosomal RNAs are marked.

coexpressed (Fig. 6). P-cadherin mRNA was strongly expressed in mouse placenta throughout pregnancy, and the expression level of P-cadherin mRNA seemed to be somewhat stronger than that of E-cadherin mRNA, corresponding well with the previous immunohistochemical data (16).

Transfection into NIH3T3 Cells

Human P-cadherin cDNA placed downstream from the chicken β actin promoter was introduced into NIH3T3 cells by cotransfection with an expression vector pβact-hP, which covers the entire coding region of λhP23 cDNA, and pSTneoB, which carries a G418-resistant gene. After ∿2 wk of incubation in the presence of G418, more than one hundred colonies appeared, and 24 colonies were picked up. Of these, 22 clones reacted with mAb NCC-CAD-299 immunocytochemically, and several positive clones were recloned. Figs. 7 and 8 show the reactivity of one transfectant, designated PN3-5, with mAb NCC-CAD-299 on immunocytochemistry and Western blot analysis, respectively. The molecule recognized by mAb NCC-CAD-299 was not identified in parental NIH3T3 cells, but it was expressed at the cell–cell border as well as in the

cytoplasm of PN3-5 cells at the same size as that in A-431 cells. In addition, PN3-5 cells appeared to adhere to one another more tightly than NIH3T3 cells, and they were dissociated by mAb NCC-CAD-299 (Fig. 9), indicating that the cadherin molecule expressed at the cell–cell border of the transfectants was functional and that the molecule detected by mAb NCC-CAD-299 is human P-cadherin.

Discussion

P-cadherin is a subclass of the cadherin family originally identified in mouse placental tissues, where its localization suggests a function of connecting the embryo to the uterus (16). In contrast, our previous immunohistochemical study suggested that the cadherin that plays the leading role in cell–cell adhesion in human placental tissue is not the P- but the E- type (20). In this study, we cloned and sequenced human P-cadherin cDNA, and investigated the RNA expression of both cadherin types in mouse and human placental tissues.

A cDNA clone, λhP23, covering the entire open reading
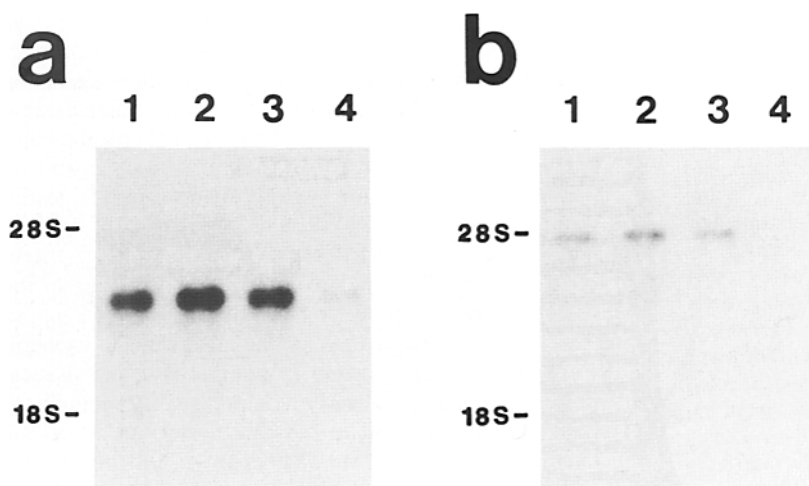
Figure 6. Northern blot analysis of poly(A)⁺ RNAs isolated from mouse placenta. (*a*) Poly-(A)⁺RNAs from fetectomied ICR mouse uterus at 10.5 d of pregnancy (lane *1*) and mouse placenta at 12.5 (lane *2*), 14.5 (lane *3*), and 16.5 d (lane *4*) of pregnancy were hybridized with a mouse P-cadherin probe. (*b*) The same filter as that in *a* was rehybridized with a mouse E-cadherin probe. The filter was exposed for 5 h in both *a* and *b*. Positions of 28S and 18S ribosomal RNAs are marked.
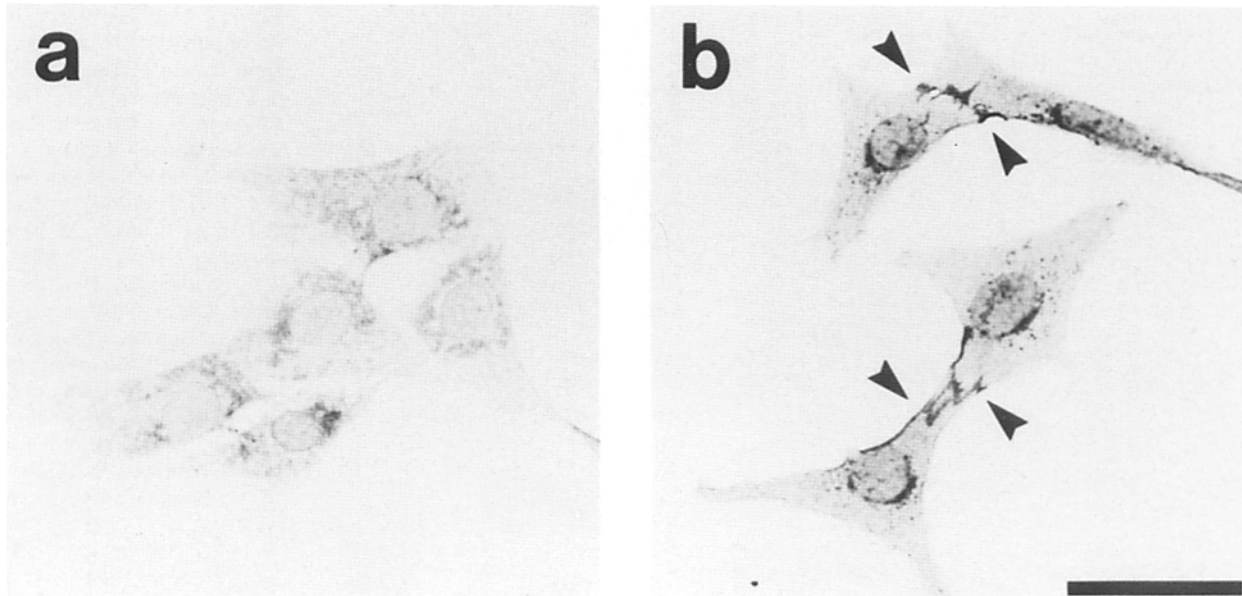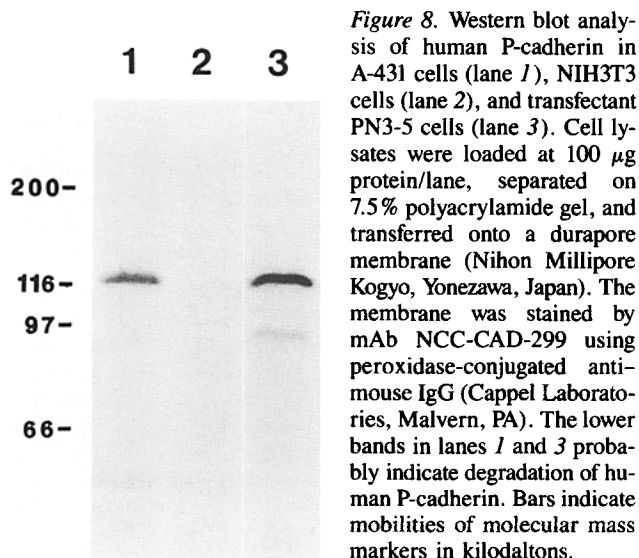
Figure 7. Immunocytochemistry of NIH3T3 cells and transfectant PN3-5 cells. NIH3T3 cells (*a*) and PN3-5 cells (*b*) were stained by mAb NCC-CAD-299 using biotinylated anti–mouse IgG and avidin-biotin-peroxidase complex, and counterstained with hematoxylin. Positive staining at the cell–cell border of PN3-5 cells, *arrowheads*. Bar, 50 μm.
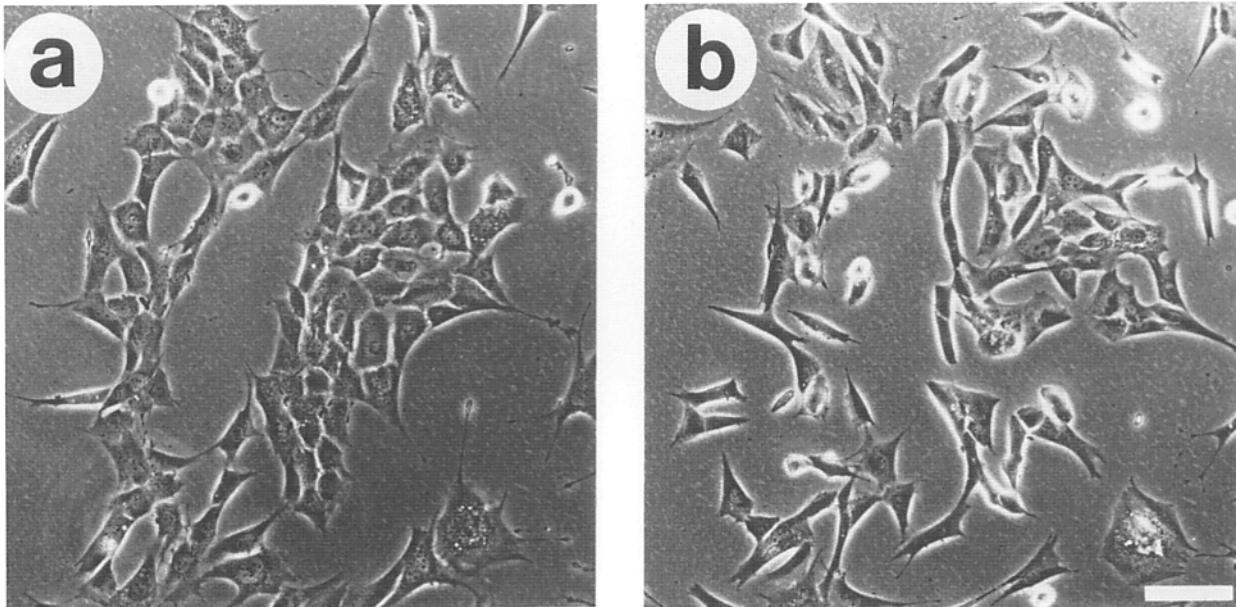
frame of human P-cadherin, was isolated by screening a λgt10 cDNA library constructed from A-431 poly(A)+RNA with a mouse P-cadherin cDNA probe under stringent conditions of hybridization. This cDNA consists of 3,171 nucleotides, and contains an open reading frame encoding a polypeptide of 829 amino acids. The deduced amino acid sequence of this protein showed that it is an integral membrane protein with structural properties common to the cadherin family. Comparison of the primary structure between this protein and other cadherins revealed a striking homology with mouse P-cadherin, indicating that λhP23 cDNA encodes a human counterpart of mouse P-cadherin. The sequence of human P-cadherin provides further information on the evolutionary conservation of each cadherin

Figure 8. Western blot analysis of human P-cadherin in A-431 cells (lane *1*), NIH3T3 cells (lane *2*), and transfectant PN3-5 cells (lane *3*). Cell lysates were loaded at 100 μg protein/lane, separated on 7.5% polyacrylamide gel, and transferred onto a durapore membrane (Nihon Millipore Kogyo, Yonezawa, Japan). The membrane was stained by mAb NCC-CAD-299 using peroxidase-conjugated anti-mouse IgG (Cappel Laboratories, Malvern, PA). The lower bands in lanes *1* and *3* probably indicate degradation of human P-cadherin. Bars indicate mobilities of molecular mass markers in kilodaltons.

subclass. The amino acid sequences of mouse P- and E-cadherin are only 58% identical (17), whereas the degree of homology between mouse and human P-cadherin is 87%. In the restricted amino acid sequences, human uvomorulin (cell-CAM 120/80) is 89% and 67% homologous with mouse E-cadherin and human P-cadherin, respectively. However, between greatly diversified species, the degree of similarity in the same subclass is quite low: chicken L-CAM shows only 65% homology with mouse E-cadherin. It is known that the amino acid sequences of cadherins among different subclasses and species are most conserved in the intracellular domain (22) and that the interaction between cadherins and the cytoskeleton must be critical for the cell–cell binding function of cadherins (7, 14). Human P-cadherin and other cadherin molecules all end in a hydrophilic and highly conserved sequence, Arg-Phe-Lys-Lys-Leu-Ala-(Asp/Glu)-(Met/Leu)-Tyr-Gly-Gly-Gly-(Glu/-)-Asp-Asp-(-/Asp). It is conceivable that this sequence may be involved in the binding of cadherins to some cytoskeletal components, which may support the tertiary structure of the extracellular domain necessary for the homophilic binding of cadherins. Analyses of the primary structure of cadherins have not provided any clear answer to the question of which sequences in the extracellular domain are responsible for the homophilic and subclass-specific binding of cadherins. However, some clue to the mechanism may be obtained in the near future by experiments using artificially designed mutant cadherin molecules.

On Northern blot analysis, the amount of P-cadherin mRNA in human placenta was very low, in contrast to the presence of large amounts of P-cadherin mRNA in mouse placenta throughout pregnancy. On the other hand, E-cadherin mRNA was definitely present in human placental tissues. These results correspond well to the previous immunohistochemical findings (20). The possibility that λhP23 cDNA encodes a P-related cadherin distinct from authentic P-cadherin, which is as strongly expressed in human placenta as

Figure 9. Phase-contrast micrographs of transfectant PN3-5 cells; *b* shows the same field as *a* at 4 h after addition of mAb NCC-CAD-299. Bar, 100 μm.

it is in mouse, is thus very unlikely. If this were the case, Northern blot analysis of human placental RNAs would have revealed the authentic P-cadherin mRNA as a stronger band distinct from the ~3.2-kb band. However, no such band was ever detected upon human placental RNA blotting even when hybridized with mouse P-cadherin cDNA at low stringency (data not shown).

The cloning of human P-cadherin cDNA enabled us to introduce the cDNA exogenously into NIH3T3 cells. The transfectants expressing ectopic human P-cadherin acquired the enhanced cell–cell binding ability mediated by this molecule, providing direct evidence that human P-cadherin is involved in cell–cell adhesion. Furthermore, the immunoreactivity of mAb NCC-CAD-299 with the transfectants showed that the molecule identified by the mAb was undoubtedly human P-cadherin.

Recently, the expression pattern of P- and E-cadherin in mouse tissues during the perimplantation period was reported in detail (9). According to that study, P-cadherin is first expressed on differentiated trophectoderm cells and decidual cells on day 5 of pregnancy and appears to be used for connection of embryonal tissues to maternal decidual cells. However, in human placental tissues both embryonal cytotrophoblast cells and maternal decidual cells express only E-cadherin and not P-cadherin after 6 wk of pregnancy (20). The finding that the main cadherin subclass in placental tissues may differ between human and mouse is very surprising. However, we cannot conclude that P-cadherin is not involved in human embryogenesis, since it is not possible to examine human placental tissues just after implantation. Three possible patterns of cadherin expression in human placental tissues are conceivable: (*a*) initial coexpression of P- and E-cadherin as in the basal layers of stratified epithelia, and cessation of P-cadherin expression in placental tissues as in the upper layers of stratified epithelia (20); (*b*) strong expression of P-cadherin even in human placental tissues at a very early stage of pregnancy, as is the case in mouse, with E-cadherin soon taking

its place. (*c*) Connection of the embryo to the uterus only by E-cadherin. The faint expression of human P-cadherin mRNA in early-stage placenta may support the first or second possibility. However, the main cadherin type responsible for human placental structures, at least from the middle to late stage of pregnancy, would be E.

Our previous study showed that the localization of human P-cadherin in normal tissues is extremely restricted (20). However, all of the forty lung tumors examined in that study expressed P-cadherin. Recently, we found immunohistochemical expression of P-cadherin in some carcinoma cells, the normal counterparts of which do not express P-cadherin (Shimoyama, Y., T. Yoshida, M. Terada, Y. Shimosato, O. Abe, and S. Hirohashi, unpublished data). This suggests that some regulatory system suppressing the expression of the human P-cadherin gene in normal epithelia is lost in the process of carcinogenesis. The nature of this regulatory mechanism, the reason for its loss in carcinogenesis, and how the newly acquired P-cadherin affects the biological behavior of carcinoma are aspects that remain to be investigated.

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