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Retinal Pigment Epithelial Cells Secrete and Respond to Hepatocyte Growth Factor

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Hepatocyte growth factor (HGF) is normally expressed by mesenchymal cells while its receptor, c-Met, is expressed in epithelial cells. Since HGF is critically involved in epithelial-mesenchyme interactions and the retinal pigment epithelium (RPE) is present at the interface between the retina and choroid, this study was initiated to determine whether the RPE expresses or responds to HGF *in vitro.* Cultured adult and fetal human RPE expressed mRNA for HGF and c-Met by RT-PCR. ELISA assay demonstrated the secretion of HGF into RPE culture supernatants. Tyrosine phosphorylation of c-Met was constitutively found in 72 hour RPE cultures and could be rapidly induced in serum-starved cells by concentrated RPE supernatants. HGF was mitogenic for cultured RPE (100 ng/ml.) and stimulated their chemotaxis (maximal response at 50 ng/ml). RPE are one of only a very limited number of epithelia that express both HGF and its receptor, suggesting the possibility of an autocrine action for this growth factor.

Key Words: **hepatocyte growth factor; c-met; retinal pigment epithelial cells; chemotaxis; autocrine growth factor.**

Hepatocyte growth factor (HGF) is a polypeptide growth factor produced by mesenchymal cells that was independently found to be a growth factor for liver cells (1,2,3), and a motility and scatter factor for epithelial cells (4). Subsequently, purification and cloning (5,6) revealed that HGF was identical to scatter factor (7), tumor cytotoxic factor (8), and fibroblast-derived epithelial growth factor (9).

HGF is a heterodimer composed of a four "kringle domain"-containing α-chain and a serine protease-like β-chain and shows structural similarities to plasminogen (10). The receptor for HGF is the membrane-spanning tyrosine kinase c-Met (11,12). Through its activation of c-Met, HGF has been found to be critical for epithelial-mesenchymal interactions with widespread effects in embryogenesis and regeneration (reviewed in 13-15).

Typically, HGF and its receptor have distinct localization patterns; HGF is expressed in specific mesenchymal cells while c-Met is expressed in epithelial cells (13-15). Recent studies, however, have suggested that the action of HGF is not limited to epithelial cells but is also extended to specific lineages of mesenchymal origin including vascular endothelial cells (16), hematopoietic precursors (17), and osteoblasts (18), and some cells of neuroectodermal derivation (19-20). This has lead to suggestions that HGF is also important in angiogenesis (21), wound healing (22) and may act as a neurotrophic factor (19,20) and axonal chemoattractant (20).

Expression of HGF in epithelial cells was not reported until very recently when the expression of both HGF and c-Met was described in human and rabbit lens epithelial cells, while other epithelial cells in the vicinity such as those of the cornea and lacrimal gland did not express HGF (23). In the brain, HGF is usually expressed by mesenchymally-derived microglia, however weak signal for HGF-mRNA has been found in neuroectodermally-derived astrocytes and in malignant cell lines of astrocytic origin (24-25).

In this paper, we report another epithelial cell type, the retinal pigment epithelium (RPE), not only expresses both HGF and c-Met, but responds chemotactically to HGF and demonstrates constitutive tyrosine phosphorylation of c-Met in vitro. The results suggest that HGF may be an autocrine growth factor for the RPE and could be involved in retinal development, wound healing response and retinal angiogenesis.

MATERIALS AND METHODS

Isolation and culture of human RPE cells. Human fetal RPE cells were isolated from donor eyes (gestational time: 23 weeks) obtained

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from the Anatomic Gift Foundation (Woodbine, GA) after therapeutic abortion. Adult human RPE were isolated from donor eyes obtained from the Lions Doheny Eye Bank. The cells were cultured (26) in Dulbecco's modified Eagle's medium (DMEM; Fisher Scientific, Pittsburgh, PA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD), 2mM glutamine (JRH, Lenexa, KS), 10 mM Hepes (Gibco BRL), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL). Cells from passage 2-4 were used in all experiments. The purity of cultured RPE was evaluated by immunocytochemical staining in which the cells were found to be positive for cytokeratin (Dako, Carpinteria, CA), and negative for von Willebrand's factor (Dako), and CD11c (Dako).

Reverse transcriptase polymerase chain reaction (RT-PCR). Poly $(A)^+$RNA was isolated from both fetal and adult RPE using Fast Track kit (Invitrogen, San Diego CA). Controls included known HGF positive and negative glioblastoma cell lines (27), c-Met positive glioblastoma cells and c-met negative fibroblasts. First strand complementary DNA (c-DNA) was synthesized at 42°C with M-MLV reverse transcriptase (Gibco BRL). PCR was performed using 2 μg cDNA/lane and previously published human HGF and c-Met primer pairs and conditions (28, 29). After 35 cycles of PCR, amplification products were resolved on 1.2% agarose gels and stained with ethidium bromide. The gel was photographed under ultraviolet illumination, and the appropriate product size determined by comparison with a 100bp DNA ladder (Gibco BRL).

Explant cultures of human eyes. Two human fetal and two adult eyes were used to determine if the intact RPE secreted HGF. The eye-cup model (30) was derived by making a circumferential incision approximately 1mm outside the limbus. The lens and lens capsule were removed along with the iris and ciliary epithelium. The vitreous was removed together with the sensory retina leaving the RPE monolayer intact within the eye cup. The inside of the explant cups was gently rinsed twice and filled with DMEM containing 10% FBS. The explant cups were incubated for up to 24 hr at 37°C and the conditioned medium collected, centrifuged and stored at -80°C before assay.

HGF enzyme-linked immunosorbant assay (ELISA). RPE cells were subcultured in 6 well plates (10^4 cells/well) in DMEM with 0.4% FBS. In triplicate experiments at 6, 24, 48 and 72 h. after plating, supernatants were collected, and assayed using a human HGF Quantikine Kit (R&D System, Inc) according to the manufacturer's protocol. Supernatants from media placed in RPE explant preparations from fetal and adult eye cups were also analyzed in a similar manner after incubation for 6-24 hours.

Scatter assay. Scatter refers to the ability of HGF to dissociate colonies of Madin-Darby canine kidney (MDCK) cells. Assay was performed using rHGF and RPE conditioned medium as previously described (31)

Immunoprecipitation. All chemicals were obtained from Sigma (St. Louis MO) unless otherwise indicated. Conditioned medium was prepared by collecting medium from several 10 cm diameter plates of 80% confluent 72 hour adult RPE cell cultures. Medium was centrifuged to clarify, and concentrated 20X using Centricon Plus-20 concentrating tubes (Millipore Corp. Bedford, MA) to a final HGF concentration of 10 ng/mL as measured by ELISA. RPE cells were grown to 80% confluence and starved overnight in serum-free medium. Cells were incubated for 10 minutes at 37°C under the following conditions: a) 50 ng/mL recombinant HGF (R&D Systems), b) non-concentrated RPE conditioned medium, c) concentrated RPE conditioned medium, d) concentrated RPE conditioned medium preabsorbed with anti-HGF polyclonal neutralizing antibody (5X ND_{50}; R&D Systems). These cultures were compared with 72 hour RPE cells grown to 80% confluence in normal medium. Stimulations were stopped by addition of lysis buffer (50 mM Tris-HCL, pH 7.5, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EGTA, 1mM Na_3VO_4, 1mM NaF, 1mM PMSF, and 1μg/mL aprotinin, leupeptin and pepstatin). Cell lysates were clarified by centrifugation at 12,000 x *g* for 10 min and pre-cleared by incubation for 1h at 4°C with agarose beads conjugated with both normal rabbit serum and normal goat serum. Samples were incubated overnight at 4°C with 2μg/ml rabbit anti-human c-Met antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) and then incubated for 2h with goat anti-rabbit IgG conjugated agarose beads (50% suspension). The agarose beads were washed 4 times with lysis buffer. Bound proteins were eluted in SDS sample buffer and boiled before samples were subjected to 10% SDS-PAGE. Proteins were electroblotted to nitrocellulose (Schleicher and Schuell, Inc). The membrane was blocked with 1% BSA, and incubated with antiphosphotyrosine antibody (RC20-HRP; Transduction Laboratories, Lexington KY) for 4 h and washed extensively. HRP bound to beads was revealed by incubating with ECL solutions (Amersham, Ltd, UK) and subsequent exposure to Hyperfilm (Amersham).

Boyden chamber assays. RPE cell migration was examined in transwell cell-culture chambers (Costar, Cambridge, MA) as previously described (32). Chemotaxis was induced by the addition of recombinant HGF (R&D System, final concentration of 1, 10, 20, 50, 100 ng/ml) to the lower compartment in the presence or absence of fibronectin coating of the insert membrane.

^{3}H-Thymidine uptake. RPE proliferation was estimated using a ^{3}H-thymidine uptake assay. RPE cells were incubated with DMEM/0.4% FCS with or without HGF (0, 1, 10, 50, 100, 200 ng/ml) for 24 or 48 hours. Cultures were pulsed with ^{3}H-thymidine (1 μCi/well; Amersham, Arlington Heights, IL) for the last 16 hours of the incubation. ^{3}H-thymidine activity was then determined as previously described (33).

Immunohistochemistry. Snap frozen cryostat sections (8 microns) of human fetal and adult retinas were fixed with acetone for 5 minutes and stained by the ABC method for immunoreactive HGF or c-Met using the ABC Elite kit (Vector, Burlingame, CA).

RESULTS

Analysis of both fetal and adult RPE by RT-PCR revealed amplification products of an appropriate size for both HGF and c-Met after 35 rounds of amplification (Figure 1). No other bands were found suggestive of alternative splicing as has been seen in other cell types for c-Met (23). Positive and negative control cell lines showed expected presence or absence of HGF and c-MET products (results not shown). Secretion of HGF by the RPE cells was demonstrated by ELISA assay of RPE culture supernatants. Secretion increased over time from 24-72 hr (Figure 2) and resulted in accumulated concentrations in the low nanogram/mL range. The adult RPE cultures tested appeared to produce more HGF than the fetal cultures when normalized for final cell number (Figure 2). Fetal eye cup secretion of HGF resulted in supernatant concentrations of 550 pg/mL over 24 hours and 1550 pg/mL after 48 hours while adult explant supernatants accumulated 700 pg/mL over 24 hours and 4400 pg/mL over 48 hours. Functional activity of RPE-secreted HGF was determined by examining for scatter activity in MDCK cells. Concentrated RPE supernatants induced scatter in the MDCK cells and this activity was lost after preabsorption with anti-HGF neutralizing antibody. Cryostat acetone-fixed sections of human retinas did not show the

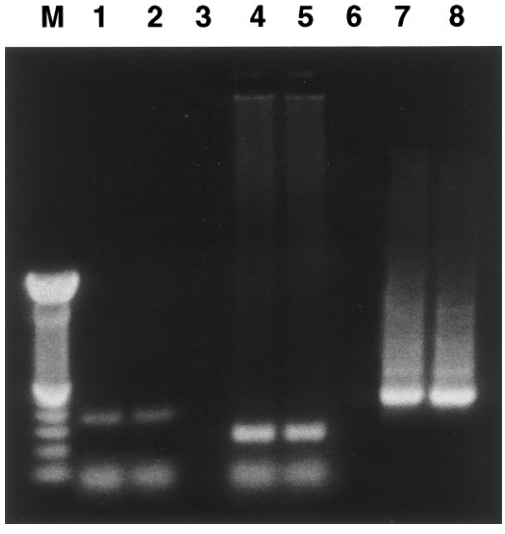

FIG. 1. RT-PCR for HGF and c-Met . mRNA was isolated from human fetal (lanes 1,4,7) and adult (lanes 2,5,8) RPE. RT-PCR was performed using specific primer pairs for HGF (lanes 1,2), c-Met (lanes 4,5), and β-actin (lanes 7,8). The Marker lane shows a 100 bp DNA ladder. The size of HGF product in 378 bp, while the c-Met product is 262 bp.

presence of any immunoreactive HGF in the outer retina or RPE; however, c-Met was observed on the apical surface of RPE cells (results not shown).

To determine the effect of HGF on the function of cultured RPE, proliferation and migration studies were performed. HGF was a mild mitogen for RPE under the conditions studied. HGF stimulated a 25% increase in ^{3}H-thymidine uptake after 48 hours of incubation at a concentration of 100 ng/ml ($p \leq 0.05$). No stimulation was present at lower concentrations and at no concentration tested did 24 hours of incubation result in a significant increase in ^{3}H-thymidine uptake. HGF stimulated a concentration-dependent chemotaxis of RPE cells in the Boyden chamber assay both in the presence and absence of a fibronectin substrate (Figure 3). Maximal response was at a HGF concentration of 50 ng/ml.

To assess potential autocrine activation of RPE by HGF, RPE cultures were examined for HGF receptor phosphorylation. rHGF induced rapid tyrosine phosphorylation of c-Met in serum starved RPE, as did concentrated RPE supernatants (Figure 4, lanes 1-2). The activity in the concentrated RPE supernatants was almost complely lost after preabsorption with neutralizing anti-HGF antibody (Figure 4 , lane 4). While non-concentrated RPE supernatants did not appear to phosphorylate receptor, actively growing 72h cultures of RPE showed constitutive phosphorylation of c-Met receptor (Figure 4, lane 5). Serum-containing medium alone did not result in phosphorylation of c-Met under these conditions.

DISCUSSION

The RPE monolayer is critically located at the interface between the delicate neurosensory retina and the vascular choroid (30). The RPE is a rich source of cytokines and growth factors which are thought to regulate cell survival and growth in the local microenvironment through autocrine and paracrine interactions (26). Because of its role as a regulator of epithelial-mesenchymal interactions, we predicted that, like many other epithelia, the RPE would express c-Met and possibly respond to HGF. Suprisingly, we found that not only did the RPE express c-Met, it also expressed and secreted HGF. Activity of the RPE-secreted HGF was demonstrated by its ability to induce scatter in MDCK cells. Previous studies had shown that with few exceptions, HGF expression was limited to mesenchymal populations (14, 23, 25, 31). Co-expression of HGF and c-Met in RPE suggests the possibility of an autocrine activation of the HGF pathway in these cells. We demonstrated that concentrated RPE supernatants induce the rapid tyrosine phosphorylation of c-Met in serum starved RPE cells; this effect was markedly inhibited by preabsorption of the supernatant with neutralizing anti-HGF antibody. Seventy-two hour cultures of RPE grown in serum-containing medium showed constitutive phosphorylation of c-Met, providing strong support for the possibility of autocrine activation of the HGF pathway in RPE. Interestingly, serum-starved RPE cells did not show tyrosine phophorylation of c-Met after stimulation with non-concentrated supernatants, suggesting that the activation of the receptor may be altered in the synchronous noncycling serum-starved population, or that the concentration of HGF may be higher in the cellular microenvironment of cell cultures than in cell-free supernatants.

The lack of immunoreactive HGF in cryostat sections

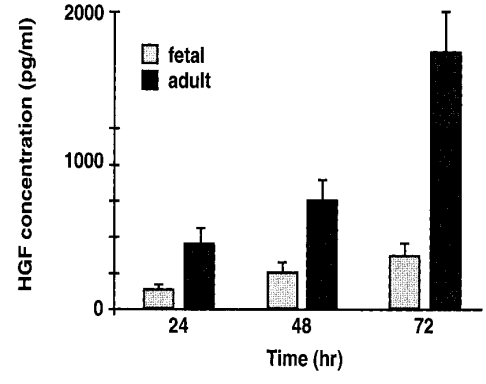

FIG. 2. ELISA for HGF of RPE Culture Supernatants. Fetal and adult RPE culture supernatants (24, 48, 72 hours) were analyzed by ELISA for the presence of immunoreactive HGF. Values are normalized to a final concentration of 10^{5} RPE. The amount of secreted HGF increases over time and is greater at each time point in the adult RPE than in the fetal RPE.

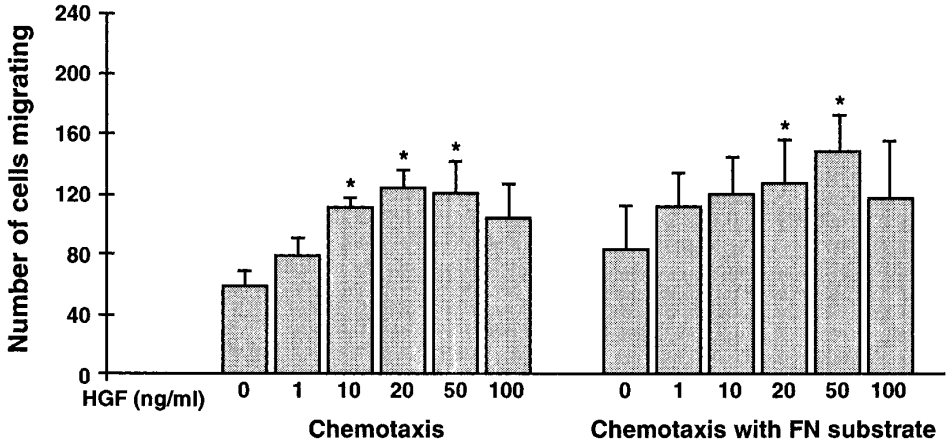

FIG. 3. Migration of RPE in response to HGF in Boyden chamber assay. RPE were placed in the upper chamber while varying amounts of HGF was added to the lower compartment. The intervening inserts were either coated or not coated with fibronectin. A dose-dependent increase in migration was found both in the presence or absence of fibronectin. Values are the average of three independent experiments, each performed in duplicate (*$p<0.05$, compared to absence of HGF).

of the RPE suggests that HGF may be expressed and secreted only when RPE are stimulated. The act of culturing the RPE, detaching the sensory retina in the explant cultures, or growth in the presence of serum or cytokines could all potentially stimulate the RPE to alter growth factor production (30). In other cell types, HGF may be induced by interleukin-1, platelet-derived growth factor, and acidic and basic fibroblast growth factor, and inhibited by transforming growth factor-β (14, 34, 35). c-Met was immunohistochemically localized to the apical surface of RPE cells. This would diminish the potential of inappropriate RPE activation due to HGF in the choroid and would position the RPE to appropriately react to HGF secreted during retinal wound healing or injury.

Whether or not HGF plays a role in the morphogenesis of the retina is unknown at this time, although it is possible since fetal RPE were also able to secrete HGF. A supporting role of HGF for differentiated photoreceptors might also be considered since HGF has been shown to be neurotrophic for motor and dopaminergic neurons, and can stimulate axonal regeneration in retinal ganglion cells (36). The upregulation of HGF by cytokines and growth factors could play a role in the dissociation, migration and proliferation of RPE found in disorders of the outer retina such as proliferative vitreoretinopathy occuring after retinal trauma or retinal detachment (37). HGF is also a pro-angiogenic factor (21) and could potentially play a role in the development of choroidal neovascularization; a common complication of age-related macular degeneration.

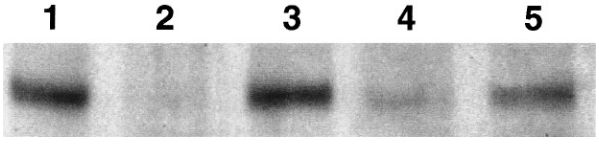

FIG. 4. Tyrosine phosphorylation of c-Met in RPE cultures. RPE cells were lysed and immunoprecipitated with anti-c-Met antibody. The immunoprecipitate was electrophoresed and electroblotted to nitrocellulose. Immunoblotting was performed using antiphosphotyrosine antibody. RPE cells grown to 80% confluence were starved overnight in serum free conditions and treated with rHGF (50 ng/ml, lane1), non-concentrated RPE supernatant (lane 2), concentrated RPE supernatant (lane 3), or concentrated RPE supernatant preabsorbed with anti-HGF antibody (lane 4). Seventy-two hour culture of RPE cells growing in serum-containing medium shown in lane 5.

Activation of the c-Met receptor by HGF results in its autophosphorylation and binding to a number of factors containing Src homology region 2 domains. Small GTP-binding proteins such as Rho and Ras are activated, as is the mitogen activated protein kinase (MAP-K) pathway (14, 19, 38). Phosphorylation of E-cadherin-associated molecules, ezrin and focal adhesion kinase is thought to lead to dissociation, cytoskeletal alterations, and migration of the epithelial cells (14, 39-42). Whether HGF activates similar signalling pathways in RPE is unknown however such studies could provide a basis for rational manipulation of HGF action. The activation of MAP-K may be of particular interest since we have recently shown that the chemotactic response of RPE to PDGF is critically regulated by this pathway (32).

This study demonstrates that human RPE can secrete active HGF and that HGF can phorphorylate the c-Met receptor and induce the proliferation and chemotaxis of RPE. The potential autocrine and paracrine actions of this factor for RPE suggests a specialization of function not found in most other epithelial sites. The known actions of HGF in wound healing and angiogenesis suggests a potential role for this factor in proliferative diseases of the outer retina.

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