Hepatocyte growth factor alters the polarity of Madin-Darby canine kidney cell monolayers

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Hepatocyte growth factor (HGF) and E-cadherin are important for epithelial morphogenetic events. We examined the effects of HGF on E-cadherin localization and interaction with β-catenin in polarized Madin-Darby canine kidney (MDCK) cell monolayers grown on filters. Surface biotinylation experiments showed that HGF increases apically accessible E-cadherin. Confocal immunofluorescence microscopy of HGF-treated cells showed localization of E-cadherin at membrane domains contacting the apical compartment and an increase in accessibility of apically applied antibodies to lateral E-cadherin below the tight junction. Coimmunoprecipitation of β-catenin/E-cadherin complexes showed that the amount of E-cadherin associated with β-catenin increased during the first 24 h of HGF treatment with a return to baseline values after 48 and 72 h. Metabolic labeling showed that HGF increased the synthetic rate of β-catenin and the amount of newly synthesized E-cadherin associated with immunoprecipitated β-catenin, with the peak effect occurring after 12 h of treatment and returning to baseline after 24 h. HGF treatment inhibited transcytosis of immunoglobulin A by the polymeric immunoglobulin receptor. We conclude that HGF treatment of polarized MDCK cells grown on filters decreases cell polarity and alters E-cadherin/β-catenin interaction and synthesis.

Hepatocyte growth factor (HGF) is a polypeptide growth factor with pleiotropic functions which, depending on target cells and tissues and stage of development, can include mitogenesis, cell motility, and the development and regeneration of organs (1). These HGF-induced events are mediated by activation of c-met, the tyrosine kinase receptor for HGF. Two in vitro models used previously for the characterization of motogenic and morphogenic events induced by HGF use the Madin-Darby canine kidney (MDCK) epithelial cell line. In the first model, MDCK cells were grown on impermeant supports as small colonies at low density. When exposed to HGF, these cells assumed a fibroblastic morphology and scatter away from the colonies (hence the synonym of scatter factor for HGF) (2, 3). In the second model, MDCK cells were grown as hollow cysts in type I collagen gels. When exposed to HGF, they formed complex branching tubules extending out from the cysts, mimicking the normal branching tubule morphogenesis that occurs during the development of many epithelial organs (4, 5).

To analyze directly the effects of HGF on polarized epithelial cell functions that may be important for epithelial cell rearrangements during HGF-induced morphogenesis and motogenesis, we tested the effects of HGF on MDCK cells cultured on permeable supports. MDCK cells cultured on permeable filter supports form well polarized monolayers with apical and basolateral membrane domains separated by a functional tight junction belt (4). This widely used model allows for apical and basolateral surface domain-specific techniques such as surface biotinylation, surface immunolabeling, and surface immunoprecipitation to study cell surface polarity. In epithelial cells, the response to HGF and cell polarity are interdependent. For example, in polarized MDCK cells, the HGF receptor, c-met, is localized to the basolateral cell surface (5), and cells respond to basolateral but not apical HGF (2). However, epithelial cells that are less well polarized, such as MDCK cells cultured on plastic, scatter in response to HGF in the apical medium and acquire a fibroblastic morphology (2, 3). We hypothesized that the permeable filter support model system would be useful for characterizing HGF-induced effects on epithelial cell polarity that might provide insight into more complex HGF-induced epithelial morphogenetic events such as tubulogenesis, organ development, and tissue repair.

These morphogenetic events are likely to involve complex changes in cell-cell interactions. E-cadherin is a 120-kDa transmembrane protein that is primarily responsible for homotypic adhesion between adjacent epithelial cells (6). In well polarized epithelia, E-cadherin is localized to the basolateral membrane below the tight junction, and the extracellular domain of E-cadherin is, therefore, inaccessible from the apical environment. E-cadherin activity is necessary to maintain the adherens junctions, which are characteristic of polarized epithelial cells, as well as for the activity of other intracellular junctions including zonula occludens (tight junctions) and desmosomes (7). E-cadherin is also believed to play a critical role in morphogenetic events through the regulation of cell-cell adhesion (8, 9). To exhibit functional activity, E-cadherin forms com-

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plexes with cytosolic proteins called catenins (α, β, and γ), which link E-cadherin to the actin cytoskeleton (10, 11, 12). β- and γ-catenin are homologous to armadillo, a Drosophila segment polarity gene (12). Both of these catenins have been shown to modulate cell-cell adhesion (13, 14). α-Catenin is involved in linking membrane proteins to the cortical cytoskeleton at sites of cell-cell contact and is also required for cell adhesion (15, 16). Because of the importance of E-cadherin in epithelial structure and its suspected role in morphogenesis, we have now studied the effect of the HGF on E-cadherin localization and interaction with β-catenin in polarized MDCK cells grown on permeable filter supports.

**Results**

**Materials and Methods.**

MDCK cells grown on Transwell filters seeded at confluency. Cell monolayers were used for experiments after 3 days of culture with daily media change. Hybridoma cells secreting mouse monoclonal E-cadherin mAb (rr1), which recognizes an extracellular epitope, were a kind gift from S. Gumbiner (Sloan Kettering, New York, NY). Rat mAb R40.76 against ZO-1, a peripheral membrane protein and its suspected role in morphogenesis, we have now studied the effect of the HGF on E-cadherin localization and interaction with β-catenin in polarized MDCK cells grown on permeable filter supports.

**Fixation and Fluorescent Labeling of Cells—**MDCK cells grown on 6-mm Transwell filters were fixed with paraformaldehyde. Cells were fixed with ice-cold 4% paraformaldehyde in PBS* for 20 min. After washing the filters three times with PBS*, the cells were quenched with 75 mM NH4Cl and 20 mM glycine, pH 8.0, with KOH (quench solution) for 10 min at room temperature. Filters were washed one time with PBS* and permeabilized with PBS* 0.7% fish skin gelatin, and 0.025% saponin (PBS) for 15 min at 37 °C. Filters were labeled with rr1 or ZO-1 mAb diluted in PBS 1:2 and 1:100, respectively, for 1 h at 37 °C. Filters were then washed four times for 5 min each with PBS at room temperature and then labeled with the appropriate secondary Ab diluted 1:100 in PBS 0.1% Tween, 0.1% TX-100, and 0.1% BSA. Filters were postfixed in 4% paraformaldehyde for 15 min at room temperature. Filters were cut from the support with a scalpel and mounted in Vectashield mounting medium (Burlingame, CA).

For surface fluorescence labeling, the living cells were cooled to 4 °C on ice and exposed to rr1-conditioned media from the apical or basolateral surface for 1 h at 4 °C. The filters were then washed three times with ice-cold PBS* and subjected to fixation and fluorescent labeling as described above.

**Scanning Laser Confocal Analysis of Fluorescently Labeled Cells—**The samples were analyzed using a krypton-argon laser coupled with a Bio-Rad MRC1000 confocal head, attached to an Optiphot II Nikon microscope with a Plan Apo 60 × 1.4 NA objective lens. The samples were scanned individually or simultaneously for fluorescein-5-isothiocyanate or Texas Red with excitation/emission wavelengths of 488/520 and 568/615, respectively. The data were analyzed using Comos software. Images were converted to tagged information file format (TIFF), and contrast levels of the images were adjusted by using the Photoshop software. Images were converted to tagged information file format (TIFF), and contrast levels of the images were adjusted by using the Photoshop software.
phogenetic events. We, therefore, examined the effect of HGF treatment on the localization of E-cadherin in polarized MDCK cell monolayers grown on filters with domain-selective surface biotinylation. Cells were plated at confluency on 24-mm Transwell filters and cultured for 3 days with daily media changes before introducing recombinant human HGF at a concentration of 100 ng/ml into the basolateral compartment. After treatment for 24 h, apical or basolateral membrane domains were surface biotinylated, and cell membrane proteins were solubilized in RIPA buffer. E-cadherin in the total cell lysates from individual filters was immunoprecipitated with an equal volume of rr1-conditioned media. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with streptavidin-horseradish peroxidase. E-cadherin is the very prominent band that is strongest in the basolateral biotinylated samples (filled arrow) but also seen well in the apically biotinylated HGF-treated sample. The fainter band above the control basolaterally biotinylated E-cadherin band was not reproducible and is likely due to an E-cadherin complex that was not completely dissociated. Data are representative of four separate experiments.

Fig. 1. HGF treatment increases apically biotinylated E-cadherin in MDCK cell monolayers. MDCK cell monolayers grown on 24-mm Transwell filters were treated with 100 ng/ml of HGF or epidermal growth factor on the basolateral surface for 24 h. Apical or basolateral membrane proteins were surface biotinylated by standard protocols. Cells were lysed in 800 μl of RIPA buffer, and total lysates from individual filters were immunoprecipitated with an equal volume of rr1-conditioned media. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with streptavidin-horseradish peroxidase. E-cadherin is the most prominent band in the lane corresponding to biotinylated biotinylated E-cadherin (Fig. 1). Thus, the increase in apically biotinylated E-cadherin band was not reproducible and is likely due to an E-cadherin complex that was not completely dissociated. Data are representative of four separate experiments.

Fig. 2. Apical or basolateral confocal immunofluorescence microscopy of control and HGF-treated MDCK cell monolayers for E-cadherin. Control (a) and HGF-treated (100 ng/ml, 24-h incubation) (b) MDCK cells were exposed to apical or basolateral rr1-conditioned media for one hour at 4°C. Fig. 2 (c and c’) shows confocal x-z section views (i.e. parallel to the plane of the filter) of the apically accessible E-cadherin (Fig. 2c’) in the 24-h treated cells. Surface immunolabeling of E-cadherin in the presence and absence of HGF were performed on at least five separate occasions with reproducible results. Bar, 20 μm.

HGF-treated control cells (Fig. 2a), basolaterally applied rr1 clearly labeled E-cadherin, whereas apically applied rr1 failed to label E-cadherin. These findings confirm that under control conditions, the MDCK cell monolayer has competent tight junctions that exclude diffusion of rr1 from the apical compartment to the basolateral compartment. In contrast, cells treated for 24 h with HGF (Fig. 2b) showed an increase (2–3% of the total number of cells) in labeling by apically applied rr1 of discrete patches of membrane at the lateral borders of individual cells of the monolayer. When viewed in the x-y axis (i.e. a section parallel to the plane of the filter), the apically accessible E-cadherin appeared to be in the lateral domain. Confocal microscopy allowed us to reconstruct x-z section views (i.e. perpendicular to the plane of the filter) of the apically accessible E-cadherin (Fig. 2c’) and the tight junction marker ZO-1 (Fig. 2c). After 24 h of HGF treatment, apically accessible E-cadherin was detected below the tight junction.

Fig. 3 shows that with extended exposures to HGF, an increasing percentage of cells expressed E-cadherin, which was accessible from the apical compartment for surface immunolabeling. Fig. 3 was taken with a conventional fluorescence microscope, because it allowed a slightly different and informative overview of the distribution of apically accessible E-cadherin in this case. After 48 h of HGF exposure, approximately 15–20% of the cells expressed apically accessible E-cadherin, whereas after 72 h of HGF exposure, 30–40% of the cells expressed apically accessible E-cadherin.

Confocal microscopy x-z section views shown in Fig. 4 of control and 48- or 72-h HGF-treated cells were co-stained for ZO-1 (Fig. 4) and either apically accessible E-cadherin (Fig. 4, b, c, and e) or basolaterally accessible E-cadherin (Fig. 4, a and d). These pictures showed that in addition to increasing access through the tight junction to E-cadherin in lateral membranes below the tight junction, the longer periods of HGF exposure (Fig. 4, c, 48 h and e, 72 h) also induced mislocalization of E-cadherin to membrane domains above the tight junction that...
are in direct contact with the apical compartment. The images of basolaterally accessible E-cadherin for control (Fig. 4a) and 72 h of HGF treatment (Fig. 4d) demonstrate that HGF treatment induced other morphological changes, i.e., increased thickness of the monolayer and more tortuous interrelationships between adjacent cells (increased lateral membrane surface).

**Effect of HGF Treatment on E-cadherin Associated with Immunoprecipitated β-catenin—**E-cadherin functional activity is dependent upon association with catenins. β-Catenin represents an important link between epidermal growth factor-induced signal transduction and cadherin function (22). We, therefore, examined the effect of HGF on the association of E-cadherin with immunoprecipitated β-catenin. Fig. 5A shows that during HGF treatment, the amount of E-cadherin associated with immunoprecipitated β-catenin increased over a 24-h period (at 24 h, the amount was 313 ± 5.7% (p = 0.0008)). Fig. 5B shows the amount of E-cadherin associated with immunoprecipitated β-catenin after 24, 48, and 72 h of HGF treatment. Again, E-cadherin associated with β-catenin is increased after 24 h of HGF treatment, but after 48 or 72 h of HGF treatment, this amount returned to approximately baseline. A slight increase in the amount of total immunoprecipitated β-catenin is detectable after HGF treatment (at 48 h, the amount was 115 ± 12% of control) but is not statistically significant (data not shown). These results demonstrate that HGF modulates E-cadherin and β-catenin interaction during morphogenetic events.

**HGF Treatment of Polarized MDCK Cell Monolayers Increases the Synthetic Rate of β-Catenin and E-cadherin Associated with β-Catenin—**Because the previous experiments provided evidence that HGF increased the amount of E-cadherin associated with β-catenin in MDCK cells and, to a lesser extent, the amount of β-catenin, we examined the effect of HGF treatment on the biosynthetic rates of β-catenin and E-cadherin associated with β-catenin. (We did not examine the biosynthesis of total E-cadherin for technical reasons.) Polarized monolayers of MDCK cells were treated with HGF for 1, 3, 6, 12, or 24 h and pulse-labeled with [35S]cysteine and [35S]methionine for 15 min, and cell lysates were immediately collected. Equal amounts of RIPA buffer solubilized protein were subjected to immunoprecipitation by β-catenin mAb. Amounts of newly synthesized E-cadherin and β-catenin in the immunoprecipitates were quantified by PhosphorImager analysis. The results (Fig. 6) show that HGF treatment of polarized MDCK cells increased the synthetic rates of β-catenin and E-cadherin found in the β-catenin immunoprecipitates. The peak effect on synthesis was observed at approximately 12 h (p < 0.003 for both E-cadherin and β-catenin) and returned to near baseline by 24 h. These results demonstrate that HGF increases the relative rate of β-catenin synthesis. Moreover, HGF also increases the amount of newly synthesized E-cadherin molecules found in β-catenin complexes. This observation suggests that HGF is stimulating the rate of E-cadherin synthesis and/or the rate at which E-cadherin interacts with new and existing pools of β-catenin.

**HGF-induced Apically Accessible E-cadherin Is Associated with β-Catenin—**The β-catenin immunoprecipitation results demonstrate that HGF modulates the amount of E-cadherin associated with β-catenin. To determine if β-catenin is associated with HGF-induced apically accessible E-cadherin, control cultures and cells treated with HGF for 24 or 48 h were subjected to apical and basolateral surface immunoprecipitation with rr1. Amounts of β-catenin associated in the immunoprecipitate complexes were determined by Western blot. Fig. 7 shows the results of such an experiment, from which we can draw the following conclusions: (a) these results confirmed that
HGF Incubation (Hrs.)

**FIG. 6.** Effect of HGF on synthetic rates of E-cadherin and β-catenin found in β-catenin immunoprecipitates. MDCK cells were grown on filters and treated with HGF (100 ng/ml) for the indicated incubation period. Cells were then metabolically labeled with Translabel as described under “Experimental Procedures.” Cells were lysed in RIPA buffer, and β-catenin-containing complexes were immunoprecipitated from 40 μg of cell protein with 0.5 μg of mouse monoclonal β-catenin antibody. Immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose. Two bands were typically observed in the molecular weight range of E-cadherin and β-catenin. The identity of each band was confirmed by Western blot analysis with specific antibodies. Amounts of 35S in the E-cadherin and β-catenin bands were analyzed by SDS-PAGE and quantitated with a PhosphorImager (Molecular Dynamics). Data are the means (bars, S.D.) of triplicate determinations and are representative of three experiments. *p < 0.003) compared with control cells.

**FIG. 7.** Analysis of β-catenin association with HGF induced apically accessible E-cadherin by surface immunoprecipitation. MDCK cell monolayers were treated with HGF (100 ng/ml) for the indicated incubation period. Surface immunoprecipitation of E-cadherin was carried out as described under “Experimental Procedures.” Immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose; the presence of E-cadherin or β-catenin was carried out as described under “Experimental Procedures.” Immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose; the presence of E-cadherin or β-catenin was determined by Western blot. Data are representative of two separate determinations. •, 116 kd; X, 97 kd. HGF Incubation: Control 0 24 48 72 HGF 0 24 48 72 E-cadherin Surface I.P.: Apical BL Apical BL

HGF increased the amount of apically accessible E-cadherin. However, this population of E-cadherin was not well visualized until 48 h of treatment, suggesting that this method is not as sensitive as surface biotinylation and surface immunofluorescence labeling; (b) these results demonstrated that both the apically and basolaterally accessible E-cadherin are associated with β-catenin. Because this protocol did not effectively immunoprecipitate apically accessible E-cadherin at 24 h, the time period in which increasing amounts of E-cadherin are associated with β-catenin, we cannot absolutely rule out changes in β-catenin associated with apically accessible E-cadherin at this time point; (c) the results show that HGF induced an increase in E-cadherin/β-catenin complexes at the basolateral accessible cell surface, consistent with the observation of increased lateral membrane surface area (Fig. 4).

Effect of HGF Treatment on pIgR Transcytosis—The HGF-induced increase in apically accessible E-cadherin suggests a significant change in cell polarity. In a preliminary attempt to gain insight into the effects of HGF on polarized membrane traffic, we examined the effect of 48-h HGF treatment on the transcytosis of iodinated dIgA by MDCK cells expressing the rabbit pIgR. Under normal conditions, these cells exhibit functional polarity by transecting dIgA from the basolateral to the apical compartment, although a small fraction of the dIgA recycles to the basolateral compartment. Fig. 8 illustrates the results of such an experiment. HGF treatment of the pIgR-expressing MDCK monolayers significantly inhibited the basolateral to apical transcytosis of 125I-labeled dIgA but did not influence basolateral recycling. To a lesser degree, a similar pattern of transcytosis inhibition was observed after 24 h of HGF treatment (data not shown). These results suggest that HGF alters the polarity of filter-grown MDCK cells, at least in part, by changing the polarized membrane traffic.

**DISCUSSION**

Our results provide evidence that HGF treatment of polarized MDCK cell monolayers grown on filters induced morphological changes, including an increase in apically accessible E-cadherin. The mechanism by which this occurs appears to be through localization of E-cadherin to the membrane domain in contact with the apical compartment and modulation of tight junction integrity. HGF treatment also modulated the association of E-cadherin with immunoprecipitated β-catenin and stimulated the de novo synthesis rates of β-catenin. In addition to altering the polarity of MDCK cell monolayers, HGF also altered polarized membrane traffic, as determined by the transcytosis of dIgA by the pIgR. Collectively, these findings demonstrate that this model of HGF treatment of polarized MDCK cells grown on filters provides a system by which complicated morphogenetic events in polarized cells can be dissected with regard to structural, biochemical, and functional polarity. This model will serve in the understanding of more complex processes, including the loss of polarity in disease processes such as carcinomas (23), regaining epithelial polarity in tissue regeneration following injuries such as acute tubular necrosis (24), and epithelial morphogenesis in normal and abnormal
(e.g. polycystic kidney disease) development of the kidney (25).

The importance of E-cadherin in epithelial morphogenetic events is not restricted to mammalian systems. Recently, two groups simultaneously reported that the Drosophila gene shot-gun that encodes the first classic cadherin isolated from invertebrates plays a crucial role in the developing Drosophila embryo (26, 27). Their results seem congruent with ours, in that they found that the continued zygotic expression of the Drosophila cadherin is required for epithelial cell rearrangement during morphogenesis. This suggests that our observed increase in the expression of E-cadherin in MDCK cells exposed to HGF may similarly be important for morphogenesis in mammalian epithelia. The Drosophila cadherin also interacts with catenins, and it is likely that these interactions are also modulated during invertebrate morphogenetic events such as embryonic development. Evidence supporting this hypothesis has been reported by two groups, demonstrating the requirement of the Drosophila β-catenin homolog Armadillo for the formation of Drosophila adherens junction and epithelial polarity (28, 29).

By inducing localization of E-cadherin to the membrane domain facing the apical compartment and increasing apical accessibility via tight junctions, HGF decreases the degree of polarization in previously well polarized MDCK cells. Because alterations in epithelial polarity are associated with pathologic states such as carcinoma (33), this model has potential in the characterization of cellular events during carcinoma induction. Recently, it was shown that estrogen activated c-JunER protein-induced loss of epithelial polarity, a disruption of intercellular junctions, and the formation of irregular multilayers in mammary epithelial cells (30). The authors of this paper suggested that these processes may be important for both normal breast development and as initiating steps in the induction of breast carcinogenesis. Epithelial invasiveness is associated with down-regulation of E-cadherin amounts (31, 32); however, the present findings showed an increase in apparent amounts of apical and basolateral E-cadherin by HGF treatment. This finding, coupled to the loss of epithelial polarity, raises the possibility of E-cadherin dysfunction in the present model and/or suggests that under certain conditions, epithelia can lose functional and structural polarity (a phenotype seen in carcinomas) with a paradoxical increase in E-cadherin amounts.

HGF also reduces transcytosis of the pIgR (33). Basolateral recycling of the pIgR by the pIgR was not affected by HGF treatment, suggesting that HGF treatment specifically alters the machinery necessary for efficient basolateral to apical translocation of the pIgR. We do not know if this is due simply to the increased distance that the pIgR and dIgA must travel across the thicker monolayer. This result is intriguing because transcytosis to the apical surface is an essential mechanism by which epithelial polarity is established and maintained, particularly during the early steps in development of the polarized phenotype (34). Clearly, HGF affects the basic mechanisms of membrane traffic involved in the development of polarity.

Treatment of polarized MDCK cells with HGF increased the amount of E-cadherin associated with immunoprecipitated β-catenin during the first 24 h of exposure with a return to baseline levels at 48 and 72 h of HGF treatment. Thus, HGF altered β-catenin protein interactions with E-cadherin in MDCK cells grown on filters. It should be noted that at 24 h, there is only a very small amount of apically accessible E-cadherin, which does not become prominent until 48–72 h. Therefore, the response to HGF can be viewed as having two phases: 1) an early phase that peaks at 24 h and is characterized primarily by an increase in the amount of E-cadherin associated with β-catenin; and 2) a late phase that peaks at 48–72 h and is characterized by the return of both β-catenin synthetic rate and association between β-catenin and E-cadherin to baseline levels, and a large amount of apically accessible E-cadherin. Assembly of E-cadherin/β-catenin complexes in polarized MDCK cells is a dynamic process (20) involving existing and newly synthesized complexes. Therefore, it is possible that HGF modifies this assembly process either through alteration in cadherin and catenin synthetic rates and/or modification of existing cadherins and catenins. We observed that HGF increases the relative rate of β-catenin synthesis. Moreover, HGF also increases the amount of newly synthesized E-cadherin molecules found in β-catenin complexes. This observation suggests that HGF is stimulating the rate of E-cadherin synthesis and/or the rate at which E-cadherin interacts with new and existing pools of β-catenin. Weidner et al. (3) reported that HGF treatment does not increase E-cadherin synthesis. However, in that study, the MDCK cells were grown nonconfluently on impermeant supports, and E-cadherin synthesis was measured between 17 and 20 h of HGF treatment, a time when effects of HGF on synthesis rates of E-cadherin may have already declined to near baseline. In human carcinoma cells, HGF has been shown to modulate the function of the cadherin-catenin system via tyrosine phosphorylation of cadherin-associated proteins, including β-catenin (35). Thus, tyrosine phosphorylation of β-catenin may play an important role in modulation of the E-cadherin/β-catenin complex assembly and composition in the HGF-treated MDCK monolayers and will be a subject for future investigation.

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