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Hepatocyte Growth Factor Stimulates Proliferation and Migration During Wound Healing of Retinal Pigment Epithelial Cells In Vitro

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Purpose: A defect in retinal pigment epithelial (RPE) cells may cause dysfunction of the neural retina, so rapid recovery of differentiated RPE cells is required after RPE injury. We investigated the effect of hepatocyte growth factor (HGF) on wound healing in RPE cells.

Methods: Confluent monolayers of bovine RPE cells were denuded, and the cells were allowed to recover in the presence or absence of HGF. The effect of HGF on RPE cell proliferation was evaluated by a 3-(4;5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetraz olium assay. In a migration assay, mitomycin C was used to inhibit proliferation, and the number of migrated cells was counted. The signaling pathways involved were examined using inhibitors of mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 (PI3) kinase and protein kinase C pathways.

Results: At 80 ng/mL, HGF stimulated the wound closure of RPE monolayers and rendered the restituted cells more epithelioid in shape. HGF at 10 ng/mL stimulated RPE cell migration the most, whereas 80 ng/mL of HGF inhibited migration, but stimulated proliferation the most. In particular, PI3 kinase and MAPK inhibitor inhibited PRE cell migration and proliferation, respectively.

Conclusions: HGF stimulated wound closure in cultured RPE cells, and rendered restituted cells epithelioid in shape. HGF may become a therapeutic candidate for RPE wound healing. **Jpn J Ophthalmol 2003;47:268–275**

Key Words: Hepatocyte growth factor, migration, proliferation, retinal pigment epithelial cell, wound healing.

Introduction

Retinal pigment epithelial (RPE) cells form the monolayer underneath photoreceptor cells and they play an important role in supporting the neural retina.[1] When the RPE cell monolayer is wounded, such as after surgical removal of the choroidal neovascular membrane in patients with age-related macular degeneration, rapid proliferation and migration of nearby RPE cells across the wound surface, a process called restitution, is desirable. Because damage to RPE cells during this surgical-procedure is thought to impede the recovery of visual function,[2] the wound should be covered by restituted RPE cells as soon as possible, and these RPE cells need to be fully differentiated to functionally support photoreceptor cells.

Recently, we have shown that hepatocyte growth factor (HGF) induces epithelial morphogenesis in RPE cells through occludin linkage to the cytoskeleton.[3] HGF, also known as scatter factor, is a mesenchyme-derived polypeptide that acts as a mitogen, motogen, morphogen and antiapoptotic factor in many epithelial and endothelial cells.[4–13] Because several reports have noted that HGF stimulates proliferation and migration of RPE cells.[14,15] We hypothesized that HGF may promote wound healing of RPE cells with an epithelial morphology that is desirable for RPE cell restitution. In this study, we investigated the effects of HGF on RPE cell wound healing, particularly on the proliferation, migration, and morphology of the cells and the signaling pathways involved.

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Materials and Methods

Cell Cultures

Bovine eyes were obtained from a local slaughterhouse, and RPE cells were isolated, as described previously.[16] Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo) supplemented with penicillin G (100 units/mL), glutamine (0.72 mg/mL), 0.5 μg/mL amphotericin B, and 10% fetal calf serum (FCS; Gibco BRL, Gaithersburg, MD, USA). The cells were incubated at 37ºC under 5% CO_2. The medium was changed every 2 days, and the cells were subcultured by trypsin-ethylenediaminetetroacetic acid digestion when they reached confluence (as assessed by phase-contrast microscopy). The purity of the cells was confirmed by immunostaining of cytokeratin with an anticytokeratin 8.13 monoclonal antibody (ICN; Lisle, IL, USA; data not shown).[16] Third passage cells were used in all experiments. Each experiment was performed at least twice on independent isolated cultures of RPE cells, and representative results are shown.

Wound and Cellular Response

One week after confluence, the tip of a 3-mm diameter micropipette was used to remove linear bands of cells from the monolayer cultures. The cells were then cultured in the presence of recombinant human HGF (R & D systems, Minneapolis, MN, USA) at 0, 10, 20, or 80 ng/mL in DMEM with 10% FCS. These cultures were observed daily with a phase-contrast microscope.

RPE Proliferation Assay

To quantify the effect of HGF on RPE cell proliferation, a 3-(4;5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay was performed with a CellTiter 96 Non-reactive Cell Proliferation Kit (Promega, Madison, WI, USA). Briefly, RPE cells were seeded onto 96-well plates at a density of 2×10^4 cells/mL. To minimize the effect of FCS and to clarify the effect of HGF on RPE cell proliferation, we reduced the concentration of FCS to 1% the following day. Twenty-four hours later, the cells were exposed to HGF (0, 5, 10, 20, 40, and 80 ng/mL) and incubated at 37ºC for 24 hours. MTS/phenazinemethosulphate solution was added to the cells for the last 4 hours of incubation, and absorbance at 490 nm was recorded with an enzyme-linked immunosorbant assay plate reader (BioRad, Hercules, CA, USA). The cells in some wells were treated with various concentrations of inhibitors of intracellular signaling pathways for 1 hour before the addition of HGF: PD98059, a mitogen-activated protein kinase (MAPK) inhibitor, at 5, 10, or 20 μM; wortmannin, a phosphatidyl inositol 3 (PI3) kinase inhibitor, at 5, 10, or 20 μM; or calphostin C, a protein kinase C (PKC) inhibitor, at 50, 100, or 200 nM (all three from Calbiochem Novabiochem, San Diego, CA, USA). The concentrations of the inhibitors used in these experiments were carefully controlled so as not to cause cell death, as confirmed by the trypan blue exclusion test (data not shown). All pharmacological inhibitors were dissolved in dimethyl sulfoxide (DMSO) and added to the medium for the final concentration of DMSO $<0.1\%$.

RPE Migration Assay

RPE cells (5×10^4 cells/mL) were seeded onto 35-mm wells in 6-well plates (Asahi Techno Glass, Chiba) that were precoated with collagen type I and were grown to confluence. Cells were cultured with 1% FCS in DMEM for 24 hours, and a cell scraper (Asahi Techno Glass) was used to denude the cultures in monolayers by removal of a linear band of cells. To inhibit the effect of cell proliferation, cells were pretreated with mitomycin C (10 μg/mL; Sigma, St. Louis, MO, USA). It was applied to the cells 2 hours before denuding and removed with three washes of Hank's balanced salt solution (HBSS) just before denuding. This treatment inhibits the proliferation of bovine RPE cells[1] and other cell types[18] without killing the cells. This antiproliferative effect of mitomycin C on RPE was confirmed with the use of a cell proliferation kit (Amersham Pharmacia Biotech, Buckinghamshire, England; data not shown). To determine the effect of HGF on RPE migration, different concentrations of HGF (0, 5, 10, 20, 40, and 80 ng/mL) were added to the dishes of DMEM with 1% FCS after denuding. Some cultures were treated with various concentrations of inhibitors from 1 hour before denuding until the end of the assay: PD98059 (5, 10, and 20 μM), wortmannin (5, 10, and 20 μM), or calphostin C (50, 100, and 200 nM). After the cultures were denuded, they were washed four times with HBSS to remove the floating cells and incubated with HGF or the appropriate inhibitor or both. After 48 hours, the cultures were fixed with 50% acetone/50% ethanol at 4ºC for 5 minutes and stained with Meyer's hematoxylin. Migration was quantified on a grid through the eyepiece of a phase-contrast microscope, and the cells that migrated into a 1-mm wide denuded area were counted. Counts were made in triplicate for each well.

Statistics

All values were tested for normal distribution after which statistical evaluations were performed with one-factor analysis of variance. A *P*-value less than .01 was considered to be significant.

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Results

HGF-stimulated Restitution of an RPE Cell Monolayer

An RPE cell monolayer at 1-week postconfluence was checked before and just after wounding. The cells displayed a cuboidal, epithelial morphology resembling that of in vivo RPE cells (Figure 1). Three days after injury, only the wound treated with 80 ng/mL HGF was closed (Figure 2G). The cells cultured with 10 ng/mL HGF seemed to have more pseudopodia and looked more widespread than the others (Figure 2C). In contrast, cells in the cultures with 20 and 80 ng/mL HGF appeared less extended, but they were more numerous than the cells in cultures with 10 ng/mL HGF (Figures 2E and G). By day 5, the wounds of the cultures treated with 10 or 20 ng/ml HGF or without HGF had also closed (Figures 2B, D, and F). At more than 20 ng/mL, and especially at 80 ng/mL, HGF seemed to make the restituted cells more concentrated and more epithelioid in shape (Figures 2F and H), whereas the cells cultured with 10 ng/mL HGF displayed fibroblastic morphology (Figure 2D).

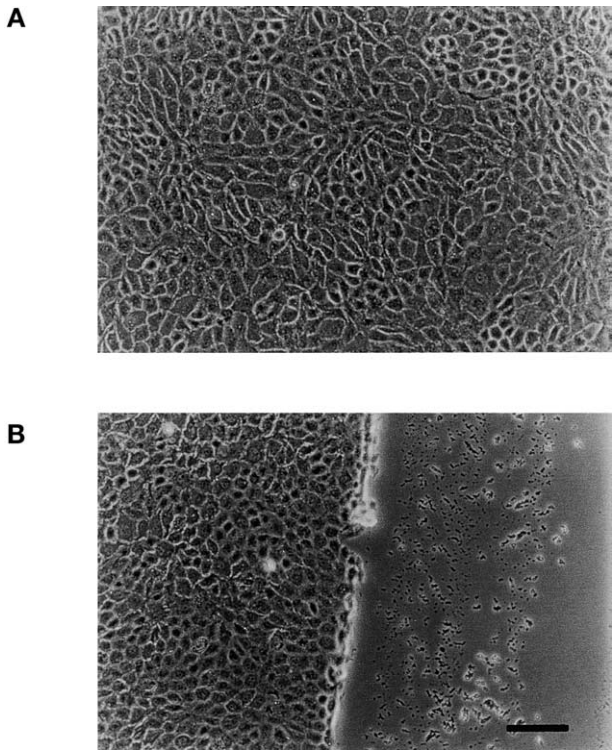

Figure 1. Retinal pigment epithelial (RPE) cell cultures before and just after injury. (**A**) Bovine RPE cells at 1-week postconfluence; (**B**) just after injury with the tip of a micropipette. (**A**, **B**) Bar = 100 μm.

HGF-promoted RPE Cell Proliferation and Migration

In an MTS assay, HGF stimulated the proliferation of RPE cells in a dose-dependent manner by 8% at 20 ng/mL, 11% at 40 ng/mL, and 23% at 80 ng/mL ($P < .0001$) (Figure 3A).

HGF also strongly stimulated RPE cell migration (Figure 3B). The stimulatory effect was greatest with 10 ng/mL HGF, which increased RPE cell migration to 218% of control ($P < .0001$). In contrast, 80 ng/mL of HGF, which had the greatest stimulatory effect on RPE cell proliferation, had an inhibitory effect on RPE cell migration (68% of control, $P < .0001$, Figure 3B).

Signaling Pathways Involved in RPE Cell Proliferation and Migration

To elucidate the intracellular signaling pathways involved in HGF actions, we investigated whether the MAPK, PI3 kinase, and PKC pathways were involved in HGF-induced RPE cell proliferation and migration; we used selective inhibitors, MAPK inhibitor (PD98059), PI3 kinase inhibitor (wortmannin), and PKC inhibitor (calphostin C), at different concentrations. PD98059 inhibited HGF-induced RPE cell proliferation, and the inhibitory effects were statistically significant at all concentrations (Figure 4A, $P < .0001$). Although PD98059 tended to inhibit RPE proliferation more at a lower concentration, no statistical significance was detected among the three concentrations. Wortmannin also inhibited HGF-induced RPE cell proliferation, but it was effective only at more than 20 nM (Figure 4B, $P < .0001$), and the inhibitory effect seemed to be smaller than that of PD98059. Calphostin C did not inhibit HGF-induced RPE cell proliferation (Figure 4C).

As shown in Figure 5A, PD98059 at 20 μM effectively inhibited HGF-induced RPE cell migration ($P < .0001$). However, at 10 μM, it did not inhibit HGF-induced cell migration. In contrast, wortmannin inhibited HGF-induced RPE cell migration more effectively at all concentrations tested (Figure 5B, $P < .0001$). Calphostin C did not inhibit HGF-induced cell migration (Figure 5C).

Discussion

Wound healing requires cell proliferation, migration, and cell-to-cell or cell–matrix contact, along with many other factors. Because HGF is reported to increase the proliferation and migration of RPE cells,[14,15] we examined the effects of HGF on wound healing in RPE monolayers in vitro.

We observed that HGF stimulated the restitution of RPE cells, and that the morphology of the covered area

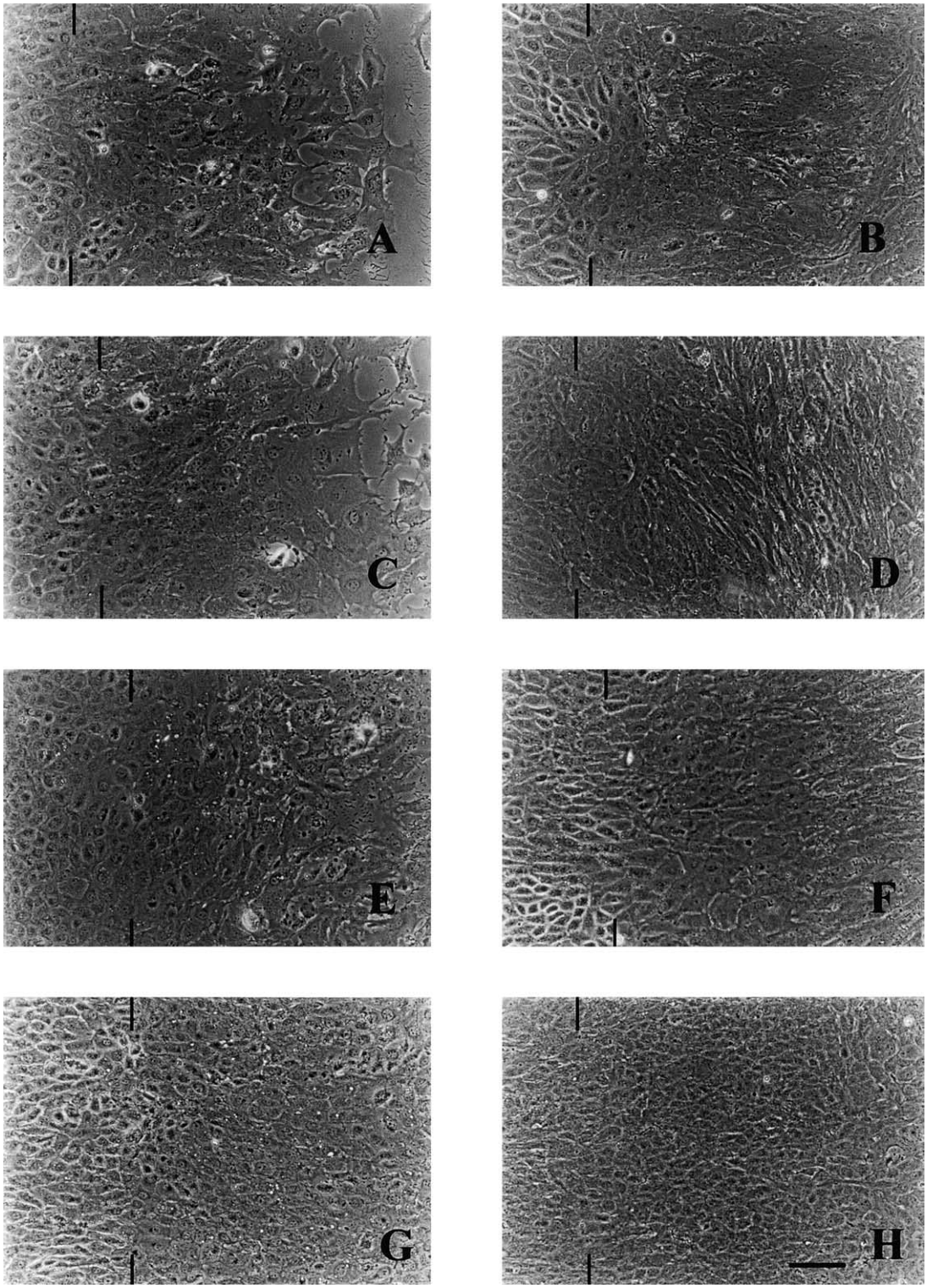

Figure 2. Effect of hepatocyte growth factor (HGF) on wound healing of retinal epithelial pigment (RPE) cells. Panels show 1-week postconfluent RPE cell cultures that have been denuded with the tip of a micropipette and incubated, with or without HGF. The cultures were photographed with a phase contrast microscope on day 3 (**A**,**C**,**E**,**G**) and on day 5 (**B**,**D**,**F**,**H**): (**A**,**B**) without HGF; (**C**,**D**) with 10 ng/mL HGF; (**E**,**F**) with 20 ng/mL HGF; (**G**,**H**) with 80 ng/mL HGF. The vertical lines on the left indicate the original wound edge; cell restitution progresses from the wound edge to the right. (**A**–**H**) Bar = 100 μm.

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varied by HGF concentration; cells cultured with 80 ng/mL HGF displayed an epithelial morphology, whereas those cultured with 10 ng/mL HGF were much less epithelioid (Figure 2). In order to clarify the reason(s) for this morphological difference by the concentration of HGF, we examined the effect of HGF on proliferation and migration separately. As shown in Figure 3A, 80 ng/mL HGF stimulated RPE cell proliferation the most. On the other hand, the stimulatory effect on migration was strongest at 10 ng/mL, and 80 ng/mL HGF had rather inhibitory effects on RPE migration (Figure 3B). These results are similar to the previous reports of He et al[14]

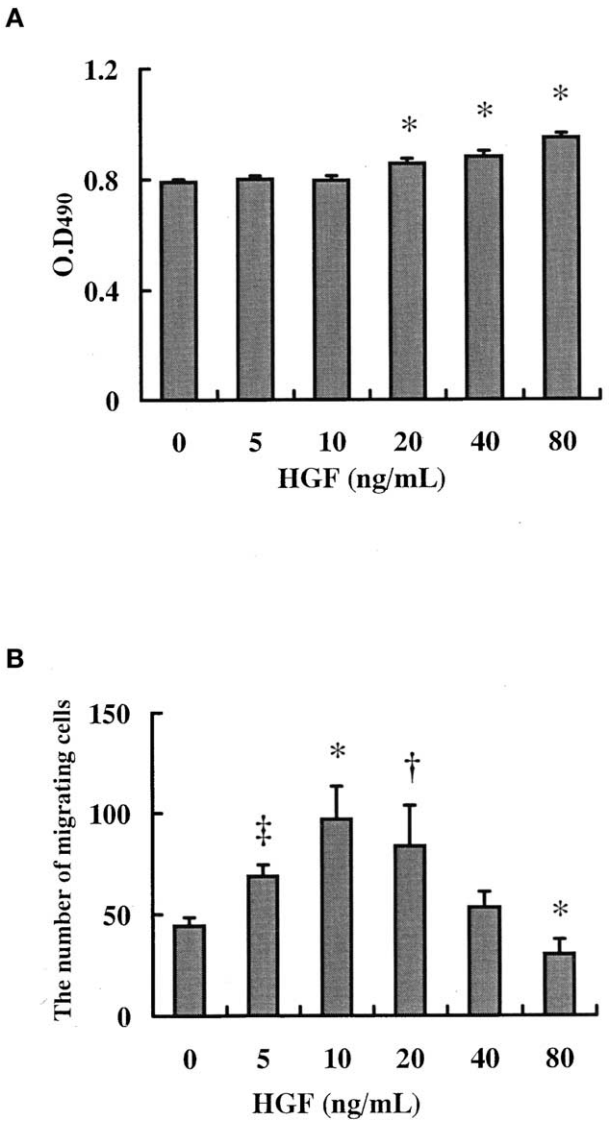

Figure 3. Hepatocyte growth factor (HGF)-induced retinal pigment epithelial (RPE) cell proliferation and migration (**A**) HGF-induced RPE cell proliferation detected by -(4;5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl) -2H-tetrazolium assay. The reaction product was measured by recording the absorbance at 490 nm ($O.D._{490}$). (**B**) HGF-induced RPE cell migration. The cells that migrated into the denuded area were counted 48 hours after denuding. $^{*}P < .0001$, $^{\dagger}P < .001$, $^{\ddagger}P < .01$ versus control (0 ng/mL HGF).

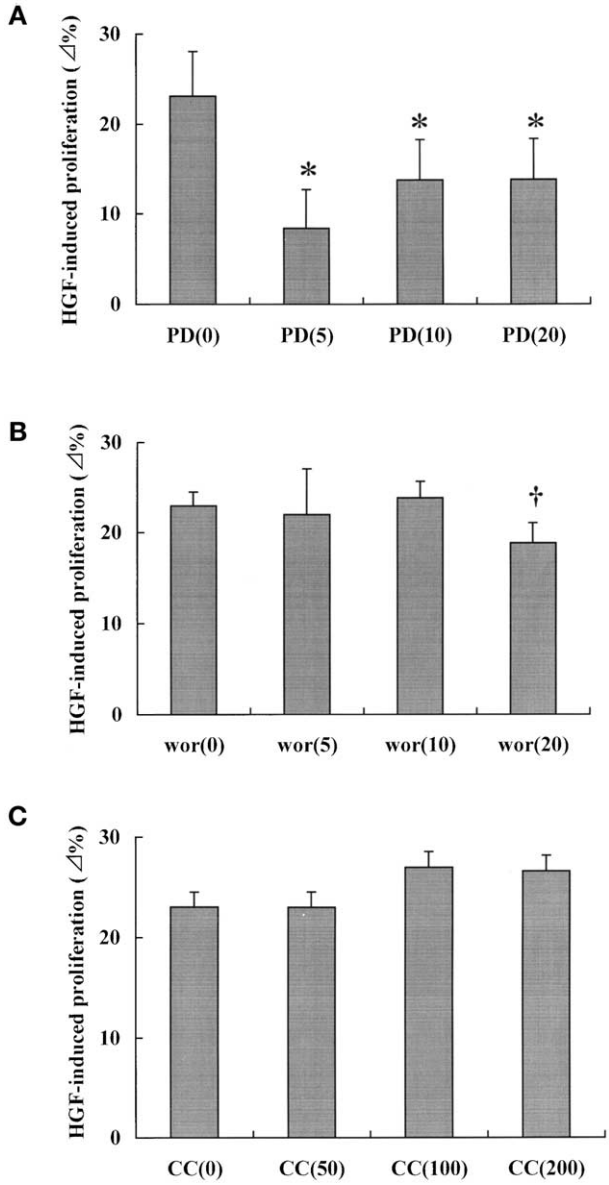

Figure 4. Effects of the inhibitors of intracellular signaling pathways on hepatocyte growth factor (HGF)-induced retinal pigment epithelial (RPE) cell proliferation. (**A**) Effect of the mitogen-activated protein kinase inhibitor, PD98059 (PD); (**B**) Effect of the phosphatidylinositol-3 kinase inhibitor, wortmannin (wor); (**C**) Effect of the protein kinase C inhibitor, calphostin C (CC). The vertical axis indicates the increased percentage of proliferation stimulated by HGF. The numbers in parentheses indicate the concentration (PD: μM, wor: μM, CC: nM) of each inhibitor used. $^{*}P < .0001$, $^{\dagger}P < .01$ versus control (without inhibitor).

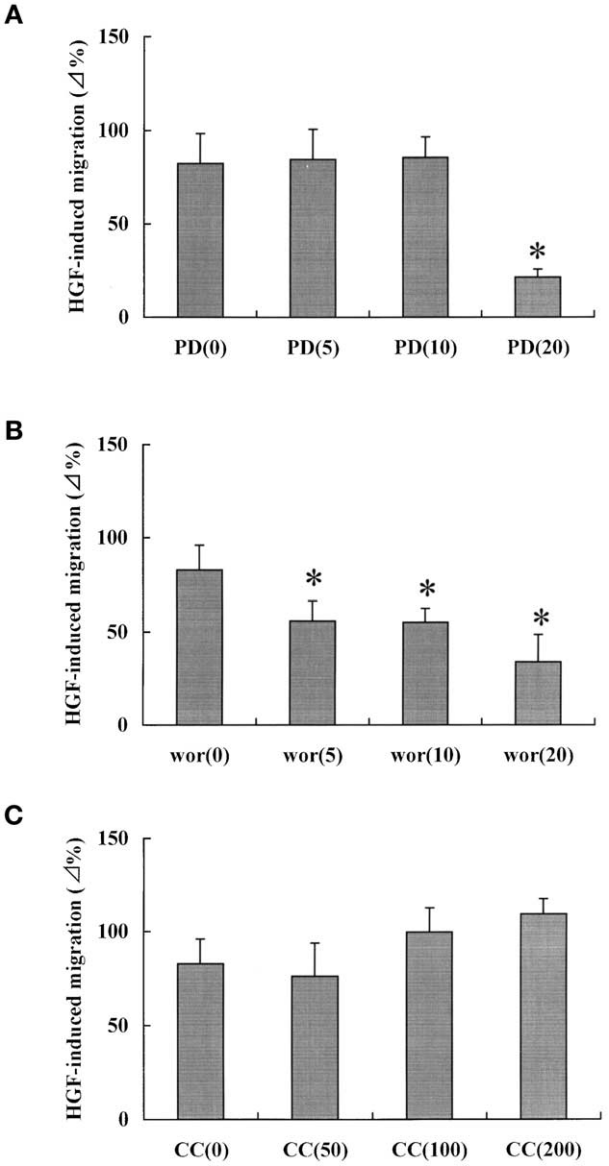

Figure 5. Effects of the inhibitors of intracellular signaling pathways on hepatocyte growth factor (HGF)-induced retinal pigment epithelial (RPE) cell migration. (**A**) Effect of the mitogen-activated protein kinase inhibitor, PD98059 (PD); (**B**) Effect of the phosphatidylinositol-3 kinase inhibitor, wortmannin (wor); (**C**) Effect of the protein kinase C inhibitor, calphostin C (CC). The vertical axis indicates the increased percentage of migration stimulated by HGF. The numbers in parentheses indicate the concentration (PD: μM, wor: μM, CC: nM) of each inhibitor used. $*P < .0001$ versus control (without inhibitor).

and Lashkari et al[15]; human RPE proliferation was stimulated most at higher concentrations of HGF (50–100 ng/mL), whereas migration, as examined by Boyden chamber assay, was at a lower concentration (10 ng/mL). In the present study, the discrepancy between the optimal concentrations for proliferation and migration may contribute to the morphological difference, ie, 80 ng/mL HGF promotes proliferation but not migration of RPE cells, and this could lead to a more epithelial morphology. This idea is supported by our previous report,[3] which demonstrated that HGF promotes epithelial morphogenesis through occludin linkage to the cytoskeleton in bovine RPE cells when cells are kept at the same density. Occludin is a major component of tight junctions, and HGF seems to induce epithelial morphogenesis in RPE cells when tight junctions start to form.[3] In the current study, 80 ng/mL HGF promoted the proliferation of RPE cells, so cell density in the wound area increased, and the cells quickly came into close contact with each other. Furthermore, this concentration of HGF inhibited RPE cell migration, so that the cells remained close together. We assume, therefore, that HGF could have easily induced occludin linkage to the cytoskeleton, which makes cells more epithelioid in shape.

We also examined intracellular signaling pathways involved in HGF-induced RPE cell proliferation and migration because, to date, there has been no report of signal transduction induced by HGF in RPE cells. According to previous reports, the signaling pathways activated by HGF vary depending upon cell type. For example, MAPK and PKC pathways are reported to be involved in HGF-induced cell proliferation in lung H441 adenocarcinoma cells,[19] and another report documents their involvement in HGF-induced cell migration in an MDCK epithelial cell line.[20] Trusolino et al showed involvement of PI3 kinase in HGF-induced cell migration in MDA-MB-231 carcinoma cells,[21] but no report has described involvement of PI3 kinase in HGF-induced cell proliferation. As for RPE cells, PKC has been reported to be involved in FCS-induced migration of bovine RPE cells.[17] In another study, researchers suggested that MAPK activation might mediate platelet-derived growth factor directed human RPE migration.[22] In our case, PD98059, an MAPK inhibitor, significantly inhibited HGF-induced RPE proliferation (Figure 4A), and wortmannin, a PI3 kinase inhibitor, inhibited it only at doses greater than 20 μM (Figure 4B), and the effect seemed to be less than that of PD98059. Calphostin C, a PKC inhibitor, did not inhibit HGF-induced RPE proliferation (Figure 4C). From these results, we conclude that the MAPK pathway may be mainly responsible for the mediation of HGF-induced RPE cell proliferation. In contrast, we found that PI3 kinase inhibitor and a high concentration of MAPK inhibitor (20 μM) inhibited HGF-induced RPE migration (Figures 5A and 5B) but calphostin C did not (Figure 5C). From these results, we assume that mainly the PI3 kinase pathway is involved in HGF-induced RPE cell migration. Our present experimental results indicate that stimulation of

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the MAPK pathway, not the PI3 kinase pathway, may induce RPE cell proliferation without stimulating its migration, which would result in quick repair of the wound, with the cells showing an epithelial morphology.

Cell conditions, such as the time period after confluence or phenotype, may change the response of RPE cells to HGF. Kaida et al showed that postconfluent cultures of human RPE cells (maintained undisturbed at confluence for approximately 8 weeks) showed a wound-healing pattern similar to that seen in vivo, but early confluent cultures displayed a slightly different pattern.[23] The restitution characteristics and HGF effects on wound healing might differ when bovine RPE cells cultured for several weeks after confluence are used. However, when bovine RPE cells are maintained in culture for more than 1 week after confluence, these cells display a fibroblastic appearance and are quite different than cells in vivo.[3] Therefore, in this study, we used RPE cells at 1-week postconfluent status. In addition to the postconfluent status, the concentration of FCS might influence the response of RPE cells to HGF. In particular, a high concentration of FCS diminishes the effect of exogenously added cytokines. Therefore, it would be desirable to conduct all of our experiments at the lowest concentration of FCS (1%) to minimize its effects. However, 1% FCS was not enough to maintain the wound cultures for longer periods, so we used 10% FCS in wound repair experiments. Although an interaction between FCS and HGF may exist, we assume that the principal effect of HGF on wound healing in RPE monolayers is similar even when FCS is reduced to less than 10%.

Our current data suggest that HGF might enhance RPE wound healing in vivo. From a clinical point of view, administration of an appropriate concentration of HGF at the site of an RPE defect may promote quick wound healing by RPE cells with epithelial morphology. The appropriate concentration of HGF is the key to success in this procedure; a concentration that stimulates proliferation but not migration should be chosen so as not to induce a condition such as proliferative vitreoretinopathy. Moreover, a drug delivery system should be developed that will maintain a desirable concentration of HGF throughout the wound closure process.

HGF might induce subretinal or retinal neovascularization because of the reported nature of angiogenesis.[24,25] Because it has been reported that HGF increases paracellular permeability in vascular endothelial cells,[26] topical HGF might increase the permeability of retinal or choroidal vessels. Furthermore, it is possible that human RPE cells do not show a response to HGF similar to that shown by bovine RPE cells. Nevertheless, we think that HGF is a promising candidate for the promotion of RPE wound healing, and further investigation of its potential clinical applications would be valuable.

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