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Cross Talk between c-Met and Epidermal Growth Factor Receptor during Retinal Pigment Epithelial Wound Healing

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Abstract

PURPOSE—The authors sought to determine how hepatocyte growth factor (HGF) receptor c-Met and epidermal growth factor receptor (EGFR) cross talk in response to injury in human ARPE-19 cells.

METHODS—A scratch wound was made on a cell monolayer of ARPE-19 cells using a sequence-comb or a pipet tip, and it was allowed to heal in the presence or absence of HGF and heparin-binding EGF-like growth factor (HB-EGF). The activation of EGFR was analyzed by immunoprecipitation with EGFR antibody, followed by Western blotting with phosphotyrosine-specific antibody. Phosphorylation of extracellular signal-regulated kinase (ERK) and AKT (a major substrate of phosphatidylinositol 3'-kinase (PI3K) was assessed by Western blotting. The release of c-Met ectodomain into the culture media was determined by Western blotting using an antibody against the extracellular region. Cell migration was assessed by Boyden chamber migration assay.

RESULTS—ARPE-19 cells underwent spontaneous wound healing in basal medium, and exogenously added HB-EGF and HGF significantly enhanced wound closure. Basal and growth factor-enhanced wound closures were attenuated but not slowed by hydroxyurea, a cell proliferation inhibitor. RPE cells expressed all four erbBs, and wounding induced EGFR transactivation and downstream ERK and PI3K phosphorylation in ARPE-19 cells. HGF also induced EGFR tyrosine phosphorylation. The EGFR kinase inhibitor AG1478 blocked wound- and HGF-stimulated EGFR transactivation and attenuated spontaneous and growth factor-induced wound closure. Wounding and EGFR ligands induced the release of c-Met into the culture media. Moreover, pretreatment of cells with HB-EGF impaired ARPE-19 migration toward HGF in a matrix metalloproteinase inhibitor-sensitive manner.

CONCLUSIONS—EGFR modulates HGF/c-Met activity by inducing c-Met ectodomain shedding, and HGF/c-Met transactivates EGFR, leading to an enhanced activation of downstream signaling pathways. Cross talk between EGFR and c-Met may play a key role in regulating RPE cell migration, proliferation, and wound healing.

In response to pathologic conditions, retinal pigment epithelial (RPE) cells initiate a wound-healing process and become transformed from a stationary epithelial state to a migratory and proliferative mesenchymal state, leading to the epiretinal membrane formation associated with the development of proliferative vitreoretinopathy (PVR).¹ It is thought that activation of several autocrine or paracrine loops by growth factors and their receptors is critical for RPE transformation and PVR progression.² Prominent among these factors are hepatocyte growth factor (HGF)/scatter factor (SF) and the epidermal growth factor (EGF) family. HGF is involved in cell scattering and migration and from epithelial to mesenchymal transition (EMT).

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^{3,4} The EGF receptor tyrosine kinase (RTK) family has been characterized in many cell systems, including RPE,⁵⁻⁷ and is known to participate in a wide variety of biological responses, including cell migration, proliferation, and differentiation.

HGF is a multipotential cytokine that has been implicated in diverse events in organ development, tissue maintenance and homeostasis, and wound healing. At the cellular level, HGF can promote other bioactivities, such as junctional breakdown, cell scattering, migration, cell survival, and invasive behavior.^{8,9} HGF is thought to be synthesized by mesenchymally derived cells, typically fibroblasts, which primarily target epithelial cells in a paracrine manner through c-Met, the only known receptor for HGF that mediates all HGF-induced biological activities.^{8,10,11} c-Met consists of an α/β heterodimer at the cell surface, with α as an extracellular subunit and β as a subunit containing an extracellular domain, a membrane-spanning domain, and a cytoplasmic tyrosine kinase domain.¹² On HGF stimulation, the c-Met receptor is tyrosine phosphorylated; this is followed by the recruitment of a group of signaling molecules, adaptor proteins, or both to its cytoplasmic domain and to its multiple docking sites. This action leads to the activation of several different signaling cascades, including extracellular signal-regulated kinase (ERK) of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3'-kinase (PI3K), that form a signaling network of intracellular and extracellular responses.

Unlike HGF, the EGFR ligand family of growth factors consists of more than 10 members, including EGF¹³ and HB-EGF.¹⁴ These factors act through the stimulation of specific cell-surface receptors of the erbB or EGFR family. There are four related RTKs: EGFR/erbB1, erbB2, erbB3, and erbB4.¹⁵⁻¹⁸ Activation of erbBs, similar to c-Met, elicits myriad signaling events, including ERK and PI3K.¹⁹⁻²¹ EGFR ligand stimulation promotes RPE cell proliferation and survival, signaling through both ERK/MAPK and PI3K pathways.^{5,6} Recently, HB-EGF has been implicated in driving the uncontrolled wound-healing process of the retina during proliferative retinopathy.⁷

Although various reactions have been described, wounding or breakdown of the tight junction barrier in vivo results in the availability of circular or otherwise segregated²² growth factors, such as HGF and EGFR ligands to their receptors, leading to the initiation of a wound healing response. Hence, the multiplicity of cell surface receptors activated by endogenous signals is contrasted by the relative uniformity of intracellular signaling pathways triggered by these receptors. In particular, the activation of EGFR and c-Met may elicit similar signal transduction pathways in cells. Thus, cross talk of these growth factor receptors may affect the strength, duration, or both of shared downstream signaling pathways. Whether c-Met and EGFR influence each other's activity and how the cross talk between these RTKs determines cell signaling remains to be fully explored. Hence, we investigated the role of HGF and HB-EGF in mediating RPE wound healing and the cross talk between these two growth factors using cultured human ARPE-19 cells.

MATERIALS AND METHODS

Materials

The following materials were used: Dulbecco modified essential medium (DMEM), penicillin/streptomycin, and trypsin (Invitrogen, Carlsbad, CA); human recombinant HGF, HB-EGF, and EGF (R&D Systems, Minneapolis, MN); GM6001, a hydroxamic acid matrix metalloproteinase (MMP) inhibitor (3-(*N*-hydroxycarbonyl)-2-(*R*)-isobutylpropionyl-L-tryptophan methylamide; Calbiochem, La Jolla, CA); antibodies against human EGFR (erbB1), erbB4, ERK 2 (p42 MAPK), phosphorylated ERK1/2 (p44/p42 MAPK), PY99, and Met (c-28; Santa Cruz Biotechnology, Santa Cruz, CA); antibodies against a major substrate of PI3K, AKT, and phospho-AKT (Cell Signaling, Beverly, MA); rabbit anti-EGFR (Tyr 845;

Biosource, Camarillo, CA); c-Met antibody that recognizes the extracellular region of Met (Upstate Biotechnology, Lake Placid, NY); antibodies against erbB2 and erbB3 (Laboratory Vision; Fremont, CA); Boyden chamber (48 wells; Neuroprobe, Cabin John, MD) and polycarbonate membranes (14- μ m pores; Osmonics, Inc., Livermore, CA); hydroxyurea, tyrphostin AG 1478, and all other chemicals (Sigma-Aldrich, St. Louis, MO).

Cell Culture, Migration, and Wounding Studies

ARPE-19,²³ the cell line most frequently used to study RPE function in vitro, was purchased (American Type Culture Collection, Manassas, VA). Cells were grown in DMEM with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) in a humidified 5% CO₂ incubator at 37°C. ARPE-19 cells were seeded onto culture dishes coated with fibronectin collagen coating mix (Biological Research Faculty and Facility, Ljamsville, MD) and then starved in serum-free DMEM overnight.

For migration assay, ARPE-19 cells were grown to 80% confluence in 12-well tissue culture plates and were wounded with a sterile 10- μ L pipet tip (TipOne; USA Scientific, Ocala, FL) to remove cells by two perpendicular linear scrapes. The debris of damaged cells was removed by washing, and the cells were refed with DMEM in the presence or absence of HGF (50 ng/mL) or HB-EGF (50 ng/mL). To determine the contribution of cell proliferation to wound closure, the cell cycle blocker hydroxyurea (100 mM) was added to the cell culture with or without growth factor. The progression of migration was photographed immediately and 17 hours after wounding at the same field near the crossing point with an inverted microscope equipped with a digital camera (SPOT; Diagnostic Instruments, Sterling Heights, MI). The extent of healing is defined as the ratio of the area difference between the original wound and the remaining wound 17 hours after injury compared with that of the original wound. The wound area was determined by the number of pixels in histogram (Photoshop CS; Adobe, San Jose, CA).

For wounding experiments, an ARPE-19 cell monolayer on 100-mm dishes was wounded by a cut of 48-well sharktooth comb for DNA sequencing gel (BioRad, Hercules, CA). The dish was then rotated, and scrapes were made in the same way at 45°, 90°, and 135° to the original scrapes, forming multiple linear scratches from one side of the dish to the other.

Western Blot for erbB Expression and Cell Signal Activation

ARPE-19 cells and human telomerase immortalized (hTERT) RPE cells²⁴ were lysed in RIPA buffer (150 mM NaCl, 100 mM Tris-HCl, pH 7.5, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 50 mM NaF, 100 mM sodium pyrophosphate, 3.5 mM sodium orthovanadate, proteinase inhibitor cocktails, and 0.1 mM phenylmethylsulfonyl fluoride), and 20 μ g protein was subjected to Western blotting with antibodies against erbB1, erbB2, erbB3, and erbB4.

To determine EGFR tyrosine phosphorylation from wounded RPE cells, serum-starved ARPE-19 cells on 100-mm dishes were wounded by sharktooth comb with nonwounded cells as control and were further cultured in DMEM for 15 minutes. Cells were then lysed, and the same amount of proteins was subjected to immunoblotting using antibodies against phosphotyrosine 845 of EGFR (Src-related site), phospho-ERK, and phospho-AKT with ERK2 levels for equal protein loading.

For HGF effects on EGFR and c-Met, serum-starved human ARPE-19 cells were stimulated with HGF (50 ng/mL) at different time points. Cells were then lysed in RIPA buffer, and protein concentration was determined with a micro-BCA kit (Pierce, Rockford, IL). For each sample, 600 μ g cell lysate was subjected to immunoprecipitation with 4 μ g EGFR or c-Met antibodies,

followed by Western blotting with PY99 antibody. The same membranes were stripped from the immunoreactivities and reprobed with EGFR or Met antibodies, respectively, to ensure that an equal amount of proteins was precipitated. Cell lysates of the same samples were subjected to immunoblotting with phospho-ERK- or phospho-AKT-specific antibodies.

To determine the role of EGFR on wounding and on HGF and HB-EGF stimulation, serum-starved ARPE-19 cells were pretreated with Tyrphostin AG1478 (1 μ M; A.G. Scientific, San Diego, CA), an EGFR inhibitor, for 1 hour and then were stimulated with wounding by sharktooth comb, HGF (50 ng/mL), or HB-EGF (50 ng/mL) for 15 minutes. Cell lysates were subjected to EGFR immunoprecipitation and then to Western blotting to determine tyrosine-phosphorylated EGFR.

Wounding or EGFR Ligand-Induced c-Met Ectodomain Shedding

Serum-starved ARPE-19 cell monolayer on 100-mm dishes was wounded by a cut of sharktooth comb. Cells with no scrape wound were treated with HGF (50 ng/mL), HB-EGF (50 ng/mL), or EGF (50 ng/mL). After 24-hour culture, the conditioned medium was collected, concentrated, normalized against its corresponding protein concentrations, and subjected to Western blotting to determine shed c-Met using antibody against the extracellular region of Met. Corresponding cell lysates were also subjected to Western blotting with the same c-Met antibody and with ERK2 antibody for equal protein loading.

Boyden Chamber Analysis for Cell Migration

A Boyden chamber of 48 wells was used to measure the migratory response of ARPE-19 cells to HGF. Cultured cells were starved in DMEM overnight, detached by 0.05% trypsin and 0.53 mM EDTA, washed with 10% fetal bovine serum to neutralize the trypsin, and adjusted to an equal cell number of 3.6×10^5 /mL. Cells were pretreated with HB-EGF (50 ng/mL) or HB-EGF, along with GM6001 (50 μ M), for 15 minutes and were then removed, with untreated cells as the control. Treated and untreated cells were plated on the top wells of the Boyden chambers, which were separated by a polycarbonate membrane from the lower chambers loaded with DMEM in the presence or absence of HGF (50 ng/mL). Cells were then incubated at 37°C in 5% CO₂ and were allowed to migrate for 4 hours. After incubation, cells on the top of the filter membrane were removed by scraping. The filter was then stained with a modified staining kit (Diff-Quik; Dade Behring Inc., Dudingon, Switzerland). Cell migration was quantified as the number of migrated cells on the lower surface of the filter membrane in three random fields under 400 $\times$ magnification.

Statistical Analysis

Results were expressed as mean $\pm$ SD. Statistical parameters were ascertained with one-way analysis of variance (ANOVA) for multiple comparisons or with unpaired Student's *t*-test for comparison between two groups. Statistical significance was set at $P < 0.05$.

RESULTS

HGF and HB-EGF Accelerating RPE Wound Healing

To study the effects of growth factors on RPE wound healing, we adopted a scratch wound model from corneal epithelial wound healing and assessed RPE wound closure in the presence of HGF and HB-EGF (Fig. 1).^{25,26} ARPE-19 cells were able to heal a scratch wound without exogenously added growth factors in serum-free DMEM, suggesting that these injured cells are able to generate autocrine factor(s) for wound healing. Consistent with previously reported studies,²⁷ HGF greatly accelerated wound closure in ARPE-19 cells. HB-EGF, a ligand for erbB1 and erbB4, also significantly enhanced epithelial wound closure, albeit to a level less

stimulatory than that for HGF. The combination of HGF and HB-EGF exhibited effects similar to those of HGF in the induction of wound closure (data not shown). To determine whether the increase in wound closure resulted from increased proliferation, increased migration, or both, ARPE-19 cells were treated with hydroxyurea, which inhibited DNA replication and thus blocked cell proliferation. Hydroxyurea inhibited wound closure in the basal medium by 45.6%. In the presence of hydroxyurea, there was a substantial inhibition of HGF and HB-EGF-induced epithelial wound closure (52.9% and 38.5% decrease in wound closure compared with HGF and HB-EGF alone, respectively). Thus, cell proliferation and migration contributed to in vitro wound closure.

Human RPE Cells Express erbB1, erbB2, erbB4, and, to a Lesser Extent, erbB3

To determine whether RPE cells express any erbBs, we used Western blotting with erbB-specific antibodies and detected all four erbBs in ARPE-19 cells and in hTERT RPE cells²⁴ (Fig. 2). Although erbB1, erbB2, and erbB4 are readily detected, erbB3 is barely detectable in human RPE cell lines. Thus, RPE cells express multiple members of the EGFR family and may be targeted by various members of the EGF family.

Wounding Induces EGFR Activation

To determine whether wounding stimulates EGFR activation and its downstream signal pathways, ARPE-19 cells were injured extensively with DNA sequencing gel comb, and the activation of EGFR was assessed by EGFR phosphorylation. As shown in Figure 3, scratch wounding induced EGFR phosphorylation at tyrosine 845 in ARPE-19 cells. Consistent with EGFR transactivation, ERK and PI3K—two key signal mediators of RTKs such as EGFR and HGF/c-Met—were also activated, as evidenced by the increased phosphorylation of ERK1/2 and AKT (a major substrate of PI3K) in wounded ARPE-19 cells.

HGF Induces EGFR Transactivation

Recent studies have shown that some growth factors, such as TGF- β ²⁸ and insulin-like growth factor,²⁹ exert some effects on cells through EGFR transactivation, resulting in the activation of ERK and PI3K pathways.^{28,29} Whether HGF is able to transactivate EGFR is unknown. Figure 4 shows that HGF elicited c-Met phosphorylation, with a maximal level reached 15 minutes after stimulation. There was a decrease in the protein levels of c-Met, detectable at 30 minutes and more apparent at 1 hour after stimulation, suggesting possible degradation of the receptor after maximal activation (15 minutes). HGF also stimulated EGFR phosphorylation in ARPE-19 cells, starting as early as 5 minutes, declining after 60 minutes, and remaining at an elevated level for up to 2 hours, whereas total levels of precipitated EGFR were unchanged during the course of the study. Downstream mediators of these RTKs, ERK and PI3K, were also activated, as evidenced by the phosphorylation of ERK2 (42 kDa) and AKT, though the patterns of the time courses of HGF-stimulated activation in these two pathways were different in ARPE-19 cells.

To determine whether phosphorylation of EGFR induced by wounding or HGF required tyrosine kinases of the EGFR, ARPE-19 cells were treated with a specific inhibitor of EGFR intrinsic tyrosine kinase activity (Tyrphostin AG1478; A.G. Scientific; Fig. 5A). As a positive control, HB-EGF elicited massive EGFR phosphorylation and degradation, as evidenced by the reduced amount of EGFR precipitated from HB-EGF-treated ARPE-19 cells. AG1478 inhibited HB-EGF-elicited EGFR phosphorylation and degradation. Wounding and HGF stimulated milder EGFR phosphorylation than did HB-EGF. The phosphorylation of EGFR elicited by these stimuli was sensitive to AG1478, suggesting that EGFR tyrosine kinase activity is required for its activation in RPE cells in response to injury or HGF stimulation.

The effects of AG1478 on ARPE-19 wound closure were also assessed in the scratch wound model (Fig. 5B). AG1478 significantly inhibited basal and HGF- or HB-EGF-induced wound healing. It inhibited wound closure in the basal medium by 30% and in HGF- and HB-EGF-induced ARPE-19 wound closure by 24.6.9% and 42.6%, respectively.

Wounding and EGFR Ligands Induce c-Met Ectodomain Shedding

c-Met belongs to the class of transmembrane proteins that can undergo ectodomain shedding, a process mediated by pathologic/physiologic effectors.³⁰⁻³² To determine whether RPE cell wounding can result in c-Met shedding, the cell monolayer was extensively injured with sharktooth sequencing comb, and the ectodomain shedding of c-Met was assayed by monitoring the appearance of the 90-kDa soluble fragment of c-Met in the culture medium by Western blotting. As shown in Figure 6, the basal amount of soluble c-Met was detectable in the control cells cultured after 24 hours. Wounding of ARPE-19 cells resulted in an increase in the accumulation of shed c-Met in the culture media when compared with control, non-wounded cells. To determine whether c-Met shedding is stimulated by other stimuli or growth factors, ARPE-19 cells were treated with EGFR ligands. HB-EGF and EGF stimulated c-Met shedding to an extent comparable to that induced by wounding, whereas exogenous HGF exhibited no apparent effects on c-Met shedding compared with the control. Steady state levels of cellular c-Met in these treated ARPE-19 cells were also determined. There was an apparent reduction in the cellular levels of c-Met in wounded and EGF-treated cells.

RPE Cell Migratory Response to HGF Is Impaired by HB-EGF Pretreatment

The observation that EGFR ligands increased c-Met shedding in ARPE-19 cells implicated that these growth factors may antagonize HGF function in RPE cells. With the use of Boyden chamber migration assay, we showed that HGF stimulated ARPE-19 cell chemotaxis (Fig. 7). However, the chemoattractant effect of HGF was significantly impaired by pretreatment of ARPE-19 cells with HB-EGF. This effect can be reversed by pretreatment of the cells with GM6001, an MMP inhibitor commonly used to block ectodomain shedding of the cell surface proteins.

DISCUSSION

In the present study, we showed that ARPE-19 cells undergo spontaneous wound closure and that the exogenously added growth factors HGF and EGFR ligands greatly accelerated in vitro wound healing. We provided evidence that RPE cells express multiple members of the EGFR family at the protein level and that wounding of ARPE-19 cells results in the activation of EGFR and its downstream ERK and PI3K/AKT signaling pathways. We also showed cross talk between c-Met and EGFR signaling pathways. HGF induced EGFR transactivation, leading to an enhanced activation of PI3K and ERK signaling pathways. EGFR ligands, on the other hand, induced ectodomain shedding of c-Met, likely leading to the downregulation of the HGF signaling in RPE cells. Consistent with this, pretreatment of cells with HB-EGF significantly attenuated the migratory response of ARPE-19 cells toward HGF in Boyden chamber migration assay. Thus, the cross talk between EGFR and c-Met may play a key role in regulating RPE cell migration, proliferation, and wound healing. Manipulation of these signaling pathways may be used to prevent or treat PVR.

The EGFR family has been a subject of extensive studies in various epithelial cells.¹⁵ However, only limited reports have been published of EGFR in RPE cells. Notably, Defoe and Grindstaff⁵ report that EGF stimulates the survival of RPE D407 cells through PI3K and MAPK/ERK pathways, and, more recently, Hollborn et al.⁷ showed that exogenous HB-EGF stimulates the proliferation and migration of RPE cells and the expression of the vascular endothelial growth factor. Using Western blot analysis, we showed expression of the four

members of the EGFR family in two human RPE cell lines, ARPE-19²³ and hTERT,²⁴ and a rapid phosphorylation (activation) of EGFR in ARPE-19 cells in response to scratch wounding, suggesting an autocrine activation of EGFR signaling in RPE cells. Furthermore, spontaneous and HB-EGF-enhanced wound closures are sensitive to EGFR inhibition with AG1478, suggesting that the EGFR signaling network plays a role in the regulation of RPE wound healing.

HGF is synthesized by mesenchymally derived cells, typically fibroblasts,⁸ which primarily target and signal epithelial cells in a paracrine manner through c-Met.^{10,11} RPE cells are unique in that they express both HGF and c-Met.^{33,34} Our study confirmed previous reports that HGF stimulates RPE and triggers a healing response and cell proliferation and migration in vitro.³⁵⁻³⁷ Because of its profound effects on RPE cells, it is likely that HGF/c-Met signaling is under tight regulation in vivo in the retina. The observation that wounding results in an increase in the release of extracellular domain of c-Met from cultured RPE cells suggests that c-Met ectodomain shedding may be one of the regulatory mechanisms for c-Met signaling. Shedding of c-Met may result in a reduction of the availability of the c-Met receptors on the cell surface or an increase in soluble Met receptor (decoy Met) that may function as an antagonist to HGF by interfering in HGF binding to Met.^{30,38} Further studies are warranted to address the physiologic significance of c-Met shedding in RPE cell response to wounding.

The most striking discovery of our study was the interplay or cross talk of c-Met and EGFR signaling pathways. EGFR emerges as a key signal transduction molecule that can be activated not only by its own ligands but also by many other growth factors, such as the ligands for G-protein-coupled receptors,³⁹ TGF- β ,²⁸ and insulin-like growth factor.⁴⁰ This study is the first to add HGF to the list of growth factors that transactivate EGFR. Because c-Met and EGFR elicit ERK and PI3K signaling pathways, EGFR transactivation by HGF may enhance HGF signaling by increasing the intensity or the duration of these receptor-mediated signals, leading to synergistic effects on RPE cells. Partial inhibition of HGF-enhanced ARPE-19 wound closure by AG1478 indicates that EGFR signaling contributes to HGF-elicited wound response in vitro. We also showed that stimulating cells with the EGFR ligand HB-EGF or EGF enhances c-Met ectodomain shedding. Hence, EGFR ligand may function as an antagonist to c-Met and thus may affect the responsiveness of RPE cells to HGF. This was confirmed by the data showing that HB-EGF pretreatment impairs the migratory response of RPE cells to HGF. The observation that GM6001, an inhibitor of MMP and ectodomain shedding, reverses HB-EGF effects on RPE chemotaxis toward HGF suggests that HB-EGF exerts its effects on HGF through the induction of c-Met ectodomain shedding. Hence, bidirectional cross talk between c-Met and EGFR implies the profound effects of temporal action of the two growth factors on the outcome of cell signaling. If EGFR is activated first, it would attenuate HGF action by inducing c-Met shedding. If, however, c-Met is activated first, EGFR would be transactivated, resulting in an enhanced activation of shared downstream signaling pathways such as MAPK and PI3K. Thus, EGFR and c-Met cross talk may be a determining factor on RPE cell signaling during wound healing. Better understanding of HGF and EGFR cross talk and their downstream signaling may provide therapeutic approaches, allowing proper wound repair but suppressing any undesired wound healing response of RPE cells and the development of PVR.

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FIGURE 1. HGF and HB-EGF accelerated RPE wound healing. Serum-starved ARPE-19 cells at confluence were injured with a 10- μ L pipet tip. Wounded cells were allowed to heal for 17 hours in the presence or absence (Basal) of HGF (50 ng/mL) or HB-EGF (50 ng/mL) with (B) or without (A) 100 mM hydroxyurea, a cell cycle blocker. Images are representative of one of the three samples and were taken at the same spot immediately after wounding (day 0) or 17 hours after wounding. (C) Changes in the extent of healing in ARPE-19 cells treated with growth factors in the presence or absence of hydroxyurea. The extent of healing (0 for no covering; 1 for complete covering of the wound bed) was calculated as described in Materials and Methods. Data are mean $\pm$ SD of three samples from one experiment. ** $P < 0.01$; one-way ANOVA.

FIGURE 2.

Expression of erbBs in human RPE cells. ARPE-19 and human telomerase immortalized RPE cells (RPE^{hTERT}) were lysed in RIPA buffer, and 20 μ g protein was subjected to Western blotting with antibodies against erbB1, erbB2, erbB3, and erbB4, with ERK2 levels as control for equal protein loading.

FIGURE 3.

Wound-induced EGFR phosphorylation. Serum-starved ARPE-19 cell monolayer cultured in 100-mm dishes was maximally wounded (W) by sharktooth comb or nonwounded as control (N) and was further cultured in DMEM for 15 minutes. Cells were then lysed and subjected to immunoblotting with the use of antibodies against phosphotyrosine 845 of EGFR, phospho-ERK, and phospho-AKT. ERK2 levels were used as control for equal protein loading.

FIGURE 4.

HGF induced the phosphorylation of EGFR, c-Met, ERK, and AKT. **(A)** Serum-starved ARPE-19 cells were stimulated with HGF (50 ng/mL) for the indicated times. Protein in equal amounts (600 μ g) was subjected to immunoprecipitation with EGFR or c-Met antibodies, followed by Western blotting with PY99 antibody (pEGFR or pMet). The same membranes were stripped from the immunoreaction and reprobed with EGFR or c-Met antibodies, respectively, to ensure that equal amounts of protein were precipitated. Cell lysates of the same samples were subjected to immunoblotting with phospho-ERK (pERK) or phospho-AKT (pAKT) antibodies. **(B)** Phosphorylation trends of EGFR, Met, ERK, and AKT after HGF stimulation were plotted on a graph. The relative amount of phosphorylated proteins in each band was quantified by gel scanning and expressed in relative light reading against different time points.

FIGURE 5.

Effects of AG1478 on EGFR phosphorylation and wound healing. **(A)** Serum-starved ARPE-19 cell monolayer was pretreated with Tyrphostin AG1478 (1 μ M) for 1 hour and then stimulated with scratch wound by sharktooth comb, HGF (50 ng/mL), or HB-EGF (50 ng/mL) for 15 minutes. Proteins of 600 μ g were subjected to immunoprecipitation with EGFR, followed by Western blotting with PY99 antibody (pEGFR). The same membrane was stripped and reprobed with EGFR antibody to ensure that equal amounts of protein were precipitated (EGFR). **(B)** Serum-starved ARPE-19 cells at confluence were injured with 200- μ L pipet tip. Wounded cells were allowed to heal for 24 hours in the presence or absence (Control) of HGF (50 ng/mL) or HB-EGF (50 ng/mL), with or without 1 μ M AG1478. The extent of healing was calculated as described in Materials and Methods. Data are mean $\pm$ SD of three samples from one experiment. ** P < 0.01, and * P < 0.05; one-way ANOVA.

FIGURE 6.

Wounding- and EGFR ligand-induced c-Met ectodomain shedding. Cultured ARPE-19 cells were wounded by sharkstooth comb (W). Unwounded cells were treated with HGF, HB-EGF, or EGF, with untreated cells as the control (C). Cells were then cultured for 24 hours. Culture supernatant was collected, concentrated, and subjected to Western blotting with anti- c-Met that recognizes extracellular domain (Extracellular). Cell lysates (20 μ g) were also subjected to Western blotting with c-MET (cellular) and ERK2 (ERK2) antibodies.

FIGURE 7.

Impaired RPE migratory response to HGF by HB-EGF pretreatment. Growth factor-starved ARPE-19 cells underwent trypsin digestion and adjustment of cell numbers. Cells were pretreated with HB-EGF (50 ng/mL) or HB-EGF along with GM6001 (50 μ M) for 15 minutes and then were removed with untreated cells as the control. Cells were plated on top of the Boyden chambers that were separated by a polycarbonate membrane of 14- μ m pore size from the lower chambers with or without HGF (50 ng/mL) in DMEM. Cells were allowed to migrate for 4 hours in the incubator. Cells migrated per field were photographed and counted. Data represent mean $\pm$ SD of counts in three wells. ** $P < 0.01$; Student's t -test.