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Effect of Hepatocyte Growth Factor on Cadherin-Mediated Cell-Cell Adhesion

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ABSTRACT. **Hepatocyte growth factor (HGF) is known to induce the dispersion of epithelial cells, as scatter factor. On the other hand, cadherins play a crucial role in connecting cells together. Two groups of cadherins are involved in epithelial cell adhesion, those locating in adherens junctions (AJ) and in desmosomes. Here, we examined the effect of HGF on the function of these cadherins in keratinocyte cell lines F and 308R, which expressed E- and P-cadherin in AJ (referred to as AJ cadherins) and desmoplakin in desmosomes. In the presence of HGF, these cells spread more extensively than in control cultures and their associations apparently loosened. However, they maintained cell-cell contacts where cadherins and desmoplakin concentrated, although the level of the concentration was reduced by HGF treatment. When antibodies to E- and P-cadherins were added to cultures of these cells without HGF, AJ cadherins were redistributed into non-junctional areas of the cells, but desmoplakin still localized at cell-cell boundaries. When HGF was added together with anti-AJ cadherin antibodies to the cultures, cell-cell contacts were now disrupted. In these cultures, not only AJ cadherins but also desmoplakin were lost at cell-cell contact sites, indicating that HGF can disrupt desmosomal cell-cell adhesion when AJ cadherins are inactive. These results suggest that, although HGF cannot block cadherin-mediated cell-cell adhesion when the entire cadherin system is intact, it might modulate the activities of cadherins, especially, of desmosomal cadherins.**

Hepatocyte growth factor (HGF) was first discovered and molecularly cloned as a potent mitogen for mature hepatocytes (Nakamura *et al.*, (16); see also a review by Matsumoto & Nakamura, (11)), and this molecule is now known to be a mesenchyme-derived humoral factor which influences not only cell growth but also cell motility and multicellular organization (Montesano *et al.*, (13): Matsumoto and Nakamura, (11)). One of the actions of HGF is to disperse epithelial cells in monolayer cultures, as scatter factor (Stoker *et al.*, (28), Weidner *et al.*, (32, 33), Konishi *et al.*, (9), Furlong *et al.*, (5), Naldini *et al.*, (18)). In the presence of HGF, the epithelial cells become fibroblastic and tend to scatter, although the sensitivity of cells in responding to this growth factor varies with cell lines. While molecular mechanisms underlying the cell-scattering action of HGF have not well been understood, it can be assumed that either of cell-cell adhesion or movement or both are affected by this growth factor.

Cell-cell adhesion is governed by multiple mechanisms. Among them, the cadherin-dependent process is central. Cadherins are Ca^{2+}-dependent homophilic cell-cell adhesion receptors, and, if they are inactivated, cells tend to lose their tight associations (Takeichi, (30)). In epithelial cells, at least two groups of cadherins are involved in their interactions, those localized in adherens junctions (AJ) and in desmosomes (Buxton and Magee, (4)). It is thus intriguing to examine if HGF has any effect on the action of these molecules in inducing cell scattering. Cadherin-mediated cell adhesion can be perturbed by various ways. A simplest way would be down-regulation of cadherin expression. Other possibilities include depletion of the cadherin-associated proteins, catenins, which are essential for cadherin function (Nagafuchi & Takeichi, (15), Ozawa *et al.*, (20), Hirano *et al.*, (8), Shimoyama *et al.*, (25)), or biochemical modification of the catenins. Concerning the latter possibility, we recently suggested that v-*src* mediated tyrosine phosphorylation of catenins may impair cadherin function (Matsuyoshi *et al.*, (12), Hamaguchi *et al.*, (6)).

In the present study, we examined the effect of HGF on cadherin-mediated cell-cell adhesion, using keratinocyte lines. E- and P-cadherin localizing in AJ (referred to as AJ cadherins) and desmoplakin (Mueller & Franke, (14)) localizing in desmosomes were used as markers for each group of the cadherin-mediated cell-cell junctions. We found that HGF altered cell morphol-

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ogy but did not disrupt cadherin-mediated cell-cell adhesion. However, when AJ cadherins were inactivated using antibodies, HGF could induce separation of the cells, removing desmoplakin from cell-cell contact sites. Without HGF treatment, desmosomal cell-cell contacts were not affected by anti-AJ cadherin antibodies. These results are described, and the possible actions of HGF on the cadherin adhesion system is discussed.

MATERIALS AND METHODS

Cell culture. Two mouse keratinocyte lines, 308R (Yuspa & Morgan, (35)) and F (Kulesz-Martin *et al.*, (10)), were used. These cells were cultured in a 1 : 1 mixture of Dulbecco's modified MEM and Ham's F12 medium supplemented with 10% FCS (DH10).

Hepatocyte growth factor and immunoreagents. Hepatocyte growth factor (HGF) used in this experiment was a human recombinant purified from culture fluids of the C-127 cells (Nakamura *et al.*, (16)). HGF was dissolved in Hepes-buffered (pH 7.4) Hanks' saline (HBS) containing 0.2% BSA.

The following antibodies were used: A rabbit antiserum to mouse E-cadherin (Shimamura & Takeichi, (24)), the rat monoclonal antibodies ECCD-1 (Yoshida-Noro *et al.*, (34)) and ECCD-2 (Shirayoshi *et al.*, (26)) to mouse E-cadherin, the rat monoclonal antibody PCD-1 to mouse P-cadherin (Nose and Takeichi, (19)), and the mouse monoclonal antibody 11-5F to desmoplakin (Parrish *et al.*, (22)) which was kindly provided by Dr. D.R. Garrod (University of Manchester). ECCD-1 was used for blocking E-cadherin, ECCD-2 for immunostaining and blotting, and others for both blocking and immunostaining or blotting. For detection of the primary antibodies, we used sheep biotinylated species-specific antibody to mouse Ig (RPN1001, Amersham International, Amersham, UK), FITC-labeled streptavidin (RPN1232, Amersham International), swine rhodamine-labeled antibody to rabbit Ig (R156, DAKOPATTS, Copenhagen, Denmark), sheep HRP-linked species-specific antibody to mouse Ig (NA931, Amersham International) and sheep HRP-linked species-specific antibody to rat Ig (NA932, Amersham International).

Microscopic observation and immunofluorescence staining. To examine morphological effects of anti-cadherin antibodies or HGF, 1×10^6 cells were plated on each of 35 mm dishes and cultured in DH10 medium with or without those reagents. Photographs were taken by a phase-contrast microscope.

Double-immunofluorescence staining for E-cadherin and desmoplakin was carried out as described previously (Shimamura *et al.*, (23)). Briefly, cells were fixed and permeabilized by treatment with methanol on ice for 5 min, and blocked with 5% skim milk. They were then treated with a mixture of the rabbit anti-E-cadherin antibodies and the mouse monoclonal antibody 11-5F to desmoplakin, biotinylated anti-mouse Ig antibodies and finally a mixture of FITC-labeled streptavidin and rhodamine-labeled anti-rabbit Ig antibodies. The samples were examined using a Zeiss Axiophot microscope.

Immunoblotting. 4×10^6 cells were plated on each 60 mm dish, cultured for 24 hr and scraped out using a rubber policeman. The cells were dissolved in the Laemmli's SDS sample buffer, and proteins were separated by 7.5% or 5% polyacrylamide gel electrophoresis, followed by transferring to nitrocellulose filters. The filters were incubated with anti-cadherin or anti-desmoplakin antibodies and then with HRP-conjugated secondary antibodies. Bound HRP was visualized by the Enhanced Chemiluminescence (ECL) system (RPN2106, Amersham International) or Immunostaining HRP Kit (IS-50B, Konica, Tokyo).

Cell Aggregation Assay. 5×10^5 cells were plated on each 60 min dish and cultured with or without HGF for 24 hr. The cell monolayers were dissociated into single cells by treatment with 0.25% trypsin (Difco laboratories, Detroit, Michigan) and 0.5 mM EDTA for 15 min at 37°C. 5×10^4 of these cells were suspended in 0.5 ml of DH10 medium and placed into each well of a 24-well plate pre-coated with 1% BSA. HGF or/and antibodies (ECCD-1 or/and PCD-1) were added to the medium when necessary. The plate was incubated for 3 hr at 37°C under rotation at 82 rpm on a gyratory shaker placed in a 5% CO_2 incubator.

RESULTS

Effect of HGF on cadherin-dependent cell aggregation. 308R and F cells grow forming epithelial colonies. Immunoblot analysis showed that these cells expressed E- and P-cadherin, of which E-cadherin was much more abundant than the other (Fig. 1). When 308R cells were dissociated into single cells and incubated in suspension on a gyratory shaker, they gradually aggregated (Fig. 2a). This aggregation was partly inhibited by monoclonal antibody to E-cadherin (ECCD-1) or P-cadherin (PCD-1) (Fig. 2c, d), of which the effect of the anti-E-cadherin antibody was stronger than the other. When both the anti-E- and P-cadherin antibodies were added together, an almost complete inhibition of cell aggregation was observed at least during the culture period tested (Fig. 2e). These results suggest that E- and P-cadherin, especially the former, are major functional AJ cadherins in these cell lines.

To test if HGF has any effect on cadherin activity, 308R cells were cultured in the presence of 1 to 10 ng/ml HGF for one day, and used for the aggregation assay. Their aggregation rate was similar to that of non-treated cells (Fig. 2b), and even in the presence of 100 ng/ml HGF, no inhibition was observed (data not shown). The anti-cadherin antibodies inhibited the aggregation of these HGF-treated cells (Fig. 2f). Thus, there was no obvious effect of this growth factor on cadherin mediated cell aggregation in suspension cultures. Essentially the same results were obtained using F cells (data not shown).

Effect of HGF and anti-cadherin antibodies on cell-

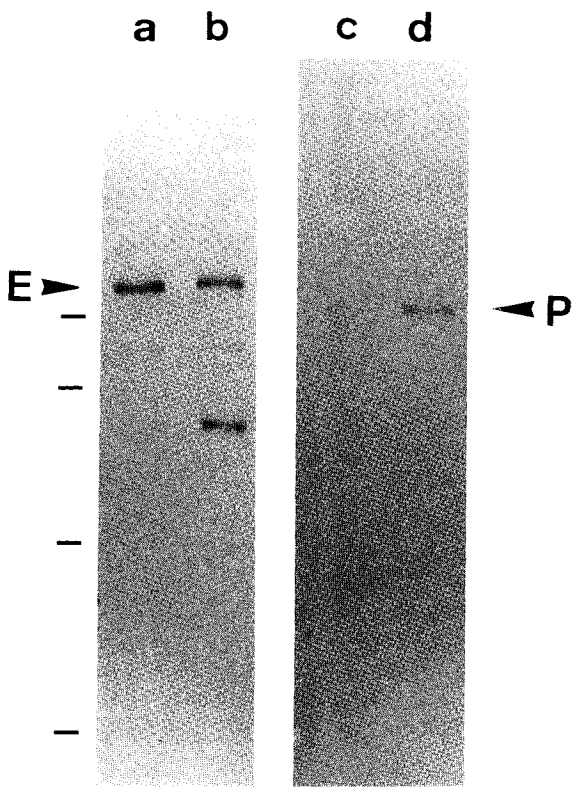

Fig. 1. Immunoblot analysis of cadherin expression. (*a, b*) E-cadherin. (*c, d*) P-cadherin. Lanes *a* and *c*, F cells; lanes *b* and *d*, 308R cells. E,E-cadherin band (124 kD); P,P-cadherin band (118 kD). Only a very small amount of P-cadherin is detected in F cells. Lower bands are probably degradation products. Positions of molecular weight markers are 116, 97, 66 and 45×10^3.

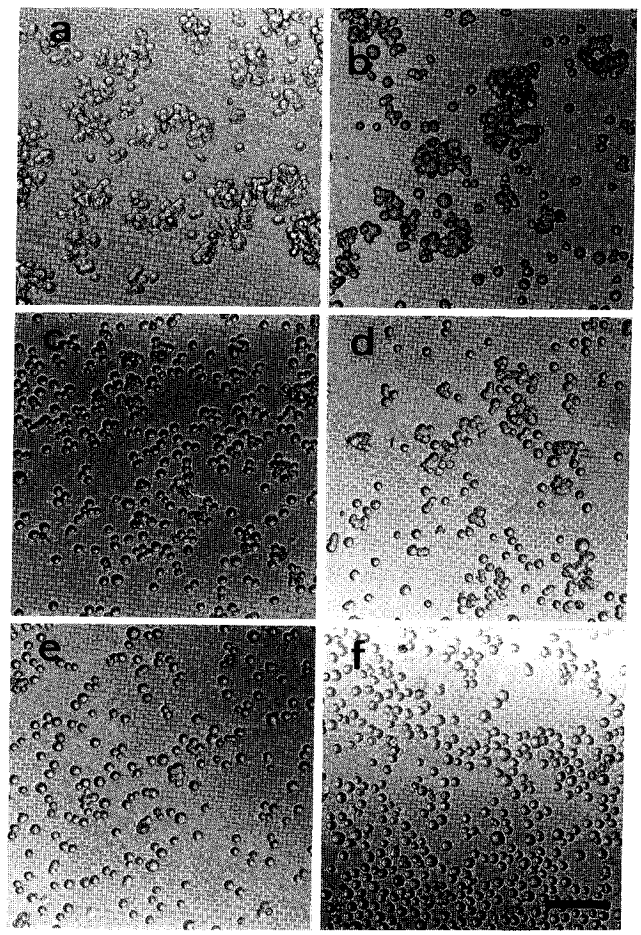

Fig. 2. Aggregation of 308R cells. (*a*) Control without any additional reagents. (*b*) With HGF. (*c*) With ECCD-1. (*d*) With PCD-1. (*e*) With ECCD-1 and PCD-1. (*f*) With HGF, ECCD-1 and PCD-1. The concentration of HGF was 5 ng/ml, and that of ECCD-1 and PCD-1 was approximately 100 μg/ml, respectively. In (*b*) and (*f*), HGF was added to the cultures 24 hr before cell harvesting and also during aggregation . Cells were dissociated and then aggregated for 3 hr, as described in Materials and Methods. Bar, 100 μm.

cell association in monolayer cultures. We examined the effect of HGF on morphology of 308R and F cell colonies. At various concentrations of HGF tested (1 to 20 ng/ml), cells stretched or spread more extensively than in controls and their associations apparently loosened during the incubation for one to two days (Fig. 3b, f). However, these cells maintained cell-cell contacts. Next, the effect of monoclonal antibodies to E-cadherin and P-cadherin was examined. We first observed that each cadherin antibody had some morphological effects on cell-cell contacts, as reported using various cell types including keratinocytes (Takeichi, (29, 30), Wheelock and Jensen, (31)). When the two antibodies were mixed, their effect was maximum. In these cultures, cell-cell boundaries became refractile under phase-contrast microscopy, and some cells in the colonies rounded up (Fig. 3c, g), suggesting that cell-cell association loosened. However, the cells still maintained their mutual contacts. We then added both HGF and the anti-E- and P-cadherin antibodies to these cultures. This treatment showed strong morphological effects. Many cells now lost their associations and separated from each other, showing a typical scattering pattern (Fig. 3d, h).

Immunofluorescence and immunoblot detection of E-cadherin and desmoplakin. The effect of HGF or/and anti-AJ cadherin antibodies on the cadherin adhesion system was studied by double-immunostaining for E-cadherin and desmoplakin, each representing the components of AJ and desmosomes, respectively. Only results obtained using 308R cells are shown below, as F cells gave similar results although this cell line exhibited a weaker staining for desmoplakin. The immunostaining for P-cadherin provided similar results to those for E-cadherin, but this staining was much weaker than E-cadherin staining (data not shown).

In untreated 308R cells, cell-cell boundaries intensely stained as sharp immunoreactive lines for E-cadherin

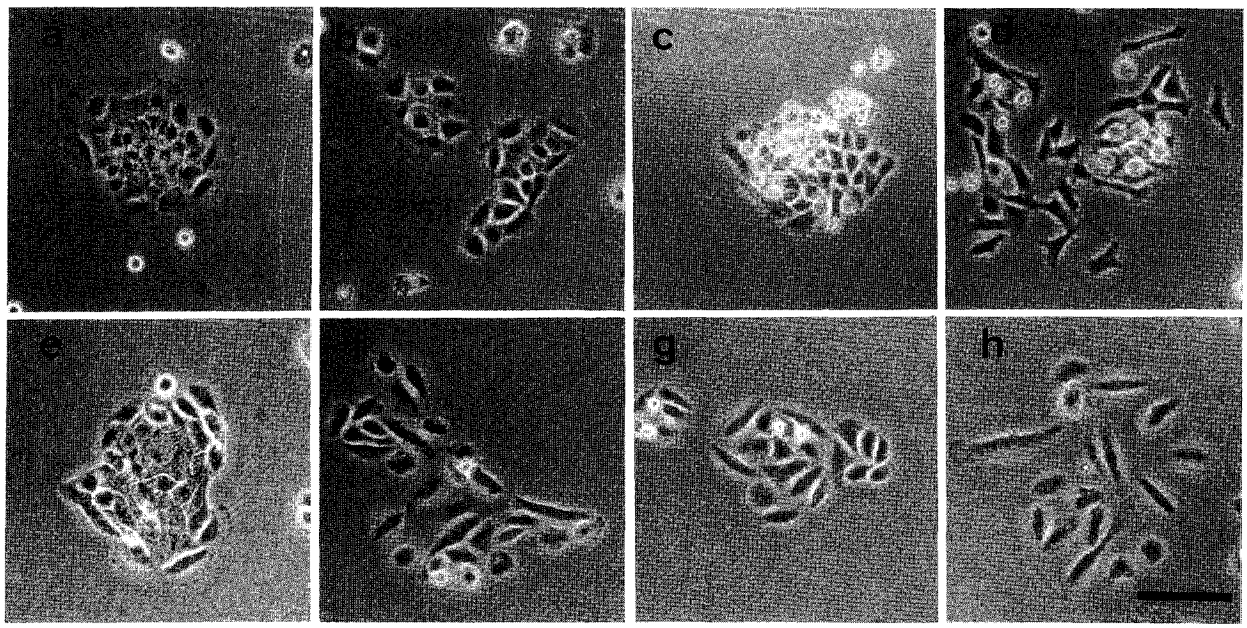

Fig. 3. Effect of HGF and anti-AJ cadherin antibodies on cell colonies. (*a*) to (*d*), F cells. (*e*) to (*h*), 308R cells. Cells were incubated for 24 hr without (*a, e*) or with HGF (*b, f*), ECCD-1 and PCD-1 (*c, g*) or HGF, ECCD-1 and PCD-1 (*d, h*). The concentrations of these reagents were the same as in Figure 2, except that 2 ng/ml HGF was added to F cell cultures. Living cells were photographed under phase-contrast optics. Bar, 100 μm.

and desmoplakin (Fig. 4a, b), although the latter showed discontinuous lines. Sometimes, cell-cell contacts were formed oblique to the substrate, and in these cases, the immunoreactive lines looked much broader. Non-junctional regions of the cell surface were generally devoid of E-cadherin, but occasionally free margins of the cells weakly stained for this molecule. Desmoplakin was detected also on the top of cell surface as small dots. Next, cells treated with HGF were examined. In these cells, the pattern of E-cadherin and desmoplakin staining was basically similar to that observed in untreated cells; they maintained E-cadherin- and desmoplakin-positive cell-cell contacts (Fig. 4c, d). However, it appeared that these molecules were less concentrated at cell-cell boundaries, and the immunoreactive cell-cell boundaries were more irregular than in controls.

When cells were treated with a mixture of monoclonal antibodies to E- and P-cadherin and subsequently stained for E-cadherin, we found that this molecule now distributed all over the cell surface, but it still tended to accumulate at cell-cell contact sites (data not shown). To obtain a stronger inhibition of cadherin function, we used a mixture of a polyclonal antiserum to E-cadherin and the monoclonal antibody to P-cadherin. In these cultures, E-cadherin disappeared from cell-cell boundaries, and scattered as small aggregates in cells(Fig. 4e). However, desmoplakin retained the distribution at cell-cell contact sites in these cells (Fig. 4f), although its distribution pattern was changed; this protein was detected only in part of the cell surface as simple straight lines in the treated cells, whereas it exhibited an honeycombed localization pattern in untreated cells (Fig. 4b). These results suggest that blocking E- and P-cadherin does not disrupt desmosomal cell-cell junctions although it alters the distribution of these junctions.

We then examined cells that had been treated with HGF together with the above mixture of anti-AJ cadherin antibodies. At cell-cell boundaries in these cultures, not only E-cadherin but also desmoplakin largely disappeared (Fig. 4g, h). Thus, under the conditions that AJ cadherins are inactive, desmosomal cell-cell junctions were sensitive to HGF treatment.

To test if the above treatments affected the expression of E and P-cadherin and desmoplakin in 308R cells, immunoblot analysis was performed. As shown in Fig.

Fig. 4. Double immunofluorescence staining for E-cadherin and desmoplakin on 308R cells. Left column, E-cadherin, and right column, desmoplakin. (*a, b*) Untreated control cells. (*c, d*) In the presence of HGF. (*e, f*) In the presence of anti-E-cadherin antiserum and PCD-1. Note that desmoplakin is present at cell-cell boundaries, in spite of the absence of E-cadherin in the same regions. (*g, h*) In the presence of HGF, anti-E-cadherin antiserum and PCD-1. Note that both E-cadherin and desmoplakin are absent at cell-cell boundaries. Cells were incubated for 24 hr. The concentrations of HGF and PCD-1 were the same as in the experiments of Fig. 2. The anti-E-cadherin antiserum was diluted 1 to 500. In each pair of the photographs, focus was changed to give a best view of the distribution of each molecule. Bar, 20 μm.

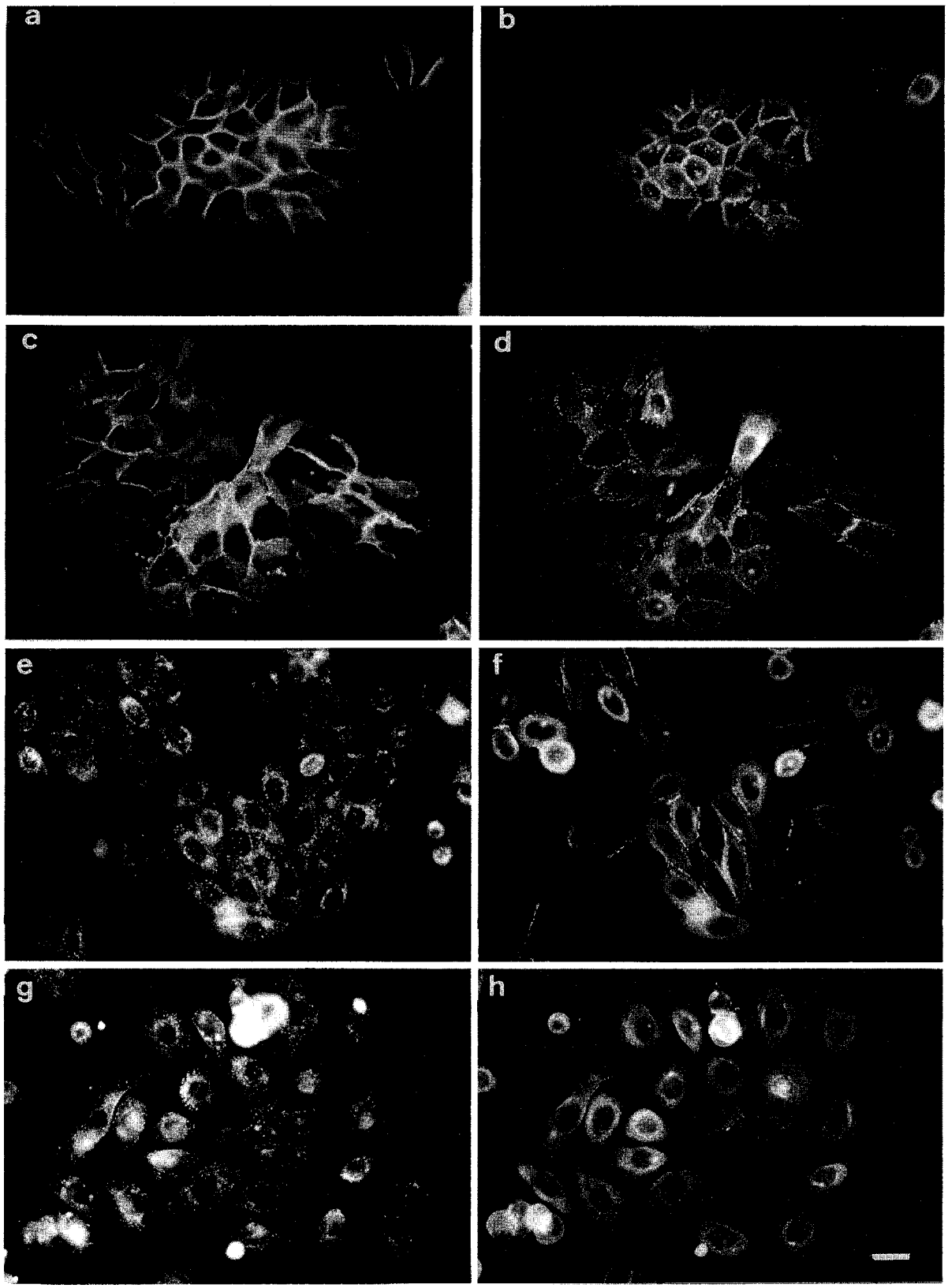

Fig. 4.

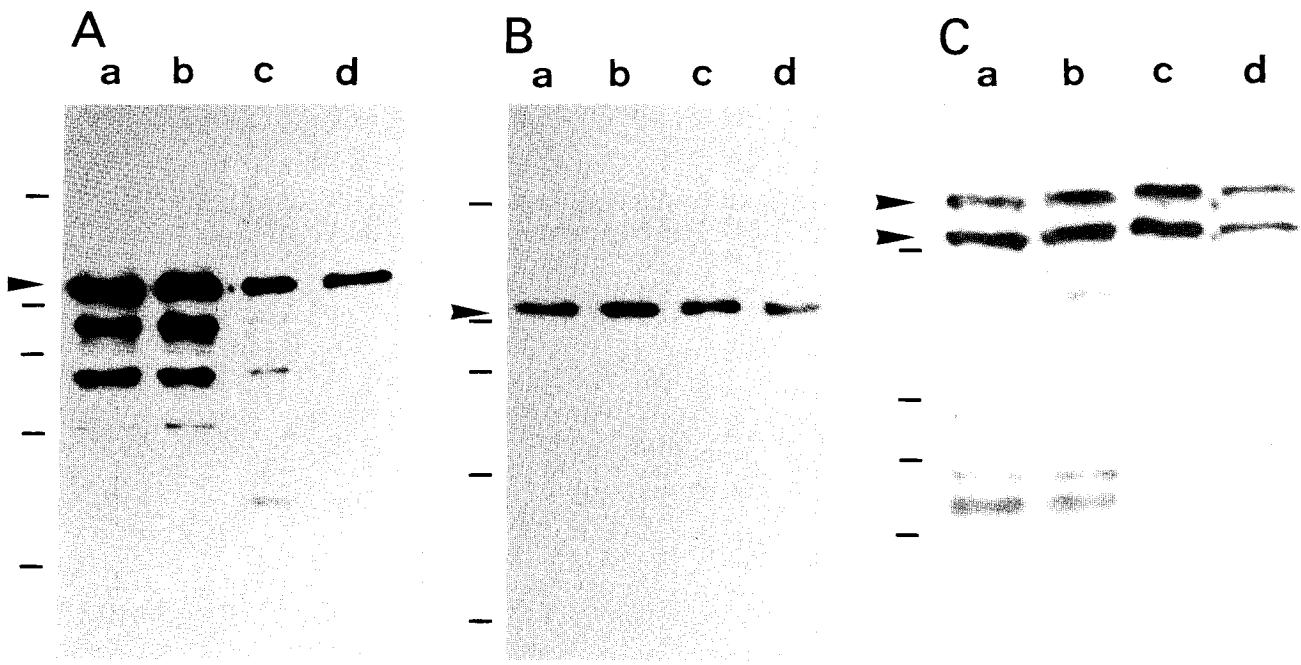

Fig. 5. Immunoblot analysis of the expression of E-cadherin and desmoplakin in 308R cells treated with HGF or/and anti-AJ cadherin antibodies. (A) E-cadherin (shown by arrowhead). Lower bands are probably degradation products. (B) P-cadherin (shown by arrowhead). (C) Desmoplakin. Two isoforms, 250 kD and 215 kD, are detected as shown by arrowheads. Lanes *a*, untreated cells. Lanes *b*, cells treated with 5 ng/ml HGF. Lanes *c*, cells treated with a mixture of the anti-E-cadherin antiserum diluted 1 to 500 and 100 μg/ml PCD-1. Lanes *d*, cells treated with a mixture of HGF and anti-E- and P-cadherin antibodies in the same concentrations as in lanes *b* and *c*. Cells were incubated for 24 hr. The same amount of total proteins was added to each lane. 7.5% and 5% polyacrylamide gels were used for (A) and (B), and (C), respectively. Positions of molecular weight markers are 200, 116, 97, 66 and 45×10^3 for (A) and (B), and 200, 116, 97 and 66×10^3 for (C).

5A–C (lanes a, b), the amount of these proteins was not altered by incubation with HGF alone, being consistent with the results of Weidner *et al.* (33). In the presence of anti-E-cadherin antiserum and anti-P-cadherin monoclonal antibody, E-cadherin was reduced either in the presence or absence of HGF (Fig. 5A, lanes c, d), but the level of P-cadherin was not significantly affected (Fig. 5B, lanes c, d). This is probably due to that the binding of E-cadherin with polyclonal antibodies enhances its degradation, as reported (Shimamura & Takeichi, (24)), but the monoclonal antibody to P-cadherin have less such effect. The level of desmoplakin was not changed by treatment with anti-AJ cadherin antibodies (Fig. 5C, lane c), but it was slightly reduced in the presence of both anti-AJ cadherins and HGF (Fig. 5C, lane d), suggesting that this protein is more readily degraded when its function is perturbed by HGF than under control conditions.

DISCUSSION

The present study was designed to ask whether HGF had any effect on cadherin-mediated cell-cell adhesion. Cell aggregation assays detected no obvious effect of HGF on this process of cell-cell adhesion. However, the studies of immunofluorescence localization for E-cadherin and desmoplakin, as representatives of the AJ and desmosomal components, respectively, suggest that HGF has some effect to interfere with the cadherin adhesion system. The following effects of HGF were observed. (1) E-cadherin and desmoplakin tended to reduce in concentration at cell-cell boundaries after HGF treatment. (2) Under the conditions that E- and P-cadherin (AJ cadherins) were blocked with antibodies, HGF inhibited the accumulation of desmoplakin to cell-cell boundaries, suggesting that desmosomes were sensitive to the action of this growth factor. Stoker and Perryman (27) found no effect of scatter factor on desmosomal cell-cell contacts, but they did not inhibit AJ cadherins in their experiments. On the other hand, desmoplakin retained its intercellular localization when AJ cadherins were blocked in the absence of HGF. This result is consistent with the previous finding of Wheelock and Jensen (31), and explains why cell-cell contacts were not entirely disrupted when only AJ cadherins were inhibited. Probably, AJ and desmosomes are structurally independent. It is however still possible that the formation or distribution of desmosomes is regulated by AJ, since desmosomal distribution was changed by the loss of AJ. In fact, we recently found that *de novo*

formation of desmosomes requires AJ cadherins (M. Watabe and M. Takeichi, in preparation).

There are two possible explanations for the above effects of HGF on cell-cell adhesion. First, HGF may have no direct effect on the cadherin adhesion system, and it indirectly interferes with it. The primary action of HGF could be only to enhance cell motility as shown by many other studies (Stoker *et al.*, (28), Weidner *et al.*, (32), Konishi *et al.*, (9), Furlong *et al.*, (5), Naldini *et al.*, (18)), and the disruption of cell-cell adhesion might be induced as a secondary effect of the enhanced cell motility. Desmosomal junctions might be sensitive to physical cell separating forces, and, therefore, HGF could disrupt cell-cell adhesion when AJ cadherins were inhibited.

Secondly, HGF might have a direct perturbing effect on the cadherin adhesion system. This notion arises from the following backgrounds. We recently found that 3Y1 cells transformed with v-*src* had an aberrant cadherin activity (Matsuyoshi *et al.*, (12)). These v-*src* transformed cells show apparently normal cadherin activity as they can aggregate using cadherins in suspension. However, these cells dissociate when cultured on solid culture substrata; such dissociation never occurs in cultures of the normal counterparts of this cell line. In these v-*src* transformed cells, β-catenin is phosphorylated at tyrosine residues. In chicken fibroblasts transformed by Rous sarcoma virus, their cadherin function is strongly inhibited, and coincidentally both α- and β-catenin are tyrosine phosphorylated (Hamaguchi *et al.*, (6)). A similar finding was recently reported using MDCK cells (Behrens *et al.*, (1)). These findings suggest that cadherin function is suppressed by an excessive tyrosine phosphorylation or related biochemical modifications of the cadherin system, and the degree of the suppression depends on the level of the modifications. A subtle perturbation of cadherin function might not be detected by cell aggregation assays, but could be detected by culturing cells on solid substrata, because, under these conditions, cells can move and this moving action might facilitate disruption of cell-cell adhesion if it is aberrant. A similar phenomenon was observed for the effect of acidic FGF on NBT-II cells (Boyer *et al.*, (3)). Also in this case, the FGF induces cell dispersion without affecting not only E-cadherin expression but also their aggregating ability in suspension. Interestingly, FGF receptor is also a tyrosine kinase.

HGF could interfere with the cadherin system via a similar mechanism. Its effect could be too subtle to detect by cell aggregation assays, but it might be detected in monolayer cultures as discussed above. HGF receptor is the c-Met tyrosine kinase (Park *et al.*, (21), Bottaro *et al.*, (2), Naldini *et al.*, (17), Higuchi *et al.*, (7)), and our recent studies demonstrated that HGF treatment induced or enhanced tyrosine phosphorylation of some cadherin-associated proteins in certain carcinoma cells (S. Shibamoto, M. Takeichi and F. Ito, in preparation). These observations support the idea that HGF has a direct effect on the activity of cadherins. Desmosomes could physiologically be more sensitive to the proposed action of HGF than AJ.

Whatever the mechanism is, we clearly demonstrated that the cell scattering effect of HGF was strongest when AJ cadherins were blocked. This finding should be important in considering the roles of HGF in tumor invasion and metastasis, as down-regulation of cadherin expression is observed in many human cancers (Takeichi, (30)).

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