MOLECULAR AND CELLULAR BIOLOGY, Mar. 1995, p. 1175–1181 Vol. 15, No. 3
0270-7306/95/$04.00+0

Loss of E-Cadherin-Dependent Cell-Cell Adhesion due to Mutation of the β-Catenin Gene in a Human Cancer Cell Line, HSC-39

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Received 3 June 1994/Returned for modification 15 July 1994/Accepted 5 December 1994

Detachment of cell-cell adhesion is indispensable for the first step of invasion and metastasis of cancer. This mechanism is frequently associated with the impairment of either E-cadherin expression or function. However, mechanisms of such abnormalities have not been fully elucidated. In this study, we demonstrated that the function of E-cadherin was completely abolished in the human gastric cancer cell line HSC-39, despite the high expression of E-cadherin, because of mutations in one of the E-cadherin-associated cytoplasmic proteins, β-catenin. Although immunofluorescence staining of HSC-39 cells by using an anti-E-cadherin antibody (HECD-1) revealed the strong and uniform expression of E-cadherin on the cell surface, cell compaction and cell aggregation were not observed in this cell. Western blotting (immunoblotting) using HECD-1 exhibited a 120-kDa band which is equivalent to normal E-cadherin. Northern (RNA) blotting demonstrated a 4.7-kb band, the same as mature E-cadherin mRNA. Immunoprecipitation of metabolically labeled proteins with HECD-1 revealed three bands corresponding to E-cadherin, α-catenin, and γ-catenin and a 79-kDa band which was apparently smaller than that of normal β-catenin, indicating truncated β-catenin. The 79-kDa band was immunologically identified as β-catenin by using immunoblotting with anti-β-catenin antibodies. Examination of β-catenin mRNA by the reverse transcriptase-PCR method revealed a transcript which was shorter than that of normal β-catenin. The sequencing of PCR product for β-catenin confirmed deletion in 321 bases from nucleotides +82 to +402. Southern blotting of β-catenin DNA disclosed mutation at the genomic level. Expression vectors of β-catenin were introduced into HSC-39 cells by transfection. In the obtained transfectants, E-cadherin-dependent cell-cell adhesiveness was recovered, as revealed by cell compaction, cell aggregation, and immunofluorescence staining. From these results, it was concluded that in HSC-39 cells, impaired cell-cell adhesion is due to mutations in β-catenin which results in the dysfunction of E-cadherin.

Cadherins are calcium-dependent cell-cell adhesion molecules that play an important role in the construction of tissues and organs (7, 33). E-cadherin is a subclass of cadherins and is responsible for epithelial intercellular adhesion. Intercellular adhesion by E-cadherin is controlled by its association with catenin family proteins (16, 20, 34). This association links the actin filaments and the cytoplasmic domain of E-cadherin molecules (18, 22, 24). In cancer tissues, detachment of cell-cell adhesion is indispensable for the first step of invasion and metastasis of cancer cells (12). It has been suggested that this detachment is related to reduced expression of E-cadherin (3, 5, 6, 9, 15, 21, 28, 29). Previously we reported on the reduced expression of E-cadherin in gastric cancer with high invasion. We also reported that tumor cells in the carcinomatous fluids from 10 of 11 cancers, including gastric, pancreatic, and lung cancers, lacked E-cadherin expression (13). However, it has recently been reported that in tumor cells from certain malignancies, disruption of cell-cell adhesion was caused by reduced function of E-cadherin even though its expression was normal (13, 17, 30). Shimoyama et al. reported an impairment of intercellular adhesion of E-cadherin in a lung cancer cell line (PC-9) resulting from the loss of expression of α-catenin, one of the catenin family proteins (30). Using the same cell line, Hirano et al. demonstrated a recovery of E-cadherin function by transfection of α-catenin cDNA (10). A similar observation of an impairment of adhesion due to a lack of α-catenin genes was made by Morton et al. with a prostate cancer cell line (PC-3) (17). Furthermore, in human esophageal cancer tissues, an immunohistological study confirmed the loss of α-catenin expression, and this loss was reported to correlate with both the degree of infiltration and the extent of lymph node metastasis (11).

As for β-catenin, another catenin family protein, a recent study demonstrated that its modification, tyrosine phosphorylation by v-*src* gene transduction, resulted in the dysfunction of E-cadherin (1, 8, 14). In addition, a more recent study showed that *APC* genes, which are linked both to familial adenomatous polyposis and to the progression of sporadic colorectal and gastric tumors, connected with β-catenin, suggesting a possible link between carcinogenesis and β-catenin expression (25, 31). In the present study, we describe a loss of intercellular adhesion of E-cadherin caused by mutations of β-catenin genes in a sparsely infiltrating signet ring cell carcinoma, HSC-39.

MATERIALS AND METHODS

Cell culture. MKN-28, a well-differentiated gastric adenocarcinoma cell line, was kindly provided by The Green Cross Co. (Osaka, Japan). HSC-39, a sparsely infiltrating signet ring cell carcinoma (35), was kindly provided by H. Yanagihara (Hiroshima University, Hiroshima, Japan). MKN-28 cells and HSC-39 cells were routinely maintained in RPMI 1640 (Nissui Co., Tokyo, Japan) and in Dulbecco's modified minimum essential medium (Nissui Co.), respectively, supplemented with 10% heat-inactivated fetal calf serum in humidified 5% CO_2–air at 37°C.

Antibody. A mouse monoclonal antibody (HECD-1) against human E-cad-

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herin (29) was kindly provided by M. Takeichi (Kyoto University, Kyoto, Japan). A mouse monoclonal antibody against a protein fragment corresponding to amino acids 570 to 782 of mouse β-catenin was purchased from Transduction Laboratories (Lexington, Ky.).

Immunofluorescence staining for E-cadherin. First, nonadherent (HSC-39) cells were evacuated by syringe onto membrane filters (PORTEC; Asahi Medical Co., Tokyo, Japan), and adherent (MKN-28 and wild-type β-catenin cDNA-transfected HSC-39) cells were attached onto SlideFlask (Nunc, Roskilde, Denmark). The cells were then fixed in 3.5% paraformaldehyde in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered saline solution (HBSS)–Ca^{2+} (37 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 5.6 mM glucose, 10 mM HEPES, 2 mM $CaCl_2$, [pH 7.4]) for 1 h. After being coated with a 5% normal goat serum-HBSS-Ca^{2+} solution, the cells were incubated with a 1:1,000 dilution of HECD-1 for 1 h and then with fluorescein isothiocyanate-labeled anti-mouse immunoglobulin G antibody (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Md.) for another hour. The membrane filters were washed extensively with HBSS-Ca^{2+} and mounted with 90% glycerol–10% HBSS-Ca^{2+} containing 0.1% *p*-phenylenediamine (13). Observations were performed with a fluorescence microscope (Axiovert 405M; Zeiss, Oberkochen, Germany).

Assay for cell aggregation. Cells were dissociated with 0.01% crystalline trypsin (type I; Sigma Chemical Co., St. Louis, Mo.) in HBSS-Ca^{2+} and washed twice in Ca^{2+}- and Mg^{2+}-free HBSS. The cells were judged more than 96% viable by trypan blue dye exclusion. Cells (10^5) resuspended in 1 ml of HBSS-Ca^{2+} were incubated for 60 min at 37°C on a gyratory shaker. After incubation, the total particle number (single cells plus cell clusters) in each cell suspension was counted with a Coulter Counter (model ZM; Coulter Electronics Inc., Hialeah, Fla.). The degree of aggregation was represented by the aggregation index N_t/N_0, where N_0 was the total particle number before incubation and N_t was the total particle number after incubation for t minutes (32).

Western blot (immunoblot) analysis of E-cadherin and β-catenin. Cells (5×10^5) were prepared by washing with phosphate-buffered saline (PBS) prior to solubilization in lysis buffer (10) containing 1% Triton X-100, 1% Nonidet P-40, 2 mM $CaCl_2$, 0.7 μg of pepstatin per ml, 50 μg of antipain dihydrochloride per ml, 0.5 μg of leupeptin per ml, and 1 mM 4-2-aminoethyl benzenesulfonylfluoride (Boehringer GmbH, Mannheim, Germany) for 1 h at 4°C. Cell extracts were denatured and reduced with a sample buffer containing 1% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol. After boiling for 5 min, the aliquot of the sample was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on a 7.5% polyacrylamide gel containing 0.1% SDS. The fractionated proteins were transferred onto Immobilon-P membranes (Millipore Co., Bedford, Mass.) at 100 mA for 1 h in Bjerrum and Sehafer-Nielsen transfer buffer (2) (48 mM Tris, 39 mM glycine, 20% methanol, 0.01% SDS, [pH 9.2]) with a semidry transfer cell unit (Bio-Rad, Richmond, Calif.). After the membrane was blocked with 5% nonfat dry milk–PBS for 1 h at room temperature, the membrane was probed with a 1:1,000 dilution of HECD-1 or 1 μg of anti-β-catenin antibody per ml and then visualized by using the Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, Calif.) and the colorimetric substrate 3,3′-diaminobenzidine tetrahydrochloride as instructed by the manufacturer.

Immunoprecipitation. Immunoprecipitation of metabolically labeled cellular proteins with HECD-1 in HSC-39 and MKN-28 cells was carried out as described by Nagafuchi and Takeichi (19), with some modifications. Cells (10^6) were labeled with [^{35}S]methionine (200 μCi/ml; Amersham International plc., Amersham, United Kingdom) for 16 h at 37°C in methionine-free minimal essential medium (Gibco, Gaithersburg, Md.) supplemented with 10% dialyzed fetal calf serum. After incubation, cells were collected and washed three times with PBS and were lysed with 1 ml of lysis buffer supplemented with 1 mg of bovine serum albumin (BSA) per ml for 1 h at 4°C. Nuclei and debris were removed by centrifugation at 14,000 × *g* for 30 min. The supernatant was incubated with 10 μg of normal mouse immunoglobulin (Inter-Cell Technologies Inc., Hopewell, N.J.) and 60 μl of protein A–Sepharose CL-4B (Pharmacia Biotech, Sollentuna, Sweden) suspended in the lysis buffer used in Western blotting for absorbing components which might nonspecifically bind to the beads. After removal of the beads by centrifugation at 13,000 × *g* for 5 min, the supernatant was incubated with a 1:1,000 dilution of HECD-1 or 5 μg of anti-β-catenin antibody in HBSS-Ca^{2+} for 1 h. Sixty microliters of protein A–Sepharose CL-4B (Pharmacia Biotech) was added, and the mixture was incubated for 1 h. The immune complexes were washed five times with lysis buffer without BSA, and proteins were released from the beads by boiling for 5 min with 30 μl of SDS-PAGE sample buffer containing 5% 2-mercaptoethanol for 5 min. The released materials were subjected to SDS-PAGE using 7.5% polyacrylamide gels. For autoradiography, gels were immersed in Enlightning (DuPont NEN Research Products, Boston, Mass.) for 30 min to enhance signals, dried, and exposed to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.).

Extraction of cellular RNA. Poly(A)$^+$ RNAs were extracted by using a Fast Track kit (Invitrogen, San Diego, Calif.) on the basis of the affinity of poly(A) tail in mRNA to oligo(dT)-cellulose.

Preparation of E-cadherin cDNA. The full-length human E-cadherin cDNA has been described elsewhere (4). Oligonucleotide reverse transcription-PCR (RT-PCR) primers were synthesized on a PCR-MATE DNA synthesizer (model 391; Applied Biosystems, Inc., Foster City, Calif.). An 18-base oligomer (PE-2; nucleotide positions 2337 to 2354), corresponding to the sequence in the human E-cadherin cDNA, was used as a primer in the reverse transcription. One microgram of mRNA extracted from MKN-28 cells was converted to the first strand of cDNA by incubation at 42°C for 30 min in buffer containing 0.5 μM PE-2 oligomer, 0.5 mM deoxynucleoside triphosphates (dNTPs), 0.01 M dithiothreitol, 200 U of SuperScript II RNase H$^-$ reverse transcriptase (Life Technologies, Inc., Gaithersburg, Md.), and 1× reverse transcriptase buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$ [pH 8.3]) with a 20-μl reaction volume. The mixture was then denatured at 95°C for 5 min and cooled on ice. For amplification of the cDNA products, the sense primer PE-1 (nucleotide positions 520 to 537) and the antisense primer PE-2 were used to amplify a 1,835-bp fragment containing an *Eco*RI site for cloning. The 50-μl reaction mixture contained 2 μl of cDNA products, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.2 μM each of the PE-1 and PE-2 primers, 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.001% gelatin [pH 8.3]), and 2.5 U of TaKaRa *Taq* (Takara Shuzo, Kyoto, Japan). The samples were overlaid with mineral oil (Sigma), and 25 cycles of amplification were performed on a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, Conn.) with a step program involving denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and polymerization at 72°C for 1.5 min. After PCR, the reaction products were electrophoresed on a 0.8% low-melting-temperature agarose gel (Gibco BRL, Gaithersburg, Md.), and a fragment of 1,835 bp in size was extracted. The fragment was digested with endonuclease *Eco*RI (New England Biolabs, Inc., Beverly, Mass.), and the resulting 1,365-bp fragment was subcloned into the *Eco*RI site of cloning vector pUC18 (Takara Shuzo) to yield pUC/HECD.

Northern (RNA) blot analysis of E-cadherin. Poly(A)$^+$-enriched RNA (20 μg) was separated by electrophoresis on a 1.0% agarose–formaldehyde gel and transferred onto a Hybond-N membrane (Amersham) by using the capillary elution method in 20 × SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) (26). The membrane was baked in a vacuum oven at 80°C for 2 h. Prehybridization was carried out in a mixture containing 50% formamide, 5× SSPE (1× SSPE is 0.15 M NaCl, 0.01 M Na_2HPO_4, and 1 mM EDTA), 2× Denhardt's solution (100× Denhardt's solution is 2% polyvinylpyrrolidone, 2% BSA, and 2% Ficoll 400), 0.1% SDS, and 100 μg of sonicated salmon testis DNA (Sigma) per ml at 42°C for 2 h. Hybridization was carried out for 24 h in the same buffer at 42°C with the ^{32}P-labeled probe at 2.5×10^6 cpm/ml. The probe used was a 1,365-bp *Eco*RI fragment from the human E-cadherin cDNA clone pUC/HECD as described above. The probe was labeled with [α-^{32}P]dCTP (Amersham) by using a *Bca*BEST labeling kit (Takara Shuzo). The membrane was washed for 20 min at room temperature in 1× SSC–0.1% SDS, washed three times from 20 min each at 65°C in 0.2× SSC–0.1% SDS, and exposed to X-Omat AR film (Eastman Kodak) with an intensifying screen. The molecular weight of the RNA examined was determined by reference to the migration of the 0.24- to 9.5-kb RNA ladder (Gibco BRL).

RT-PCR amplification of β-catenin mRNA. A pair of primers was designed to amplify the entire coding region of β-catenin mRNA isolated from MKN-28 and HSC-39 cells. A sense primer, Pr-1, corresponding to nucleotides −18 to +10 of the β-catenin cDNA (accession number Z19054 in the EMBL/GenBank data libraries), and a antisense primer, Pr-2, corresponding to nucleotides +2338 to +2361, were used. In each primer, two nucleotides were mutated to construct the *Xba*I restriction site and to facilitate subcloning in the following experiments. The sequences of these primers are as follows (underlined sequences are *Xba*I restriction sites): Pr-1 (sense), 5′-GAA AAT CTA GAG TGG ACA ATG GCT ACT C-3′; Pr-2 (antisense), 5′-CTT CTA GAG GAT GAT TTA CAG GTC-3′. Otherwise, the procedure for RT-PCR was the same as that for E-cadherin as described above. Five microliters of the PCR product was then electrophoresed on a 1% agarose gel along with size markers. The expected amplification product consisted of 2,379 bp containing 18 bases of the 5′ untranslated region, the entire coding region, and 18 bases of the 3′ untranslated region of the human β-catenin message.

Sequencing of β-catenin cDNA. A pair of primers was designed to amplify the N-terminal region of β-catenin mRNA (see Results). The Pr-1 primer described above was used as a sense primer, and an antisense primer, Pr-3, corresponding to nucleotides +501 to +524, which was mutated to construct the *Xba*I restriction site, was synthesized. Otherwise, the procedure for RT-PCR was the same as that for E-cadherin as described above. The reaction products were separated by gel electrophoresis using a low-melting-temperature agarose, and the fragment of interest was extracted. The fragment was digested with endonuclease *Xba*I (New England Biolabs) and subcloned into the *Xba*I site of cloning vector pUC118 (Takara Shuzo). The single-stranded vector containing β-catenin cDNA was purified from the supernatant of a bacterial culture infected by the helper phage M13K07 (Takara Shuzo). The purified DNA was sequenced by the dideoxy-chain termination method, using Sequenase version 2 enzyme and the biotinylated primer (United States Biochemical Co., Cleveland, Ohio) as instructed by the manufacturer. Sequencing reaction products were heated at 90°C for 2 min, loaded on a 6% polyacrylamide gel containing 7 M urea in 1× TBE (0.18 M Tris-borate, 4 mM EDTA [pH 8.0]), and electrophoresed at 30 W for 3 h. After electrophoresis, the sequence ladders were transferred to a positively charged nylon membrane by using the contact blot method. The immobilized sequence ladders were detected by the enzyme-catalyzed light reaction method, using an Imaging high-chemiluminescence detection kit (United States Biochemical Co.) as instructed by the manufacturer.

Construction of an expression vector for human β-catenin. A β-catenin cDNA encoding the protein-translating region was cloned by RT-PCR using β-catenin mRNA extracted from MKN-28 cells. After PCR, the amplification products

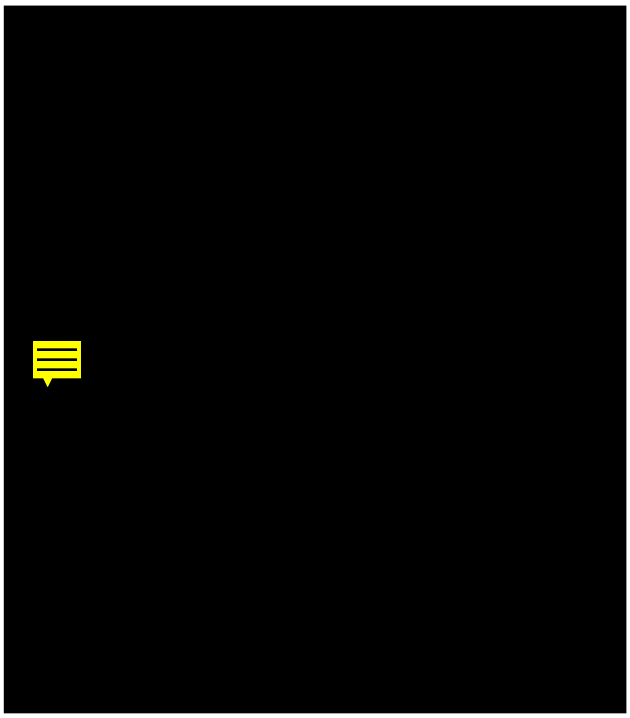

FIG. 1. Phase-contrast microscopic appearances of MKN-28 cells and HSC-39 cells and immunofluorescence staining pattern for E-cadherin. A phase-contrast photograph showed tight intercellular adhesion of MKN-28 cells (A) and loose attachment with no apparent cell-cell contact of HSC-39 cells (B). For immunofluorescence staining using a monoclonal antibody to the N-terminal portion of E-cadherin (HECD-1), 10^4 HSC-39 cells (D) or MKN-28 cells (C) were either applied by syringe onto membrane filters or attached onto Slide-Flask, respectively, and the cells were fixed and stained as described in Materials and Methods. Magnifications, ×388 (A and B) and ×776 (C and D).

were separated by low-melting-temperature agarose gel electrophoresis, and a 2.4-kb fragment was extracted. After digestion of this fragment with endonuclease *Xba*I, the resulting fragment contained six bases of the 5′ untranslated region, the entire coding region, and seven bases of the 3′ untranslated region of the β-catenin message. The restriction fragment was inserted into the eukaryotic expression vector pRc/CMV (Invitrogen) to yield pRc/CMV/HCA-β. The orientation of the β-catenin cDNA in the vector was determined by the digestion of the recombinant vector with endonuclease *Bgl*II (New England Biolabs).

Transfection of β-catenin cDNA into HSC-39 cells. HSC-39 cells (10^6/ml of Opti-MEM) in a 35-mm-diameter culture dish were transfected with 5 μg of the β-catenin mRNA expression vector linearized with endonuclease *Sca*I (New England Biolabs), using Lipofectin (Life Technologies) as described previously (27). After exposure to the Lipofectin-DNA complex for 6 h, cells surviving after exposure to 200 μg of G418 sulfate (Life Technologies) per ml were pooled and cloned by the limiting-dilution technique, and four clones thus obtained were examined in subsequent experiments.

Southern blot analysis of β-catenin. For preparation of high-molecular-weight DNAs from HSC-39 cells and human placenta, cultured cells or placenta samples cut into small pieces were lysed with a lysis buffer containing 0.5% SDS, 10 mM Tris-HCl (pH 7.5), 0.1 M NaCl, and 1 mM EDTA and were then digested with 100 μg of proteinase K (Merck, Darmstadt, Germany) per ml at 50°C for 3 h. DNAs were then purified by extraction with phenol-chloroform as described previously (26). Purified cellular and placental DNAs were digested with endonucleases *Eco*RI and *Hin*dIII (New England Biolabs) for 2 h under the conditions recommended by the supplier. Ten-microgram samples of the DNA digests were subjected to electrophoresis in 0.7% agarose gels. The DNAs were transferred to Hybond-N membranes by using the capillary elution method in 10× SSC, and the membrane was baked at 80°C for 2 h. Prehybridization was carried out in a mixture containing 50% formamide, 6× SSC, 5× Denhardt's solution, 0.5% SDS, and 100 μg of sonicated salmon testes DNA per ml at 42°C for 2 h. Hybridization was carried out for 24 h in the same buffer at 42°C with a ^{32}P-labeled probe. The probe used was an *Xba*I fragment from the human β-catenin cDNA clone, pRc/CMV/HCA-β, and the fragment was labeled by the method used for Northern blot analysis as described above. The membrane was washed once for 5 min and once for 15 min at room temperature in 2× SSC–0.5% SDS. The membrane was then washed twice for 30 min each time at 37 and 68°C in 0.1× SSC–0.5% SDS. After washing, the membrane was exposed to Kodak X-Omat AR film with an intensifying screen.

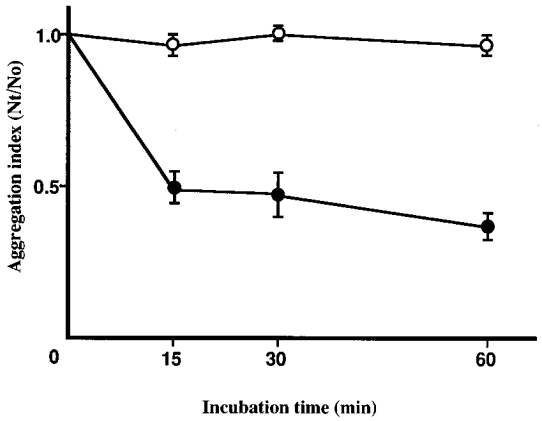

FIG. 2. Cell aggregation assay for MKN-28 cells and HSC-39 cells. The degree of aggregation was quantified by the aggregation index N_t/N_0, where N_0 was the total particle number (free cell number plus aggregated cell number) before incubation and N_t was the total particle number after incubation for t minutes in the presence of Ca^{2+}. HSC-39 cells (○) showed almost no aggregation in the presence of Ca^{2+}, while the control cell line MKN-28 (●) clearly exhibited mutual aggregation.

RESULTS

Phase-contrast microscopy and immunofluorescence staining for E-cadherin of HSC-39 and MKN-28 cells. Phase-contrast microscopic examination of an MKN-28 cell line derived from a well-differentiated adenocarcinoma revealed a typical cell compaction which was considered to be a characteristic of cell-cell adhesion by E-cadherin (Fig. 1A). However, the HSC-39 cell line, derived from a sparsely infiltrating signet ring cell carcinoma, showed a cluster with no apparent compaction (Fig. 1B). Immunofluorescence staining of E-cadherin with HECD-1 revealed the same level of E-cadherin expression in both cell lines (Fig. 1C and D). The staining patterns of these two cell lines, however, were clearly different. In HSC-39 cells, E-cadherin was stained uniformly along the cell membrane. In contrast, the E-cadherin of MKN-28 cells was concentrated at the cell-cell contact site. Consequently, dysfunction of E-cadherin in HSC-39 cells was suspected.

Cell aggregation assay of HSC-39 and MKN-28 cells. To examine calcium-dependent cell-cell adhesiveness, a cell aggregation assay was performed. As shown in Fig. 2, with MKN-28 cells, which were used as a control, cell aggregation was clearly demonstrated by recalcification of the culture medium, while no aggregation was seen with HSC-39 cells during the observation period (60 min). It was therefore evident that the calcium-dependent cell-cell adhesion was completely negated in HSC-39.

Western blot analysis of E-cadherin in HSC-39 and MKN-28 cells. To determine whether the E-cadherin molecule is structurally abnormal, the molecular size of E-cadherin was examined by Western blot analysis. A band of 120 kDa, the same size as native E-cadherin, was observed in the preparation of HSC-39 cells (Fig. 3, lane 2). The band of MKN-28 cells also showed the same mobility as that of HSC-39 cells (Fig. 3, lane 1). No apparent difference was observed in the molecular size between these two cell lines.

Northern blot analysis of E-cadherin in HSC-39 and MKN-28 cells. We constructed a 1,835-bp cDNA probe for human E-cadherin, and Northern blot analysis was performed with

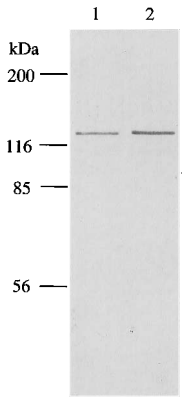

FIG. 3. Western blot analysis of E-cadherin expression on HSC-39 cells and MKN-28 cells. The cell extracts were electrophoresed on a 7.5% polyacrylamide gel containing 0.1% SDS, proteins were transferred to an Immobilon-P membrane, and the membrane was probed with HECD-1. Bands corresponding to full-size E-cadherin (120 kDa) were equally demonstrated in MKN-28 (lane 1) and HSC-39 (lane 2) cells. Positions of the molecular weight standards are shown at the left.

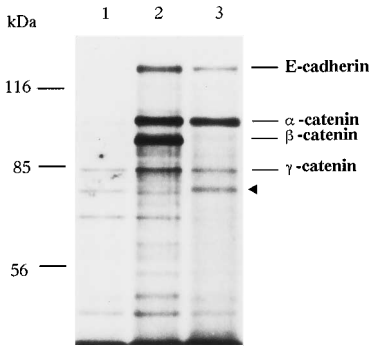

FIG. 5. Autoradiograph of immunoprecipitates from metabolically labeled HSC-39 cells and MKN-28 cells. Cells were incubated with [^{35}S]methionine for 16 h at 37°C and, after a wash with PBS, were lysed to be immunoprecipitated with HECD-1. The immunoprecipitates from MKN-28 (lane 2) and HSC-39 (lane 3) cells were analyzed by electrophoresis on a 0.1% SDS–7.5% polyacrylamide gel. The gel was dried and autoradiographed. In lane 1, the precipitate with normal mouse immunoglobulin G in the MKN-28 cell lysate was electrophoresed. The arrowhead indicate a 79-kDa band.

this probe. As shown in Fig. 4, HSC-39 cells demonstrated an intense signal in the same position as normal E-cadherin, 4.7 kb (lane 2). In MKN-28 cells, a signal was also detected in the same position as that in HSC-39 cells (lane 1). These results suggested that expression of E-cadherin was insignificantly altered in HSC-39 compared with MKN-28 cells.

Immunoprecipitation of E-cadherin in HSC-39 and MKN-28 cells. To examine E-cadherin-associated cytoplasmic proteins, soluble proteins metabolically labeled with [^{35}S]methionine were immunoprecipitated by using HECD-1, and then SDS-PAGE was performed. As shown in Fig. 5, when the cell lysate of MKN-28 cells was treated with HECD-1, four distinct bands corresponding to E-cadherin (120 kDa), α-catenin (100 kDa), β-catenin (95 kDa) and γ-catenin (82 kDa) were detected (lane 2). However, when the cell lysate of HSC-39 cells was treated with HECD-1, the bands corresponding to E-cadherin, α-catenin, and γ-catenin were detected but the band corresponding to normal β-catenin was not, although a 79-kDa band was detected (lane 3). This result suggests that β-catenin is absent from HSC-39 cells, is present in an altered molecular size, or is present but does not interact with E-cadherin.

Western blot analysis of β-catenin in HSC-39 and MKN-28 cells. To examine the status of the β-catenin protein in HSC-39, we conducted a Western blot analysis using antibodies to β-catenin. As shown in Fig. 6, bands of 79 and 95 kDa were detected in HSC-39 cells (lane 2) and MKN-28 cells (lane 1), respectively. This result was consistent with that of the immunoprecipitation experiment, indicating that the 79-kDa band represented truncated β-catenin.

RT-PCR of β-catenin mRNA and partial sequencing of its 5′-terminal fragment in HSC-39 cells. Since the β-catenin was truncated in HSC-39 cells, we extended our investigation to explore the possibility that the truncation reflected a deletion in its mRNA. On the basis of the previously reported sequence of β-catenin mRNA (GenBank accession number Z19054), we synthesized primers which were designed to cover the whole

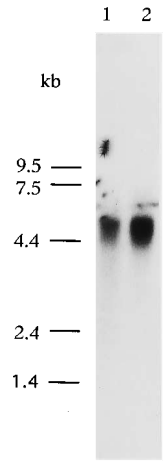

FIG. 4. Northern blot analysis of E-cadherin transcripts of HSC-39 cells and MKN-28 cells. Poly(A)$^+$-enriched RNA (20 μg) was electrophoresed in the presence of formaldehyde on a 1% agarose gel, transferred onto a nylon membrane, and hybridized with a ^{32}P-labeled *Eco*RI fragment of human E-cadherin cDNA. No difference was observed in the mobility of transcript signals of MKN-28 (lane 1) and HSC-39 (lane 2) cells. The 4.7-kb transcripts corresponding E-cadherin mRNA were equally demonstrated in the two cell lines. The positions of marker RNAs in the gel are indicated on the left.

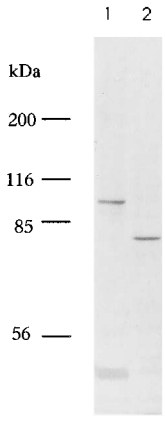

FIG. 6. Western blot analysis of β-catenin in MKN-28 and HSC-39 cells. The cell extracts were electrophoresed on a 7.5% polyacrylamide gel containing 0.1% SDS, proteins were transferred to an Immobilon-P membrane, and the membrane was probed with an anti-β-catenin antibody. Bands corresponding to full-size β-catenin (95 kDa) were identified in MKN-28 cells (lane 1), whereas abnormal small-sized β-catenin (79 kDa) was detected in HSC-39 cells (lane 2).

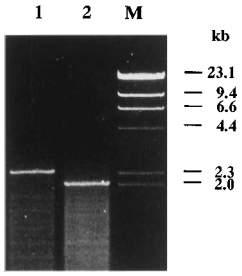

FIG. 7. RT-PCR analysis of β-catenin mRNA in HSC-39 and MKN-28 cells. mRNAs prepared from both cells were subjected to RT-PCR using a specific primer for human β-catenin. The cDNA was amplified for 25 cycles as described in Materials and Methods. PCR products were electrophoresed on a 1.0% agarose gel, stained with ethidium bromide, then visualized, and photographed under a UV illuminator. A band of mRNA for HSC-39 cells was detected at the 2.0-kb position (lane 2) which was approximately 0.3 kb shorter than that expected from the full sequence of mature mRNA for β-catenin. Lane 1, MKN-28 cells; lane 2, HSC-39 cells. A molecular weight marker (*Hin*dIII digest of λDNA) is present in lane M.

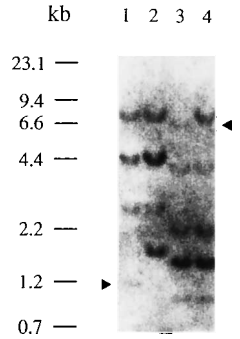

FIG. 9. Southern blot analysis of the β-catenin DNA from HSC-39 cells. Ten-microgram samples of high-molecular-weight DNA were digested with *Eco*RI (lanes 1 and 2) and *Hin*dIII (lanes 3 and 4) and then run on a 0.7% agarose gel, transferred onto a nylon membrane, and hybridized with a ^{32}P-labeled 2.4-kb fragment of β-catenin cDNA. Lanes 1 and 3, HSC-39 cells; lanes 2 and 4, placental DNA. The arrowheads indicate bands apparently different between HSC-39 cells and placental DNAs. The numbers on the left are lengths of size markers.

open reading frame and performed RT-PCR with these primers. The size of the mRNA for β-catenin was found to be 2.0 kb (Fig. 7, lane 2), which was approximately 0.3 kb smaller than that expected from the full sequence of mature mRNA for β-catenin. To determine the partial deletion site in β-catenin mRNA, agarose gel electrophoresis of PCR-amplified β-catenin cDNA after digestion with *Eco*RI, *Hin*dIII, *Nco*I, and *Sac*I was first carried out. Comparison of our results with the restriction fragment maps which were deduced from the previously reported cDNA indicated that the deletion was located at the 5′-terminal fragment (data not shown). Next, the 5′-terminal fragment which thought to contain the deletion site was synthesized by PCR using the Pr-1 and Pr-3 primers. The products was then sequenced by the dideoxy-chain termination method as described in Materials and Methods. Comparison of this sequence with the normal β-catenin sequence confirmed the deletion of 321 bases from nucleotides +82 to +402. This deletion does not alter the reading frame and should be equivalent to the amino acid deletion in the 107 codons from positions 28 to 134 (Fig. 8).

Southern blot analysis of HSC-39 cells. After digestion of high-molecular-weight DNA with restriction enzymes, Southern blot analysis was performed with β-catenin cDNA as a probe. As shown in Fig. 9, the band pattern in HSC-39 cells (lanes 1 and 3) was apparently different from that of the placental DNA as a germ line (lanes 2 and 4) when DNAs were digested with *Eco*RI (lanes 1 and 2) and *Hin*dIII (lanes 3 and 4), respectively. Each of the bands (indicated by arrowheads) showed approximately a 500-base deletion in HSC-39 cells. β-Catenin mRNA sequence analysis demonstrated a 321-base deletion in HSC-39 cells. Taken together, the deletion in the genome of HSC-39 cells might be 321 bases in the region of the exon and approximately 180 bases in the region of the intron, although the possibility of polymorphism of the β-catenin gene was not completely ruled out. Because we used cDNA as a probe, the signal intensities of smaller bands (as indicated by arrowheads) in which 321 bases of exon were deleted might be weak compared with other bands.

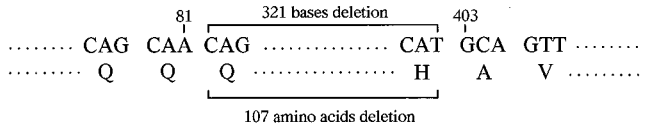

FIG. 8. Nucleotide sequence of the deleted position in β-catenin mRNA in HSC-39 cells. Products amplified by RT-PCR using primers specific to human β-catenin were purified and then subcloned into pUC118. After infection of the plasmid with a helper phage, the single-stranded plasmid containing β-catenin cDNA was sequenced by the dideoxy-chain termination method using Sequenase and then compared with that reported in the EMBL/GenBank database. The comparison confirmed the deletion of 321 bases from nucleotides +82 to +402. This deletion corresponded to that of the amino acid deletion from positions 28 to 134.

Characterization of wild-type β-catenin gene transduced HSC-39 cells. To directly prove that the dysfunction of E-cadherin is indeed due to the mutation of β-catenin in HSC-39 cells, a β-catenin expression vector was constructed by inserting cDNA into pRc/CMV and transfected into HSC-39 cells to characterize their aggregation properties. We obtained four clones after selection with G418 and limiting dilution of the transfectants. The cell aggregation assay to examine calcium-dependent cell-cell adhesiveness was carried out with these clones. As shown in Fig. 10, the clones exhibited the recovery of reduced cell-cell adhesion with various magnitudes of aggregation. On the other hand, with *neo* gene (pRc/CMV) transfectants, all five clones selected in G418 medium showed

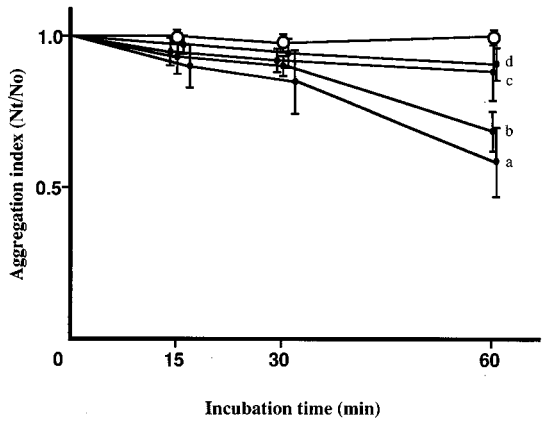

FIG. 10. Restoration of cell aggregation in β-catenin-transfected HSC-39 cells. Wild-type β-catenin expression vector pRc/CMV/HCA-β was transfected into HSC-39 cells, and four clones (a, b, c, and d) were selected by limiting dilution. The cell aggregation assay was carried out as described in the legend to Fig. 2. Two (a and b) of the four clones showed significant cell aggregation compared with parental cells (○).

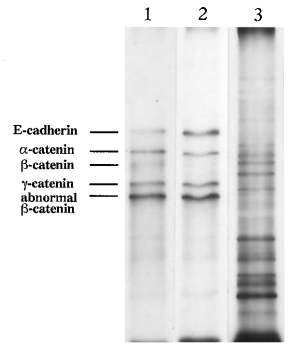

FIG. 11. Expression of wild-type β-catenin protein in pRc/CMV/HCA-β-transfected HSC-39 cells. The cells (clones a and d as indicated in Fig. 10) were metabolically labeled with [^{35}S]methionine, and immunoprecipitation using HECD-1 was performed as described in Materials and Methods. In clone a, a 95-kDa band of β-catenin was coprecipitated with E-cadherin, α-catenin, γ-catenin, and small abnormal β-catenin (lane 1), while the 95-kDa band of β-catenin was undetectable in clone d (lane 2). In lane 3, the precipitate with normal mouse immunoglobulin G in the cell lysate was electrophoresed.

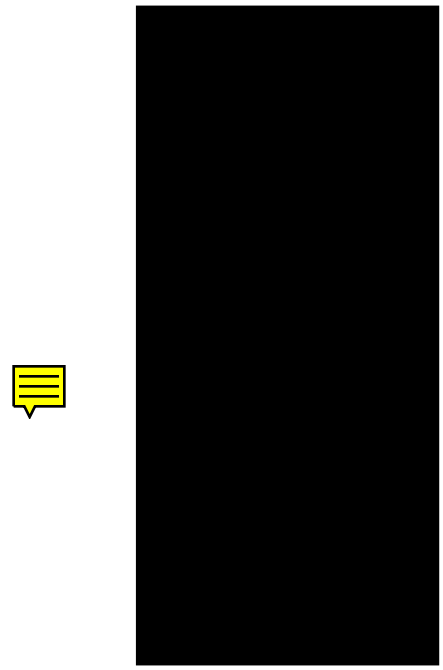

FIG. 12. Restoration of E-cadherin-dependent cell-cell adhesion and cell compaction. Phase-contrast microscopy of β-catenin protein in pRc/CMV/HCA-β-transfected HSC-39 cells (clone a as indicated in Fig. 10) clearly demonstrated cell compaction (A). For immunofluorescence staining using HECD-1, 10^4 cells were attached onto SlideFlask for the clone a, fixed, and stained as described in Materials and Methods. This transfectant also showed typical E-cadherin-dependent cell-cell adhesion (B).

no aggregation (data not shown). The kinetics of the aggregations of restored clones markedly differed from that of MKN-28 cells, reflecting the difference in cellular origin and/or the insufficiency of wild-type β-catenin as an E-cadherin-associated cytoplasmic protein in competing with mutated β-catenin.

To confirm that wild-type β-catenin is indeed expressed in the aggregating clones and to examine whether the variability of aggregation is due to the difference in expression levels of wild-type β-catenin in these clones, we immunoprecipited metabolically labeled cellular proteins with HECD-1, using two representative clones, one showing apparent aggregation (clone a) and the other with very little aggregation (clone d). As shown in Fig. 11, in addition to the bands of E-cadherin, α-catenin, γ-catenin, and abnormal β-catenin, a weak but discrete band of 95-kDa β-catenin was observed in clone a (lane 1), while the expression of 95-kDa β-catenin was undetectable in clone d (lane 2).

Clone a exhibited cell compaction by phase-contrast microscopy (Fig. 12A) and, by immunofluorescence staining, the concentrated expression of E-cadherin at the cell-cell adhesion sites (Fig. 12B).

DISCUSSION

In this investigation, we first reported on a human gastric cancer cell line with impaired E-cadherin function due to the mutation of β-catenin. A human HSC-39 cell line, derived from a sparsely infiltrating signet ring cell carcinoma, showed neither cell compaction nor aggregation. However, the existence of E-cadherin on the membrane of this cell was clearly demonstrated by immunofluorescence staining. Furthermore, Western blot and Northern blot analyses revealed respective bands of protein and mRNA which corresponded to those of normal E-cadherin. These results strongly suggested an impaired function of E-cadherin in this particular cell. By subsequent investigation, mutation was observed in a cytoplasmic protein β-catenin which is known to sustain the function of E-cadherin in concert with other catenin family proteins, α-catenin and γ-catenin. The reduced function of cell-cell adhesiveness in HSC-39 cells was therefore theorized to have been caused by this mutation. A cell aggregation assay of HSC-39 cells transfected with the β-catenin gene demonstrated the recovery of the reduced cell-cell adhesion, although the magnitude of aggregation varied among the transfectants (four established clones). On the contrary, all clones (five established clones) transduced with the *neo* gene showed no aggregation. Theses results suggested that the restored aggregation in β-catenin transfectants is due not to the clonal variability of parental HSC-39 cells but to the transfection of the wild-type β-catenin gene. The variability of aggregation magnitude in the rescued HSC-39 clones appeared to reflect the differences in expression levels of wild-type β-catenin among different transformants, since transfection of a gene quite often results in diversity of its expression levels. In fact, when the immunoprecipitation with HECD-1 was performed, clone a, which showed the most apparent restoration of E-cadherin-dependent cell-cell adhesion, expressed wild-type β-catenin in detectable amounts, while wild-type β-catenin in clone d, which showed very weak aggregation, was below the detectable level. The differences in aggregation kinetics between β-catenin transfectants and MKN-28 cells may be due partly to the difference in origins of the two types of cells and partly to the insufficiency of wild-type β-catenin as an E-cadherin-associated cytoplasmic protein in competing with mutated β-catenin as shown in Fig. 11. Thus, the data obtained in this study clearly indicate that the reduced intercellular adhesion of HSC-39 cells is indeed caused by mutation in β-catenin.

Some reports have been published on the relationship between β-catenin expression and carcinogenesis. Two reports showed that a product of the *APC* gene, one of the candidates for tumor suppressor genes for colorectal and gastric carcinoma, was connected to β-catenin, indicating a link between tumor initiation and cell adhesion (23, 30). Others showed that the reduced function of cell-cell adhesion was brought about by tyrosine phosphorylation of β-catenin with v-*src* gene trans-

fection (1, 8, 14). That result suggested a mechanism by which v-*src* counteracts junctional assembly and thereby promotes invasiveness and dedifferentiation of epithelial cells through phosphorylation of the E-cadherin–catenin complex. However, structurally altered β-catenin which is responsible for impaired cell-cell adhesion has not been reported yet.

Although it has been demonstrated that the catenins which bind to the region of the cadherin cytoplasmic domain required normal cell-cell adhesion function (16, 23, 24), the structure of the E-cadherin–catenin complex is not fully understood. In this context, the results of the present study provide some valuable information on the interaction of E-cadherin and catenin family proteins. The N-terminal domain of β-catenin is not essential for its interaction with E-cadherin since the β-catenin of HSC-39 cells from which the N-terminal domain (codons 28 to 134) had been deleted was coimmunoprecipitated with HECD-1 (Fig. 5). Furthermore, as α-catenin and γ-catenin were also coprecipitated with HECD-1, it is clear that the N-terminal domain was not essential for their interaction. However, the fact that HSC-39 did not exhibit any tight compaction or cell aggregation, despite the apparent association of the N-terminal-domain-deleted β-catenin with E-cadherin, indicates the requirement of the N-terminal domain of β-catenin for normal cadherin function. It is likely that β-catenin deleted of the N-terminal domain loses its activity with conformational distortion. Further studies using epitope-specific antibodies or site mutagenetically engineered proteins will be needed to understand the complete structure of the E-cadherin–catenin complex.

The question could be addressed as to whether this type of abnormality is common in cancer cells. Accumulated data from similar investigations would provide an answer to this question.

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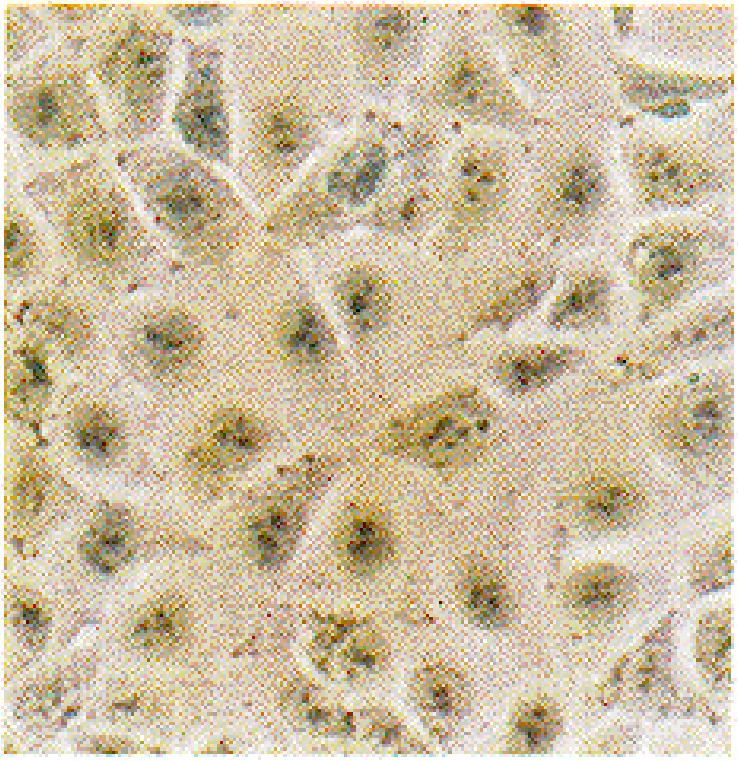

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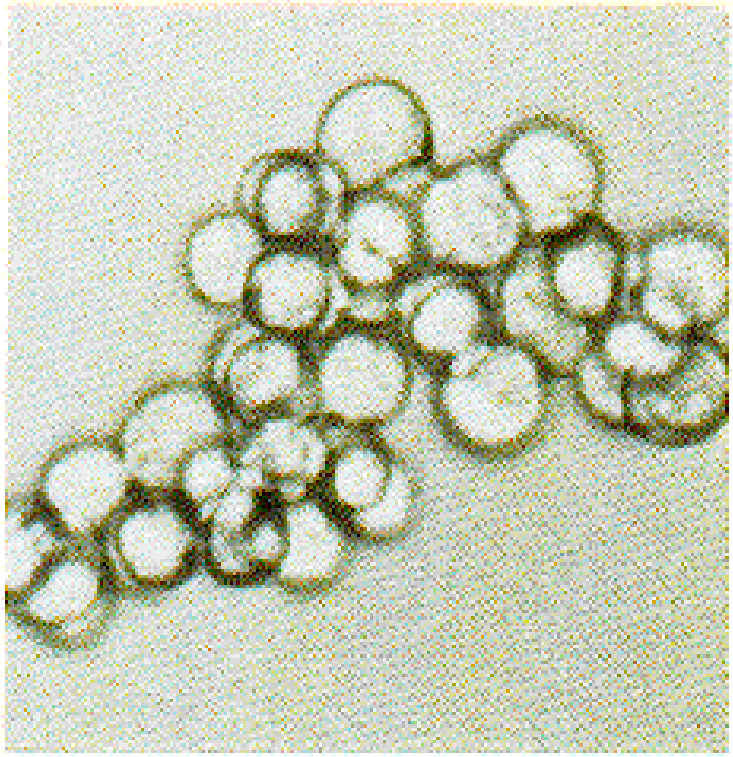

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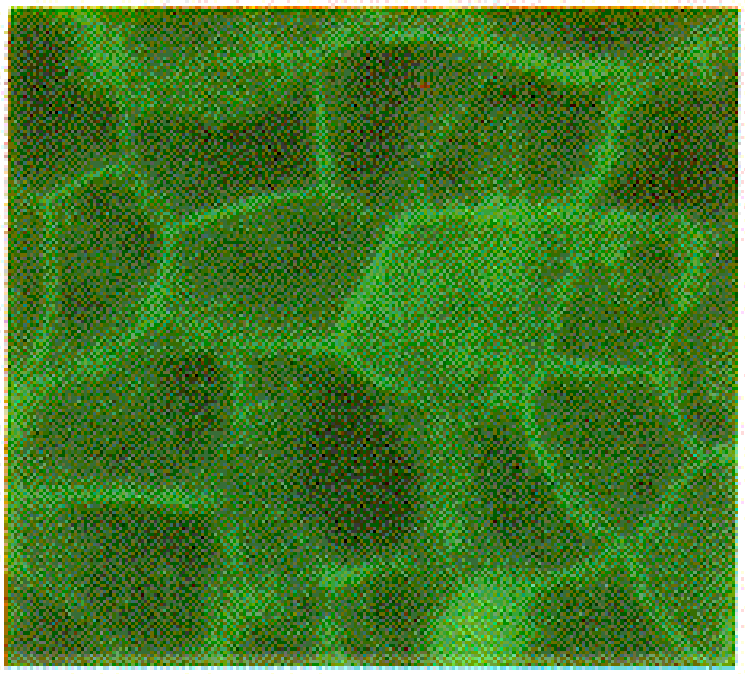

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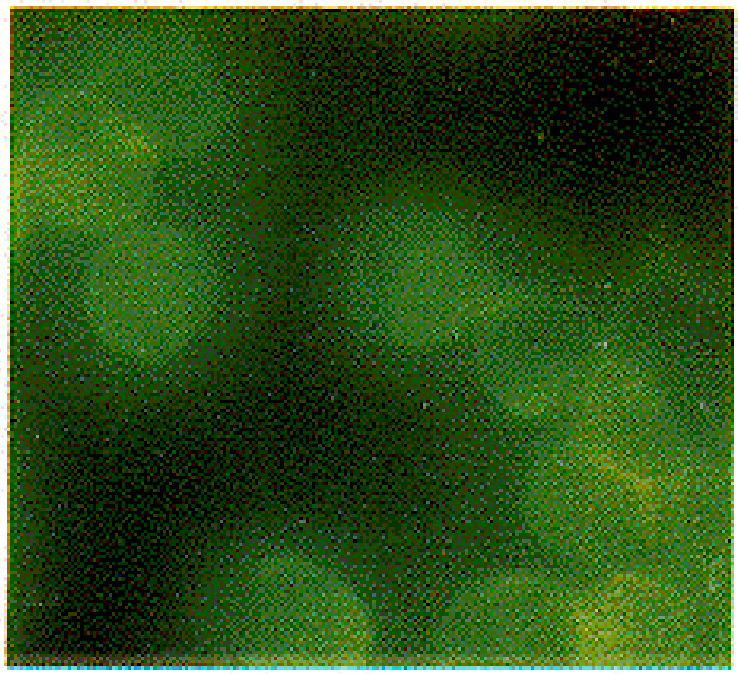

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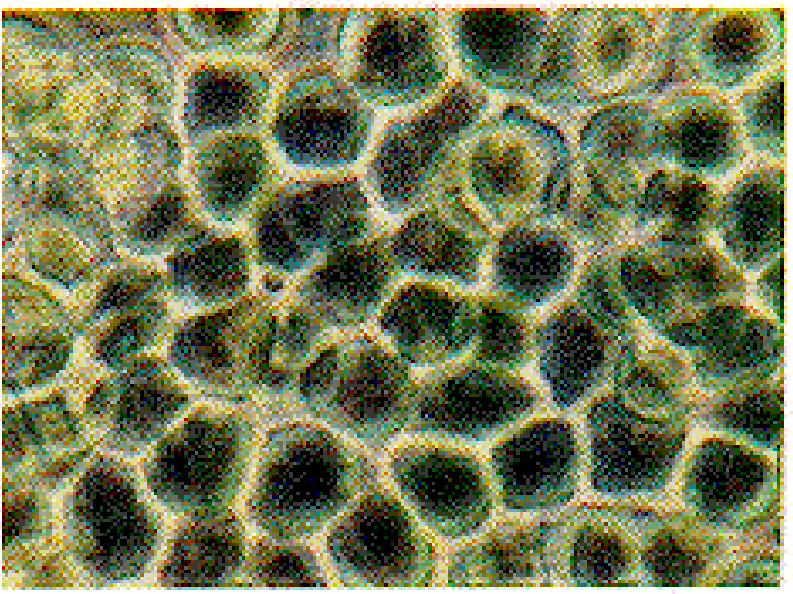

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