Hepatocyte growth factor induces MDCK cell morphogenesis without causing loss of tight junction functional integrity

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Hepatocyte growth factor induces MDCK cell morphogenesis without causing loss of tight junction functional integrity. Am J Physiol Cell Physiol 286: C482–C494, 2004. First published October 30, 2003; 10.1152/ajpcell.00377.2003.—Hepatocyte growth factor (HGF) induces mitogenesis, motogenesis, and tubulogenesis of cultured Madin-Darby canine kidney (MDCK) epithelial cells. We report that in addition to these effects HGF stimulates morphogenesis of tight, polarized MDCK cell monolayers into pseudostratified layers without loss of tight junction (TJ) functional integrity. We tested TJ functional integrity during formation of pseudostratified layers. In response to HGF, the TJ marker ZO-1 remained in morphologically complete rings and functional barriers to paracellular diffusion of ruthenium red were maintained in pseudostratified layers. Transepithelial resistance (TER) increased transiently two- to threefold during the morphogenetic transition from monolayers to pseudostratified layers and then declined to baseline levels once pseudostratified layers were formed. In MDCK cells expressing the trk/met chimera, both HGF and NGF at concentrations of 2.5 ng/ml induced scattering. However, 2.5 ng/ml HGF did not affect TER. The peak effect of HGF on TER was at a concentration of 100 ng/ml. In contrast, NGF at concentrations as high as 25 µg/ml had no effect on TER or pseudostratified layer morphogenesis of trk/met-expressing cultures. These results suggest that altered presentation of the stimulus, such as through HGF interaction with low-affinity sites, may change the downstream signaling response. In addition, our results demonstrate that HGF stimulates pseudostratified layer morphogenesis while inducing an increase in TER and maintaining the overall tightness of the epithelial layer. Stimulation of epithelial cell movements by HGF without loss of functional TJs may be important for maintaining epithelial integrity during morphogenetic events such as formation of pseudostratified epithelia, organ regeneration, and tissue repair.

MORPHOGENETIC CELL REARRANGEMENTS are stimulated by hepatocyte growth factor (HGF), a fibroblast-derived growth factor (77) that has paracrine actions on a variety of cell types. In vivo, HGF is important for the development of the placenta, liver, limbs, and kidney (6, 28, 67, 71, 72, 82, 87) and induces morphogenesis of blood vessels (9, 22, 47, 70). In addition, both HGF and the c-met protooncogene (c-met) (31, 86), a tyrosine kinase receptor (11, 19, 51, 54) to which HGF binds with high affinity (25, 32), are regulated in response to organ injury and stimulate epithelial morphogenesis that contributes to tissue repair and regeneration in liver, kidney, lung, gastric mucosa, and muscle (12, 38, 40, 66, 81, 88). In vitro, many of the pleiotropic effects of HGF can be modeled and show a dependence on both the target cell type and environmental context: paracrine actions of HGF increase hepatocyte mitogenesis (20, 41, 49, 50, 90), inhibit growth of tumor cells (69, 78), activate motogenesis and invasiveness of epithelial and endothelial cells (9, 18, 63, 64, 70, 75, 84, 85), stimulate wound repair (53), and induce morphogenesis of either epithelial tubes or pseudostratified layers from polarized epithelial monolayers (4, 42, 43, 56, 73).

The morphogenetic effects of HGF suggested that HGF may affect tight junctions (TJs). Several previous studies analyzed the effects of HGF on the assembly of TJs during the formation of a polarized epithelial monolayer (23, 55). The results of these studies indicated that HGF could inhibit assembly of newly forming epithelial cell-cell junctions. Treatment of polarized endothelial or epithelial monolayers with HGF had varying effects, either increasing or decreasing transepithelial resistance (TER) (26, 27, 34, 53). However, the time frame or conditions of HGF treatment in these studies were not reported to induce morphogenetic cell rearrangements.

The effect of HGF on TJ function during morphogenetic cell rearrangements is not well understood. Initial models of HGF-induced morphogenesis proposed that stimulation of cell dissociation is important for cell rearrangements. However, we showed previously (4, 56) that during HGF-induced tubulogenesis or formation of pseudostratified layers from polarized monolayers cell-cell contacts are maintained and the TJ marker ZO-1 remains localized at sites of contact between cells that are rearranging. In light of these results we hypothesize that TJ function may be maintained during HGF-induced cell rearrangements. A two-dimensional model system in which polarized Madin-Darby canine kidney (MDCK) cell monolayers are plated on permeable filter supports and stimulated by HGF to form pseudostratified layers (4, 91) allows functional aspects of TJs to be studied during morphogenetic cell movements.

In this study we analyzed the effect of HGF on TJ function during stimulation of morphogenetic cell rearrangements. We report that HGF induced cells of a polarized MDCK cell monolayer to crawl over each other to form a pseudostratified layer. During the transition from a monolayer to a pseudostratified layer HGF caused a transient increase in MDCK cell TER and preserved a barrier to paracellular diffusion of solutes. In the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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addition, we show that the effects of HGF on TER were not inducible by direct activation of c-met and may require interaction of HGF with low-affinity binding sites. Our analysis of TJ integrity during pseudostratified layer formation reveals that maintenance of functionally intact TJs is an important component of HGF-stimulated morphogenesis that is likely to be critical for tissue morphogenesis and repair.

**MATERIALS AND METHODS**

**Reagents.** Recombinant human (rh)HGF was generously provided by R. Schwall (Genentech, San Francisco, CA). NGF was generously supplied by W. Mobley (University of California, San Francisco). Rabbit polyclonal anti-HGF antibody was obtained from J. Rubin (National Cancer Institute, Bethesda, MD). Mouse monoclonal antibody DO24 (Mab DO24) was kindly provided by T. Crepaldi, M. Prat, and P. Comoglio (University of Turin, Turin, Italy). Hybridoma cells secreting mouse anti-E-cadherin MAb (rl1; Ref. 24) were a kind gift from B. Gumbiner (University of Virginia, Charlottesville, VA). Rat MAB R40.76 against ZO-1, a TJ peripheral membrane protein (2, 74), was obtained from B. R. Stevenson (Salk Institute, La Jolla, CA) or Chemicon International (Temecula, CA). Affinity-purified guinea pig polyclonal antibody against rabbit polymeric immunoglobulin receptor (plgR) secretory component was described previously (7, 8). Secondary antibodies for immunofluorescence were goat anti-mouse-FITC, goat anti-rat-FITC, and goat anti-guinea pig–lissamine rhodamine sulfonyl chloride from Jackson Immunoresearch Laboratories (West Grove, PA). Propidium iodide (pI) was purchased from Sigma Chemical (St. Louis, MO).

**Cells and culture conditions.** MDCK type II cells, either nontransfected or expressing the wild-type rabbit plgR (45), were maintained in MEM containing Earle’s balanced salt solution (MEM-EBSS; Cellgro, Mediatech, Washington, DC) supplemented with 5% FBS (HyClone, Logan, UT), 100 U/ml penicillin, and 100 mg/ml streptomycin in 5% CO2–95% air. MDCK type II cells transfected with pSV2-neo and either a β-actin promoter expression vector (pB眼泪) alone (46) or a pBAT vector containing a trk/met hybrid receptor cDNA (WT12) (86) were kindly provided by M. Weidner and W. Birchmeier (Max Delbrück Center for Molecular Medicine, Berlin, Germany). These cells were maintained as described above except that 700 μg/ml G418 was included in the medium.

**Assay for TER.** Cells were seeded at confluent density onto Transwells (catalog no. 3401, polycarbonate membrane, 12-mm diameter, 0.4-μm pore size; Costar, Cambridge, MA) and grown in MEM-EBSS–5% FBS for 4–6 days, with daily medium changes, before use. Confluent, polarized monolayers were treated basally with HGF, MAB DO24, or NGF that was diluted into MEM-EBSS–5% FBS. For antibody inhibition of HGF, rabbit polyclonal anti-HGF antibody was diluted to a final concentration of 27 ng/ml into MEM-EBSS–5% FBS containing 100 ng/ml HGF and rotated for 2 h at 4°C before use on cell cultures.

To determine TER, cultures were washed one time quickly and then equilibrated for 10 min with MEM containing Hanks’ balanced salts, 0.6% BSA, and 20 mM HEPES, pH 7.3 (MEM-BSA) at 37°C. We used a Millicell-ERS instrument (Millipore Continental Water Systems, Bedford, MA) to measure TER, being careful to keep distances from the bottom of the well and between the electrodes standardized. Resistances were calculated after subtracting background values obtained from blank Transwells that had been cultured in parallel.

**Inulin diffusion measurements.** Apical to basolateral 14C-labeled inulin leakage was measured across MDCK cell cultures grown on 12-mm Costar Transwells. MDCK cell cultures were plated at confluent density and grown 4–5 days in MEM–5% FBS followed by 24 h in MEM–5% FBS alone or MEM–5% FBS + 100 ng/ml rhHGF. To measure inulin diffusion, cultures were washed once with MEM-BSA at 37°C and then 0.5 ml of MEM-BSA containing 1.25 × 104 cpm of [14C]inulin was placed in the apical compartment and 1 ml of MEM-BSA in the basal well. These volumes were chosen because they result in matching fluid levels across the filter. Cultures were maintained at 37°C, and aliquots were collected, 20 μl from the apical side and 40 μl from the basal side at 1, 2, 4, and 8 h after addition of [14C]inulin. Controls included both blank filters and MDCK cultures that were grown as above in MEM–5% FBS and transferred 12 h before the diffusion assay to MEM suspension medium (S-MEM, Gibco-BRL, Gaithersburg, MD) containing 5% diazoyed FBS and 2 μM CaCl2 (low-Ca2+ cultures). The inulin diffusion assay for low-Ca2+ cultures was carried out in MEM-BSA containing 2 μM CaCl2. The aliquots were counted in a liquid scintillation counter (Beckman Instruments, Irvine, CA).

**Assay for cell scattering.** Cells were trypsinized from confluent 10-cm tissue culture plates, resuspended in MEM-EBSS–5% FBS, and counted in a hemocytometer. Two milliliters of medium containing cells at a density of 5 × 105 cells/ml was replated into each 35-mm well. Cells were cultured for 8–9 h in a 5% CO2–95% air incubator to allow cells to attach and form small islands containing 5–20 cells each. Cultures were then treated for 24–28 h with HGF, MAB DO24, or NGF and photographed with a Nikon camera attached to a Zeiss inverted microscope outfitted with phase and Hoffman Modulation Contrast optics.

**Morphogenesis assay.** Cells were cultured on Transwells as described for the TER analysis. For coculture experiments, MDCK cells expressing plgR were mixed with nontransfected MDCK cells before plating onto Transwells, so that 10% of the cells plated contained plgR. Polarized monolayers were treated for 20–24 h with 100 ng/ml HGF. Alternatively, polarized monolayers of nontransfected MDCK cells or MDCK cells transfected with plgR, pSV2-neo/pB眼泪 vector, or trk/met chimeric receptor were treated as above with HGF or NGF at concentrations between 2.5 and 2,500 ng/ml. All samples were processed for immunofluorescence or electron microscopy as described in Immunofluorescence and confocal microscopy and Electron microscopy.

**Immunofluorescence and confocal microscopy.** Transwell filter cultures were rinsed at room temperature with PBS, pH 7.4, containing 1 mM CaCl2 and 0.5 mM MgCl2 (PBS–), fixed for 30 min with 4% paraformaldehyde in PBS–, permeabilized for 30 min with 0.025% saponin in PBS–, rinsed with PBS–, and quenched for 10 min with 75 mM NH4Cl–20 mM glycine in PBS–, pH 8.0. Nonspecific binding sites were blocked by rocking for 10 min in PBS–0.025% saponin–0.7% fish skin gelatin (block buffer) followed by 10 min in block buffer with 0.1% (w/v) bovine serum albumin (BSA) and 0.05% sodium azide (block buffer). For antibody dilution in block buffer, either for 60 min in a humidified chamber at 37°C or overnight at 4°C. Primary antibody concentrations were as follows: 1: MAb supernatant, 3:1; R40.76 anti-ZO-1 ascites, 1:150; R40.76 anti-ZO-1 MAb supernatant, 3:1; guinea pig anti-secretory component, 1:130. After extensive washing with PBS––saponin and blocking buffer, cells were incubated for 30 min at 37°C in a humidified chamber in a block buffer solution containing fluorophore-conjugated secondary antibodies, diluted 1:100, and pI, diluted 1:1,000 from a 3–4 mg/ml stock. Samples were washed extensively, postfixed with 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, and mounted in Vectashield (Vector Labs, Burlingame, CA).

Confocal images were collected with a krypton-argon laser with K and K2 filter sets coupled to a Bio-Rad MRC600 confocal head and an Optiphot II Nikon microscope with a Plan Apo 60×/1.4 NA objective. Transwell filter samples were imaged either in the X-Y plane or in the Z-Z plane with a motor step size of 0.5 or 1 μm, Kalman filtering with 5 frames/image, and diaphragm set at 1/3 open. The data were analyzed with Comos software. Images were converted to TIFF format, and composites of images were prepared with Adobe Photoshop (Adobe, Mountain View, CA) on a Macintosh computer (Apple Computer, Cupertino, CA).
Electron microscopy. For ultrastructural analysis, MDCK cells on Transwell filters were fixed 60 min on ice with 1.3% glutaraldehyde, 1 mM CaCl₂, 1 mM MgCl₂, 0.05% ruthenium red, and 67 mM sodium cacodylate, pH 7.4, on the apical side of the Transwell and 67 mM cacodylate, pH 7.4, on the opposing side. These samples were then postfixed for 3 h at room temperature in 1.7% OsO₄, 0.05% ruthenium red, and 67 mM sodium cacodylate, pH 7.4, counterstained overnight with 0.5% uranyl acetate, dehydrated in ethanol, and embedded in epon. Thin sections were cut in the X-Z plane of the Transwell with a diamond knife (Diatome, Fort Washington, PA) and were observed at 80 kV in a Zeiss EM-10 electron microscope.

Statistical analysis. Data are expressed as means ± SE. Statistical analysis was performed by Student’s t-test, one-way ANOVA, and Tukey honestly significant difference multiple-comparison test. P < 0.05 was considered statistically significant.

RESULTS

We previously showed (4) that HGF induces polarized MDCK cell monolayers to form pseudostratified layers. However, the dynamics of cell rearrangements during the transition of a uniform monolayer into a pseudostratified layer are not well understood. To examine the HGF-induced mechanism of cell rearrangements we used Transwell filter cocultures of polarized monolayers that contained 90% nontransfected MDCK cells and 10% MDCK cells transfected with plgR. The use of a low percentage of cells expressing plgR enabled us to mark individual cells and distinguish them from adjacent cells so as to detect movements of cells relative to their neighbors. After stimulating for 20 h with HGF we collected X-Z confocal sections of untreated and HGF-treated cultures (Fig. 1, A and B, respectively) stained with ppl to detect nuclei (arrows, Fig. 1, A’ and B’) and for plgR to distinguish individual cell cytoplasmas (arrowheads, Fig. 1, A’ and B’). Cell borders were labeled with E-cadherin (Fig. 1, A and B), which was shown previously to be localized in regions of cell-cell and cell-substrate contact in both monolayers and pseudostratified layers (4). We found that individual cells extend over each other while maintaining a common cell-cell border. In addition, analysis of serial X-Z confocal images (not shown) indicated that extending cells retain tortuous connections to both the filter and apical medium, confirming that HGF treatment resulted in formation of pseudostratified layers. These results suggest that HGF induced MDCK cells within a tight, polarized monolayer to crawl over each other and restructure into a pseudostratified layer without cell-cell dissociation.

TJs are important cell-cell junctions that encircle individual epithelial cells and function to maintain segregated apical and basolateral subdomains and provide paracellular permeability barriers between epithelial cells in vivo (39). We hypothesized that during morphogenetic cell movements these cell-cell junctions are altered. Therefore, we examined the effect of HGF on TJs during formation of pseudostratified layers. The localization of ZO-1, a cytoplasmic plaque protein of TJs, was determined with confocal microscopy. We examined sections through control and HGF-treated Transwell filter-grown MDCK cell cultures in both X-Z and X-Y planes. In X-Z cross sections through untreated MDCK monolayers ZO-1 was localized at the apical-most aspect of lateral cell-cell borders (Fig. 2A, arrow). After HGF-induced morphogenesis, ZO-1 appeared in X-Z confocal images as punctate spots at sites of cell-cell contact at several levels through the pseudostratified layer (Fig. 2B). In contrast to untreated monolayers, HGF-treated cultures often had multiple spots of ZO-1 along an individual cell border (Fig. 2B’, arrowhead). To determine whether HGF affected the formation of TJ rings that normally surround epithelial cells, we collected X-Y confocal sections through basal, central, and apical planes of untreated and HGF-treated MDCK Transwell cultures. ZO-1 staining appeared within a discrete apical plane in untreated MDCK cell monolayers, whereas it formed a bright ring that outlined the

Fig. 1. Hepatocyte growth factor (HGF) induces polarized Madin-Darby canine kidney (MDCK) cells to extend over each other to reshape a monolayer into a pseudostratified layer. Examples shown are from cocultures containing 90% nontransfected and 10% polymeric immunoglobulin receptor (plgR)-transfected MDCK cells. Cells were plated at confluent density on Transwell filters and cultured to form a polarized monolayer before 20-h treatment with MEM (−; A, A’) or HGF (+; B, B’). FITC, propidium iodide (ppl), and tetramethylrhodamine isothiocyanate (TRITC) images were simultaneously collected in X-Z confocal sections and represent E-cadherin, nuclei, and plgR, respectively. The images were split so that E-cadherin is shown in A and B and both plgR and nuclei are shown in A’ and B’. In A and B, E-cadherin staining (arrowheads) is confined to the lateral membrane in polarized monolayers and surrounds cells within the pseudostratified layer. In A’ and B’, plgR staining fills the cytoplasm of transfected cells (arrowheads) but is distinguishable from the nuclei (arrow). A and A’: in a polarized monolayer E-cadherin outlines the lateral borders of a transfected cell in which plgR staining fills the apical cytoplasm. B and B’: after HGF treatment E-cadherin staining at cell-cell borders of a plgR-transfected cell demonstrates that this cell extends over its neighbor, suggesting that HGF causes cells of the monolayer to crawl over each other while retaining cell-cell contacts. Bar: 10 μm.
plasma membrane of each cell-cell border (Fig. 2, C, E, and G). After HGF-induced formation of pseudostratified layers, X-Y confocal images showed that ZO-1 was found in all planes of the culture—basal, central, and apical (Fig. 2, D, F, and H). ZO-1 staining in individual X-Y planes of HGF-treated pseudostratified layers appeared discontinuous. However, by examining projections of confocal images, obtained by summing all X-Y confocal sections through a sample, we found that there were complete rings of ZO-1 around each cell in both monolayers and pseudostratified layers (Fig. 2, I and J). This indicates that, although HGF induced changes in cell shape and the localization of ZO-1, staining of ZO-1 in pseudostratified layers was maintained.
layers depicts morphologically intact TJ belts. These results raised the possibility that TJs remain functionally intact during morphogenesis despite their irregular organization.

To determine whether the morphologically complete rings of ZO-1 represent functionally intact TJs we tested the integrity of TJs during HGF-induced morphogenesis. Ruthenium red is a heavy metal conjugate that is too large to cross a functionally intact TJ and can be visualized with electron microscopy (35, 36, 83). Ruthenium red was added to the apical side of either untreated or HGF-treated Transwell filter cultures of MDCK cells during fixation and processing for electron microscopy. X-Z sections were prepared of MDCK cell monolayers and pseudostratified layers, and images were collected to determine whether ruthenium red crossed the cell layers. In electron micrographs, shown in Fig. 3, we observed that TJs between cells in HGF-treated cultures were no longer localized solely at the apical aspect of the plasma membrane but were also found at different levels along the lateral membrane (Fig. 3B, arrows), in agreement with the detection of ZO-1 in different planes of section mentioned above. However, ruthenium red remained at the apical side of both untreated and HGF-treated MDCK cell layers. In addition, ruthenium red added basally never reached the apical cell surface, although it appeared to encircle portions of cells that angled through the pseudostratified layer underneath the apical-most cells and were sectioned below the level of the TJs (not shown). Together, our results indicate that TJs are morphologically and functionally intact, maintaining paracellular permeability barriers during HGF-induced rearrangement of a monolayer into a pseudostratified layer.

To quantitatively measure the functional integrity of TJs during HGF-induced cell rearrangements we measured TER in response to treatment with HGF. In low-resistance MDCK strain II cells, TER is comprised of transcellular and paracellular resistors in parallel for which the transcellular resistance is two orders of magnitude greater than the paracellular resistance. Therefore, for these cells TER is a direct indicator for tightness of the paracellular seal and an instantaneous measure of paracellular permeability (21, 61). MDCK cells were plated and maintained at confluent density on Transwell filters to form electrically tight, polarized monolayers and then cultured for an additional 20 h in the presence or absence of 100 ng/ml rhHGF. TER measurements are shown in Fig. 4A. In the absence of HGF, TJs between MDCK cells in monolayers maintained a TER of $\sim 100 \ \Omega \cdot \text{cm}^2$. Surprisingly, the TER of MDCK cell cultures treated with HGF was increased more than twofold compared with untreated cultures. Similar results were obtained with a clone of MDCK cells transfected with plgR, demonstrating that the effect of HGF on TER is not clone specific. In addition, HGF treatment from the basal side of the filter alone was sufficient to induce a maximal increase in TER (data not shown; Ref. 13). Therefore, all further experiments were carried out by treating monolayers with HGF on the basolateral side only. To directly test that the effect on TER was due specifically to HGF we treated the basolateral side of filter-grown MDCK cell monolayers with medium that had been preincubated with both 100 ng/ml HGF and anti-HGF polyclonal antibodies. Figure 4A shows that antibody pretreatment almost completely blocked the HGF-induced increase in TER, confirming that the effect on TER is due to HGF. These results indicate that HGF activation of a basolaterally localized receptor mechanism causes an increase in MDCK cell monolayer TER.

To determine the time course of the effect of HGF on TER we cultured polarized Transwell filter-grown monolayers of MDCK cells for various time periods in the presence and absence of continuous HGF treatment. We found (Fig. 4B) that HGF caused a transient increase in TER, peaking around 24 h and then declining to baseline by 48 h. A similar time course of effect was found with the plgR-expressing clone of MDCK, which had a baseline TER of $96 \pm 3 \ \Omega \cdot \text{cm}^2$ and an HGF-induced peak TER at 24 h of $251 \pm 17 \ \Omega \cdot \text{cm}^2$ (not shown), demonstrating that this effect is not clone specific. Thus the HGF-stimulated rise in TER correlates in time with the morphological transition of a monolayer into a pseudostratified layer and then declines to baseline.

To analyze the effect of HGF on the dynamic function of TJs during HGF-stimulated cell rearrangement we measured apical to basolateral $^{14}$C-inulin leakage across Transwell filter-grown MDCK cell cultures during stimulation of pseudostratified layer formation. In contrast to TER, which is an instantaneous measure of TJ functional integrity, $^{14}$C-inulin diffusion is measured over a period of several hours and therefore may be a more sensitive measure of apical to basolateral leakage.

Fig. 3. Tight junctions (TJs) of HGF-treated MDCK cell layers are impermeable to ruthenium red. MDCK cells grown as confluent monolayers on Transwell filters were treated for 24 h with or without HGF before fixing in the presence of apically applied ruthenium red and processing for electron microscopy. Representative sections cut in the X-Z plane of the Transwell filter are shown. Electron-dense ruthenium red labels the apical side of both untreated (A) and HGF-treated (B) MDCK cell layers. Arrows indicate TJs at multiple levels across the HGF-treated cell layer. Ruthenium red is not detected on basal membranes of either untreated or HGF-treated cultures. Bars: 10 $\mu$m.
Tight, polarized MDCK cell monolayers were treated for 24 h in the absence or presence of HGF, and treatment was continued as apical to basolateral diffusion was measured by including \([\text{14}^\text{C}]\text{inulin}\) in the apical medium and collecting aliquots of both apical and basolateral medium at various time points (Fig. 5). We found that there was no significant increase in the amount of apical to basolateral \([\text{14}^\text{C}]\text{inulin}\) diffusion within the first 2 h of measurement. A small but statistically insignificant increase in the amount of basolateral \([\text{14}^\text{C}]\text{inulin}\) was detectable 4–8 h after apical application of \([\text{14}^\text{C}]\text{inulin}\). In contrast, in samples treated with low-Ca\(^{2+}\) medium complete equilibration of \([\text{14}^\text{C}]\text{inulin}\) is detected, demonstrating that TJ function is completely lost. This suggests that the overall integrity of TJs is maintained in HGF-treated cultures during the 8-h time period in which \([\text{14}^\text{C}]\text{inulin}\) diffusion was measured.

Our initial studies suggested that the concentration of HGF required to cause an increase in TER was much higher than that required to induce scattering (not shown). To analyze the amount of HGF required to stimulate these different activities we tested different concentrations of rhHGF (0–250 ng/ml) for effects on TER vs. scattering. In Fig. 6, we show that MDCK cells plated and allowed to form small colonies (Fig. 6A) elicited a full scattering response when treated with either 2.5 (Fig. 6B) or 100 (Fig. 6C) ng/ml HGF. In contrast, the dose-response of TER to HGF (Fig. 6D) demonstrates that 2.5 ng/ml HGF had no effect on TER and that peak effects were induced by HGF concentrations of ~100 ng/ml.

The c-met protooncogene is identified as the HGF receptor that transduces all known functions of HGF (86). This receptor is basolaterally localized in polarized MDCK cells (13), suggesting it as a likely candidate to transduce the HGF signal, resulting in an increase in TER. To test whether direct activation of c-met causes an increase in TER similar to that seen with HGF we took the following two approaches: 1) stimulation of MDCK cells with an activating antibody of c-met, DO24 (57–59), and 2) NGF stimulation of transfected MDCK cells expressing a trk/met chimeric receptor containing the ligand binding domain of the NGF receptor and the transmembrane and tyrosine kinase domains of c-met (86).

Fig. 4. HGF stimulates a transient increase in transepithelial resistance (TER) of polarized MDCK cell monolayers through a basolaterally localized signaling mechanism. A: anti-HGF blocks the HGF-stimulated increase in TER. Polarized monolayers of Transwell filter-grown MDCK cells were cultured for 20 h ± HGF. Monolayers were treated basally with MEM (control), 100 ng/ml HGF, or 100 ng/ml HGF pretreated with 27 μg/ml rabbit anti-human HGF polyclonal antibodies. TER was assayed as described in MATERIALS AND METHODS; n = 3 for each treatment. Data were analyzed by 1-way ANOVA and Tukey honestly significant difference (HSD) multiple-comparison test. **P < 0.0005 compared with −HGF. *P < 0.0005 compared with +HGF and P = 0.218 compared with −HGF. B: effect of HGF on TER transient. Polarized monolayers of Transwell filter-grown MDCK cells were treated basally ± 100 ng/ml HGF for various amounts of time before measurement of TER. Each bar is the average from 1 representative experiment of duplicate samples for each condition. Data were analyzed by Student’s t-test. *P < 0.05 compared with paired control. Similar results were obtained in 4 separate experiments with nontransfected or pIgR-transfected MDCK cells.

Fig. 5. Inulin diffusion measurements show that TJ functional integrity is maintained during pseudostratified layer formation. Apical to basolateral inulin diffusion was measured across Transwell filters either alone or cultured with polarized MDCK cell monolayers treated ± HGF. An additional control was also tested, consisting of Transwell filter cultures of MDCK (as above) whose TJs were disrupted by 12-h Ca\(^{2+}\) depletion before addition of inulin (“low” calcium). Diffusion was measured at 1, 2, 4, and 8 h after addition of inulin to the apical side. Data were analyzed by 1-way ANOVA and Tukey HSD multiple-comparison test. *P < 0.02, **P < 0.0005 compared with −HGF; n = 3 for all treatment conditions.
The scattering activity of 0, 2, and 10 nM DO24 is shown in Fig. 7, A–C. DO24 induced a complete MDCK cell scattering response at a concentration of 2 nM (compare Fig. 7A with Fig. 7, B and C). The effect of DO24 on TER was tested at 2, 10, 100, and 200 nM, concentrations that included and far exceeded the doses necessary to induce scattering. Results in Fig. 7D show that DO24 induced a small increase in TER that was similar at all concentrations tested, demonstrating that direct antibody activation of c-met produced only a partial effect on TER.

Weidner et al. (86) showed previously that all known biological effects of HGF, including stimulation of cell scattering, invasiveness, morphogenesis, and proliferation, are inducible by NGF treatment of MDCK cells that are expressing a trk/met chimeric receptor. This demonstrated that direct activation of c-met transduces these cellular responses. The effects of both HGF and NGF could be tested with trk/met-transfected MDCK
cells because they retain expression of the endogenous c-met receptor. Therefore, we compared the effects of HGF and NGF on the TER of control (pSV2-neo/pBAT vector) or trk/met-transfected MDCK cells to determine whether the same mechanism, direct activation of c-met, also transduces this response. We first assayed scattering as a functional measure of HGF and NGF activity in our culture system. Similar to the results shown above for nontransfected or pIgR-transfected MDCK cells, treatment of control transfected (Fig. 8C) or trk/met (Fig. 8D) clones with 2.5 ng/ml HGF produced a complete scattering response compared with untreated cultures (Fig. 8, A and B). As expected, treatment with either 2.5 or 250 ng/ml NGF had no effect on scattering of control clones (compare Fig. 8, A, E, and G). However, 2.5 ng/ml NGF induced a complete scattering response with trk/met-expressing MDCK cells (compare Figs. 8, B, F, and H) similar to the effect of 2.5 ng/ml HGF (compare Fig. 8, D and F). This confirmed that stimulation of the trk/met chimera with NGF fully activated c-met transduction of the scattering response.

We then compared the effects of NGF and HGF on TER. Figure 8f shows that HGF caused an increase in the TER of monolayer cultures of both control-transfected and trk/met MDCK cells, with peak effects at 100 ng/ml. The peak effects of HGF on TER of control-transfected MDCK (pSV2-neo/pBAT vector) were similar to those in nontransfected and pIgR-transfected MDCK clones, resulting in a two- to threefold increase in TER. trk/met-Transfected MDCK cells responded to HGF with a three- to fourfold increase in TER. In contrast, NGF, at concentrations between 2 and 25,000 ng/ml, did not have any effect on TER of either control or trk/met-transfected MDCK cell cultures (Fig. 8J). In addition, the ability of HGF (2.5, 25, 50, 100, 250, and 500 ng/ml) or NGF (2.5, 25, 250, 500, and 2,500 ng/ml) to induce trk/met MDCK cell pseudostratified layer morphogenesis was tested. Representative X-Z confocal sections are shown in Fig. 9. In untreated trk/met MDCK cell monolayers the nuclei were basally located and E-cadherin was basolateral. Cells remained in polarized monolayers after treatment with HGF at 2.5 ng/ml. At 25 and 50 ng/ml HGF, nuclei appeared more uneven but cells were still in monolayers (not shown). Pseudostratified layers were formed at 100, 250, and 500 ng/ml HGF. In contrast, NGF did not induce pseudostratified layer morphogenesis at any concentration tested. Therefore, our data provide the surprising evidence that direct activation of the trk/met chimeric receptor with NGF induces scattering but is not sufficient to cause pseudostratified layer morphogenesis or an increase in TER.

**DISCUSSION**

Our results demonstrate that TJ function is maintained during HGF-stimulated morphogenesis of pseudostratified layers...
from well-polarized MDCK cell monolayers. HGF induced individual cells of the monolayer to extend and crawl over each other to form a pseudostratified layer. During this process TJPs between cells in HGF-treated cultures were no longer localized specifically at the apical aspect of the plasma membrane but were found at different levels along the lateral membrane. In addition, multiple TJs were observed along the lateral border of individual cells. TJs at different levels were linked into morphologically complete rings that were functionally intact, as shown by a transient increase in TER and the absence of leakage of ruthenium red across the HGF-treated layers. A slight increase in inulin permeability during HGF-induced morphogenesis suggests that TJs “breathe” as cells move to form new structures, but the overall tightness of the cell layer is maintained. Together, these results demonstrate that TJs are modified during HGF-induced rearrangement of a polarized monolayer into a pseudostratified layer but continue to maintain intact permeability barriers and retain the overall tightness of the epithelial layer during morphogenesis (Fig. 10).

We had initially hypothesized that HGF would cause a decrease in MDCK cell TER during morphogenesis. This hypothesis was based on the previously reported ability of HGF to induce scattering of MDCK cells (75, 76), disrupt TJ function (53), or inhibit TJ assembly after calcium switch (23, 26). Instead, after testing four different subclones of MDCK type II cells with recombinant human HGF concentrations ranging from 2.5 to 500 ng/ml, we found that HGF increased TER (Figs. 4, 6, and 8). Our results were not due to alterations in transcellular resistance, because HGF does not affect the function of ion channels in the transcellular pathway (53). The increase in TER was transient, peaking at 24 h and then declining to baseline (Fig. 4). Treatment of Transwell filter-grown cultures of MDCK cells with HGF for up to 72 h did not decrease TER below baseline. The timing of the HGF-induced increase in TER paralleled that of HGF stimulation of morphogenesis, demonstrating that HGF induced cell rearrangements without loss of TJ functional integrity during the transition of a polarized MDCK cell monolayer into a pseudostratified layer.

The increase in TER that we observed in response to HGF contrasts with studies by Nusrat et al. (53) in which recombinant human HGF caused a decrease in TER of monolayers of the human intestinal cell line T84 and mouse HGF caused a similar decrease with MDCK cells. In our hands, culturing MDCK cells similarly to Nusrat et al. on cross-linked or non-cross-linked collagen-coated filters lowered the baseline TER but fold increases in TER in response to either 100 or 500 ng/ml rhHGF were similar to those observed with uncoated Transwell filters. Nusrat et al. (53) reported that cell polarity was not altered by HGF treatment, suggesting that cell rearrangements were not induced. Therefore, the difference between our TER results and those of Nusrat et al. may depend on whether cell rearrangements were induced by HGF.

HGF may have differential effects depending on the initial state of cell polarity and cell-cell and cell-substrate adhesion. Variable effects of HGF on TER of endothelial and epithelial monolayers have been reported by others, showing a decrease (26, 27), an increase (34), or no effect (34) on TER. Culture environment, cell type, initial cell polarity, cell-substrate and cell-cell adhesion, dose of HGF, and treatment times varied...
showed that HGF decreases transcytosis (4). The small in-
cell junctional integrity is lost. This effect is not due to
Our results con-
rm that the effects of HGF on inulin perme-
stances, transient openings and closings of individual TJs may
dynamic changes in TJs during cell rearrangements. For in-
ance, transient openings and closings of individual TJs may
initially trap and subsequently release inulin from between the
multiple TJs that develop along a single lateral cell-cell border
during morphogenesis.

In the present study, we used multiple approaches to analyze
cordately both morphological and functional effects of HGF
on TJs from polarized epithelial monolayers as cells were
stimulated to rearrange. Three-dimensional (3D) analyses of
morphological alterations in both X-Y and X-Z planes provided
a clearer understanding of how HGF alters TJs during cell
rearrangements. For example, if we had analyzed only individ-
ual confocal sections in single planes we might have concluded
that ZO-1 staining is disorganized and discontinuous after HGF
treatment. In contrast, immunofluorescence microscopy with-
out sectioning can give the impression that HGF did not affect
ZO-1, similar to a projection of summed X-Y confocal sections.
The 3D morphological analyses demonstrated that, in response
to HGF, ZO-1 is rearranged but tight junctional rings encir-
cling each cell are morphologically complete (Fig. 2). Our
morphological and quantitative measures of TJ functional
integrity together provide strong evidence that TJ functional
integrity is maintained during HGF-induced morphogenesis.

Our results demonstrate that HGF induces an increase in
TER and crawling of cells from a polarized MDCK monolayer
to form a pseudostratiﬁed layer. However, the downstream
signaling pathway that mediates these HGF-induced effects is
less clear. Our results show that MAb DO24 activation of
endogenous c-met or NGF activation of a trk/met chimera
stimulated all of the previously identiﬁed effects of HGF on
MDCK cells but did not affect TER or pseudostratiﬁed layer
formation. Prat et al. (58) showed previously that the MAbs
DN30 and DO24 both bind c-met and activate receptor dimer-
ization and phosphorylation but the biological responses to
these two ligands are very different. These results suggested
that speciﬁc interactions of ligands with the c-met receptor can
alter downstream responses. Our results indicate that direct
activation of the c-met signaling pathway by non-HGF ligands
that are known to induce motility, invasion, tubulogenesis,
proliferation, and cell survival is not sufﬁcient to mediate an
increase in TER or pseudostratiﬁed layer formation.

We observed that the concentration of HGF required to
stimulate an increase in TER is much higher than that required
to stimulate scattering. Concentrations of HGF that are sufﬁ-
cient to stimulate an increase in MDCK cell TER are at
saturating levels for the high-afﬁnity HGF receptor c-met,
which has a $K_D$ equal to 20 pM (79). However, MDCK cells
also contain lower-afﬁnity, higher-capacity HGF binding sites
(79, 89). These sites have an afﬁnity that is 10-fold lower than
the high-afﬁnity receptor. The concentrations of HGF that
induce an increase in TER are close to the $K_D$ of the low-
afﬁnity MDCK cell surface HGF binding sites. During tissue
repair and disease the concentration of HGF increases signif-
icantly over baseline in both injured tissue and plasma (40, 66,
88). On the basis of our results we surmise that local concen-
trations of HGF surrounding cells in injured tissue are in-
creased and interactions with low-afﬁnity sites may affect HGF
activity to stimulate cell movement and alterations in TER that
contribute to tissue repair.

Previous studies determined that in some assay systems
low-afﬁnity binding interactions of HGF with the cell surface
could be inhibited or disrupted by the presence of an excess of
heparin (>1 $\mu$g/ml), suggesting that these sites are composed of
glycosaminoglycans (48, 52, 89). Subsequently, speciﬁc
HGF-binding heparan sulfate structures and sulfoglycolipids
were identiﬁed. These sites are endogenously expressed at cell
surfaces of various tissues, and their interactions with HGF are
heparin sensitive (3, 30, 37). c-met was also found to contain
potential glycosaminoglycan binding domains (10), suggesting
that trimeric complexes may form between cell surface heparan
sulfate proteoglycans or sulfoglycolipids, HGF, and c-met.
Several studies have shown that glycosaminoglycans affect HGF function. For example, the presence of soluble or substrate-bound heparin or other heparin-like sulfated oligosaccharides has been found to increase the mitogenic potential of HGF, induction of cell motility by HGF, oligomerization of HGF, and autophosphorylation of c-met (1, 14, 29, 48, 60, 65, 90, 92). In addition, recent evidence shows that binding of syndecan-1, a heparan sulfate proteoglycan, to HGF enhances c-met-induced downstream signaling responses (15). The results of these studies suggest that low-affinity HGF binding sites are important regulators of HGF-induced signaling. We found that low concentrations (0.1 µg/ml) of heparin potentiate the effect of submaximal doses (25 or 50 ng/ml) of HGF on MDCK cell TER (unpublished data). NGF and MAb D024 do not bind heparin and most likely do not interact with low-affinity HGF binding sites. Naka et al. (48) showed previously that 0.1 µg/ml heparin does not disrupt binding of HGF to cell surface receptors. It is possible that 0.1 µg/ml heparin in our system increased oligomerization of HGF and potentiated HGF activity without interfering with HGF interaction with endogenous glycosaminoglycans or c-met, similar to effects shown by Zioncheck et al. (92) on hepatocyte mitogenic potency. We suggest two possible HGF signaling mechanisms to explain our results. Low-affinity sites may alter responses to HGF by 1) providing an independent HGF receptor pathway or 2) acting as cofactors in signaling pathways that involve c-met. Our results, together with those of others, suggest that interactions of HGF with low-affinity binding sites may be important for mediating the TER and pseudostratiﬁed layer morphogenesis responses to HGF.

In summary, we demonstrate that TJ functional integrity is maintained during HGF-induced epithelial morphogenesis. We show that in response to HGF individual cells of a polarized epithelial monolayer were stimulated to move relative to their neighbors to form a pseudostratiﬁed layer. Individual TJs may be modiﬁed during this process, but the overall function of the epithelial layer as a barrier to paracellular transport is maintained. In addition, the mechanism of HGF-induced increases in TER and stimulation of pseudostratiﬁed layer formation is not activated by non-HGF ligands that directly activate c-met. We suggest that low-affinity HGF binding sites are involved in mediating the HGF-speciﬁc effects. The ability of polarized epithelial cells to migrate in response to HGF without loss of TJ functional integrity is consistent with a critical role of this mechanism in processes such as wound healing. During wound healing, the context of cells adjacent to a wound margin is different from that of cells that are farther from the wound edge. Our results suggest that cells in these different contexts may modulate their response to HGF and contribute to wound healing without disrupting the overall TJ functional integrity of the epithelium.

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REFERENCES


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Scatter factor is a hepatocyte growth factor/scatter factor that plays a critical role in epithelial interactions. An epithelial scatter factor released by the tight junction (zonula occludens) in a variety of epithelia stimulates DNA synthesis and cell proliferation of human mammary myoepithelial-like cells by hepatocyte growth factor/scatter factor. This factor has potent anti-proliferative activity in various tumor cell lines.

Hepatocyte growth factor (HGF) or scatter factor is a polypeptide growth factor that binds to the c-met tyrosine kinase receptor, which is essential for liver development. HGF inhibits growth of hepatocellular carcinoma and plays a role in liver regeneration after acute liver injury.

Roles of scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, are critical in epithelial cell development, proliferation, differentiation, and morphogenesis. HGF binds to its receptor and transduces motility, proliferation, and morphogenic signals in epithelial cells.

The role of cellular migration and proliferation is also regulated by HGF, which is upregulated in various tumors. HGF may act as a pulmotrophic factor on lung regeneration after acute lung injury.

HGF increases TER during morphogenesis, playing a crucial role in the regulation of cell growth and motility by hepatocyte growth factor and receptor expression in various cell species. This effect is essential for liver development and hepatocyte growth factor/scatter factor is essential for liver development.

In vivo activation of met receptor transduces motility, proliferation, and morphogenic signals of scatter factor/hepatocyte growth factor in epithelial cells. Adult rat hepatocytes in primary monolayer culture exhibit structural characteristics of intercellular contacts and cell membrane differentiations.

HGF increases TER during morphogenesis, indicating its importance in the repair process of gastric epithelial cells. Regulation of cell growth and motility by hepatocyte growth factor and receptor expression in various cell species is essential for liver development and hepatocyte growth factor/scatter factor is essential for liver development.