Calcium-dependent Cell-Cell Adhesion Molecules Common to Hepatocytes and Teratocarcinoma Stem Cells

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ABSTRACT The molecules involved in Ca^{2+}-dependent cell-cell adhesion systems (CDS) in mouse hepatocytes were characterized and compared with those in teratocarcinoma cells. Fab fragments of antibody raised against liver tissues (anti-liver) inhibited Ca^{2+}-dependent aggregation of both liver and teratocarcinoma cells. A monoclonal antibody raised against teratocarcinoma CDS (ECCD-1) also inhibited the Ca^{2+}-dependent aggregation of these two cell types equally. These antibodies induced disruption of cell-cell adhesion in monolayers of hepatocytes. Thus, CDS in these two cell types are not immunologically distinctive. Immunochemical analyses with these antibodies showed that CDS in both hepatocytes and teratocarcinoma cells involved at least two classes of cell surface proteins with molecular weights of 124,000 and 104,000. ECCD-1 selectively bound to hepatocytes but not to fibroblastic cells in liver cell cultures. Thus, the molecular constitution of CDS in hepatocytes and teratocarcinoma stem cells is identical. As ECCD-1 reacts with other classes of embryonic and fetal cells, the molecules identified here could have a major role in cell-cell adhesion in various tissues at any developmental stage of animals.

Cell-cell adhesion is a fundamental process in the construction of multicellular animals, and a number of studies have been undertaken to elucidate its molecular mechanisms. A series of experiments from this and other laboratories has revealed that a single cell is provided with multiple cell-cell adhesion systems, classified into Ca^{2+}-dependent and Ca^{2+}-independent (see reference 1 for review). Our recent studies have focused on the identification of molecules involved in Ca^{2+}-dependent cell-cell adhesion systems (CDS), and have implicated the presence a cell surface protein (p140) in CDS of teratocarcinoma cells (2, 3). This protein displays a unique Ca^{2+}-sensitive property in that it is digested by treatment with various proteases in the absence of Ca^{2+} but not digested when the protease solutions contain Ca^{2+} (3). This property is well correlated with the often reported protease-sensitivity of CDS.

We confirmed the above observation using a monoclonal antibody, ECCD-1, which causes disruption of cell-cell adhesion in monolayers of teratocarcinoma cells when added to cultures, specifically inhibiting the function of CDS (C. Yoshida-Noro, N. Suzuki, and M. Takeichi, manuscript submitted). Immunochemical studies revealed that ECCD-1 recognizes multiple cell surface proteins, among which p140 was a major antigen. The molecular weight of p140 was determined to be ~124,000 in the study with ECCD-1, although it was formerly reported to be 140,000 (3).

We also showed that ECCD-1 reacts with not only teratocarcinoma cells and early embryonic cells but also with certain types of differentiated epithelial cells such as hepatocytes and epidermal cells. In the present study, we investigated the properties of CDS in mouse hepatocytes, and showed that hepatocytes are provided with the same Ca^{2+}-dependent cell-cell adhesion molecules as teratocarcinoma cells, suggesting that these molecules are of general importance to cell-cell adhesion in a variety of tissues reactive with ECCD-1 (C. Yoshida-Noro, N. Suzuki, and M. Takeichi, manuscript submitted).

MATERIALS AND METHODS

Saline Solutions and Cell Lines Used: The following saline solutions were used: Ca^{2+}- and Mg^{2+}-free Puck's saline buffered to pH 7.4 with *N*-2-hydroxylethyl piperazine-N'-2-ethane sulfonic acid (HCMF) (4), and HCMF supplemented with 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (HBSS). Two teratocarcinoma cell lines, AT805 (2) and F9 (5) were used. Both lines were maintained in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal calf serum (DEM-10F).

Primary Cultures of Fetal Mouse Liver: To study the adhesive behavior of hepatocytes, we first attempted to use cell suspensions prepared by treating freshly collected liver tissues with protease solutions. We found, however, that this preparation contained numerous blood cells, which could interfere with hepatocyte adhesion. To eliminate these blood cells and also to enrich

Abbreviations used in this paper: CDS, Ca^{2+}-dependent cell-cell adhesion systems; HCMF, *N*-2-hydroxylethylpiperazine-N'-2-ethane sulfonic acid; HBSS, HCMF supplemented with 2 mM $CaCl_2$ and 1 mM $MgCl_2$.

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hepatocytes, we used the following procedure to culture liver cells.

Livers were collected from ICR mouse fetuses of 15 to 17 days post coitus. After mincing with scissors, the tissue fragments were incubated in 0.5% crude trypsin (1:250; Difco Laboratories, Detroit, MI) in HCMF supplemented with 1 mM ethylene diamine tetraacetic acid for 30 min at 37°C, and dissociated into small cell clusters or single cells by pipetting. After centrifugation at 70 *g* for 1 min, the pellet was dispersed by pipetting and inoculated with DEM-10F into plastic culture dishes precoated with rat tendon collagen. After overnight incubation at 37°C, cells floating or attached loosely to the upper surface of hepatocytes (mainly blood cells) were removed by rinsing the cultures with HBSS. About 80% of the cells remaining attached directly to the culture substratum were hepatocytes (liver parenchymal cells), as judged by morphological and immunohistochemical observations. These cultures were used for further experiments.

Antibodies: Antibody against fetal mouse liver (anti-liver) was prepared as follows. Fetal mouse livers were mixed with an equal volume of Freund's complete adjuvant, and homogenized by a Homo blender (Sakuma Seisakusho, Tokyo). About 5 ml of this sample was injected subcutaneously along the back of a rabbit. 3 wk later, the liver tissue mixed with Freund's incomplete adjuvant was injected. This injection was repeated five times at weekly intervals. 7 d after the last injection, antiserum was collected and heat-inactivated before use. Fab fragments of anti-liver IgG (anti-liver Fab) were prepared as described (6) and the final preparation was dialyzed against HBSS.

ECCD-1, a monoclonal antibody recognizing CDS in teratocarcinoma cells, was raised in our laboratory (C. Yoshida-Noro, N. Suzuki, and M. Takeichi, manuscript submitted). The hybridoma clone producing this antibody was obtained by fusion of mouse myeloma P3-X63-Ag8 cells and lymphocytes of a rat that received injections of F9 cells; ECCD-1 was recognized by anti-rat IgG. Antibody in the culture supernatant of the hybridoma producing ECCD-1 was precipitated by 50% saturation of ammonium sulphate and dissolved in HBSS, the volume of which was adjusted to 1/20 of the original volume. This sample was dialyzed against HBSS.

Protease Treatments of Cells: Teratocarcinoma cells were harvested by trypsin treatments leaving CDS intact or inactivating CDS as follows. Cells with intact CDS were collected by treatment with 0.01% crystallized trypsin (Type I, Sigma Chemical Company, St. Louis, MO) and 1 mM $CaCl_2$ in HCMF (denoted TC-treatment), and cells without CDS were collected by treatment with 0.01% crystallized trypsin and 1 mM EGTA in HCMF (denoted TE-treatment), as described previously (2, 3). Trypsin was replaced with 0.01% pronase in the above solution when used to dissaggregate teratocarcinoma cells for the aggregation experiments.

Liver cells cultured overnight were harvested by TC- or TE-treatment for 30 min at 37°C. For some aggregation experiments, liver cells were first treated with 0.05% collagenase (Type I, Sigma Chemical Co.) in HCMF supplemented with 5 mM $CaCl_2$ at 37°C for 15 min, then crystallized trypsin was added to make a final concentration of 0.01%, followed by further incubation for 15 min. Treated cells were rinsed three times with 0.05% soybean trypsin inhibitor (Sigma Chemical Co.) in HCMF supplemented with 1 mM $CaCl_2$. After two further washings with HCMF, the cells were dissociated by pipetting in HCMF. All steps for rinsing cells were carried out at 4°C.

Aggregation Assay and Radiolabeling of Cells: To examine the aggregation of dissociated cells, 5×10^4 cells in 0.5 ml of HCMF, supplemented with 1 mM $CaCl_2$ and/or antibodies when necessary, were put into each well of a Linbro 24-well flat bottom plate (76-033-05, Flow Laboratories, McLean, VA) that had been coated with 1% bovine serum albumin in HCMF, and incubated on a gyratory shaker (70 rpm) at 37°C. Cells in preconfluent cultures were washed with methionine-free Eagle's minimal essential medium and incubated overnight with 40–100 μCi/ml of [^{35}S]methionine in methionine-free Eagle's minimal essential medium supplemented with 10% fetal calf serum. The labeled cells were used for immunoprecipitation experiments.

Immunoprecipitation of Cell Surface Antigens: Cells in cultures or harvested with trypsin were rinsed three times with HBSS and then incubated with 10% anti-liver in HBSS at 4°C for 1 h. After removing the antibody solution, the cells were rinsed at least five times with HBSS. The cells were then solubilized with 0.5% SDS in 10 mM Tris-HCl (pH 7.5) (in a minimum amount), followed by the addition of an equal volume of 2% Nonidet P-40 (NP40) in 10 mM Tris-HCl (pH 7.5). These detergent solutions contained 1 mM phenylmethylsulfonyl fluoride, 1 mM *p*-tosyl-L-arginine methyl ester, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. Then 10 u/ml of deoxyribonuclease I (DN-CL, Sigma Chemical Co.) were added. To this preparation, heat-inactivated and formalin-fixed *Staphylococcus aureus* (Pansorbin, Calbiochem-Behring Corp. LaJolla, CA) was added (~20 μl of Pansorbin pellet per sample collected from a 10-cm dish) and incubated for 1 h at 4°C. The Pansorbin was then washed several times with a 1:1 mixture of the above SDS and Nonidet P-40 solutions, once with 62.5 mM Tris-HCl (pH 6.8), and the sample prepared for electrophoresis.

Electrophoretic Analysis of the Immunoprecipitates: The antigen-antibody complexes were released from the Pansorbin by boiling for 3 min in 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.002% bromphenol blue in 62.5 mM Tris-HCl (pH 6.8) and analyzed by SDS-PAGE using Laemmli's method (7). ^{35}S-labeled antigens separated in the gels were detected by fluorography as described elsewhere (8).

To detect molecules reacting with the monoclonal antibody ECCD-1, cell surface antigens reactive with anti-liver were collected and electrophoresed as above, and transferred electrophoretically to a nitrocellulose sheet (BA85, 0.45 μm, Schleicher and Schuell, Dassel, West Germany) (9). The sheet was precoated with bovine serum albumin and immersed overnight in ECCD-1 solution (containing 2 mM $CaCl_2$) at room temperature. After washing in cold HBSS for 15 min, the sheet was treated with 1.75% glutaraldehyde in HBSS for 15 min at 4°C followed by another wash with HBSS for 15 min. The sheet was again coated for 1 h with a 10% nonimmune sheep serum diluted in HBSS, and then incubated with 1 μCi/ml/lane ^{125}I-labeled sheep anti-rat Ig F(ab')$_2$ (Amersham International, Amersham, United Kingdom) in HBSS with 10% sheep serum. After washing with HBSS, the nitrocellulose sheet was processed for autoradiography.

RESULTS

Dissociation and Aggregation of Hepatocytes

Liver cell cultures were used as a hepatocyte source throughout the experiments. Hepatocytes were distinguished from fibroblastic cells co-existing in the liver cell cultures, by immunohistochemical staining with an antibody against alpha-fetoprotein which is known to be a marker of hepatocytes in mouse fetus (10).

It was difficult to dissociate the monolayers of liver cells into single cells by treatment with trypsin in the presence of Ca^{2+} (TC-treatment). We found, however, that the treatment of liver cell cultures with collagenase in the presence of Ca^{2+} prior to TC-treatment (collagenase-TC-treatment) facilitates the dissociation of cells, although most of cells still remained in small clusters. On the other hand, suspensions of single

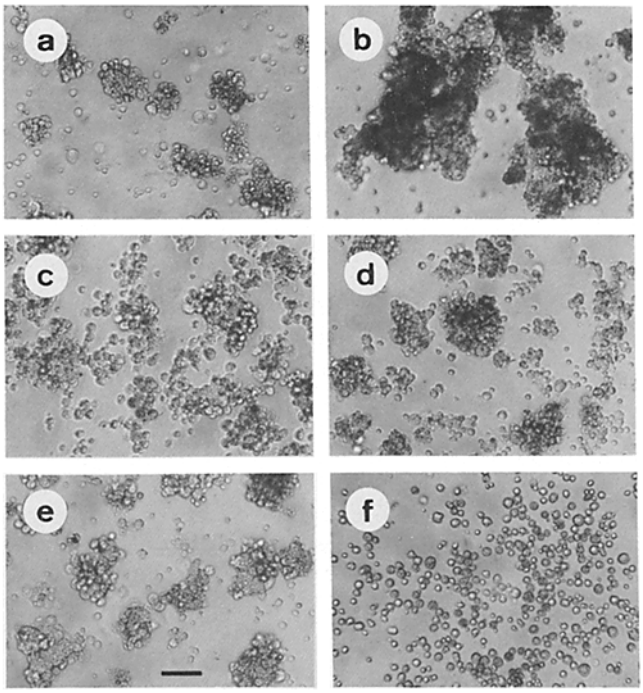

FIGURE 1 Aggregation of liver cells. (*a–e*) Collagenase-TC-treated cells: Cells before aggregation (*a*); cells incubated for 30 min in the presence of 1 mM Ca^{2+} without antibody (*b*), with anti-liver Fab (1.75 mg/ml) (*d*) and with ECCD-1 (diluted 1:10) (*e*), and in the absence of Ca^{2+} without antibody (*c*). (*f*) TE-treated cells incubated for 30 min in the presence of 1 mM Ca^{2+}. Bar, 150 μm. × 33.

cells were easily obtained by treatment with trypsin in the presence of EGTA (TE-treatment) of the monolayers.

Fig. 1 shows the aggregation profile of liver cells. Cells harvested by collagenase-TC-treatment aggregated into larger clusters in the presence of Ca^{2+} (Fig. 1 *a* and *b*), but not in the absence of Ca^{2+} (Fig. 1 *c*). TE-treated cells did not aggregate under either condition (Fig. 1 *f*). These results suggested the presence of typical Ca^{2+}-dependent cell-cell adhesion systems (CDS), which are resistant to protease in the presence of Ca^{2+} but not in the absence of Ca^{2+}, in liver cells.

Effect of Antibodies on Cell-Cell Adhesion

Anti-liver Fab and ECCD-1 equally inhibited the aggregation of liver cells dissociated by collagenase-TC-treatment (Fig. 1, *d* and *e*). This Fab also inhibited the aggregation of teratocarcinoma F9 and AT805 cells prepared by treatment with pronase and Ca^{2+} leaving their CDS intact (Fig. 2).

These antibodies were added to monolayer cultures of liver cells and AT805 cells. Anti-liver caused a rounding-up of hepatocytes (data not shown) and AT805 cells (Fig. 3, *a* and *b*), apparently disrupting their cell-cell adhesion. When ECCD-1 was added to the cultures of hepatocytes, the effect was not clear during the first few hours of incubation. However, when the cells were cultured overnight with ECCD-1, a remarkable effect was observed. Colonies of hepatocytes were dispersed into smaller cell clusters, although not into single cells (Fig. 3, *c* and *d*). Such an effect was most conspicuous when the antibody was added to the cells from the start of the cultures. We then examined which cell types in liver cell cultures reacted with ECCD-1 using an autoradiographic technique. Fig. 4 shows that ECCD-1 binds only to hepatocytes, not to fibroblastic cells.

Immunological Identification of Cell-Cell Adhesion Molecules

Cell surface antigens reactive with anti-liver were collected from liver cells treated with trypsin in the presence of Ca^{2+} (TC) or EGTA (TE), by immunoprecipitation. Fig. 5, *a* and *b* show that one cell surface protein with an approximate molecular weight of 124,000 (124-kdalton component) is present only in TC-treated cells. The molecular weight of this protein was the same as that of "p140" identified as a component of CDS in teratocarcinoma cells using an antibody raised against F9 cells (anti-TC-F9) in our previous work (3). Another protein, approximate molecular weight 100,000, was

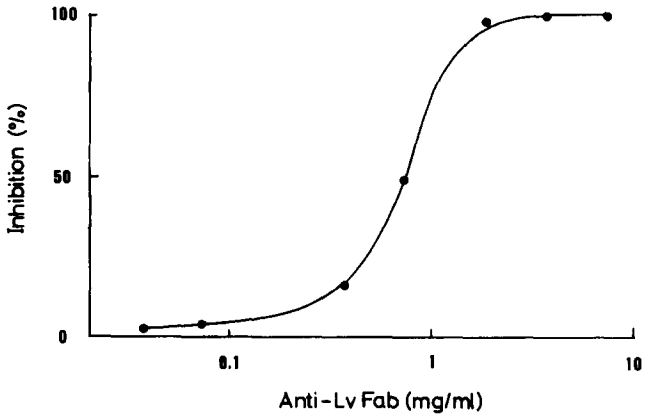

FIGURE 2 Effect of anti-liver Fab on aggregation of AT805 cells treated with pronase and Ca^{2+}. Cells were incubated for 30 min in the presence of 1 mM Ca^{2+} and various concentrations of anti-liver Fab. The degree of inhibition of aggregation was calculated as described elsewhere (6).

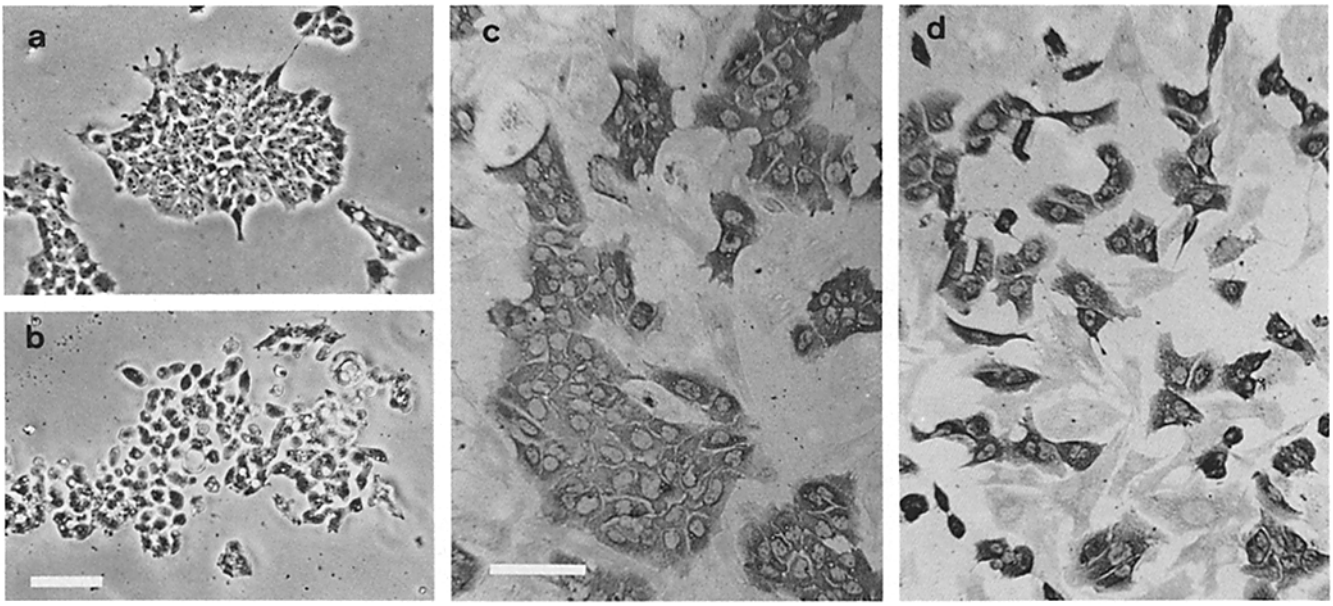

FIGURE 3 Effect of antibodies on monolayer cultures of cells. AT805 cells were incubated for 4 h without (a) and with (*b*) anti-liver Fab (2 mg/ml). Liver cells were cultured overnight without (c) and with (*d*) ECCD-1 (diluted 1:5). In c and *d*, alpha-fetoprotein was stained to identify hepatocytes: The cultures were fixed with absolute methanol at −20°C and incubated with rabbit anti-mouse alpha-fetoprotein diluted 1:20 (Miles Laboratories, Elkhart, IN), then with goat anti-rabbit IgG diluted 1:10 (Cappel Laboratories, Cochranville, PA), and finally with rabbit peroxidase-anti-peroxidase diluted 1:50 (Cappel Laboratories), all of which were diluted with PBS. The bound antibodies were detected by staining as described by Boenisch (15). (*a* and *b*), phase contrast microscopy; (c and *d*), transmitted light microscopy. Bars, 100 μm. (*a* and *b*), × 100; (c and *d*), × 126.

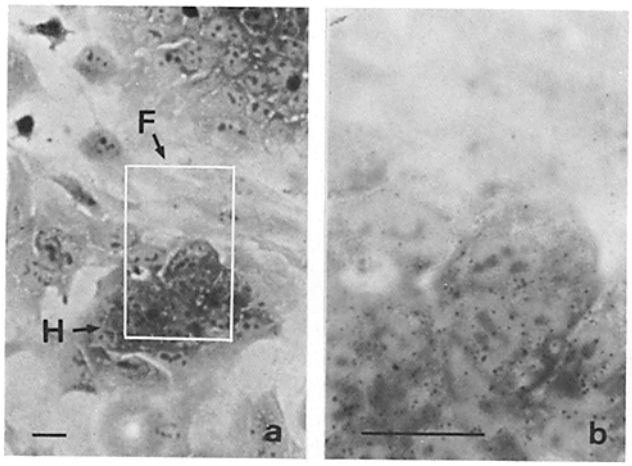

FIGURE 4 Autoradiographic detection of ECCD-1 bound to liver cell culture. Cells were incubated in ECCD-1 solution diluted 1:4 (containing 2 mM $CaCl_2$) for 30 min at 4°C, and after washing with cold HBSS, they were incubated with 1 μCi/ml ^{125}I-labeled anti-rat Ig F(ab')$_2$ in HBSS for 30 min. After washing with HBSS, the cells were fixed with 2% glutaraldehyde in HBSS and washed again with HBSS, followed by a coating with Sakura autoradiographic emulsion NR-M2. A portion of *a* enclosed by a square was enlarged in *b* in which photographs were focused on silver grains. *H* indicates hepatocytes and *F* indicates fibroblastic cells. Cells were stained with Giemsa's solution. Bar, 25 μm. (*a*) × 167; (*b*) × 540.

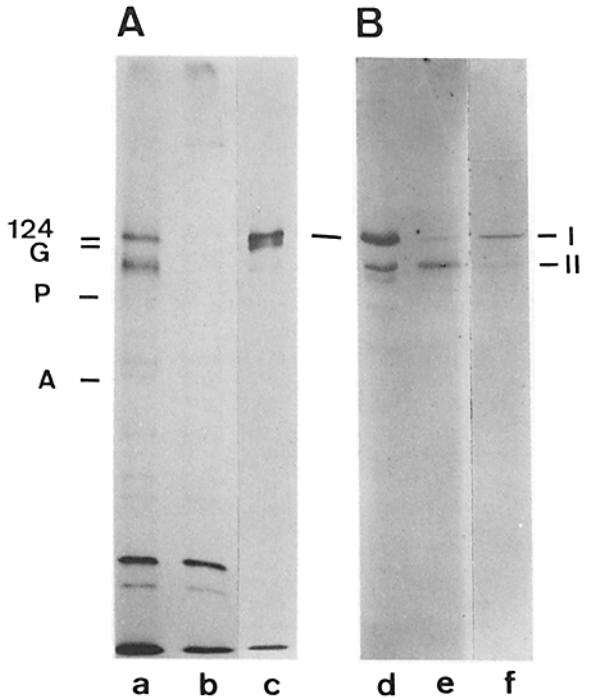

FIGURE 5 Detection of antigens reacting with anti-liver (*A*) and ECCD-1 (*B*). In *A*, antigen samples were collected from TC-treated liver cells, (a), TE-treated liver cells (*b*),and monolayers of liver cells (*c*). In *B*, antigens were collected from monolayers of F9 cells (*d*) and of liver cells (e and *f*). Molecular weight markers were *G*, *E. coli* β-galactosidase (116,000); *P*, phosphorylase-b (94,000); *A*, BSA (68,000). Positions of these markers are shown only for *A*.

also found to be specific to TC-treated cells. When the antigen samples were collected from nontrypsinized monolayer cultures of liver cells, the 124-kdalton component was again detected (Fig. 5 *c*). Interestingly, this 124-kdalton component was the major cell surface antigen reactive with anti-liver. The 124-kdalton component was also detected in TC-treated and nontrypsinized F9 and AT805 cells as an antigen reacting with anti-liver but not in TE-treated cells (data not shown).

In detecting the target molecules for ECCD-1, the immunoprecipitation technique used in the above experiments was not applicable as described elsewhere (C. Yoshida-Noro, N. Suzuki, and M. Takeichi, manuscript submitted), so we utilized "Western blots." As an antigen source for this analysis, the whole cell lysate was not useful, since it did not contain amounts of antigen detectable by ECCD-1. To prepare concentrated antigen samples, we collected cell surface molecules immunoreacting with anti-liver from monolayer cultures of F9 and liver cells by the same method used in the above experiments (except that the cells were not radiolabeled), and used them for Western blot analysis. The rationale of this method was based on the assumption that the target molecule(s) for ECCD-1 would be one(s) of those for anti-liver, and this was supported by the observation that binding of radiolabeled ECCD-1 to cell surfaces is inhibited by the presence of anti-liver (data not shown).

Fig. 5 *d* shows that ECCD-1 binds to multiple bands in the sample of F9 cells, bands I (mol wt 124,000) and II (mol wt 104,000) being the first and second major components, respectively, The molecular weight of band I corresponded to the 124-kdalton band detected in the above experiment. This was consistent with results obtained using an antibody raised against F9 cells to collect cell surface antigens, described elsewhere (C. Yoshida-Noro, N. Suzuki, and M. Takeichi, manuscript submitted).

In liver, ECCD-1 reacted with the same components (bands I and II) as in F9 cells (Fig. 5, *e* and *f*). However, the ratio of bands I and II varied from experiment to experiment, i.e., in some cases, band I was the major component and in other cases, band II was. It appeared that the amounts of these two components were present in inverse proportions.

DISCUSSION

The results described in this paper showed that hepatocytes in mouse liver have Ca^{2+}-dependent cell-cell adhesion systems, as found in various other cell types. The analysis of cell-cell adhesion in hepatocytes, however, seems to be more complicated than that in other cells studied thus far, i.e., fibroblasts (4), neural retina cells (6), and teratocarcinoma cells (2). It was difficult to prepare a suspension of single liver cells retaining intact CDS by treatment with protease solutions containing Ca^{2+}. Consistent with this finding, ECCD-1 did not completely dissociate colonies of hepatocytes into single cells in their monolayer cultures, indicating the presence of another mediator(s) for cell-cell adhesion. These mediators could be tight junctions, other junctional structures, or some unknown molecules in Ca^{2+}-independent cell-cell adhesion systems. Even if there are several different cell-cell adhesion mediators in hepatocytes, it seems certain that CDS play a major role in maintaining the physiological functions of the tissues based on cell-cell adhesion. Thus, we observed that animals receiving an injection of ECCD-1 or the hybridoma producing ECCD-1 into the peritoneal cavity die shortly afterwards (unpublished data).

Immunological analysis in the present study showed that CDS in hepatocytes and teratocarcinoma cells are composed of essentially the same components. Two classes of cell surface

proteins recognized by ECCD-1 in teratocarcinoma cells were also detected in liver. This suggests that the other tissues reacting with ECCD-1, such as epidermis, are also provided with the same adhesion molecules. Since ECCD-1 reacted with various tissues, always affecting their cell-cell adhesion (C. Yoshida-Noro, N. Suzuki, and M. Takeichi, manuscript submitted), the molecules identified in the present study should be of general importance to cell-cell adhesion in many kinds of cells. Although the 124-kdalton (and 104-kdalton) components are probably the major constituents of CDS in the tissues studied here, we cannot rule out the possibility of the presence of other components of CDS recognizable by ECCD-1, since we detected antigens reactive with ECCD-1 only from materials immunopreciptated with anti-liver.

We observed some difference between liver and teratocarcinoma in the electrophoretic pattern of antigens reacting with ECCD-1. In teratocarcinoma, band I was the predominant component in the Western blot. On the other hand, in liver, band I was sometimes detected as a minor component and was even undetectable in some electrophoretic runs, whereas band II became prominent as band I diminished. The most reasonable explanation would be to assume that band II is a degradation product of band I. Although protease inhibitors were always included in the solutions for extraction of antigens, certain classes of proteolytic enzymes might be able to cleave the band I component into smaller molecules under certain conditions. Teratocarcinoma cells may have less of such enzymes.

The present results are consistent with the recent findings of Gallin et al. (11). They characterized a cell adhesion molecule involved in chick embryonic liver, termed L-CAM. The molecular weight of a major component recognized by antibodies against L-CAM was 124,000. L-CAM showed a Ca^{2+}-sensitive property when cleaved with proteases. Thus, the overall properties of L-CAM are quite similar to those of the Ca^{2+}-dependent cell adhesion molecules studied here. Although we did not detect a tryptic fragment with a molecular weight of 81,000 as described in their paper, and they did not detect a component corresponding to band II described in the present study, the discrepancy between these results could only be due to the difference in the method of detection of antigens. Gallin et al. (11) also reported that L-CAM is not detected in fibroblasts, consistent with our findings. We can, therefore, conclude that mammalian and avian cells are provided with Ca^{2+}-dependent cell-cell adhesion molecules of the same molecular weight and probably with the same cell-type-specificity.

Ocklind et al. (12) have suggested the involvement of a glycoprotein with a molecular weight of 105,000 (cell-CAM 105) in rat hepatocyte cell-cell adhesion. This molecule is quite similar in size to band II of our present study, suggesting an identity for these two molecules. If the adhesion system that these authors investigated was identical to the one we studied, they should have found the band I component. It might, however, be that this component is easy to miss owing to degradation during preparation of samples. On the other hand, Ocklind et al. (13) recently reported that an antibody against cell-CAM 105 does not react with skin, whereas ECCD-1 strongly reacted with epidermis of this tissue (C. Yoshida-Noro, N. Suzuki, and M. Takeichi, manuscript submitted), suggesting the possibility that these antibodies may recognize the different antigens.

ECCD-1 selectively reacted with hepatocytes and other epithelial cells, but not with fibroblastic cells, in cultures of various tissues, as shown in the present and other studies (C. Yoshida-Noro, N. Suzuki, and M. Takeichi, manuscript submitted). The cell adhesion molecules described here, therefore, should work only for connecting particular cell types. Previous studies demonstrated that fibroblastic cells also have CDS but with different functional and immunological specificities from those in teratocarcinoma cells (2). We also showed that mouse embryos at cleavage stage, even at one-cell stage, are provided with CDS of the same specificity as in teratocarcinoma cells (14). Therefore, a lineage of cells with ECCD-1-sensitive CDS is initiated at the start of embryonic development and continues to late developmental stage (probably to adult stage), being inherited by certain classes of epithelial cells. Other lineages of cells with CDS of different specificities, such as fibroblasts, must be generated by diverging from this initiative lineage. It will be the important subject in future studies to elucidate the developmental significance and the genetic control mechanisms of such divergence of cell lineages with different types of CDS.

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