Regulation of RPE Intercellular Junction Integrity and Function by Hepatocyte Growth Factor

Manlin Jin, Ernesto Barron, Shikun He, Stephen J. Ryan, and David R. Hinton

PURPOSE. To evaluate the effect of hepatocyte growth factor (HGF) on the integrity and function of tight junctions and adherens junctions in the retinal pigment epithelial (RPE) monolayer.

METHODS. Fresh bovine eyes were dissected to obtain 2- to 3-mm2 explants of intact RPE with underlying choroid and sclera. Explants were cultured with or without HGF (20 ng/mL) for various periods (20 minutes to 72 hours). Junction integrity was assessed by transmission and scanning electron microscopy; localization, expression, and phosphorylation of junction proteins; and measurement of transepithelial resistance (TER), diffusion of fluorescent labeling in the plasma membrane, and the migration of RPE cells from the monolayer.

RESULTS. Untreated explants consisted of polarized cells with apical microvilli and well-developed tight and adherens junctions. After HGF treatment, the explants showed loss of tight and adherens junctions ultrastructurally, diffusion of fluorescent label from apical to lateral membrane domains, and increased chemotactic migration of RPE cells from the monolayer. Primary cultures of confluent RPE cells showed a progressive decrease in TER. Western blot analysis showed rapid tyrosine phosphorylation of ZO-1, occludin, and β-catenin within 20 minutes of stimulation. There was a marked loss of ZO-1 protein within 1 hour of HGF treatment. After 6 hours of treatment with HGF, occludin, claudin-1, and β-catenin were redistributed from the membrane to the cytoplasm.

CONCLUSIONS. Treatment of RPE explants with HGF results in rapid disassembly of tight and adherens junctions associated with loss or redistribution of junctional proteins, decreased TER, and increased migration of RPE cells from the monolayer. (Invest Ophthalmol Vis Sci. 2002;43:2782–2790)

Retinal pigment epithelial (RPE) cells function, in part, to provide a permeability barrier between retina and choroid and to enable vectorial transport between these layers. Their highly specialized junctional complexes separate cell surface membrane proteins and lipids into apical and basolateral membrane domains. The tight junction, which is the most apical component of the junctional complex, represents the anatomic substrate of the outer blood–retinal barrier.

A number of tight junction–associated proteins have been identified and cloned, including the cytoplasmic anchor proteins ZO-1, -2, and -3 and the transmembrane protein occludin. Occludin has a domain that binds to both ZO-1 and -2, which in turn bind to the cytoskeleton. The extracellular domain of occludin binds to another occludin molecule on an adjacent cell, forming the tight junction responsible for the permeability barrier. The claudin family of transmembrane proteins has also been identified as a critical component of this tight junction barrier function.

Adherens junctions bind to a continuous belt of actin filaments (the adhesion belt), where they function to hold neighboring cells together through the family of Ca2+-dependent cell–cell adhesion molecules known as cadherins. There are many members of the classic cadherin family, but E-cadherin in epithelial tissue is the best studied member in the context of stable adhesion. Both continued expression and functional activity of E-cadherin are required for cells to remain tightly associated in the epithelium.

To exhibit functional adhesive activity, cadherins must form complexes with cytoplasmic plaque proteins called catenins and with the actin cytoskeleton. β-Catenin is a critical regulatory component of the adherens junction, and its tyrosine phosphorylation correlates with diminished adhesion in response either to growth factors or to cell transformation. Cultured RPE cells can form an intact monolayer with defined barrier functions, and they can express junctional proteins, including ZO-1, occludin, catenins, and cadherins.

The expression of E-cadherin by RPE has been controversial. In comparison with other epithelial cells, RPE cells have been reported not to express E-cadherin and to express N-cadherin instead. Burke et al. and have reported that E-cadherin is found in adult human and bovine RPE in situ and in postconfluent RPE cell cultures and that high E/P-cadherin expression is associated with decreased apical polarity of Na,K-adenosine triphosphatase (ATPase).

Little is currently known about the regulation of junctions in the intact RPE monolayer. Serum inhibits the formation of tight junctions in cultured RPE, do interferon-γ and tumor necrosis factor-α. In cultures of other cell types, barrier function is regulated by a variety of factors, including hepatocyte growth factor (HGF), vascular endothelial growth factor, and histamine. However, the effect of these growth factors on preformed cell junctions in an intact epithelial monolayer has not been determined.

Hepatocyte growth factor/scatter factor is a glycoprotein, usually produced by mesenchymal cells, that induces the proliferation, survival, dissociation, motility, and invasiveness of epithelial and endothelial cells. The receptor for HGF is the membrane-spanning tyrosine kinase c-met. The HGF/c-met system is critical for epithelial–mesenchymal interactions and has widespread effects in embryogenesis and regeneration.

The scatter effect of HGF was first found in the Madin-Darby canine kidney (MDCK) cell line. When MDCK epithelial cells are grown as small colonies at low density on impermeant supports and then exposed to HGF, the cells assume a fibroblastic morphology and scatter away from the colonies. HGF decreases the expression of occludin and transepithelial re-

From the Departments of 1Pathology and 3Ophthalmology, the 2Beckman Macular Research Center, and the 3Doheny Eye Institute, Keck School of Medicine of the University of Southern California Los Angeles, California.

Supported in part by Grant EY02061 and Core Grant EY03040 from the National Eye Institute, a grant from the Arnold and Mabel Beckman Foundation, and a grant to the Department of Ophthalmology from Research to Prevent Blindness.

Submitted for publication October 9, 2001; revised March 14, 2002; accepted April 25, 2002.

Commercial relationships policy: N.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: David R. Hinton, Department of Pathology, Keck School of Medicine of the University of Southern California, 2011 Zonal Ave, HMR 209, Los Angeles, CA 90033; dhinton@hsc.usc.edu.
sistance in vascular endothelial cells and alters the polarity of MDCK monolayers in two-dimensional culture, suggesting that HGF is a critical regulator of monolayer functions. In vivo, HGF is involved in angiogenesis and the branching morphogenesis of epithelial cells in the development of many organs. In pathologic conditions, HGF is associated with several benign and malignant proliferative disorders. Its expression is increased in a variety of cancers, and it is believed to be related to the invasion and metastasis of tumor cells.

We and others have shown that cultured RPE cells express the HGF receptor c-met and that they respond to HGF by proliferation and chemotactic migration. C-Met is also expressed on RPE in epiretinal membranes from patients with proliferative vitreoretinopathy (PVR), and HGF is elevated in PVR vitreous fluids, suggesting that HGF/c-met may be involved in the pathogenesis of PVR. We initiated a study to examine the effects of HGF on the intact monolayer and to elucidate the mechanism by which RPE cells that adhere tightly to one another in the monolayer can separate and migrate to participate in membrane formation. In the current study, HGF dramatically altered junctional integrity and function in the RPE monolayer, and HGF promoted the ability of RPE to separate and participate in membrane formation.

**MATERIALS AND METHODS**

**RPE Explant Culture and Treatment**

Fresh bovine eyes were obtained from Shamrock Meats, Inc. (Vernon, CA). Rat eyes were enucleated from albino rats (Charles River, Boston, MA). All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The eyes were immersed in Hanks' balanced salt solution (HBSS; Irvine Scientific, Santa Ana, CA) containing 1% penicillin and streptomycin for 10 minutes. Under a dissecting microscope in sterile conditions, the vitreous humor and neural retina were carefully removed without disturbing the RPE layer. The eyes were then cut into 2- to 3-mm² explants, each with an intact RPE layer, choroid, and sclera. The explants were immediately placed in high-glucose Dulbecco's modified Eagle's medium (DMEM; Irvine Scientific) supplemented with penicillin G (100 U/mL), t-glutamine (0.292 mg/mL), and 1% fetal bovine serum (FBS; Irvine Scientific). Explants were cultured with or without HGF (20 ng/mL; R&D Systems, Minneapolis, MN) for various amounts of time, up to 72 hours. We have shown that these explants remain viable and can respond to growth factor stimulation for at least 72 hours.

**Electron Microscopy**

Explants were fixed in 0.25% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2-7.4) at 4°C for 48 hours. Specimens prepared for transmission electron microscopy were postfixed in 1% osmium tetroxide for 2 hours, dehydrated in a series of graded alcohols, embedded with an epoxy resin kit (Polysciences, Inc., Warrington, PA), sectioned, stained with uranyl acetate, and examined with a transmission electron microscope (model EM10B; Carl Zeiss, Thornwood, NY). Specimens prepared for scanning electron microscopy were postfixed in 1% osmium tetroxide, dehydrated, critical point dried, coated with gold palladium, and examined with a scanning electron microscope (model S-570; Hitachi, Tokyo, Japan). For TEM and SEM, at least three control- and three HGF-treated explants were examined. For TEM, at least 10 sections were examined for each explant.

**Fluorescent Lipid Labeling of the Plasma Membrane**

The diffusion barrier between the apical and basolateral domains was evaluated by using the fluorescent probe FM1-43 (Molecular Probes, Inc., Eugene, OR). FM1-43 inserts into the outer leaflet of the lipid bilayer, but it cannot flip-flop to the inner layer; thus, it cannot permeate an intact tight junction. When incubation periods are short, FM1-43 is a valuable marker of an intact tight junction. After removal of the retina, FM1-43 (2 μM) was added to the culture medium bathing the bovine eyecup. After a 5-minute incubation, the eyecup was washed with cold phosphate-buffered saline (PBS), dissected, and embedded in optimal cutting temperature (OCT) compound (Tissue-Tek, Torrance, CA). Eight-micrometer serial sections were prepared and evaluated with a confocal microscope (model LSM510; Zeiss) with a 40× oil-immersion objective. Three independent experiments were performed.

**Transepithelial Resistance**

Fresh bovine RPE cells were isolated, as previously described and plated at a density of 2 × 10⁶ cells/cm² on a 24-mm permeable membrane insert (12-mm diameter, 0.4-μm pore size, Transwell, Costar, Cambridge, MA) coated with laminin (5 μg/cm²; Becton Dickinson Laboratory, Franklin Lakes, NJ) in DMEM with 10% FBS. After the RPE cells became confluent, the medium was changed to DMEM with 1% FBS until the transepithelial resistance (TER) stabilized. At this stage of culture, the RPE cells expressed prominent membrane-associated ZO-1 and occludin (results not shown). The TER was monitored with an epithelial voltohmmeter (World Precision Instruments, Sarasota, FL) at regular intervals and was corrected for background resistance contributed by the blank filter and culture medium. Three independent experiments were performed. Results are expressed as mean ohms · cm² ± SD and compared by Student's t-test. Significance was set at P < 0.05.

**Migration Assay**

Migration of RPE cells from the monolayer was detected by Boyden chamber assay, as previously described, modified for explant culture. Briefly, 24-well cell culture inserts (Fisher Scientific, Fair Lawn, NJ) were coated with fibronectin (5 μg/cm²). Explants were placed RPE side down in the upper compartment. Platelet-derived growth factor (PDGF, 20 ng/mL; R&D Systems) was added to the lower compartment as a chemotaxis stimulus. After 12 hours of incubation, the inserts were washed three times with PBS, fixed with pure cold methanol for 10 minutes, and counterstained with hematoxylin for 20 minutes. Cells on the top surface of the insert membrane were removed by wiping with a cotton swab. The number of migrating cells was determined by phase-contrast microscopy at 320× high-power field. Four randomly chosen fields were counted per insert. Three independent experiments were performed. Comparisons were made by Student's t-test with significance set at P < 0.05.

**Immunofluorescence Microscopy**

The expression and localization of the tight junction proteins were examined by qualitative analysis of immunofluorescence staining. Sections from at least five explants were used in all cases. Explants were fixed in 2% paraformaldehyde in PBS for 15 minutes at room temperature, permeabilized in 0.2% Triton X-100 in PBS for 15 minutes, and incubated with the primary antibody for 60 minutes at 4°C and the secondary antibody for 30 minutes at 4°C. Primary antibodies were diluted in PBS: anti-ZO-1 (1:100; Chemicon, Temecula, CA), anti-occludin (1:100; Zymed Laboratory Inc, San Francisco, CA), anti-claudin-1 (1:100, Cat. No. 51-9000; Zymed Laboratory Inc.), anti-E-cadherin (clone 34, 1:100; Transduction Laboratories, Lexington, KY), and anti-β-catenin (1:100; Transduction Laboratories). The secondary antibodies were rhodamine-conjugated goat-anti-rabbit (1:400; Chemicon) and FITC-conjugated goat-anti-mouse (1:400; Chemicon). The slides were washed in PBS, mounted, and examined with a confocal microscope (Carl Zeiss) with a 40× oil-immersion objective.

**Immunoprecipitation and Western Blot Analysis**

**Western Blot Analysis**

The explant was homogenized in 100 μL modified RIPA buffer (50 mM Tris-HCl [pH 7.4], 1% Triton X-100,
0.2% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol, 2 mM EGTA, 4 mM EDTA, 2 mM sodium orthovanadate, and 100 mM NaCl) with fresh protease inhibitors, including 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/mL each of aprotinin, pepstatin A, and soybean trypsin inhibitor (Sigma, St. Louis, MO). Samples were incubated at 4°C for 30 minutes to solubilize proteins, and insoluble materials were pelleted by centrifugation at 14,000g for 10 minutes. Protein concentration was determined with a protein assay (Bio-Rad, Richmond, CA), and equal total protein content was loaded into 7.5% SDS-polyacrylamide gels (SDS-PAGE; Bio-Rad). Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), blocked by 1% bovine serum albumin for 60 minutes, and incubated with the primary antibodies of anti-ZO-1, anti-occludin, anti-claudin-1, anti-E-cadherin, and anti-β-catenin, followed by horseradish peroxidase-conjugated secondary antibody. The membranes were then washed and developed with the addition of a chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ).

**RPE explants were treated with HGF for 60 minutes, the RPE explant (A) shows polygonal cells with well-developed apical microvilli. After treatment with HGF for 60 minutes, the RPE explant (B) showed retraction of apical membrane, loss of apical microvilli, and focal areas of RPE separation, appearing as a hole in the center of the image. Transmission electron micrograph of control RPE explants (C) shows well-developed tight and adherens junctions, and abundant, long apical microvilli (mv). High magnification shows an intact junctional complex (D). After treatment with HGF for 60 minutes, the RPE cell showed fewer apical microvilli (D). High magnification shows loss of tight and adherens junction structure, a reduced junction-associated actin belt, and focal separation between cells in the region of the junction (E). Arrows: tight junction; arrowheads: adherens junction. Bar applies to (A) and (B). Magnification: (C–F) ×2000.**

**RESULTS**

**HGF-Induced Disassembly of Tight and Adherens Junctions**

SEM of the control explants (n = 3) revealed that the RPE cells had uniform polygonal shape and abundant apical microvilli (Fig. 1A). Dramatic changes in the ultrastructural appearance of the RPE cells were observed after 60 minutes of HGF treatment of the explants (n = 3). The RPE cells showed a loss of apical microvilli and retraction of the apical membrane, allowing us to see the lateral membranes and areas in which RPE cells separate from each other (Fig. 1B).

TEM (three explants, 10 sections each) revealed that the control RPE explant cells had well-developed apical RPE tight and adherens junctions and a junction-related actin filament belt. The apical surface of the cells had abundant, long microvilli (Fig. 1C, 1E). After treatment with HGF for 60 minutes, TEM sections (five explants, 10 sections each) revealed that the RPE cells had lost their defined tight and adherens junction structures. The RPE cells showed a reduced junction-associated actin belt and fewer apical microvilli (Fig. 1D, 1F).

Tight junctions contribute to the establishment of epithelial cell surface polarity and inhibit the diffusion of lipids in the outer leaflet of the plasma membrane between apical and basolateral membrane domains. To confirm the HGF-induced disruption of barrier function, the fluorescent lipid probe FM1-43 was used to pulse label the apical plasma membranes. The control cells showed only apical membrane labeling of FM1-43 (Fig. 2A). After HGF treatment of the RPE for 60 minutes, pulse label of the apical membrane resulted in prominent labeling of both apical and lateral membranes (Fig. 2B), indi-
cating loss of the diffusion barrier between these domains. Three independent experiments were performed.

HGF-Induced Decrease in TER

The loss of tight junctions is associated with a decrease in TER. The TER of cultured bovine RPE cells was measured at 30 minutes and 1, 3, 24, 48, and 72 hours after treatment with HGF (Fig. 3). The TER of control RPE cells remained stable (180 Ω/cm²) throughout the experiments. A reduction of TER was first seen after 60 minutes of treatment with HGF (P < 0.05). Continuous culture of RPE cells with HGF for longer periods resulted in further reduction of TER, with a maximum effect after 24 hours of treatment (P < 0.01) that was maintained to 72 hours (P < 0.01).

HGF-Induced Migration of RPE Cells from the Explant Monolayer

The adherens junction plays a critical role in intercellular adhesion and functions to hold neighboring cells together, thus preventing easy dissociation of RPE from the monolayer. We modified the Boyden chamber assay to test the migration of RPE cells from the monolayer in response to a chemotactic agent after HGF treatment. To maximize the migration of RPE cells from the control explant, we used optimal conditions, as previously published, including the use of PDGF as a chemotaxis stimulus and the use of fibronectin as a coating for the intervening porous membrane. Under those conditions, very few cells migrated from the control explant. After 6 hours of HGF treatment, the RPE explant showed a sixfold increase in the number of migrating cells (Fig. 4, P < 0.001).

Immunofluorescent Staining of Tight Junction Proteins

The expression and localization of the tight junction proteins were examined by qualitative assessment of immunofluorescent staining. Results shown are representative of five independent experiments. The control RPE explant cells showed a clear membrane-associated staining pattern for ZO-1, and the cells had a uniform polygonal shape (Fig. 5A). After 60 minutes of HGF treatment, the RPE explant cells showed decreased continuous membrane staining (Fig. 5B). Empty spaces surrounded by three or more abnormally shaped RPE cells were occasionally observed, suggesting that an individual cell had been displaced from the monolayer. Quantitative analysis revealed that such empty spaces represented 2% to 3% of the RPE cell population after 60 minutes of HGF treatment. After treatment with HGF for 6 hours, ZO-1 staining was much weaker (Fig. 5C). The control RPE explant cells showed a membrane-associated staining pattern for occludin similar to that seen with ZO-1, but the pattern with occludin was more granular (Fig. 5D). After 60 minutes of HGF treatment, occludin staining was still intact (Fig. 5E). After 6 hours of HGF treatment, occludin showed a loss of association with the membrane, appearing instead in a diffuse, cytoplasmic pattern (Fig. 5F). The control RPE explant cells showed a membrane-associated pattern for claudin-1 staining (Fig. 5G). After 60 minutes of HGF treatment, claudin-1 staining was still intact (Fig. 5H). After 6 hours of HGF treatment, claudin-1 staining showed a loss of linear membrane-associated pattern, with the development of punctate aggregates (Fig. 5I).
After 60 minutes of HGF treatment, β-catenin staining was intact (Fig. 6B). After 6 hours of HGF treatment, there was loss of membrane β-catenin staining, which instead appeared as diffuse cytoplasmic staining (Fig. 6C). The control explants showed a membrane-associated localization for E-cadherin (Fig. 6D). After 60 minutes of HGF treatment, E-cadherin staining was intact (Fig. 6E). After 6 hours of treatment, E-cadherin staining was weaker with cytoplasmic aggregation (Fig. 6F).

Western Blot Analysis of Junctional Proteins

Quantitation of junctional proteins was determined by Western blot analysis. ZO-1 was identified as a single 220-kDa band, occludin as two bands of approximately 60 to 65 kDa, claudin-1 as a single 22-kDa band, E-cadherin as a 120-kDa band, and β-catenin as a 95-kDa band. As shown in Figure 7, the amount of ZO-1 protein detected by the Western blot analysis was decreased by 50% (P < 0.01) and by 80% (P < 0.01), after treatment with HGF for either 20 minutes or 40 minutes. The amount of ZO-1 protein was not significantly changed after treatment with HGF for either 20 minutes or 40 minutes. The amount of ZO-1 protein detected by the Western blot analysis was decreased by 50% (P < 0.01) and by 80% (P < 0.01), after treatment with HGF for 60 minutes and 6 hours, respectively. After 6 hours of HGF treatment, the amount of occludin protein was also decreased by 25% (P < 0.05). There was no significant change in the amount of claudin-1, β-catenin, and E-cadherin protein after HGF treatment. All experiments were repeated three times; densitometric data are shown as a percentage of the control in Table 1.
0.05) protein in the insoluble pool. Blots were quantified by densitometric analysis, and measurements were expressed as a percentage of total density of both fractions (I/S + I) (Fig. 8B).

**Table 1. Densitometry Data for Western Blot of Junctional Proteins**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>HGF (20 min)</th>
<th>HGF (40 min)</th>
<th>HGF (1 h)</th>
<th>HGF (6 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZO-1</td>
<td>100</td>
<td>95.1 ± 2.8</td>
<td>90.1 ± 8.6</td>
<td>55.1 ± 16.8*</td>
<td>20.2 ± 10.9†</td>
</tr>
<tr>
<td>Occludin</td>
<td>100</td>
<td>93.6 ± 11.4</td>
<td>95.8 ± 5.7</td>
<td>94.2 ± 5.9</td>
<td>75.6 ± 9.5*</td>
</tr>
<tr>
<td>Claudin-1</td>
<td>100</td>
<td>97.4 ± 9.5</td>
<td>97.3 ± 3.1</td>
<td>92.8 ± 9.9</td>
<td>90.2 ± 11.2</td>
</tr>
<tr>
<td>β-Catenin</td>
<td>100</td>
<td>100.8 ± 8.6</td>
<td>97.4 ± 5.0</td>
<td>95.7 ± 2.1</td>
<td>91.3 ± 5.5</td>
</tr>
<tr>
<td>E-Cadherin</td>
<td>100</td>
<td>96.7 ± 4.6</td>
<td>91.4 ± 4.0</td>
<td>87.7 ± 9.1</td>
<td>86.5 ± 7.1</td>
</tr>
</tbody>
</table>

Densitometric analysis after treatment with HGF revealed a significant decrease in the amount of ZO-1 protein after HGF treatment for 1 hour and 6 hours, and amount of occludin protein after HGF treatment for 6 hours. Data are representative of mean results in three separate experiments and are shown as normalized percentage to control.  
* P < 0.05.  
† P < 0.01.

**HGF-Induced Increase in Tyrosine Phosphorylation of ZO-1, Occludin, and β-Catenin**

Because junctional protein function and solubility are highly regulated by phosphorylation, HGF-induced phosphorylation may represent the mechanism by which the tight junctions are disassembled. RPE explants were treated with HGF for various amounts of time and extracted with a buffer containing both 1% Triton X-100 and 0.2% SDS. Cell extracts were then immunoprecipitated with anti-ZO-1, anti-occludin, or anti-β-catenin antibody. Western blots of the immunoprecipitates were probed with anti-phosphotyrosine antibody. There was a significant increase in the phosphotyrosine content of phosphorylated ZO-1, occludin, and β-catenin after as little as 20 minutes of HGF treatment (Fig. 9A). Blots were quantified by densitometric analysis, and data are shown as a percentage of the control in Table 2. In a parallel experiment repeated three times, the immunoprecipitation blot was stripped and re-probed with ZO-1 antibody to confirm the concurrent decrease in ZO-1 protein in the sample after 60 minutes of HGF treatment (Fig. 9B). No tyrosine phosphorylation of claudin-1 and E-cadherin was detected (data not shown).

**Figure 8.** Analysis of junctional protein solubility in Triton-X after treatment with HGF. Triton X-100 soluble (S) and insoluble (I) fractions were separated by 7.5% SDS-PAGE and transferred to membranes. (A) Blots probed with antibodies specific for ZO-1, occludin, claudin-1, E-cadherin and β-catenin. Data are representative of results in three separate experiments. After treatment with HGF for 1 hour and 6 hours, a significant decrease of ZO-1 protein in the insoluble pool (P < 0.01) was detected. After treatment for 6 hours, a significant decrease of occludin (P < 0.01), claudin-1 (P < 0.05), β-catenin (P < 0.01), and E-cadherin (P < 0.05) protein was seen in the insoluble pool. (B) Densitometric quantification of blots, with measurements expressed as a percentage of total density of both fractions (I/S + I). *P < 0.05 and **P < 0.01 versus control.

**Figure 9.** Effect of HGF on the tyrosine phosphorylation of junctional proteins in RPE explants. (A) The tyrosine phosphorylation of ZO-1, occludin, and β-catenin showed a significant increase after treatment with HGF for as long as 60 minutes. Data are representative of results in three separate experiments. Data from densitometric analysis of blots are shown in Table 2. (B) In a parallel experiment, blots used to detect the tyrosine phosphorylation (PY) state of ZO-1 were placed in protein stripping buffer and then reprobed with anti-ZO-1 antibody, confirming that increased tyrosine phosphorylation of ZO-1 was associated with a decreased amount of ZO-1 protein.
endothelial and epithelial cell polarization, proliferation, and
normalized percentage to control.

The RPE monolayer constitutes the outer blood-
retinal barrier; thus, its integrity is critical to the normal functioning of the
neural retina. This monolayer is characterized by well-orga-
nized intercellular tight and adherens junctions between RPE
cells.\(^1\) The RPE explant is a good model for investigating
disruption of these junctions. Compared with other cell cul-
ture monolayer models, such as confluent MDCK cells or vas-
cular endothelial cells, the junctions of RPE explants are pre-
formed and are closer to the normal physiological condition.

With this model, we provide evidence that HGF treatment of
the RPE monolayer induces disassembly of tight and adherens
junctions, both morphologically and physiologically.

This effect appeared to be mediated primarily by the action
of HGF on the cytoplasmic membrane plaque protein ZO-1.
ZO-1 is a peripheral membrane protein localized to the tight
junction complex in epithelial and endothelial cells. Anchoring
of ZO-1 with the underlying cytoskeleton is required for local-
ization of occludin and claudin in the tight junction.\(^2\) ZO-1, -2,
and -3 contain three PDZ domains, one SH3 domain, and one
guananyl kinase-like domain (GuK). Through its GuK domains,
ZO-1 binds directly to the carboxyl terminus of claudins and
occludin and may function as an adaptor at the cytoplasmic
surface of the tight junction to recruit other proteins, including
cytoskeletal and signaling molecules.\(^4\) These components can
form a huge macromolecular complex at the cytoplasmic sur-
face of tight junctions and may be involved in the regulation of
endothelial and epithelial cell polarization, proliferation, and
differentiation.\(^4\) As an adaptor, ZO-1 is a critical regulatory
protein between occludin or claudin and the cytoskeleton or
signaling molecules. Loss of ZO-1 may break down this linkage
and lead to the rapid morphologic and physiological changes
induced by HGF. The most notable early change in this study
was the rapid loss of ZO-1 protein after treatment with HGF for
60 minutes. The loss of ZO-1 protein preceded the redistribution
of occludin and claudin-1 from the tight junction. The time
frame of this redistribution was consistent with the detected
dynamic and physiological changes. These changes, there-
fore, suggest that HGF may first target and induce degradation
of ZO-1 protein and that this degradation is followed by the
redistribution of occludin and claudin and the physiological
disruption of the tight junctions. The loss of junctional proteins
has been shown to play a critical role in the disassembly of
tight junctions in other cells. Agents such as histamine induce
a rapid decrease in ZO-1 protein, leading to an increase of
paracellular permeability in retinal endothelial cells.\(^17\)
Phosphorylation of ZO-1 is also a critical regulator in the assembly
and disassembly of tight junctions.\(^36,37\) The tyrosine-phospho-
rylated form of ZO-1 may cause a dismantling of tight junc-
tions.\(^38,39\) Our study found that HGF induced a marked increase
in tyrosine phosphorylation, but there was no detectable phos-
phorylation of serine and threonine on immunoprecipitated
ZO-1 (data not shown). The hyperphosphorylation of ZO-1
may contribute to its degradation, similar to the degradation
of p27\(^kip1\) and p53\(^kip2\).\(^40\) Proteasome inhibitors have been shown
to stabilize the HGF-induced disassembly of junctions in MDCK
cells, consistent with the notion that protein degradation plays
a part in junction breakdown.\(^41\)

Occludin is a critical paracellular component of tight junc-
tion barrier function. Expression of occludin is high in vessels
with tight barriers, such as those of the brain.\(^42\) overexpression
of occludin by transfection increases TER in kidney cells,\(^43\) and
microinjection of COOH-terminal truncated occludin into oocy-
tes causes a dominant negative effect on micromolecular
tissue barrier formation.\(^44\) In our experiment, treatment with
HGF altered the function of the tight junctions in the RPE
monolayer. This altered function may be caused either by the
rapid phosphorylation and redistribution of occludin from
membrane to cytoplasm or by a slower loss of occludin protein
content. The phosphorylation status of occludin has been
shown to be a critical regulator of its distribution in tight
junctions and cytoplasm. Phospho-aminobutyric acid analysis suggests
that occludin is phosphorylated predominantly on serine-threo-
nine residues in MDCK cells.\(^45\) However, more recent studies
have shown that tyrosine phosphorylation of occludin is im-
portant for tight junction reassembly.\(^32,46\) In MDCK cells, oc-
cludin is heavily tyrosine phosphorylated, with a time course
similar to that of the formation of tight junctions after the
application of a calcium switch.\(^46\) In vascular endothelial cells,
HGF has been shown to induce increased tyrosine and serine-
threonine phosphorylation of occludin associated with de-
creased TER.\(^15\)

In our studies, HGF stimulation was associated with rapid
tyrosine phosphorylation of occludin. Preliminary immuno-
 blot experiments using anti-phosphoserine antibody (Sigma)
demonstrated weak serine phosphorylation of occludin, but this
did not appear to be modulated by stimulation with HGF
(results not shown). Further characterization of serine-threo-
nine phosphorylation sites on RPE-derived occludin and the
modulation of these sites by HGF by gel shift assay\(^57,45\) or
improved phosphoserine-threonine antibodies would be of in-
terest. In RPE, the effect of HGF on occludin may relate in part
to the confluence of the monolayer. It has been reported that
subconfluent RPE cultured on type I collagen and treated with
HGF for 1 week maintain an epithelial phenotype and show
increased occludin expression localized to the cell mem-
brane.\(^47\) Although occludin has been shown to be essential in
the barrier function of tight junctions, other studies indicate
that one or more other proteins are involved in the formation
of tight junctions in the absence of occludin. Occludin-defi-
cient embryonic stem cells differentiate into polarized epithe-
lial cells with well-developed tight junction strands.\(^48\)

Claudin-1 and -2 have recently been identified as transmem-
brane proteins localizing at tight junctions of epithelium, and
more than 20 claudin-like proteins have been identified.\(^4\) We
showed that RPE explants express claudin-1 and that HGF
induces similar changes in claudin-1 and occludin, including
loss of the distinct membrane-associated pattern and redistri-
bution away from the junctional area.

Loss of tight junctions correlates with the loss of polarity
in the RPE monolayer, as shown in SEM by the retraction of apical
microvilli and separation between the cells. The mechanism
of the changes observed in apical microvilli is unknown, but it
could be related to changes in expression or phosphorylation
of ezrin. Ezrin is a member of the ezrin-radixin-moesin family,
which localizes to the microvilli of RPE cells in vivo and
regulates their morphology.\(^59\) Ezrin has been shown to be a
substrate of HGF receptor and plays a critical role in HGF-
induced morphogenesis in kidney cells.\(^50\)

### Table 2. Densitometry Data for PY Content of Junctional Proteins

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HGF (20 min)</th>
<th>HGF (40 min)</th>
<th>HGF (1 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZO-1</td>
<td>100</td>
<td>188.8 ± 30.2</td>
<td>184.0 ± 25.6</td>
<td>2155.2 ± 24.0</td>
</tr>
<tr>
<td>Occludin</td>
<td>100</td>
<td>193.9 ± 12.2</td>
<td>189.7 ± 14.5</td>
<td>250.2 ± 22.3</td>
</tr>
<tr>
<td>β-Catenin</td>
<td>100</td>
<td>179.0 ± 27.6</td>
<td>245.8 ± 33.9</td>
<td>212.0 ± 21.8</td>
</tr>
</tbody>
</table>

Densitometric analysis after treatment with HGF revealed a signif-
ificant increase in tyrosine phosphorylation of ZO-1, occludin and
β-catenin after HGF treatment for up to 60 minutes. Data are repre-
sentative of mean results in three separate experiments and shown as
normalized percentage to control.

**DISCUSSION**

<table>
<thead>
<tr>
<th>Control</th>
<th>HGF (20 min)</th>
<th>HGF (40 min)</th>
<th>HGF (1 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occludin</td>
<td>100</td>
<td>193.9 ± 12.2</td>
<td>189.7 ± 14.5</td>
</tr>
<tr>
<td>ZO-1</td>
<td>100</td>
<td>188.8 ± 30.2</td>
<td>184.0 ± 25.6</td>
</tr>
<tr>
<td>β-Catenin</td>
<td>100</td>
<td>179.0 ± 27.6</td>
<td>245.8 ± 33.9</td>
</tr>
</tbody>
</table>
Intercellular adhesion, which plays a key role in maintaining tissue integrity and morphology of RPE cells, is facilitated primarily by the adherens junction. Because the adherens junction is critical for cell–cell adhesion, it has been extensively investigated in cancer biology. Both in vitro and in vivo studies have shown that a loss or a reduction of E-cadherin or β-catenin expression is associated with invasion and metastasis in various cancers. In our study, HGF induced disassembly of the adherens junctions, a rapid increase in tyrosine phosphorylation of β-catenin (20 minutes), and a redistribution of β-catenin and E-cadherin from membrane to cytoplasm (6 hours). The function of E-cadherin is mediated intracellularly by its interaction with cytoplasmic catenins. At the cell surface, E-cadherin forms an adherens junction that is intracellularly linked to the actin cytoskeleton by the catenins. Data from other cell types have shown that HGF-induced phosphorylation of β-catenin may cause dissociation of β-catenin from the E-cadherin complex to the cytoplasm, leading to abnormal function of the adherens junction. Disassembly of adherens junctions leads to the loss of adhesion between RPE cells in the monolayer and greatly increases the ability of RPE cells to migrate from the monolayer in response to a chemotactic agent PDGF. Both fibronectin and PDGF are prominently expressed in PVR epiretinal membranes. This work suggests that prior exposure to HGF could dramatically facilitate the ability of PDGF and fibronectin to induce migration of RPE cells from the monolayer.

We have demonstrated that HGF induces disassembly of tight and adherens junctions in an intact RPE monolayer and that this disassembly is associated with a decreased TER and an increased ability to migrate from the monolayer. The effect of HGF is likely to be targeted primarily at the ZO-1 protein. These results, in conjunction with studies showing increased HGF expression in PVR, suggest that HGF may play a critical role in the pathogenesis of PVR.

Acknowledgments

The authors thank Laurie Labree for statistical consultation and Chris Spee for technical support.

References


