Hepatocyte Growth Factor Induces Retinal Vascular Permeability via MAP-Kinase and PI-3 Kinase without Altering Retinal Hemodynamics

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PURPOSE. Although vascular endothelial growth factor (VEGF) is a key mediator of retinal vascular permeability (RVP), there may be additional humoral contributors. Hepatocyte growth factor (HGF) induces endothelial cell separation, regulates expression of cell adhesion molecules and is increased in the vitreous fluid of patients with proliferative diabetic retinopathy. The purpose of this study was to evaluate the in vivo effects of HGF on RVP and retinal hemodynamics and delineate the signaling pathways.

METHODS. RVP was assessed by vitreous fluorescein fluorophotometry in rats. Time course and dose–response were determined after intravitreal HGF injection. MAP kinase (MAPK), phosphatidylinositol 3-kinase (PI-3 kinase), and protein kinase C (PKC) involvement were examined by using selective inhibitors. Retinal blood flow (RBF) and mean circulation time (MCT) were evaluated by video fluorescein angiography.

RESULTS. HGF increased RVP in a time- and dose-dependent manner. HGF-induced RVP was evident 5 minutes after injection, and reached maximal levels after 25 minutes ($+107\%$ versus vehicle, $P = 0.002$). This effect was comparable to that of maximum VEGF stimulation ($134\% \pm 128\%$ at 25 ng/mL). Selective inhibitors of MAPK (PD98059) and PI-3 kinase (LY294002) suppressed HGF-induced RVP by $86\% \pm 44\%$ ($P = 0.015$) and $97\% \pm 59\%$ ($P = 0.021$), respectively. Non–isoform-selective inhibition of PKC did not significantly decrease HGF-induced RVP. Although VEGF increases RBF and reduces MCT, HGF did not affect either.

CONCLUSIONS. HGF increases RVP in a time- and dose-dependent manner at physiologically relevant concentrations with a magnitude and profile similar to that of VEGF, without affecting retinal hemodynamics. Thus, HGF may represent another clinically significant contributor to retinal edema distinct from the actions of VEGF. (*Invest Ophthalmol Vis Sci.* 2006; 47:2701–2708) DOI:10.1167/iovs.05-0071

Retinal vascular leakage is a vision-threatening complication associated with numerous ocular conditions, including diabetic macular edema, retinal vein occlusions, retinopathy of prematurity, and exudative age-related macular degeneration. Various cytokines are thought to mediate these permeability changes. Vascular endothelial growth factor (VEGF),[1] also referred to as vascular permeability factor (VPF),[2] is an endothelial cell mitogen with angiogenic and vasopermeability activity thought to play a critical role in this regard.[3–8] Involvement of additional humoral factors in mediating these complex events is likely, although the contribution of other factors is not fully understood.

Hepatocyte growth factor (HGF), or scatter factor (SF), was originally described as a fibroblast-derived paracrine modulator of epithelial cell mobility.[9] HGF has a disulfide-linked heterodimer structure comprising a 62-kDa α-chain and a 32-kDa β-chain.[10] Its biological effects are mediated through the receptor c-MET, a cell surface tyrosine kinase receptor encoded by the c-MET proto-oncogene.[11,12] The c-MET receptor has been localized at intercellular junctions, and its expression is induced by cytokines, PMA, and HGF itself.[13] On receptor binding, HGF induces autophosphorylation of the receptor at tyrosine residues in both the kinase region and regulatory carboxyl-terminal tail,[14] resulting in subsequent altered expression of proteins responsible for cell–cell adhesion and eventual separation of endothelial cells at intercellular junctions.[15–17] Expression of the tight-junction–forming proteins occludin, claudin-1, -2, and -5; JAM-1 and -2; and the cell adhesion molecule cadherin are reduced by HGF.[17–20] HGF increases the association of the adhesion protein complex E-cadherin/catenin with cMet,[21] resulting in the loss of β-catenin from the E-cadherin/catenin complex and subsequent decreased cell–cell adhesion.[22]

HGF is also a potential mediator of retinal neovascularization,[23] as evidenced by its mitogenic and motogenic effects on retinal endothelial cells in culture.[24–26] HGF expression is upregulated under hypoxic conditions,[27] and its concentration is significantly higher in the vitreous fluid of diabetic patients with active PDR compared with the vitreous fluid of nondiabetic patients, nondiabetic patients with proliferative vitreoretinopathy, diabetic patients without PDR, or diabetic patients with quiescent PDR.[28,29] In cultured human epidermal keratinocytes, HGF induces VEGF expression.[30]

Studies have demonstrated that retinal endothelial cells express HGF and its receptor.[25,26,31] HGF induces MAP kinase (MAPK) phosphorylation in bovine retinal endothelial cells in culture, a response partially mediated by phosphatidylinositol 3-kinase (PI-3 kinase) and, to a lesser extent, protein kinase C (PKC).[26] The effector protein Grb2 is thought to link downstream mediators with the receptor cMet.[32] In other cell types,

HGF signaling may involve PI-3 kinase, ERK, p38 MAPK, PKC, and Rho GTPases.[33,34]

Although previous cell culture and biochemical data suggest that HGF may act as a regulator of angiogenesis and vasopermeability, the in vivo effect of HGF on retinal vascular permeability and retinal hemodynamics, the magnitude of its effect in relation to known important permeability factors in the eye, and the pathways mediating these effects have not been previously evaluated.

Our results demonstrate that HGF increases retinal vascular permeability in a dose-dependent manner, resulting in ocular permeability at physiologically relevant concentrations similar to that observed for VEGF. Unlike VEGF, however, retinal mean circulation time and blood flow remain unaltered. In addition, inhibition of PI-3 kinase or MAPK, but not PKC, suppresses HGF-induced retinal vascular permeability, suggesting that both PI-3 kinase and MAPK are important in mediating HGF's retinal permeability, whereas the role of PKC may be less significant.

METHODS

Animals

Male albino Sprague-Dawley rats weighing 200 to 250 g were obtained from Taconic (Germantown, NY). All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Vitreous Fluorescein Fluorophotometry

The instrumentation for vitreous fluorescein fluorophotometry has been described.[3] Briefly, the fluorophotometer consists of a modified slit lamp (Haag-Streit, Köniz, Switzerland) which provides both illumination and collection optics. A 488-nm argon laser (Omnichrome, La Jolla, CA) provides the excitation wavelength. Focused onto a fiber optic, the laserlight is delivered to the vitreous under direct visualization. The emitted fluorescence is collected through a fiber optic and delivered to a monochromator (SPEX Industries, Metuchen, NJ). The emitted light is dispersed along an intensified 1024 photodiode array, and the spectra displayed graphically onscreen (Princeton Instruments, Trenton, NJ). Each spectrum is analyzed by determining the ratio of the integrated fluorescence centered at 520 nm to the intensity of the unshifted laser line. This ratio represents a normalized vitreous fluorescence level with respect to attenuation of both the incident excitation and emitted fluorescence intensities.

Each animal had a 13-cm length of polyvinyl catheter (Renathane MRE-033; Braintree Scientific, Braintree, MA) surgically implanted into the right jugular vein 24 hours before the experiment. The catheter was subcutaneously located along the shoulder for later access from the back of the animal. On the day of the experiment, the fluorophotometer was calibrated with a fluorescent standard. Animals were anesthetized using pentobarbital (50 mg/kg) and both eyes were dilated with 1% tropicamide. Background fluorescence spectra were obtained from each animal. The jugular catheter was exposed and connected to a 100-μL syringe (Hamilton, Reno, NV) containing 65 μL of 10% sodium fluorescein.

HGF Time Course and Dose Response

The dose–response and time course of intravitreal HGF injection on in vivo retinal vascular permeability was examined by injecting 10 μL of recombinant human HGF (R&D Systems, Minneapolis, MN) dissolved in filtered 0.1% bovine serum albumin and phosphate-buffered saline (PBS) into the vitreous of one eye. The final concentration of HGF was calculated based on the vitreous volume, per Hughes.[35] Intravitreal injections were performed under direct visualization, by using a 10-μL syringe (Hamilton) with a 32-gauge needle inserted 2 mm posterior to the limbus. Contralateral eyes received an intravitreal injection of 10

μL vehicle alone. Sodium fluorescein (65 μL of 10%) was infused intravenously 10 minutes after HGF injection. As a positive control, 2 ng (0.48 nM final) of recombinant human VEGF (R&D Systems) was injected into the vitreous of one eye in another set of animals. Vitreous fluorophotometry measurements were obtained at 5, 15, and 25 minutes after fluorescein infusion.

For dose–response experiments, HGF was injected into the vitreous of one eye and vehicle into the contralateral eye. Vitreous fluorophotometry measurements were obtained 25 minutes after fluorescein infusion.

HGF Signal Transduction

A 10-μL volume of inhibitors of protein kinase C (GFX; Sigma-Aldrich, St. Louis, MO), MEK (PD98059, 20 μM final), or PI-3 kinase (LY294002, 50 μM final) was injected into the vitreous of one eye, and vehicle alone was injected into the vitreous of the contralateral eye. After 10 minutes, a 10-μL volume containing 5 ng (0.69 nM final) of HGF was injected into the vitreous of both eyes. After 10 minutes, 65 μL of 10% sodium fluorescein was infused, and vitreous fluorophotometry measurements were obtained 25 minutes later.

Western Blot of In Vivo Retinal HGF-Induced MAPK Activity

Protein was extracted from rat retinas using RIPA buffer with protease inhibitors. Total protein concentration was determined by protein assay (Bio-Rad, Hercules, CA). Total protein (50 μg) was separated by 12% SDS-PAGE, transferred to nitrocellulose (Bio-Rad), and incubated with phospho-p44/42 MAPK or p42 MAPK primary antibody (Cell Signaling, Beverly, MA) overnight at 4°C. Proteins were visualized with a chemiluminescence system (GE Healthcare, Piscataway, FL).

Mean Circulation Time and Retinal Blood Flow

Video fluorescein angiography was used to determine retinal blood Flow (RBF). The video fluorescein angiography system has been described.[36] Briefly, the system consists of a fundus camera (Nikon, Tokyo, Japan) coupled with a low light-level SIT camera. The NTSC output signal was digitized by a (Targa 2000) frame grabber from Truevision (Indianapolis, IN). The images were digitized at a rate of 30 frames/s with a resolution of 640 × 480 × 8-bit gray scale and stored as a sequential series of TIFF images. Analysis of the digitized angiograms was performed on a frame-by-frame basis with a program written in commercial software (MatLab; The MathWorks, Natick, MA).

The procedure for measurement of RBF has been described.[37] Animals underwent surgical implantation of a polyvinyl catheter into the right jugular vein. After 24 hours, the eyes were dilated and angiograms obtained by rapidly injection of 5 μL of 10% sodium fluorescein. Analyses of the digitized angiograms were performed as detailed previously.[36] Dye dilution curves were obtained from the primary arteries and veins at a distance of 1.5 optic disc diameters. The dye dilution curves were fit by a log-normal distribution function and the curve-fit parameters used to determine the mean arterial and venous circulation times. The MCT was defined as the difference between the mean venous and arterial filling times. The diameters of the primary vessels were measured at the defined sample sites by a boundary-crossing algorithm. Segmental RBF was calculated by the sum of the squares of the retinal artery and vein diameters divided by the mean circulation time.

Statistics

Results are expressed as the mean ± SD, unless otherwise indicated. Statistical analysis was performed on computer (SigmaStat; SPSS, Chicago, IL). Statistical analysis used the Student's *t*-test to compare quantitative data populations with normal distributions and equal variance. The paired test was used for comparison of eyes from the same animal and the unpaired test was used for interanimal comparisons. The
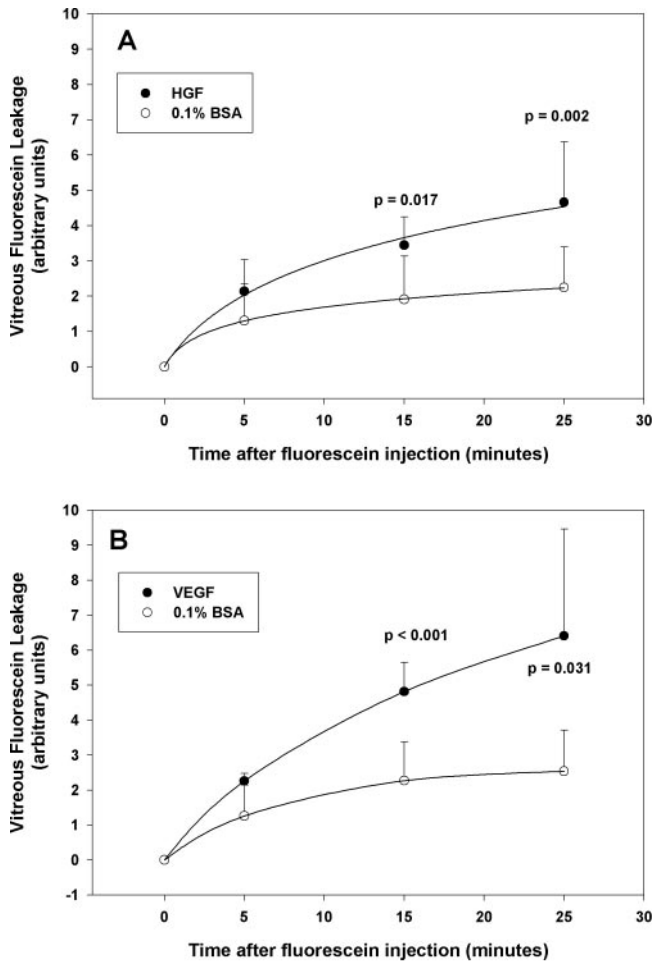
FIGURE 1. HGF induces retinal vascular permeability in vivo. Vitreous fluorescein leakage was measured at the indicated times after intravenous fluorescein injection in eyes that had been pretreated for 10 minutes with (**A**) HGF (5 ng/eye, 0.69 nM final), (**B**) VEGF (2 ng/eye, 0.48 nM final), or vehicle control (0.1% BSA).

Mann-Whitney rank-sum test was used for populations with non-normal distributions or unequal variance.

RESULTS

Characterization of HGF-Induced Retinal Vascular Permeability

The effect of HGF on retinal vascular permeability (RVP) in vivo was assessed by vitreous fluorophotometry. HGF (5 ng/eye, 0.69 nM final) or vehicle alone was injected intravitreally, and 10 minutes later sodium fluorescein was injected intravenously and retinal vascular leakage was assessed after an additional 5, 15, and 25 minutes. In eyes receiving vehicle alone (0.1% BSA), vitreous fluorescence increased 1.26 ± 0.87, 1.91 ± 1.23, and 2.25 ± 1.15 AU after 5, 15, and 25 minutes, respectively (Fig. 1A), consistent with low basal levels of fluorescein leakage from an intact retinal vasculature, as reported previously.[3] In eyes receiving HGF, vitreous fluorescence increased 2.14 ± 0.89, 3.45 ± 0.81, and 4.66 ± 1.71 AU after 5, 15, and 25 minutes, respectively. Thus, HGF increased permeability compared with vehicle alone by $62\% \pm 68\%$, $81\% \pm 42\%$ ($P = 0.017$), and $107\% \pm 76\%$ ($P = 0.002$) after 5, 15, and 25 minutes, respectively. After 60 minutes, the effect of HGF on

permeability had declined to $57\% \pm 33\%$ above that of vehicle-treated eyes (data not shown).

VEGF is a well-recognized potent mediator of RVP thought to play a central role in ocular conditions associated with vascular leakage. To evaluate the relative magnitude of the HGF response, VEGF-induced RVP was measured under equivalent conditions. VEGF at doses previously shown to provide the maximal effect (2 ng/eye, 0.48 nM final)[3] increased RVP $79\% \pm 17\%$, $99\% \pm 34\%$ ($P < 0.001$), and $134\% \pm 128\%$ ($P = 0.031$) compared with vehicle response, at 5, 15, and 25 minutes after fluorescein injection, respectively (Fig. 1B). Thus, the magnitude of the HGF-elicited response was $83\% \pm 89\%$, $64\% \pm 34\%$, and $65\% \pm 46\%$ of the VEGF maximum stimulatory effect at 5, 15, and 25 minutes after infusion, respectively. The magnitude of the HGF-induced stimulated retinal leakage was not statistically different from that observed for VEGF at any time point.

HGF Dose Response

To determine the dose–response of HGF-induced RVP, intravitreal injections of HGF were given in one eye at the concentrations listed in Figure 2, whereas the contralateral eye received vehicle alone. Maximum stimulation by HGF was observed at 5 ng/eye, 0.69 nM final (4.66 ± 1.72 vs. 2.25 ± 1.14; $P = 0.002$), yielding a $143\% \pm 113\%$ increase in vitreous fluorescein leakage. Permeability was increased, $61\% \pm 70\%$, $91\% \pm 58\%$ ($P = 0.043$), and $133\% \pm 131\%$ ($P = 0.036$) at HGF doses of 0.05, 0.1, and 1.0 ng/eye (0.007, 0.014, and 0.140 nM), respectively. The half-maximal HGF effect occurred between 0.1 and 1.0 ng/eye (0.014 and 0.140 nM). In comparison, VEGF at a maximal stimulatory concentration (2 ng/eye, 0.48 nM final) increased vitreous fluorescein leakage by $200\% \pm 89\%$ (7.74 ± 4.13 vs. 2.75 ± 0.76; $P = 0.035$.) The VEGF response was not significantly different from that observed for 1, 5, or 50 ng/eye HGF.

To determine whether the effect on permeability was generalized or more specific to HGF and VEGF, pigment epithelium derived factor (PEDF), a similarly sized endogenous molecule with survival factor and antiangiogenic and antipermeabilty properties,[38] was injected into the vitreous. A 10-μL volume of PEDF (200 ng) was injected intravitreally into one eye, and balanced saline solution into the contralateral eye.

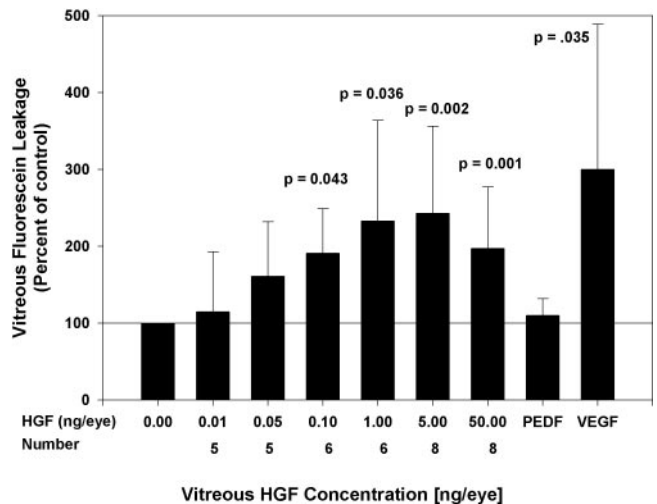**FIGURE 2.** HGF increased retinal vascular leakage in vivo at physiologic concentrations and in a dose-dependent manner. Vitreous fluorescein leakage was measured 25 minutes after a 10-minute intravitreal pretreatment with HGF at the concentrations indicated. Retinal vascular leakage after 2 ng/eye VEGF and 200 ng/eye PEDF under maximal effective conditions were also evaluated for comparison.

As shown in Figure 2, at 25 minutes after fluorescein infusion, no difference was observed in the permeability response for vehicle and PEDF (2.35 ± 0.23 vs. 2.56 ± 0.38, respectively).

HGF Signal Transduction

To investigate the mechanisms by which HGF stimulates RVP, selective inhibitors of PI-3 kinase (LY294002), protein kinase C (GFX), and MAPK (PD98059) were evaluated. Each of these inhibitors have been used successfully in this animal model.[3] Inhibitors' concentrations were determined from in vitro experiments with HGF using bovine retinal microvascular endothelial cells.[26] LY294002 (0.5 or 50 μM) was injected into the vitreous of one eye, and the contralateral eye received vehicle alone. HGF (5 ng/eye, 0.69 nM final) was injected into both eyes 10 minutes later. One group of rats received intravitreal injections of PBS in both eyes followed by HGF or BSA (HGF vehicle control). PBS/HGF increased vitreous fluorescein leakage 106% ± 66% (4.64 ± 1.53 vs. 2.23 ± 1.12; $P < 0.001$) compared with PBS/BSA-treated eyes. LY294002, at doses of 0.5 (3.08 ± 0.76, $n = 8$; $P = $ NS) and 50 (2.36 ± 0.93, $n = 5$) μM, suppressed HGF-induced RVP by 57% ± 97% and 96% ± 59% ($P = 0.021$), respectively, compared with the contralateral PBS/HGF-treated eye (Fig. 3).

In additional experiments, intravitreal injection of the MEK specific inhibitor PD98059 (20 μM) was performed in one eye and PBS alone performed in the opposite eye. HGF (5 ng/eye) was injected into both eyes 10 minutes later. The PBS/HGF-treated eyes increased vitreous fluorescein leakage by 113% ± 79% (4.79 ± 1.78; $P = 0.005$) compared with PBS/BSA treated eyes. MAPK pathway inhibition suppressed HGF-induced permeability 86% ± 44% compared with the PBS/HGF-treated eye (Fig. 4A; $P = 0.015$). To confirm activation of the MAPK pathway by HGF in vivo, 10 ng/mL of HGF was injected intravitreally in one eye and saline control delivered to the contralateral eye. Retinas were excised 5 minutes after intravitreal injection. Intravitreal HGF increased retinal p42/44 ERK phosphorylation by 54% after 5 minutes compared with contralateral eyes injected with saline control (data not shown). In contrast, intravitreal injection of the non–isoform-selective PKC inhibitor GFX (0.5 μM final) in one eye followed by
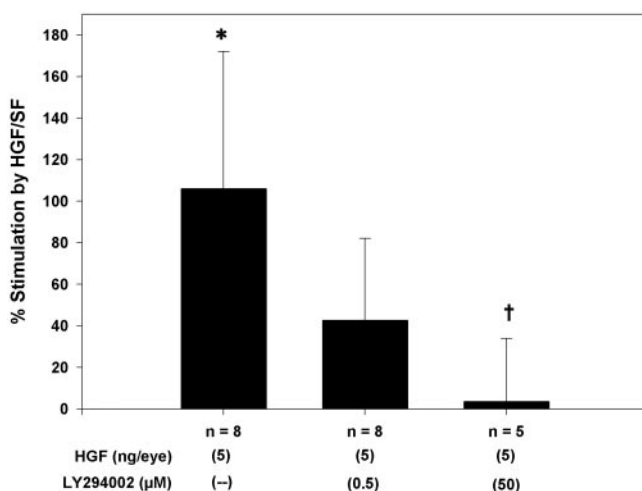

FIGURE 3. HGF-induced retinal vascular permeability was partially mediated by PI-3 kinase in vivo. The effect of HGF injection (5 ng/eye) on vitreous fluorescein leakage was evaluated after a 10-minute pretreatment with the PI-3 kinase inhibitor LY294002. Pretreatment with vehicle alone followed by HGF injection established basal (100%) levels. (*$P = 0.005$ compared with eyes pretreated with BSA and injected with BSA, †$P = 0.021$ compared with eyes pretreated with BSA and injected with HGF.)
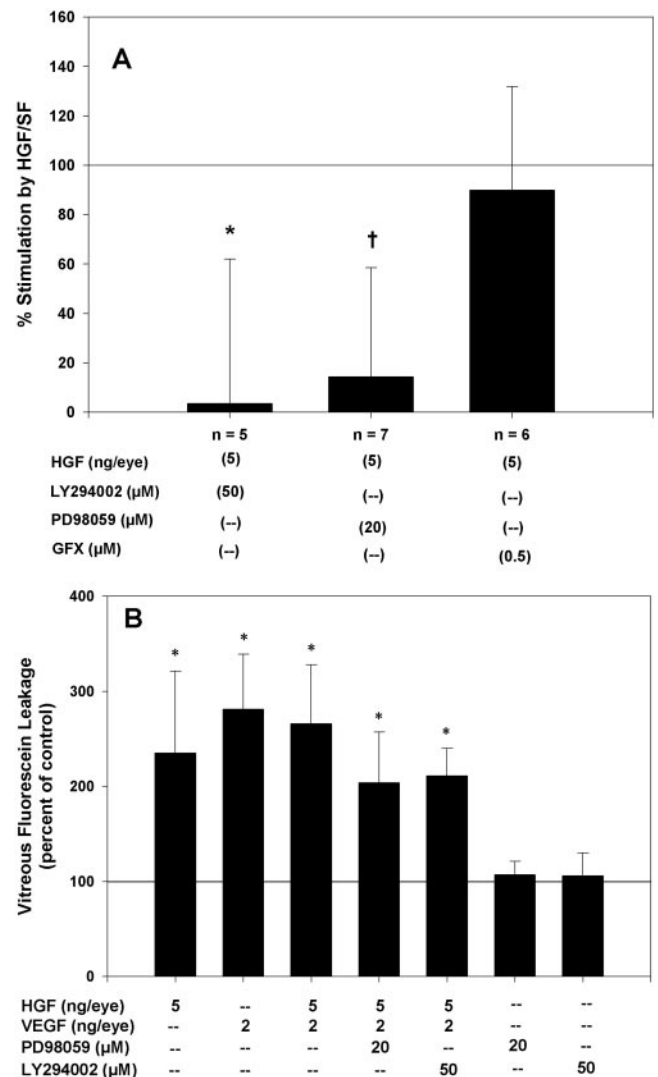

FIGURE 4. (**A**) HGF-induced retinal vascular permeability was partially mediated in vivo by PI-3 kinase and MAPK, but not by protein kinase C. The effect of a 10-minute pretreatment with PI-3 kinase inhibitor (LY294002 at 50 μM), MEK-specific inhibitor (PD98059 at 20 μM), or non–isoform-selective protein kinase C inhibitor (GFX at 0.5 μM) on vitreous fluorescein leakage after HGF injection (5 ng/eye) was evaluated. Pretreatment with BSA established the basal (100%) HGF/SF leakage stimulation level. (*$P = 0.005$; †$P = 0.015$ compared with eyes pretreated with BSA and injected with HGF.) (**B**) HGF/VEGF's effect on retinal vascular permeability was not additive in vivo. The effect of a 10-minute pretreatment with PI-3 kinase inhibitor (LY294002 at 50 μM) or MEK-specific inhibitor (PD98059 at 20 μM) on vitreous fluorescein leakage after an HGF (5 ng/eye)/VEGF (2 ng/eye) combination injection was evaluated. Pretreatment with balanced saline established basal (100%) leakage stimulation level. (*$P < 0.01$ compared with eyes pretreated with balanced saline and injected with BSA.)

injections of 5 ng/eye HGF after 10 minutes did not significantly reduce retinal leakage (10% ± 42%, $P = $ NS) compared with the opposite eye receiving PBS/HGF. GFX used in this manner and at this concentration has been shown to reduce VEGF-induced RVP by 98%.[3]

To evaluate whether the retinal permeability effects of HGF and VEGF were additive, a 10-μL volume containing both HGF (5 ng/eye) and VEGF (2 ng/eye) was injected into the vitreous of one eye and a 10-μL volume of 0.1% BSA into the contralateral eye. Each eye had been pretreated for 10 minutes by intravitreal injection of balanced saline, the MAPK inhibitor

TABLE 1. Circulation Parameters with the Three Study Treatments

Time after Injection (min)	MCT (s)	Artery Diam. (pixels)	Vein Diam. (pixels)	RBF (pixels squared/s)
HGF/SF (5 ng/eye)				
0	0.86 ± 0.11	5.8 ± 0.1	6.9 ± 0.3	96 ± 15
5	0.89 ± 0.13	5.8 ± 0.2	7.0 ± 0.4	96 ± 19
15	0.90 ± 0.10	5.8 ± 0.2	7.0 ± 0.4	94 ± 10
VEGF (2 ng/eye)				
0	0.83 ± 0.09	5.8 ± 0.1	6.7 ± 0.5	97 ± 17
5	0.69 ± 0.08*	5.9 ± 0.2	7.2 ± 0.5	124 ± 21*
15	0.67 ± 0.08*	5.6 ± 0.3	7.1 ± 0.5	123 ± 12*
Vehicle (0.1% BSA)				
0	0.87 ± 0.19	5.9 ± 0.1	7.1 ± 0.1	102 ± 25
5	0.85 ± 0.13	5.9 ± 0.1	7.0 ± 0.2	102 ± 17
15	0.86 ± 0.05	5.9 ± 0.1	7.2 ± 0.2	101 ± 6

MCT, arterial and venous diameters, and RBF were unchanged in rats treated with intravitreous injection of HGF, compared with those treated with vehicle alone. MCT decreased and RBF increased in rats treated with VEGF, when compared with those treated with vehicle. *$P < 0.05$ compared with baseline. Diam., diameter.

PD98059 at 20 μM, or the PI-3 kinase inhibitor LY294002 at 50 μM. Balanced saline/HGF- and balanced saline/VEGF-treated eyes increased vitreous fluorescein leakage by 135% ± 86% ($P = 0.004$) and 182% ± 58% ($P < 0.001$), respectively compared with balanced saline/BSA-treated eyes (Fig. 4B). Eyes treated with balanced saline and combined HGF/VEGF increased vitreous leakage by 166% ± 61% ($P = 0.041$, $n = 6$). The combined HGF/VEGF effect was reduced by 37% ± 32% ($n = 6$) with pretreatment by PD98059 and 33% ± 17% ($n = 5$) by LY294002, but remained significantly elevated ($P < 0.01$) compared with the response of balanced saline/BSA-treated eyes. Treatment with saline followed by either PD98059 or LY294002 had no effect on basal leakage.

Retinal Blood Flow

VEGF increases RBF, a physiologic abnormality observed in diabetic patients with more established stages of diabetic retinopathy.[42] To evaluate whether HGF also affects RBF, video fluorescein angiography (VFA) was performed on rats receiving maximally effective intravitreal injections of HGF (5 ng/eye, five rats), VEGF (2 ng/eye, seven rats), and vehicle alone (six rats) as shown in Table 1. VFA measurements were made at 5 and 15 minutes after the intravitreal injections. Baseline MCT was equivalent between all groups (0.87 ± 0.19, 0.83 ± 0.0.09, and 0.86 ± 0.11 for HGF, VEGF, and vehicle treated groups, respectively). Primary artery and vein diameters, MCT, and RBF were not altered by injection of vehicle alone or HGF (Table 1). In contrast, VEGF treatment reduced MCT 17% ± 10% ($P = 0.004$) and 19% ± 10% ($P = 0.01$; Fig. 5A) and increased RBF by 28% ± 22% ($P = 0.004$) and 27% ± 13% ($P < 0.001$; Fig. 5B) after 5 and 15 minutes, respectively. No differences were observed in the primary artery and vein diameters after intravitreal VEGF injection (Table 1).

DISCUSSION

Numerous ocular disorders result in a vision-threatening increase in RVP, presumably mediated by various cytokines. Studies have demonstrated that retinal endothelial cells express HGF and its receptor c-MET.[25–27] HGF causes endothelial cell separation,[15,16] an effect credited to its decreasing occluding tight junction protein[18] and the cell adhesion molecule cadherin.[19] In addition, HGF has been shown to decrease transendothelial resistance and increase paracellular permeability in HUVEC monolayers,[18,39] breast cancer cells,[17] and mammalian renal epithelial cells.[20] This effect is associated

with a decrease in the expression of the cytoplasmic plaque protein ZO-1, and several tight junction molecules including claudin-1, 2, and 5; occludin; and JAM-1 and -2.[17,20,39] These findings suggest that HGF could induce RVP.

To our knowledge, the present studies are the first direct demonstration of HGF-induced retinal permeability in vivo and the first to demonstrate that this effect is of a magnitude similar to that induced by VEGF. Combined with data showing that HGF expression is upregulated under hypoxic conditions,[27] vitreous and serum concentrations are higher in eyes of patients with proliferative diabetic retinopathy than in patients with nondiabetic diseases,[28,29,40] and that photocoagulation decreases HGF expression,[41] these results strongly suggest that HGF may contribute to the retinal vascular leakage characteristic of ocular disorders such as diabetic macular edema. These effects would be in addition to its potential contribution to retinal neovascularization,[23] as suggested by the mitogenic and motogenic effects on retinal endothelial cells.[24–26]

We demonstrated that HGF induced RVP at concentrations as low as 0.1 ng/eye (0.8 ng/mL; $P = 0.043$) with a maximum stimulatory concentration of 5 ng/eye (41.6 ng/mL; $P = 0.002$). From Figure 2, the half-maximal stimulatory concentration of HGF is approximately 0.3 ng/eye (6.6 ng/mL). Patients with active proliferative diabetic retinopathy have HGF serum concentrations of 0.213 ng/mL compared with 0.04 ng/mL for diabetic patients with quiescent retinopathy or patients with no diabetic retinopathy. Seventy percent of patients had serum HGF concentrations higher than 0.1 ng/mL.[40] HGF concentrations in the vitreous are much higher on average (6.0 ng/mL),[28,29] achieving concentrations as high as 22.2 ng/mL in some patients with PDR.[28] Our data suggest that these concentrations could easily elicit retinal permeability regardless of contributions from the serum through any preexisting diabetes-induced alterations of the blood–retinal barrier.

Growth factors such as VEGF increase RBF, a physiologic abnormality in diabetic patients associated with advancing stages of diabetic retinopathy.[41,42] The present study confirmed that VEGF alters retinal MCT and RBF.[42] However, unlike VEGF, HGF did not alter retinal hemodynamics. This finding suggests that HGF signaling may be independent of that of VEGF, a possibility supported by the finding of no correlation between vitreous concentrations of HGF and VEGF in patients with PDR and in patients with nondiabetic ocular disease.[28] We demonstrated in an earlier study that HGF's effects on retinal angiogenesis are additive with VEGF[26] and that VEGF mediates its in vivo vasopermeability effects pre-
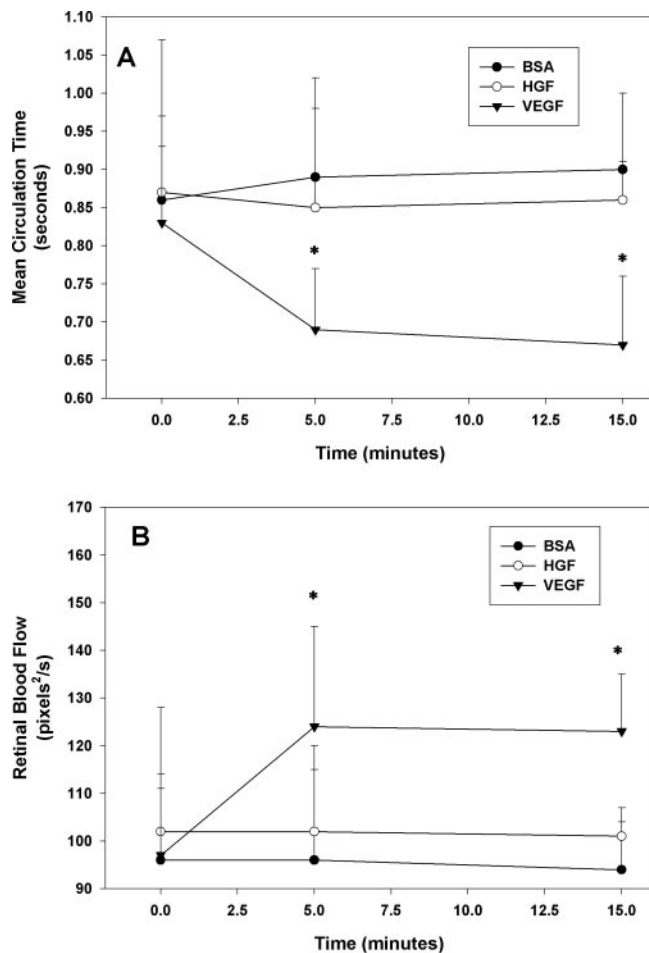
FIGURE 5. HGF, unlike VEGF, did not decrease mean circulation time or increase retinal blood flow. Retinal hemodynamics were assessed by video fluorescein angiography at baseline and after intravitreal injection of HGF (5 ng/eye), VEGF (2 ng/eye), or vehicle alone (0.1% BSA). MCT (A) was reduced and RBF (B) was increased at 5 and 15 minutes ($P < 0.05$) after intravitreal injection of VEGF. No change was observed with HGF or vehicle compared with baseline.

dominantly through the β isoform of PKC and the high-affinity kinase insert domain-containing receptor (KDR).[3] HGF mediates its effects through the cell surface tyrosine kinase receptor encoded by the c-MET proto-oncogene,[11,12] thought to be linked with downstream mediators by the effector protein Grb2.[32] However, despite independent pathways, HGF and VEGF together did not exert an additive effect on vasopermeability in vivo. Because treatment with inhibitors suppressed combined HGF/VEGF leakage by only 30% compared with a 90% suppression when treated with HGF alone, it is possible that VEGF continues to induce permeability at these concentrations, perhaps through activation of PKC.[3]

HGF/SF is a potent mitogenic and angiogenic factor that is associated with many types of cancer. One of the biological effects of HGF is to promote the matrix invasion of tumor cells by disruption of cell–cell tight junctions. HGF expression by malignant gliomas increases blood–brain barrier permeability.[43] In the eye, this effect would impair the integrity of the blood–retina barrier and contribute to the accumulation of plasma proteins in the surrounding matrix. The main signal-transduction pathways by which HGF/c-MET exerts its effects are the PI-3 kinase pathway, associated with cellular migration, and the Grb2/SOS/MAPK pathway, associated with proliferation. In bovine retinal endothelial cells in vitro, we have dem-

onstrated that HGF induces both MAPK phosphorylation and an increase in PI-3 kinase activity where MAPK phosphorylation was partially mediated by PI-3 kinase and to a lesser extent PKC.

In the present study, PI-3 kinase inhibition suppressed HGF-induced retinal permeability by 97% $\pm$ 59% ($P = 0.021$) without affecting basal activity. Thus, the increase in retinal permeability by HGF may be through the PI-3 kinase pathway with downstream activation of PKB/AKT and nitric oxide (NO). Okano et al.[44] demonstrated that the proliferative action of HGF is predominantly mediated through activation of PI-3 kinase and AKT in rat oval cells. Furthermore, stimulation of aortic endothelial cells with HGF leads to a PI-3-kinase/AKT-dependent phosphorylation of endothelial nitric oxide synthase (eNOS) within 5 minutes that would result in enzyme activation and NO production.[45] Shen et al.[46] found that HGF increases eNOS protein levels in adrenal capillary endothelial cells by the same extent as VEGF. The production of NO could activate the cyclic GMP/cyclic GMP-dependent kinase (PKG) pathway that can regulate intercellular junctional proteins.[47] Also a direct effect of NO on retinal permeability was demonstrated by the dose-dependent increase in vivo on retinal vascular permeability by the NO donor S-nitroso-N-acetylpenicillamine (SNAP) (Bursell S-E, et al. IOVS 1997;38:ARVO Abstract 1135). Although mechanistic studies based on inhibitors are not conclusive, our data showing marked inhibition of HGF-induced retinal permeability by inhibition of PI-3 kinase is consistent with these data, as it would effectively block the PKB/AKT/NO signaling cascade.

On receptor binding, HGF initiates the phosphorylation of MAPK through the Grb2/SOS/ras/raf pathway. Specifically, HGF binding phosphorylates both p44/p42 (ERK1/2) and p38 MAPKs. Experimental evidence suggests that HGF-induced MAPK activation initiates cell motility by targeting adherens junctions. Although the specific target proteins for HGF-induced junctional disruption have not been fully identified, the inhibition of ERK1/2 in ras-transformed MDCK cells leads to recruitment of the adherens proteins occludin, claudin-1, and ZO-1.[48] In the present study, HGF caused a rapid (<15 minutes) increase in RVP that was suppressed 86% $\pm$ 44% ($P = 0.015$) by inhibition of MAPK in vivo, suggesting that HGF is modulating RVP through a rapid signaling mechanism rather than junctional protein synthesis.

In contrast to the effect of HGF on retinal vascular leakage presented in the present study, other data have suggested that HGF can enhance endothelial cell barrier function and may tighten cell junctions.[34] In a study by Liu et al.,[34] transendothelial resistance of EC monolayers was increased after treatment with HGF. However, these results are in contrast to the HGF-induced increase in paracellular leakage observed by Jiang et al.[18] The opposing conclusions may arise due to differences in cell lines and the fact that in vitro EC monolayers may not correspond directly to responses in whole tissues in vivo. Microvascular endothelial cells require contact with vascular smooth muscle cells and pericytes for proper cellular communication and may respond differently compared with the arterial endothelial cells used in the study by Liu et al. Specifically, the activation PI-3 kinase has been linked to phosphorylation of AKT and eNOS which are thought to be associated with an enhancement in paracellular leakage.[49] Indeed, Shen et al.[46] demonstrated that treatment of capillary endothelial cells with HGF increases eNOS protein expression by the same magnitude as VEGF.

Inhibition of PKC using the non–isoform-specific GFX (0.5 μM) did not significantly suppress HGF-induced RVP. This is in contrast to another potent permeability agent, histamine, which activates PKC isoforms in addition to MEK and ERK, and whose action is suppressed by GFX.[3,50–53] PKC activation

increases vascular leakage and regulates vascular tone.[54] In diabetes, PKC activation occurs through the de novo synthesis of glucose and is activated locally by growth factors such as VEGF. Both diabetes-induced and receptor-mediated activation of PKC have been shown to modulate retinal vascular leakage specifically through the β isoform[3,42,55] HGF/SF binding to c-MET can phosphorylate PLC-γ, leading to the formation of IP3 and diacylglycerol in hepatocytes.[56] The production of IP3 and DAG increases intracellular Ca^{2+} and activates PKC. In fact, stimulation of bovine retinal endothelial cells with HGF led to a 37% increase in PKC activity in situ within 10 minutes.[26] In addition, studies by Sharma et al.[57] and Kermorgant et al.[58] have demonstrated that HGF activates PKCα and PKCε. However, the activation of PKCα by c-MET has been associated with the recycling of c-MET receptor through intracellular microtubules and modulation of the MAPK pathway rather than direct functional control.[26,58,59] HGF also induces PKCα translocation in corneal epithelial cells.[57] Thus, although HGF can induce PKC activation in cells, the specific isoforms and extent of PKC activation in retinal tissue is unknown, and our data suggest that HGF can induce retinal vascular permeability predominantly independent of its effects on PKC. Also, as demonstrated by Bursell et al.,[37] PKC plays a prominent role in the regulation of retinal blood flow in diabetes. The lack of c-MET modulated PKC action on vascular hemodynamics is supported by our finding of no HGF-induced alterations in retinal blood flow.

In summary, this study demonstrates that HGF increases retinal vascular permeability in a time- and dose-dependent manner, resulting in ocular permeability at physiologically relevant concentrations similar to that induced by VEGF. In contrast, retinal hemodynamics remain unaltered by HGF. Effects of HGF on retinal permeability appear to be mediated by PI-3 kinase and MAPK, but not predominantly by PKC. Thus, the effects of HGF and VEGF on RVP may be mediated by different pathways, and further studies to elucidate the mechanisms by which HGF induces these effects may lead to novel therapeutic targets for ocular conditions characterized by retinal vascular leakage.

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