RESEARCH ARTICLE

FASEB BioAdvances   WILEY

Hepatocyte growth factor is upregulated in ischemic retina and contributes to retinal vascular leakage and neovascularization

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Abstract

In patients with macular edema due to ischemic retinopathy, aqueous levels of hepatocyte growth factor (HGF) correlate with edema severity. We tested whether HGF expression and activity in mice with oxygen-induced ischemic retinopathy supports a role in macular edema. In ischemic retina, HGF was increased in endogenous cells and macrophages associated with retinal neovascularization (NV). HGF activator was increased in and around retinal vessels potentially providing vascular targeting. One day after intravitreous injection of HGF, VE-cadherin was reduced and albumin levels in retina and vitreous were significantly increased indicating vascular leakage. Injection of VEGF caused higher levels of vitreous albumin than HGF, and co-injection of both growth factors caused significantly higher levels than either alone. HGF increased the number of macrophages on the retinal surface, which was blocked by anti-c-Met and abrogated in chemokine (C-C motif) ligand 2 (CCL2)$^{-/-}$ mice. Injection of anti-c-Met significantly decreased leakage within 24 hours and after 5 days it reduced retinal NV in mice with ischemic retinopathy, but had no effect on choroidal NV. These data indicate that HGF is a pro-permeability, pro-inflammatory, and pro-angiogenic factor and along with its activator is increased in ischemic retina providing support for a potential role of HGF in macular edema in ischemic retinopathies.

KEYWORDS
diabetic macular edema, hepatocyte growth factor activator, retinal vein occlusion, vascular leakage

1 | INTRODUCTION

Ischemic retinopathies are diseases in which damage to retinal blood vessels results in areas of poorly perfused retina causing stabilization of hypoxia-inducible factor-1 (HIF-1) which stimulates expression of multiple hypoxia-regulated genes.[1,2] The two most common ischemic retinopathies are diabetic retinopathy and retinal vein occlusion (RVO) and in both the most common cause of vision loss is macular edema. Both diabetic macular edema (DME) and macular edema due to

Abbreviations: AMD, age related macular degeneration; C57BL/6, C57 Black; CCL2, chemokine (C-C motif) ligand 2; c-MET, tyrosine-protein kinase Met; DME, diabetic macular edema; GFAP, glial fibrillary acidic protein; HGF, hepatocyte growth factor; NV, neovascularization; OIR, oxygen-induced ischemic retinopathy; RVO, retinal vein occlusion.

RVO are substantially benefitted by suppression of VEGF,[3-7] but some patients with these disease processes have residual edema despite frequent anti-VEGF injections. Intraocular steroids, such as the dexamethasone implant (Ozurdex®), are second line treatment for patients with DME or RVO who respond sub-optimally to VEGF suppression and are felt to work by reducing the production of other undetermined pro-permeability factors. In the *O*zurdex for *R*etinal *V*ein *O*cclusion (ORVO) study, patients with macular edema poorly responsive to VEGF suppression had aqueous samples taken before and after intravitreous injection of a dexamethasone implant.[8] Most of these patients showed substantially greater edema reduction after injection of a dexamethasone implant than after injection of a specific inhibitor of VEGF. In many such patients, protein arrays demonstrated that hepatocyte growth factor (HGF) was high at baseline, decreased as edema decreased and increased as edema returned. A similar experimental design was used in patients with DME poorly responsive to VEGF suppression in the *D*iabetic *M*acular *E*dema treated with *O*zurdex (DMEO) trial and once again several patients had high aqueous levels of HGF at baseline that decreased as edema improved and increased as edema worsened.[9]

Hepatocyte growth factor is best known for its effects in cancer. It was isolated from platelets and found to stimulate hepatocyte growth.[10] Subsequently it was found to be identical to scatter factor, a fibroblast-derived stimulator of epithelial cell motility.[11,12] The receptor for HGF, tyrosine-protein kinase Met (c-Met), was first isolated from a human osteosarcoma cell line in which there was a DNA rearrangement fusing the translocated promoter region to the *MET* gene resulting in high expression of c-Met.[13] Many tumors, particularly melanomas, have mutations in *MET* that result in high expression or constitutive stimulation, and high levels of c-Met and/or HGF are associated with poor prognosis in many types of cancer.[14-16] The activities of HGF/c-Met signaling most linked to tumor progression are mitogenesis, migration, invasive growth, and metastasis[17,18]; however, stimulation of angiogenesis has also been implicated.[19-23] HGF and c-Met are increased in hypoxic tissue[2,24] and have been implicated as a potential contributors to proliferative diabetic retinopathy,[25-27] but there has been little evidence to support a role in macular edema. In this study, we sought to determine if the expression and activities of HGF in mice with ischemic retinopathy support a role for HGF in macular edema in hypoxic retina.

2 | MATERIALS AND METHODS

2.1 | Mouse model of OIR

Mice were treated in accordance with the Association for Research in Vision and Ophthalmology Guidelines on the care and use of animals in research. Oxygen-induced ischemic retinopathy (OIR) was produced by placing litters of C57BL/6 mice in $75 \pm 3\%$ oxygen at P7 and returning them to room air at P12.[28] The retina immediately becomes hypoxic upon return to room air as demonstrated by upregulation of HIF-1α within 6 hours[1] and remains hypoxic beyond P17, the peak of neovascularization (NV). Mice with OIR were euthanized at P13, P15, or P17 for studies described below.

2.2 | Mouse model of choroidal NV due to laser-induce rupture of Bruch's membrane

Laser photocoagulation-induced rupture of Bruch's membrane was used to generate choroidal NV as previously described.[29] Briefly, 4- to 5-week-old C57BL/6 mice were anesthetized, pupils were dilated with 1% tropicamide (Alcon Labs, Inc), and Bruch's membrane was ruptured at 9, 12, and 3 o'clock positions of the posterior pole with 532 nm diode laser photocoagulation (75 μm spot size, 0.1 s duration, 140 mW) using the slit lamp delivery system of an OcuLight GL Photocoagulator (Iridex, Mountain View) and a handheld cover slide as a contact lens.

2.3 | Effect of HGF or anti-c-Met on retinal and choroidal NV

Intravitreous injections were done in mice with OIR at P12 and in adult mice immediately after rupture of Bruch's membrane. A pulled glass micropipette was used to penetrate the sclera 1 mm posterior to the limbus and a foot switch activated a pump to inject 1 μL containing 3 mmol/L anti-c-Met (R&D Systems) or 6.25 mmol/L recombinant human HGF (Peprotech) in one eye, and 3 mmol/L mouse IgG (Equitech-Bio, Kerrville, TX, USA) or 7.5 mmol/L albumin (Sigma-Aldrich) in the fellow eye.

Mice with OIR were euthanized at P17, eyes were fixed in 10% phosphate-buffered formalin for 4 hours at 22°C, retinas were dissected out intact, stained with Alexa Fluor 488-conjugated *Griffonia simplicifolia* agglutinin (GSA) lectin (1:250; Invitrogen) for 45 minutes, flat mounted and examined by fluorescence microscopy. Photographs were obtained at 5× magnification and merged into a single image to show the entire retina using Photoshop CS5.1. An observer masked with regard to treatment group measured the area of NV per retina by image analysis using image analysis with Image-Pro Plus software (Media Cybernetics).

Mice with rupture of Bruch's membrane were euthanized 7 days after laser, eyes were fixed in 10% phosphate-buffered formalin for 4 hours at 22°C, retinas were removed and eyecups containing retinal pigmented epithelium, choroid, and sclera were stained with GSA lectin and flat mounted. Flat

mounts were examined by fluorescence microscopy and the area of choroidal NV at each Bruch's membrane rupture site was measured by a masked investigator and the three measurements in each eye were averaged to provide a single experimental value.

2.4 | Immunostaining of ocular sections from mice with OIR

Mice with OIR or controls were euthanized at P15 or P17 and eyes were frozen in optimum cutting temperature compound (Sakura-Finetek) and stored at −80°C. Ten μm sections were fixed in 4% paraformaldehyde (PFA) for 5 minutes at 22°C, washed with PBS and blocked 45 minutes in PBST containing 10% chicken serum (Sigma-Aldrich). Sections were incubated overnight at 4°C with goat anti-mouse HGF antibody (1:80; R&D Systems), goat anti-mouse HGF activator (HGFA) antibody (1:50; R&D Systems), or rabbit anti-glial fibrillary acidic protein (GFAP) (1:100; Dako). The anti-mouse HGFA antibody predominantly binds the inactive precursor of HGFA, pro-HGFA, and binds HGFA will lower affinity. Sections were washed and incubated in Alexa Fluor 647- or 594-conjugated GSA, Alexa Fluor 594-conjugated chicken anti-rabbit (1:650), Alexa Fluor 488-conjugated chicken anti-goat (1:650) or Alexa Fluor 594-conjugated chicken anti-goat (1:650) for 1 hour at 22°C; and then incubated for 5 minutes in Hoescht (1/3000; Sigma-Aldrich). Sections were washed once with PBST and twice with PBS and then mounted and visualized using a confocal laser-scanning microscope.

2.5 | Immunostaining of retinal flat mounts

Mice with OIR or controls were euthanized at P17 and eyes were fixed with 4% PFA for 1 hour at 22°C. Retinas were dissected intact and blocked and permeabilized in phosphate-buffered saline (PBS) containing 10% horse serum (Sigma-Aldrich) and 0.3% Triton-X-100 (PBST) for 1 hour at 22°C. Then the tissues were incubated in anti-mouse HGF antibody (1:100; R&D Systems) and brilliant violet 421-conjugated anti-mouse F4/80 (1:100; Biolegend) overnight at 4°C in PBST. Retinas were washed with PBST and incubated with Alexa Fluor 594- or 488-conjugated GSA lectin (1:250; Invitrogen), and anti-mouse F4/80 Brilliant blue 421-conjugated primary (1:150; Biolegend) overnight at 4°C. The retinas were washed with PBS and examined using a confocal laser-scanning microscope (Zeiss LSM 710; Carl Zeiss, Inc). For the quantification of F4/80 positive cells, complete retinal whole mount images were analyzed and the total number of positive F4/80 cells in each retina was quantified using *ImageJ software* (National Institutes of Health).

2.6 | Quantitative real-time PCR

Mice with OIR or controls were euthanized at P13, P15, or P17, retinas were dissected and homogenized in 200 μL Trizol. To test the effect of overexpression of VEGF on expression of HGF, P21, and P30 rho/VEGF transgenic mice in which the rhodopsin promoter drives high expression of VEGF in the retina[30] were euthanized and retinas were dissected and homogenized in 200 μL Trizol. In other experiments, adult C57BL/6 mice were anesthetized and given an intraocular injection of 1 μL of 6.25 mmol/L HGF or 6.25 mmol/L VEGF (R&D Systems) in one eye and PBS in the fellow eye. Mice were euthanized at 1 or 3 days after injection and retinas were removed and homogenized in Trizol. Total RNA was extracted with Direct-zol™ RNA kits (Zymo Research) in which centrifuge columns were pre-treated with DNAase to remove contaminating genomic DNA. Total RNA was suspended in RNAase free water and placed in RNAase-free tubes. The concentration of extracted RNA was measured with the *NanoDrop 2000* spectrophotometer (Thermo Fisher Scientific), normalized to the lowest concentration, reverse transcribed with Superscript III (Invitrogen). All cDNA samples were aliquoted and stored at −80°C. PCR primers targeting mouse HGF (*F: TGACCTGCAATGGTGAAAGC, R: GGGTCAAGAGTGTAGCACCA*), VEGF (*F: CACGGGA GACAATGGGATGA, R: CTGGAAGTGAGCCAATGTGC*), mouse CCL2 (F: TTAAAAACCTGGATCGGAACCAA, R: GCATTAGCTTCAGATTTACGGGT), and cyclophilin A as housekeeping gene, were used. Standard curves were made for each product before quantification. Real-time quantitative PCR was performed using a Rotor-Gene Q instrument and Rotor-Gene SYBR Green PCR Kit (Qiagen). The mRNA for each target gene was normalized to 10^6 copies of cyclophilin A. A standard curve was made for each molecule before quantification and fold changes in genes expression were calculated using ΔΔCt values.[31]

2.7 | Measurement of HGF effect on retinal vascular leakage

Adult C57BL/6 mice were anesthetized and had intraocular injection of 1 μL of 6.25 μmol/L HGF or VEGF$_{165}$ (R&D Systems) in one eye and PBS in the fellow eye. At 1 and 3 days after injection, some of the mice were anesthetized and chests were opened to expose the heart; a 27-gauge cannula was inserted into left ventricle, the right atrium was cut for drainage, and the vasculature was flushed with PBS for 3 minutes, and retinas were dissected intact. Retinas or vitreous samples were sonication in PBS/0.3% Triton buffer containing phosphatase and protease inhibitors (Roche), centrifugation at 9200 *g* for 10 minutes, and supernatants were stored at −20°C. Albumin levels in retinal or vitreous samples were

measured using the albumin ELISA kit (ab108791, Abcam). The protein concentrations of retinal lysates were measured by the BioRad protein assay and 2 μg of total protein was added to each well. One μL of each vitreous sample was added to each well. Plates were read at 450 and at 570 nm and albumin levels were determined by plotting values on a standard curve.

2.8 | Effect of anti-c-Met on vascular leakage in mice with OIR

At P17, mice with OIR were given an intraocular injection of 1 μL of 3 mmol/L anti-c-Met in one eye and 1 μL of 3 mmol/L mouse IgG in the fellow eye. After 24 hours, mice were euthanized, vitreous samples were collected and vitreous albumin levels were measured as described above.

2.9 | Measurement of retinal leukostasis

Adult C57BL/6 mice were given an intravitreous injection of 1 μL of 6.25 mmol/L HGF or 6.25 mmol/L VEGF in one eye and 1 μL of PBS in the fellow eye. Twenty-four hours after injection, leukostasis was measured as previously described.[32] Briefly, mice were anesthetized, the chest was opened to expose the heart, the right atrium was cut for drainage, a 27-gauge cannula was inserted into left ventricle, and the vasculature was flushed with PBS for 3 minutes to removal all nonadherent cells. Mice were then perfused over a span of 2-3 minutes with rhodamine- or FITC-labeled conA (20 μg/mL in PBS, 5 mg/kg; RL-1002 and FL-1001; Vector Labs Thermofisher). The vasculature was flushed for 4 minutes with PBS to remove residual unbound con A. In some experiments, the vasculature was also perfused with fluorescein-labeled dextran (Sigma-Aldrich). Eyes were removed and fixed in 2% PFA for 2 hours at room temperature and retinas were dissected and flat mounted. Retinas were examined by fluorescence microscopy and the number of intravascular leukocytes throughout the entire retina were counted by an investigator masked with respect to treatment group.

2.10 | Immunoblots

Fifty μg of retinal lysates were added to Bolt LDS Sample Buffer (Thermo Fisher Scientific), resolved by 4%-12% gradient SDS-PAGE and transferred to PDVF membranes for immunoblot analysis. Membranes were probed with primary antibodies against mouse VE-Cadherin (1:2500; R&D Systems); mouse HGFA (1:5000; R&D Systems) and actin (1:2000; Cell Signaling). Secondary antibodies were donkey

anti-rabbit (1:10 000, GE Healthcare) and rabbit anti-goat (1:20 000, Thermo Fisher Scientific).

2.11 | Statistics

In experiments in which an experimental group was compared to its own control group, statistical comparisons were made by unpaired Student's t test. In experiments in which multiple experimental groups were compared, comparisons were made by 2 way-ANOVA with using Newman-Keuls Multiple Comparison Test or one way-ANOVA with Bonferroni's correction for multiple comparisons.

3 | RESULTS

3.1 | HGF is increased in ischemic retina

There was faint immunofluorescent staining for HGF in the retinas of room air-raised P15 mice (Figure 1A, left column) that could be related to retinal vascular development, because it was less in the retinas of P17 mice raised in room air (Figure 1A, third column). The retinas of mice with OIR[28] mimic aspects of the retinas of patients with ischemic retinopathies including retinopathy of prematurity, proliferative diabetic retinopathy, and RVO. Staining for HGF was increased in ischemic retina of mice with OIR at P15 and P17 (Figure 1A, top row). The difference in HGF staining between non-ischemic and ischemic retina at P17 is best seen in the magnified view in Figure 1B, top row. Visualization of retinal vascular endothelial cells by staining with GSA lectin shows normal superficial, intermediate, and deep capillaries in the retinas of P15 and P17 retinas from room air-raised mice and retinal NV on the surface of the retina of OIR mice that was mild at P15 and severe at P17 (Figure 1A,B, second row). Some of the HGF staining co-localized with staining for GFAP, indicating that it was present in astrocytes on the surface of the retina and Muller cells throughout the inner retina, but there were also many HGF-stained cells that were not GFAP-positive, indicating that other cells in the inner retina also expressed HGF (Figure 1A,B, third row). Quantitative real time PCR confirmed a significant increase in expression of *hgf* mRNA in retinas of P15 and P17 OIR mice compared with RA-reared mice (Figure 1C).

Immunofluorescent staining of retinal flat mounts from P17 room air-reared mice showed HGF-positive (Figure 2A), F4/80-positive (Figure 2B) macrophages on the surface of the retina with some in close association with superficial retinal vessels (Figure 2C, arrowheads). Retinal flat mounts from P17 mice with OIR showed an increase in HGF-positive (Figure 2D), F4/80-positive (Figure 2E)
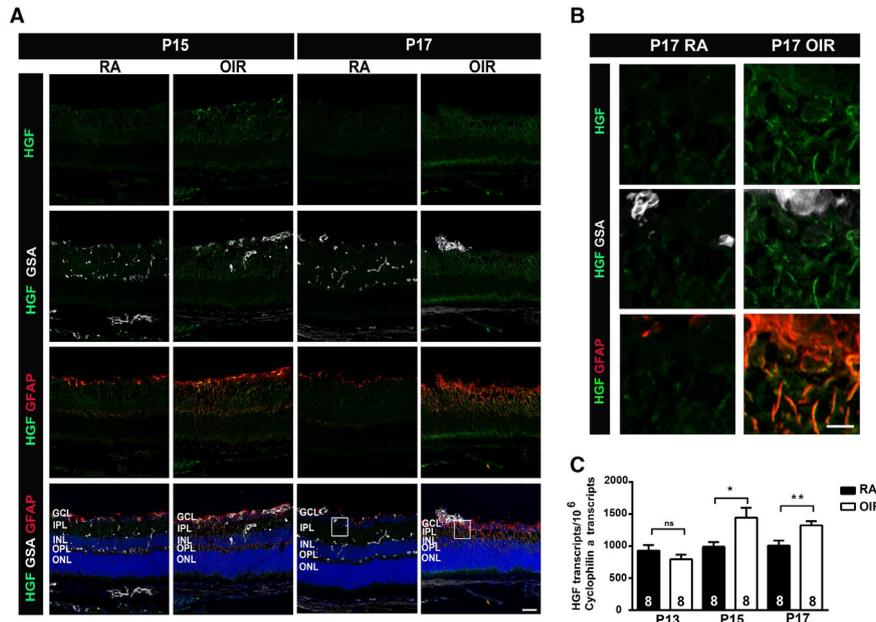
FIGURE 1 HGF is increased in ischemic retina. At P15 and P17, mice with oxygen-induced ischemic retinopathy and control mice raised in room air were euthanized and 10 μm ocular frozen sections were immunostained for HGF (green) and GFAP (red), histochemically stained with GSA lectin (white) which stains vascular cells, and Hoechst (blue) to stain nuclei (A). The retinal layers, GCL, IPL, INL, outer plexiform layer, and ONL, are labeled in the panels of the bottom row (scale bar = 50 μm). A magnified view of the regions within the rectangles are shown in (B) (scale bar = 25 μm). Retinas were dissected from P13, P15, and P17 mice with OIR and RA controls (n = 8 for each time point) and quantitative real time PCR was used to measure the number of HGF transcripts per 10^6 cyclophillin A transcripts (C). Bars represent mean (± SEM). **$P < .05$, *$P < .01$ for difference from control by unpaired t test. GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; HGF, hepatocyte growth factor; INL, inner nuclear layer; IPL, inner plexiform layer; OIR, oxygen-induced ischemic retinopathy; ONL, outer nuclear layer; RA, room ari
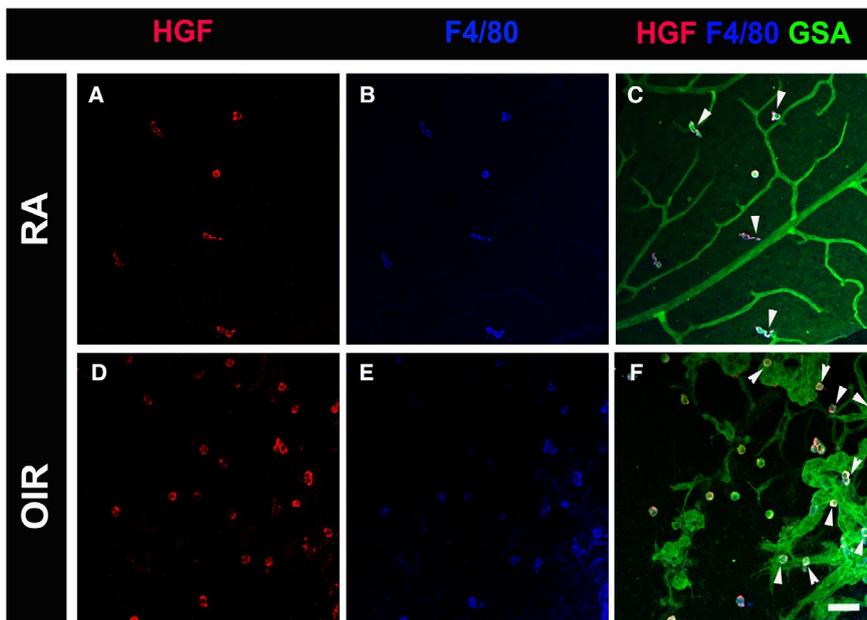

FIGURE 2 Ischemic retina contains increased HGF-positive macrophages in close proximity to NV. At P17, mice with OIR or RA controls were euthanized and retinas were dissected intact, stained with anti-HGF (red) and F4/80 (blue), flat mounted, and examined by confocal microscopy. Retinas of RA control mice had sparse HGF-positive (A), F4/80-positive (B) macrophages on the surface of the retina in close association with GSA (green) lectin-stained blood vessels (C). Retinas of OIR mice showed increased numbers of HGF-positive macrophages (D, E) on the surface of the retina in close proximity to NV (F). Scale bar = 50 μm. GSA, *Griffonia simplicifolia* agglutinin; HGF, hepatocyte growth factor; NV, neovascularization; OIR, oxygen-induced ischemic retinopathy; RA, room air

macrophages many of which were closely associated with NV (Figure 2F).

3.2 | The precursor of HGFA is increased in ischemic retina

Hepatocyte growth factor is synthetized in an inactive pro-form (pro-HGF) and cleavage by a serine protease is required to convert pro-HGF to active, mature HGF.[33] A particularly important protease is HGFA,[34] which is increased in injured or hypoxic tissues.[35,36] HGFA is derived from an enzymatically inactive precursor, pro-HGFA, which is proteolytically cleaved to HGFA.[34] Immunoblots with an antibody that binds pro-HGFA with high affinity and HGFA with lower affinity, showed a more prominent band in retinal homogenates from OIR mice vs those from room air-raised mice (Figure 3A). Densitometry showed a significant increase in the pro-HGFA/actin ratio at P13 and P15 in retinas from mice with OIR vs those from control mice (Figure 3B). There was no detectable immunofluorescent staining for pro-HGFA in the retinas of P15 control mice at low (Figure 3C) or high magnification (Figure 3D), but there were many lightly stained cells primarily in the inner retina of P15 OIR mice (Figure 3E) best seen with high magnification (Figure 3F). Compared with GSA lectin-stained retinal vessels in P15 control mice (Figure 3G,H), vessels in P15 mice with OIR were dilated and showed staining for pro-HGFA (Figure 3I,J). While staining was strongest in vascular cells, there was also staining for pro-HGFA in non-vascular cells around vessels. Staining of nuclei with Hoesch allowed identification of all of the retinal layers (Figure 3K-N) and much of the non-vascular staining for pro-HGFA in retinas from mice with OIR was in the inner plexiform layer (Figure 3M,N). The predominantly vascular localization is interesting and may provide a means of targeting active HGF to endothelial cells.

3.3 | HGF causes retinal vascular leakage

One or 3 days after intravitreous injection of 1 μL of 6.25 mmol/L HGF or 6.25 mmol/L VEGF in one eye and PBS in the fellow eye, C57BL/6 mice were perfused through the left ventricle with PBS to remove intravascular proteins and the concentration of albumin in retinal homogenates was measured by ELISA. At 1 day after injection, the mean retinal concentration of albumin was significantly increased in eyes injected with HGF compared with fellow eyes injected with PBS (Figure 4A). Extravascular albumin diffuses into the avascular vitreous and albumin measurements in vitreous samples eliminate the need for perfusion to remove all intravascular albumin. The experiment was repeated and

albumin was measured in vitreous samples. One day after intravitreous injection of 1 μL of 6.25 mmol/L HGF, mean albumin concentration in the vitreous was significantly higher than that in PBS-injected fellow eyes, but not as high as that in eyes injected with an equimolar amount of VEGF (Figure 4B). The VEGF-induced leakage was transient and the excess albumin was cleared from the vitreous within days, because at 3 days after injection, mean albumin level in VEGF-injected eyes was similar to that in fellow eye controls; however, there was still significant elevation above controls in HGF-injected eyes suggesting more prolonged barrier disruption. Co-injection of VEGF and HGF resulted in a significantly higher mean vitreous albumin concentration than either alone (Figure 4C), suggesting additive effects on vascular leakage. One day after intravitreous injection of 1 μL of 6.25 mmol/L HGF or PBS, immunoblots of retinal homogenates showed a significant reduction in VE-cadherin in retinas from HGF-injected eyes compared with those from eyes injected with PBS (Figure 4D). Compared with retinas from eyes injected with PBS, those from eyes injected with 1 μL of 6.25 mmol/L HGF showed no difference in mean *vegf* mRNA level at 1 day ($P = .16$) or 3 days ($P = .90$) after injection (Figure 4E). Conversely, retinas from eyes injected with 1 μL of 6.25 mmol/L VEGF had no difference in mean level of *hgf* mRNA compared with retinas from eyes injected with PBS at 1 ($P = .46$) or 3 days ($P = .28$) after injection (Figure 4F). Similar to an injection of VEGF, sustained expression of VEGF did not increase expression of HGF in the retina, because at P21 and P30, mean retinal level of *hgf* mRNA was not significantly different in rho/ VEGF transgenic mice with increased expression of VEGF in the retina compared with age-matched wild-type mice (Figure 4F, $P = .66$ at P21 and $P = .24$ at P30) Therefore high levels of HGF do not increase expression of VEGF in the retina, and high levels of VEGF do not increase expression of HGF.

3.4 | HGF increases extravascular macrophages in the retina, but does not cause leukocytic plugging in retinal vessels

One day after intravitreous injection of 1 μL of 6.25 mmol/L HGF, the density of F4/80-positive macrophages on the retinal surface was dramatically greater than that seen after injection of PBS (Figure 5A, top two rows and Figure 5B). Compared with eyes injected with HGF, those injected with anti-c-Met prior to injection of HGF showed a striking reduction in macrophages on the retinal surface (Figure 5A, row 3 and Figure 5B), indicating that stimulation of c-Met on macrophages was necessary for their recruitment. Interestingly, chemokine (C-C motif) ligand 2 (CCL2)$^{-/-}$ mice also failed to show HGF-induced macrophage recruitment into the
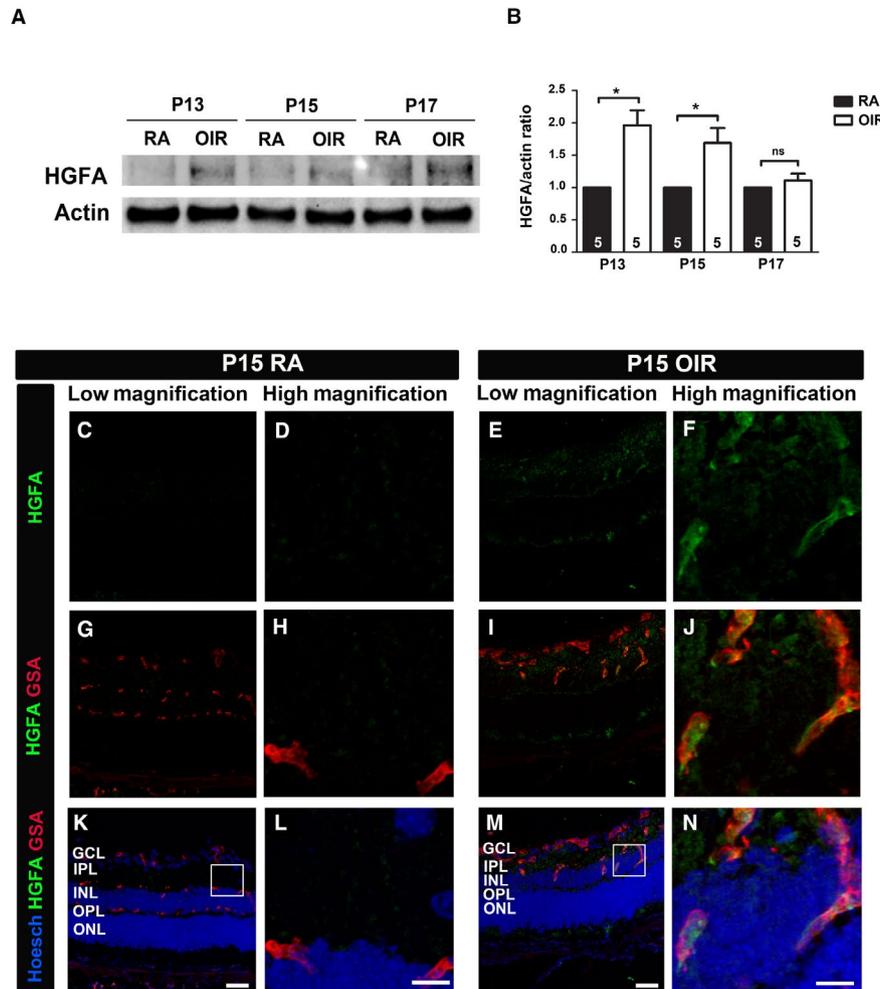
FIGURE 3 HGFA is increased in ischemic retina primarily in and around retinal blood vessels. At P13, P15, and P17, mice with OIR or RA controls were euthanized and immunoblots showed increased levels of HGFA in retinal homogenates of OIR mice compared with RA mice at P13 and P15 (A and B). Bars show the mean (± SEM) HGFA/Actin ratio of densitometry readings for blots from five mice in each group (*$P < .05$ for difference from corresponding RA control by unpaired t test). Frozen ocular sections from P15 RA controls and OIR mice were immunostained for HGFA (green, C-N), histochemically stained with GSA lectin to stain vascular cells (red, G-N), and nuclei were stained with Hoechst (blue, K-N). Low magnification images (C, E, G, I, K, M; scale bar = 50 μm) and high magnification images of the region within the rectangle in K (D, H, L; scale bar = 15 μm) and M (F, J, N; scale bar = 15 μm) are shown. There was no detectable staining for HGF in retinas from RA control mice at low (C, G, K) or high magnification (D, H, L), but clearly identifiable staining in the retinas of OIR mice (E, F), primarily in and around retinal vessels (I, J). The retinal layers, GCL, IPL, INL, outer plexiform layer, and ONL, are labeled in the panels with nuclear stain (K-N). GCL, ganglion cell layer; GSA, *Griffonia simplicifolia* agglutinin; HGF, hepatocyte growth factor; HGFA, HGF activator; INL, inner nuclear layer; IPL, inner plexiform layer; OIR, oxygen-induced ischemic retinopathy; ONL, outer nuclear layer; RA, room air

retina, indicating that prior activation of macrophages with CCL2 was necessary for macrophage responsiveness to HGF (Figure 5A bottom row and Figure 5B). In wild-type mice, retinal mRNA for CCL2 was significantly increased after intraocular injection of HGF (Figure 5C).

We have previously demonstrated that increased intraocular levels of VEGF increase intravascular adherent leukocytes in the retina causing leukocytic plugging of retinal vessels,[32] however extravascular F4/80-positive macrophages were not increased by intraocular injection of 1 μL of 6.25 mmol/L VEGF (Figure 5B). Conversely, intravascular leukocytes were not increased by intraocular injection of 1 μL of 6.25 mmol/L HGF (Figure 6A,B). However, high

magnification views of retinal flat mounts from eyes injected with HGF showed macrophages adherent to the outer wall of retinal vessels. Many of the macrophages in PBS-injected or HGF-injected eyes stained with GSA lectin as well as F4/80 as previously described[37] and were closely associated with retinal vessels (Figure 6C).

3.5 | HGF contributes to ischemia-induced retinal NV and retinal vascular leakage

At P12 in mice with OIR, the onset of the ischemic period, eyes were given an intravitreous injection of 1 μL containing

A Retina

B Vitreous

C Vitreous

D

E

F
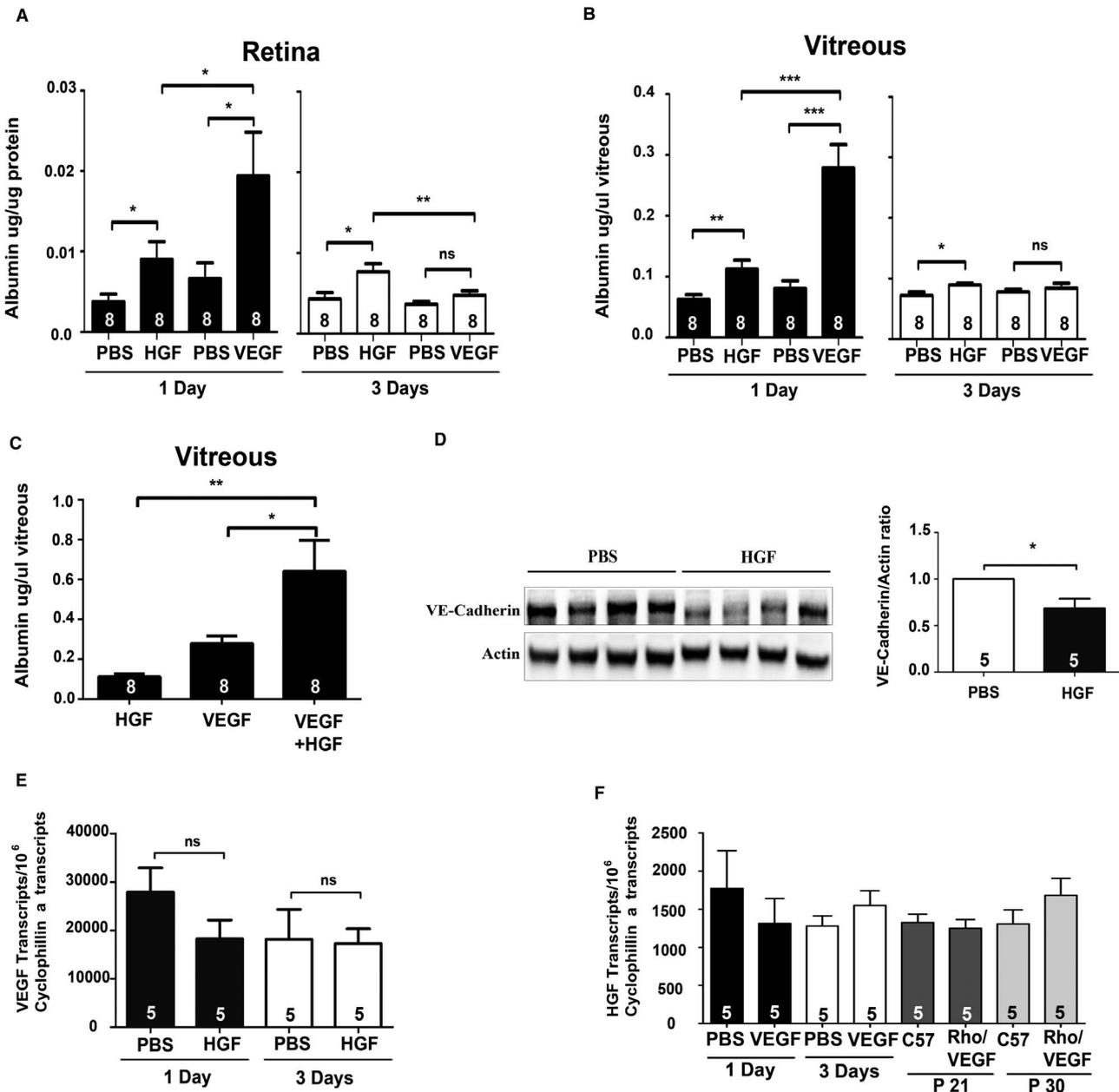
6.25 mmol/L HGF, 7.5 mmol/L albumin, 3 mmol/L anti-c-Met antibody, or 3 mmol/L control IgG. At P17, the area of retinal NV per retina was measured on retinal flat mounts. Compared with eyes injected with albumin, those injected with HGF had a significant increase in the mean area of retinal NV (Figure 7A, top row), and compared with those injected with control IgG, those injected with anti-c-Met had a significant reduction in mean area of retinal NV (Figure 7B). This suggests that HGF contributes to ischemia-induced retinal NV.

To test the effect of HGF on vascular leakage in mice with OIR, it is necessary to use a brief treatment period that is too short to have an effect on NV. At P17 mice with OIR were given an intraocular injection of 1 µL of 3 mmol/L anti-c-Met in one eye and 1 µL of 3 mmol/L control IgG in the fellow eye. After 24 hours, the concentration of vitreous albumin

was significantly less in mice treated with anti-c-Met (Figure 7C).

3.6 │ Exogenous HGF stimulates choroidal NV at Bruch's membrane rupture sites, but endogenous HGF is not a major contributor

Laser-induced rupture of Bruch's membrane in mice is a widely used model that predicted the importance of VEGF in neovascular age-related macular degeneration.[29,38,39] Immediately after rupture of Bruch's membrane, eyes were given an intravitreous injection of 1 µL containing 6.25 mmol/L HGF, 7.5 mmol/L albumin, 3 mmol/L anti-c-Met, or 3 mmol/L control IgG and after 7 days the area of choroidal NV at Bruch's membrane rupture sites was

FIGURE 4 HGF reduces VE-cadherin and causes retinal vascular leakage. Adult C57BL/6 mice were given an intravitreous injection of 1 μL of 6.25 mmol/L HGF or 6.25 mmol/L VEGF in one eye and 1 μL of PBS in the fellow eye. At 1 or 3 days after injection, the mice were perfused through the left ventricle with PBS and retinas were removed (A), or vitreous samples were obtained (B). Retinal lysates had measurement of protein levels and measurement of albumin by ELISA. The bars show the mean ($\pm$ SEM) albumin level per μg protein with n shown within bars (A). In (B), the bars show the mean ($\pm$ SEM) albumin level per μL of vitreous measured by ELISA. (C) In an independent experiment, mice were given an intravitreous injection in one eye of 1 μL of 6.25 mmol/L HGF, 6.25 mmol/L VEGF, or 6.25 mmol/L of each. Vitreous samples were obtained 1 day after injection and vitreous albumin levels were measured by ELISA. The bars show the mean ($\pm$ SEM) albumin level per μL of vitreous. (D) One day after injection of 1 μL of 6.25 mmol/L HGF in one eye and PBS in the fellow eye, retinal lysates were immunoblotted for VE-Cadherin and actin. Bars represent the mean ($\pm$ SEM) VE-Cadherin/Actin ratio of densitometry readings for blots from five mice in each group. (E) One or 3 days after intravitreous injection of 1 μL of 6.25 mmol/L HGF in one eye and PBS in the fellow eye, retinas were isolated and quantitative real time PCR was done to measure mRNA for VEGF and cyclophillin. Bars represent the mean ($\pm$ SEM) *vegf* transcripts per 10^6 *cyclophillin* transcripts and there was no significant difference between retinas from HGF-injected and PBS-injected eyes. (F) One or 3 days after intravitreous injection of 1 μL of 6.25 mmol/L VEGF in one eye and PBS in the fellow eye, retinas were isolated and quantitative real time PCR was done to measure mRNA for HGF and cyclophillin. Bars represent the mean ($\pm$ SEM) *hgf* transcripts per 10^6 *cyclophillin* transcripts and there was no significant difference between retinas from VEGF-injected and PBS-injected eyes. At P21 and P30, transgenic mice in which the *rhodopsin* promoter drives expression of VEGF in the retina (*rho/VEGF* mice) and control wild-type mice were euthanized, retinas were isolated, and quantitative real time PCR was done to measure mRNA for HGF and cyclophillin. Bars represent the mean ($\pm$ SEM) *hgf* transcripts per 10^6 *cyclophillin* transcripts in the retina and there was no significant difference between retinas from rho/VEGF and wild-type (C57) mice at P21 or P30. *$P < .05$, **$P < .01$, and ***$P < .001$ for difference from control by ANOVA with correction for multiple comparisons using Newman-Keuls Multiple Comparison Test (A-C) or by unpaired *t* test (D-E). HGF, hepatocyte growth factor

measured. Compared with eyes injected with albumin, the mean area of choroidal NV was significantly greater in eyes injected with HGF (Figure 7D), indicating that HGF stimulates the growth of choroidal NV. However, compared with eyes injected with control IgG, the mean area of choroidal NV was not significantly less in eyes injected with anti-c-Met (Figure 7E), suggesting that endogenous HGF is not a major contributor in this model.

4 | DISCUSSION

Recent clinical trials have implicated HGF as a potential contributor to macular edema in patients with DME or RVO in whom suppression of VEGF has a suboptimal response.[8,9] The primary purpose of this study was to determine if the expression and activity of HGF in mouse models provide support for such a role for HGF. In mice with ischemic retinopathy, expression of HGF was increased in both glial and neuronal cells throughout the inner retina and in mononuclear cells that closely associated with NV on the surface of the retina. Compared with normal control mice, the retinal level of *hgf* mRNA was increased in mice with OIR confirming the findings of Columbo et al.[25] It is also consistent with studies that have measured increased levels of HGF in the vitreous of patients with proliferative diabetic retinopathy[26,27] or central RVO[40] compared with controls. However, increased levels of immunoreactive HGF or mRNA for HGF is not necessarily indicative of increased HGF activity, because an inactive form of HGF, pro-HGF is secreted from hypoxic cells, and must be cleaved by serine proteases to form active HGF.[33] The primary serine protease responsible for this is HGFA, which is also derived from a larger inactive

protein, pro-HFGA. There was no detectable pro-HGFA in normal retina, but it was present in ischemic retina, predominantly in and around vascular cells, which could serve to target active HGF to endothelial cells. Homing of HGF-positive macrophages to vessels (Figure 2) may play a similar role.

Previous studies focused on possible contribution of HGF to ischemia-induced retinal NV. Our data support this possibility because suppression of HGF signaling by blocking c-Met reduced retinal NV in mice with OIR confirming the findings of Columbo et al.[25] However, we were particularly interested in whether HGF could contribute to macular edema in ischemic retinopathies, in which VEGF is important, but is not the only stimulator of retinal vascular leakage. Compared with injection of PBS, intravitreous injection of HGF caused greater vascular leakage as evidenced by elevated albumin in the retina or vitreous. We have recently demonstrated that albumin levels in the vitreous correlate well with assessment of vascular leakage severity by fluorescein angiography.[41] Additional validation of the usefulness of this approach is provided in the current study because the relative changes in vitreous albumin levels after HGF injection mirrored the changes in retinal albumin levels of perfused mice in which all intravascular albumin was eliminated. These results are consistent with the observation that intravitreous injection of HGF in rats causes increased leakage measured by vitreous fluorophotometery.[42] Compared with intravitreous injection of HGF, injection of an equimolar amount of VEGF caused more than twice as much leakage, but interestingly co-injection of HGF and VEGF caused significantly greater leakage than either alone. HGF has previously been shown to act synergistically with VEGF to enhance endothelial cell survival and tube formation in collagen gels, and corneal angiogenesis,[43] and the current study indicates that VEGF and HGF also work cooperatively in promoting vascular leakage in
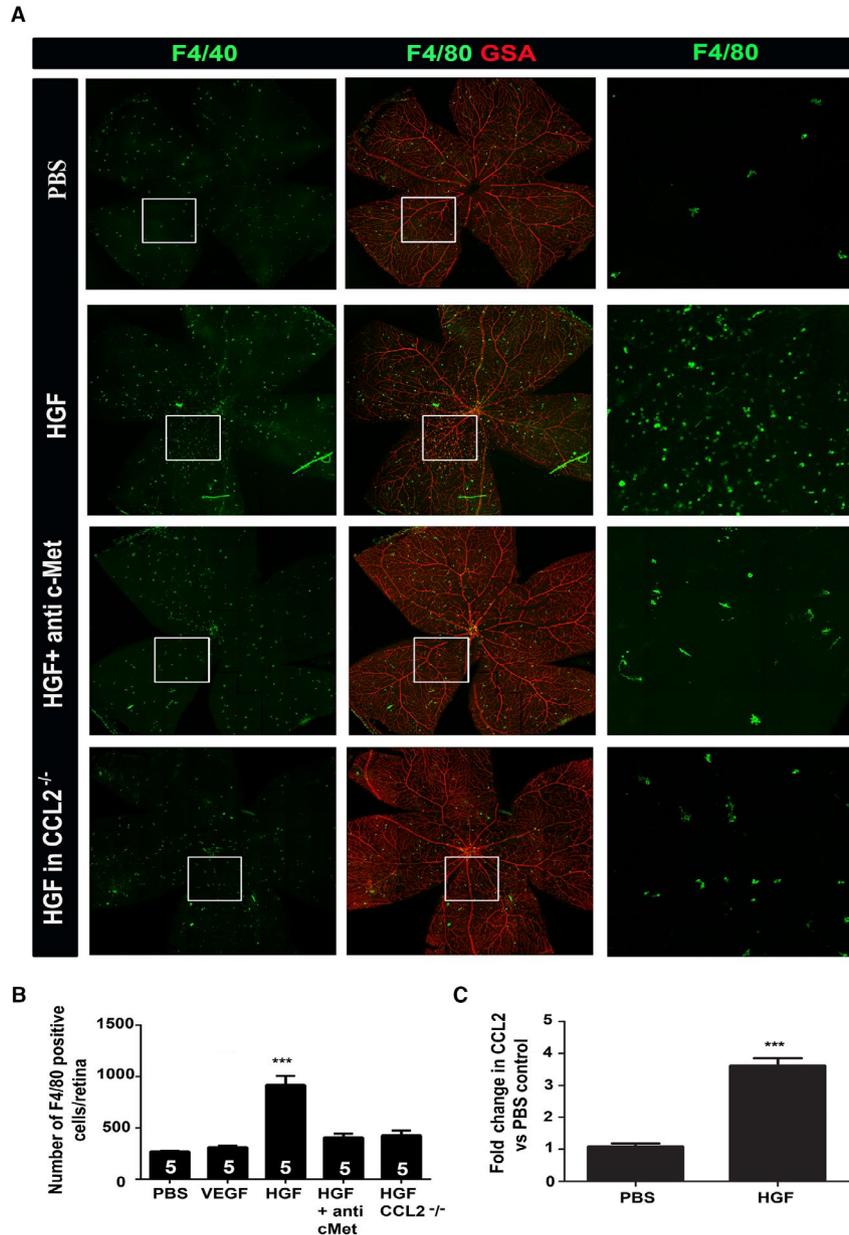
FIGURE 5 HGF recruits macrophages into the retina through stimulation of c-Met and requires CCL2. (A) Adult C57BL/6 mice (n = 5) or CCL2$^{-/-}$ mice in a C57BL/6 background (n = 5) were given an intravitreous injection of 1 µL of 6.25 mmol/L HGF in one eye and 1 µL of PBS in the other eye. Another group of C57BL/6 mice (n = 5) was given an injection of 3 mmol/L anti-c-Met in each eye and 3 days later received an intravitreous injection of 1 µL of 6.25 mmol/L HGF in one eye and 1 µL of PBS in the other eye. Each group of mice was euthanized 1 day after injection of HGF, retinas were dissected and stained with *Griffonia simplicifolia* lectin (red) and F4/80 (green). Fluorescent mages were captured with a confocal microscope (scale bar = 500 µm). The region within the box in first two columns was enlarged in third column to provide better visualization of the macrophages (scale bar = 70 µm). (B) The mean (± SEM) number of macrophages per retina was determined by image analysis by an investigator masked with regard to treatment group. ***$P < .0001$ for difference from all other groups by one way-ANOVA with Bonferroni's correction for multiple comparisons. (C) One day after injection of 1 µL of 6.25 mmol/L HGF in one eye and 1 µL of PBS in the other eye, mice (n = 5) were euthanized and the level of mRNA for CCL2 was measured by quantitative real time PCR. ***$P < .0001$ for difference from PBS by unpaired *t* test. CCL2, chemokine (C-C motif) ligand 2; HGF, hepatocyte growth factor

the retina. There was severe leakage in mice with ischemic retinopathy that was significantly reduced by anti-c-Met indicating that HGF contributes to vascular leakage in ischemic retina.

Previous studies have shown that HGF stimulates angiogenesis in vitro[19,20] and acts directly on confluent monolayers of vascular endothelial cells to reduce transendothelial resistance, increase permeability, and reduce levels of the tight junction protein occludin.[21] We found that HGFA was undetectable in normal retina and was upregulated in retinal vessels of ischemic retina and that intraocular injection of HGF reduced levels of VE-cadherin, an endothelial-specific gene. Taken together, these findings suggest that HGF
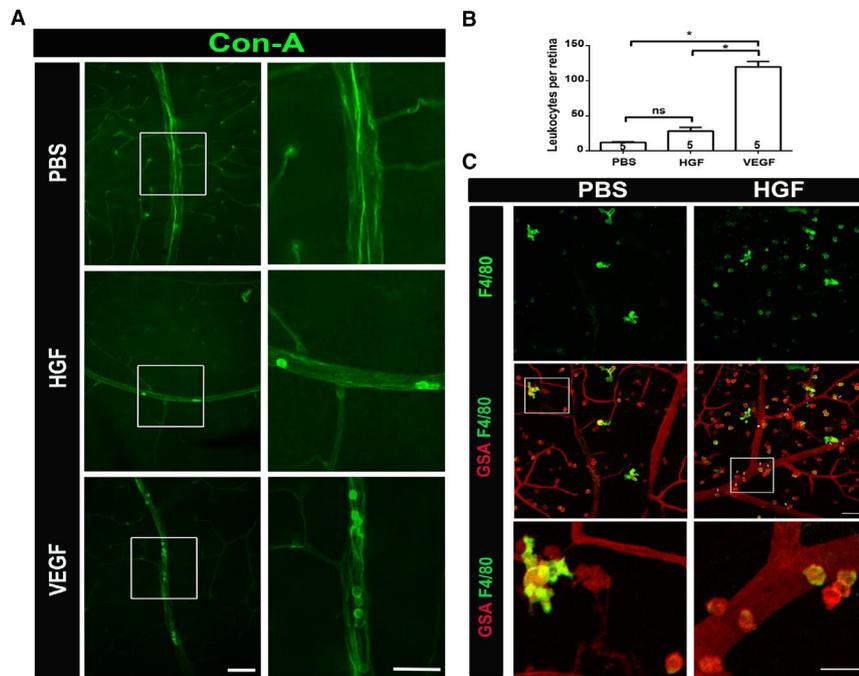
FIGURE 6 HGF does not increase leukocytic plugging, but recruits macrophages that associate with outer walls of retinal vessels. (A) Adult C57BL/6 mice were given an intravitreous injection of 1 µL of 6.25 mmol/L HGF or 6.25 mmol/L VEGF in one eye and 1 µL of PBS in the fellow eye. Twenty-four hours later, the mice were perfused with PBS and then FITC-concanavalin A, retinas were flat mounted, and the number of adherent intravascular leukocytes per retina was measured. Representative low (column 1, scale bar = 100 µm) and high power images (column 2, scale bar = 25 µm) show few leukocytes in vessels from PBS- and HGF-injected eyes, and many in vessels from VEGF-injected eyes. (B) The number of intravascular adherent leukocytes per retina were counted by an investigator masked with regard to treatment group. Bars represent the mean (± SEM) with n = 5 per group. *P = .001 by ANOVA with Bonferroni's correction for multiple comparisons. (C) Flat mounted retinas of adult C57BL/6 mice that received an intraocular injection of 1 µL of 6.25 mmol/L HGF in one eye and PBS in the fellow eye were stained with F4/80 (green) and *Griffonia simplicifolia* lectin lectin (red). Scale bar for top two rows = 50 µm. High magnification of boxes in second row images are shown in the third row. (Scale bar = 15 µm). HGF, hepatocyte growth factor

acts directly on retinal endothelial cells to disrupt junctional proteins and increase vascular permeability.

In addition to direct effects on the vasculature to increase permeability, the HGF signaling system may contribute indirectly through recruitment of macrophages which produce pro-permeability factors that augment the direct effects of HGF on endothelial cells. Interestingly, HGF-induced recruitment of macrophages was reduced in CCL2$^{-/-}$ mice suggesting that CCL2 may be involved. This is consistent with the observations that CCL2 is increased in ischemic retina in association with macrophage influx[44] and that HGF increases production of CCL2 by fibroblast-like synovial cells and increases macrophage infiltration of joints.[45] Intracoronary administration of Ad-HGF increased serum levels of CCL2 and the percentage of CD34(+) and CD117(+) endothelial cell progenitor cells, a proposed mechanism for HGF-induced benefit in myocardial infarction.[46] However, the effects of HGF on CCL2 differ in different tissues, because in kidney it reduces CCL2 production and is anti-inflammatory.[47] Our data demonstrate that HGF is pro-inflammatory in the retina, an effect mediated at least in part by CCL2. In the retinas of diabetic rats, there is upregulation of CCL2 associated

with monocyte/macrophage infiltration and breakdown of the blood-retinal barrier.[48] Thus, HGF may act directly and indirectly through CCL2 to promote retinal vascular leakage. In addition, the increase in HGFA in ischemic retina may contribute to macrophage recruitment, because HGFA cleaves inactive macrophage stimulating protein into its active form,[49] which directly stimulates macrophage chemotaxis and sensitizes macrophages to other chemoattractants.[50]

Age-related macular degeneration (AMD) is a prevalent cause of moderate and severe vision loss in elderly individuals.[51] Some patients with AMD develop choroidal NV that grows under the retina and leaks fluid that collects under or within the retina causing distortion and reduction of vision. Studies in animal models of choroidal NV indicated that VEGF plays a critical role[38,39,52] and were confirmed in clinical trials that demonstrated marked improvement in vision of patients with neovascular AMD treated with monthly injections of a VEGF antagonist.[53,54] While VEGF antagonists are highly efficacious in most patients with neovascular AMD, some respond sub-optimally to monthly injections suggesting that factors other than VEGF may also contribute to choroidal NV growth and leakage in those
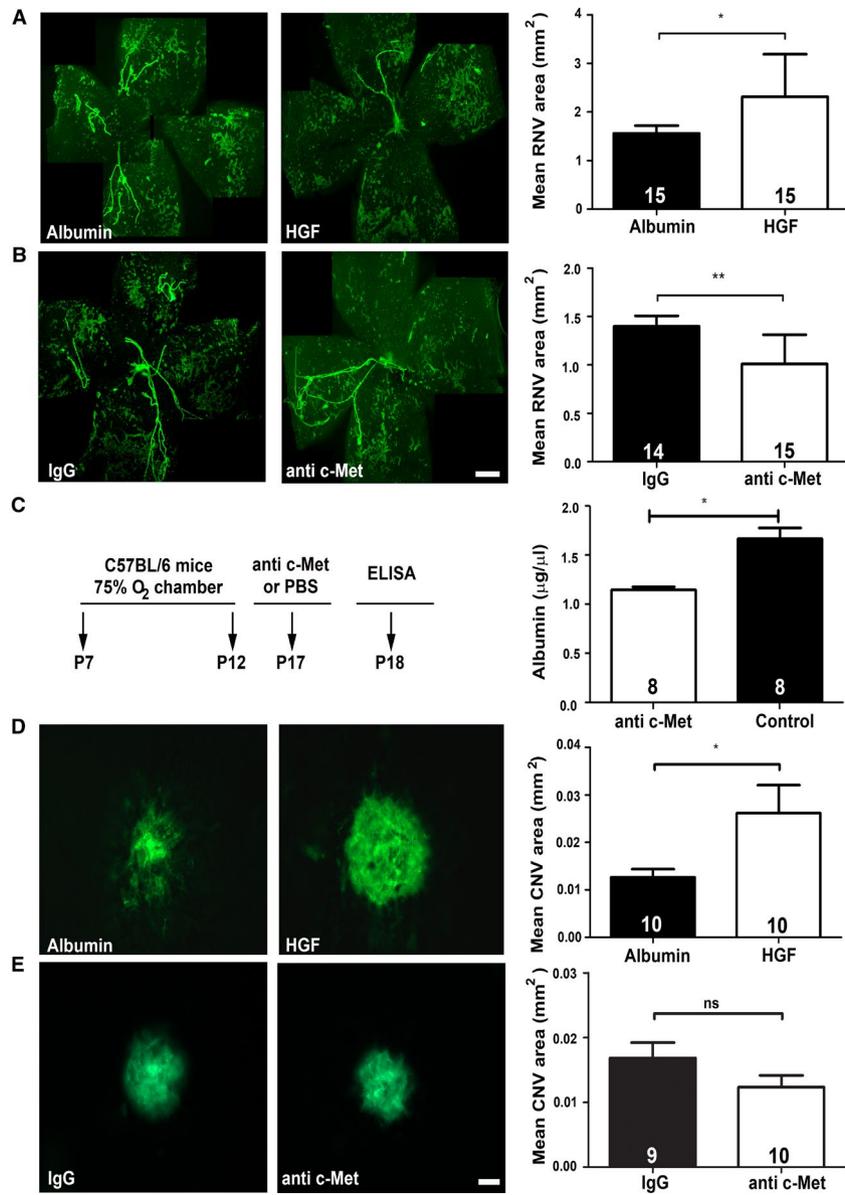
A

Albumin | HGF

Mean RNV area (mm^2)
Albumin 15 | HGF 15
*

B

IgG | anti c-Met

Mean RNV area (mm^2)
IgG 14 | anti c-Met 15
**

C

C57BL/6 mice
75% O$_2$ chamber | anti c-Met or PBS | ELISA
P7 → P12 → P17 → P18

Albumin (µg/µl)
anti c-Met 8 | Control 8
*

D

Albumin | HGF

Mean CNV area (mm^2)
Albumin 10 | HGF 10
*

E

IgG | anti c-Met

Mean CNV area (mm^2)
IgG 9 | anti c-Met 10
ns

FIGURE 7 Suppression of HGF signaling reduces ischemia-induced retinal NV, but does not reduces choroidal NV at Bruch's membrane rupture sites. At postnatal day (P) 12, mice with oxygen-induced ischemic retinopathy were given an intravitreous injection of 1 µL of 6.25 mmol/L HGF in one eye (n = 15) and 1 µL of 7.5 mmol/L albumin in the fellow eye (n = 15), 1 µL of 3 mmol/L anti-c-Met in one eye (n = 15) and 1 µL of 3 mmol/L control IgG in the fellow eye (n = 14, one eye was damaged and could not be used). At P17, mice were euthanized and retinal flat mounts were stained with GSA and the area of retinal NV was measured by a masked investigator. Representative images and graphs in which bars represent the mean (± SEM) indicate a significant increase in the RNV area in HGF-injected vs albumin-injected eyes (A) and a significant reduction in eyes injected with anti-c-Met compared with those injected with control IgG (B). Bars represent mean (± SEM). *$P < .05$, **$P < .01$ for difference from control by unpaired t test. Scale bar = 500 µm. (C) At P17 mice with OIR (n = 8) were given and injection of 1 µL of 3 mmol/L anti-c-Met in one eye and 1 µL of 3 mmol/L control IgG in the fellow eye. After 24 hours, vitreous samples were obtained and the level of albumin was measured by ELISA. There was a significant reduction in vitreous albumin in anti-c-Met-injected eyes compared with IgG controls. Bars represent mean (± SEM). *$P < .05$ for difference from control by unpaired t test. C57 Black mice had laser-induced rupture of Bruch's membrane at three locations in each eye followed by intravitreous injection of 1 µL of 6.25 mmol/L HGF in one eye (n = 10) and 1 µL of 7.5 mmol/L albumin in the fellow eye (n = 10), or 1 µL of 3 mmol/L anti-c-Met in one eye (n = 10) and 1 µL of 3 mmol/L control IgG in the fellow eye (n = 9, one eye was damaged and could not be used). After 7 days, mice were euthanized and choroidal flat mounts were stained with *Griffonia simplicifolia* lectin. The area of choroidal NV at Bruch's membrane ruptured sites was measured by a masked investigator and the three values for each eye were averaged to provide a single experimental value. Representative images and graphs in which bars represent the mean (± SEM) indicate a significant increase in the size of CNV in HGF-injected vs albumin-injected control eyes (D) and no significant difference in eyes injected with anti-c-Met vs IgG controls (E). *$P < .05$ by unpaired t test for difference from control. Scale bar = 100 µm. GSA, *Griffonia simplicifolia* agglutinin; HGF, hepatocyte growth factor; NV, neovascularization

patients. Thus, just like ischemic retinopathies, neovascular AMD is primarily driven by VEGF, but other factors may contribute. However, unlike the situation in ischemic retinopathy, we were unable to demonstrate a possible role for HGF in choroidal NV, because while exogenous HGF is capable of stimulating growth of CNV at Bruch's membrane rupture sites, blockade of HGF signaling failed to significantly reduce choroidal NV. This does not rule out a role for HGF because this model may not mimic all aspects of neovascular AMD, but regardless our data provide no support.

In summary, in the search for other factors that contribute to macular edema in patients with ischemic retinopathy who respond sub-optimally to suppression of VEGF, clinical trials have identified HGF as a suspect. The current study provides additional circumstantial evidence because HGF is increased in ischemic retina as is its activator which is increased predominantly in and around retinal vessels. Furthermore HGF promotes retinal vascular leakage in an additive fashion with VEGF and suppression of HGF signaling reduce vascular leakage in ischemic retinopathy. Thus, the hypothesis that HGF contributes to macular edema in ischemic retinopathies has been supported, but to definitively test it, clinical studies are needed using specific HGF antagonists in patients with DME and RVO.

CONFLICT OF INTEREST

AUTHOR CONTRIBUTIONS

V. E. Lorenc and P. A. Campochiaro designed research; V. E. Lorenc, R. Lima e Silva, S. F. Hackett, S. D. Fortmann, and Y. Liu performed research; V. E. Lorenc, R. Lima e Silva, and P. A. Campochiaro analyzed the data; V. E. Lorenc and P. A. Campochiaro wrote the manuscript, all authors edited and approved the manuscript.

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