Flt-1 Intraceptor Induces the Unfolded Protein Response, Apoptotic Factors, and Regression of Murine Injury–Induced Corneal Neovascularization

Nirbhai Singh,[1] *Pooja D. Jani,*[1] *Tushar Suthar,*[1] *Shivan Amin,*[1] *and Balamurali K. Ambati*[1,2]

Purpose. To determine whether Flt24K, a recombinant construct of domains 2 to 4 of VEGFR-1 (Flt) coupled with an endoplasmic reticulum retention signal (KDEL) can bind VEGFR-2 and induce apoptosis, unfolded protein response (UPR), and regression of injury-induced corneal neovascularization.

Methods. Human microvascular endothelial cells were transfected with pCMV.Flt24K and subjected to hypoxia. Cell lysates underwent Western blot analysis with anti-XBP-1 antibody and RT-PCR for CHOP. Human malignant melanoma cells (which express VEGFR-2 but not Flt), were transfected with pCMV.Flt24K, and lysates underwent immunoprecipitation with anti-FLT antibody, and Western blot analysis for VEGF and VEGFR-2. Mouse corneas sustained injury induced by topical NaOH and mechanical scraping and were injected with pCMV.Flt24K 2 weeks later. Corneas were harvested 2 days later for Western blot analysis for XBP-1 and caspase-3 or 1 week later for quantification of neovascularization and TUNEL staining. Saline and empty pCMV vector were used in control experiments.

Results. The mean percentage area of corneal neovascularization in mice 3 weeks after corneal injury and 1 week after intrastromal injection of empty pCMV vector or pCMV.Flt24K was 55.4% ± 2.7% vs. 19.3% ± 6.1%, respectively ($P < 0.001$). Flt24K was found to bind VEGFR-2 and upregulate activated XBP-1 and CHOP in vitro. In vivo, pCMV.Flt24K upregulated activated XBP-1 and caspase-3. Apoptosis was observed in corneal neovascular endothelium in corneas treated with pCMV.Flt24K but not in the control.

Conclusions. The Flt24K intraceptor can bind VEGFR-2 within cells, induce the unfolded protein response in vitro and in vivo, elicit apoptosis of vascular endothelial cells in vivo, and induce regression of corneal neovascularization in vivo. (*Invest Ophthalmol Vis Sci.* 2006;47:4787–4793) DOI:10.1167/iovs.06-0419

Angiogenesis, the growth of new blood vessels, is a fundamental biological process that plays a central role in the pathogenesis of cancer, diabetic retinopathy, and macular degeneration, in which vascular overgrowth is detrimental. In the eye, vision-threatening angiogenesis can be caused by diabetes mellitus, age-related macular degeneration, rejection of corneal transplants, chemical burns, and infections such as trachoma, Stevens-Johnson syndrome, and other disorders.[1] The cornea is normally avascular, to permit optimal visual clarity. However, in pathologic conditions, neovascularization can occur, compromising visual clarity and thus acuity. Corneal neovascularization is a central feature in the pathogenesis of many blinding corneal disorders and is a major sight-threatening complication in corneal infections and chemical injury and after keratoplasty, in which neovascularization adversely affects the corneal transplant's survival.[1] New approaches to inducing the regression of corneal neovascularization are therefore needed.

Vascular endothelial growth factor (VEGF) has been demonstrated to be a key mediator of angiogenesis in many models.[2–13] Corneal angiogenesis is driven by increased secretion of VEGF.[4] Although several studies have shown that VEGFR-2/KDR is the signal transducer for VEGF-induced mitogenesis, chemotaxis, and cytoskeletal reorganization and thus is the principal receptor involved in angiogenesis,[7,14–16] VEGFR-1/Flt-1 has a 10-fold higher binding affinity. Domain-deletion studies have shown that a subunit construct of domains 2 to 3 binds VEGF with near wild-type affinity and that domain 1 serves as a secretion signal sequence. Domain 4 is also thought to participate somewhat in VEGF binding and heterodimerization with VEGFR-2/KDR.[15,17,18]

Strategies to inhibit VEGF include VEGF receptors with blocking antibodies, decoy receptors for VEGF, and anti-VEGF antibodies.[19–26] These strategies generally reduce neovascularization by only 30% to 50%. We believe it important to target VEGF intracellularly, as several cell types respond to their own VEGF production in an autocrine fashion. VEGF autocrine loops have been demonstrated in endothelial cells.[27,28] Further, VEGF can upregulate its own receptor, VEGFR-2.[22,29] Intracellular autocrine loops would render these cell types resistant to modalities targeting VEGF extracellularly. Intracellular disruption of VEGF signaling may represent a powerful addition to the antiangiogenic arsenal, by sabotaging VEGF secretion and intracellular autocrine loops.

We have developed "intraceptors," recombinant constructs of the VEGF-binding domains of the Flt-1 receptors coupled with the endoplasmic reticulum (ER) retention-signaling sequence KDEL and have demonstrated that Flt intraceptors can bind VEGF intracellularly, inhibit hypoxia-induced VEGF secretion in corneal cells in vitro, and suppress injury-induced corneal angiogenesis.[30] We now propose to test whether intraceptors can induce regression of corneal neovascularization, a more clinically relevant endpoint. We coupled domains 2 to 4 of Flt with KDEL to produce an intraceptor (Flt24K) that has been shown to sequester VEGF but which we also expect would dimerize with the principal angiogenic VEGF receptor-2 (VEGFR-2) and retain it in the ER, where VEGFR-2 would theoretically be degraded[31] (as just mentioned, domain 4 is thought to be involved in heterodimerization with KDR[12]). We

From the Departments of [1]Ophthalmology and [2]Cell Biology and Anatomy, Medical College of Georgia, Augusta, Georgia.

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Corresponding author: Balamurali K. Ambati, Medical College of Georgia, Department of Ophthalmology, 1120 15th Street, CB1611, Augusta, GA 30912; bambati@mail.mcg.edu.

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hypothesized that accumulation of sequestered intraceptor–VEGF complexes in the ER would lead to ER overload and the triggering of the unfolded protein response (UPR). The UPR could cause apoptosis of endothelial cells, since the presence of unfolded proteins in the ER leads to a stress response including release of proapoptotic factors such as active caspase-3, spliced XBP-1, and CHOP.[32–37]

In the present study, we sought to determine whether our Flt24K intraceptor could bind VEGFR-2, induce apoptotic factors associated with the UPR, and cause apoptosis and regression of corneal neovascular vessels induced by mechanical alkali trauma.

Materials and Methods

All experiments were conducted in accordance with the Declaration of Helsinki and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Vector Construction

A vector was constructed containing domains 2 to 4 of VEGFR1 (FLT) with the ER retention signal tag, a four-amino-acid protein sequence linked to the 3′ end. Human Flt-1 cDNA was used as the template DNA for PCR reactions (Open Biosystems, Huntsville, AL). Primers were designed for attachment of the retention signal tag to the truncated receptor sequences. Primers flt2 to 4(+) (5′-TAG GAT CCA TGG ATA CAG GTA GAC CTT TCG TAG AG-3′) and flt2 to 4(–) (5′-TAG AAT TCT ATT ACA GCT CGT CCT TGG CCT TTT CGT AAA TCT GG-3′) were used to amplify flt2-4/KDEL. The amplified PCR products were digested with *Eco*RI/*Bam*H1 and were ligated into a commercial vector (pCMV Script; Stratagene, La Jolla, CA). The pCMV Script FLT2-4KDEL was transfected into competent *Escherichia coli* (DH1α) cells and selected by using kanamycin antibiotics. Desired colonies were tested for the presence of the insert through screening PCR with T3 and T7 primers. Finally, the orientation of the insert was checked with DNA sequencing (3730 XL 96-capillary sequencer; Applied Biosystems [ABI], Foster City, CA). The clone was cultured in Luria's broth (containing kanamycin) and maxiplasmid preparations were made (Eppendorf, Westbury, NY). Puc19 was used throughout the transformations, as a positive control.[30]

Cell Culture Experiments

Human microvascular endothelial cells (HMECs; gift of Helen Pappas, Centers for Disease Control, Atlanta, GA) and A375 human malignant melanoma cells (HMMCs; CRL-1619; ATCC, Manassas, VA) were grown on culture plates precoated with 0.01 mg/mL fibronectin, 0.01 mg bovine serum albumin (BSA; both from Sigma-Aldrich, St. Louis, MO), and 0.03 mg/mL bovine collagen type I (Vitrogen-100; Invitrogen-Cohesion, Palo Alto, CA) in keratinocyte serum-free medium (ATCC) with 5 ng/mL human recombinant endothelial growth factor, 0.05 mg/mL bovine pituitary extract (both from Invitrogen-Gibco, Grand Island, NY), 0.005 mg/mL insulin, and 500 ng/mL hydrocortisone (both from Sigma-Aldrich). After passage 3, the cells were used for experiments at 30% confluence. Cell culture experiments were performed in triplicate.

For hypoxia experiments, the cells were placed in 12- or 24-well culture plates in a hypoxia chamber (Coy Laboratory Products, Inc., Grass Lake, MI) programmed for 5% oxygen-5% carbon dioxide-90% nitrogen, which studies have shown is optimal for inducing VEGF without impairing cell viability.[38,39] When at 30% confluence, the cells were incubated with pCMV.Flt23K or pCMV.Flt24K+transfection reagent (siPORT; Ambion, Austin, TX). Forty-eight hours after transfection, the cells were placed in hypoxic conditions (5% O_2) in a hypoxia chamber (Coy Laboratory Products, Inc.). Nontransfected cells and cells transfected with empty pCMV vector served as control cultures. The former were placed in the hypoxia chamber 48 hours after reaching 30% confluence, whereas the latter were placed in the chamber 48 hours after transfection, on schedule with the cells transfected with pCMV.Flt23K or pCMV.Flt24K.

To determine whether intraceptors sequester VEGF and whether Flt24K (not Flt23K) binds KDR, A375 HMMCs, known to express KDR naturally but not Flt[40] (thus allowing us to avoid confounding our results by intrinsic Flt-VEGF complexes or Flt-KDR heterodimers), were transfected with PBS, empty pCMV, pCMV.Flt23K, or pCMV.Flt24K and subjected to hypoxia for 48 hours. Two days later, the cellular fraction underwent immunoprecipitation with anti-FLT antibody followed by Western blot analysis with anti-VEGF or anti-KDR antibody. To determine whether the Flt intraceptor induces the UPR in vitro in endothelial cells, we transfected HMECs with pCMV.Flt24K (controls including empty pCMV vector and PBS) and, 48 hours later, we placed the cells in 5% hypoxia for 6 hours. Cell lysates were analyzed by Western blot with anti-XBP-1 antibody (Chemicon, Temecula, CA) or by RT-PCR for CHOP.

Animal Experiments

To test the hypothesis that intraceptors induce regression of corneal neovascularization, mouse corneas underwent mechanical alkali trauma, and color photographs were taken before intrastromal injections of PBS, empty pCMV vector, and pCMV.Flt24K at 14 days after injury. Another set of photographs was taken 7 days after injections, before harvesting for quantitation of neovascularization.

To determine whether intraceptors induce the UPR in vivo, we performed corneal injury, injected the corneas with pCMV.Flt24K or control 2 weeks later, and harvested corneas 2 days later for Western blot analysis for XBP-1.

To determine whether intraceptors induce activated caspase-3, we injected mouse corneas with pCMV.Flt24K or empty pCMV vector 2 weeks after mechanical alkali trauma, harvested corneas 1 week afterward, and performed Western blot analysis for caspase-3.

To determine whether regression could be due to apoptosis of vascular endothelial cells, we injected mouse corneas with pCMV.Flt24K or control (empty pCMV vector or saline) 2 weeks after corneal injury and harvested mouse corneas 1 week afterward. TUNEL staining was performed after fixation.

Corneal Intrastromal Injection

Under direct microscopic observation, a nick in the epithelium and anterior stroma of a BALB/c mouse cornea was made in the midperiphery with a 0.5-in., 30-gauge needle (BD Biosciences, Franklin Lakes, NJ). A 0.5-in., 33-gauge needle with a 30° bevel on a 10-μL gas-tight syringe (Hamilton, Reno, NV) was introduced into the corneal stroma and advanced 1.5 mm to the corneal center. Two microliters of plasmid solution (pCMV.Flt24K, empty vector, or saline) was forcibly injected into the stroma, to separate corneal lamellae and disperse the plasmid.[41]

Mechanical Alkali Trauma Model of Corneal Neovascularization

Topical proparacaine and 2 μL of 1 M NaOH were applied to both corneas of each mouse. The corneal and limbal epithelia were removed with a Tooke corneal knife (Katena Products, Denville, NJ) in a rotary motion parallel to the limbus. Erythromycin ophthalmic ointment was instilled immediately after epithelial denudation.

Labeling of Corneal Neovascularization

Immunohistochemical staining for vascular endothelial cells was performed on corneal flatmounts. Fresh corneas were dissected, rinsed in PBS for 30 minutes, and fixed in 100% acetone (Sigma-Aldrich) for 20 minutes. After corneas were washed in PBS, nonspecific binding was blocked with 0.1 M PBS, 2% albumin (Sigma-Aldrich) for 1 hour at room temperature. Incubation with FITC-coupled monoclonal anti-mouse CD31 antibody (BD-PharMingen, San Diego, CA) at a concentration of 1:500 in 0.1 M PBS, 2% albumin at 4°C overnight was

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followed by subsequent washes in PBS at room temperature. Corneas were mounted with an antifading agent (Gelmount; Biomeda, Inc., San Francisco, CA) and visualized with a fluorescence microscope (Leica, Heidelberg, Germany).

Quantification of Corneal Neovascularization

Digital quantification of corneal neovascularization has been described.[42] Images of the corneal vasculature were captured with a charge-coupled device (CCD) camera (CD-330; Dage-MIT, Inc., Michigan City, IN) attached to a fluorescence microscope (MZ FLIII; Leica Microsystems Inc., Deerfield, IL). The images were analyzed on computer (LSM-510; Carl Zeiss MicroImaging Inc., Thornwood, NY), resolved at 624 × 480 pixels, and converted to tagged information file format (TIFF) files. Neovascularization was quantified by setting a threshold level of fluorescence above which only vessels were captured. The entire mounted cornea was analyzed to minimize sampling bias. The quantification of the neovascularization was performed in masked fashion. The total corneal area was outlined, with the innermost vessel of the limbal arcade used as the border. The total area of neovascularization was then normalized to the total corneal area, and the percentage of the cornea that was covered by vessels was calculated.

Immunoprecipitation and Western Blot Analysis

Western blot analysis of HMMCs transfected with pCMV.Flt23K and pCMV.Flt24K first involved freeze fracturing with a mortar and pestle, and placement in 200 μL RIPA buffer (Tris-HCl, NaCl, NP-40, Na-deoxycholate, and protease inhibitors). After incubation in RIPA buffer for 1 hour, cell samples were sonicated on ice four times at 15-second intervals at level-4 intensity. Immunoprecipitation of HMMCs with anti-sFLT antibody (epitope specific for domains 2 and 3; Santa Cruz Biotechnology, Santa Cruz, CA) began with pipetting 50 μL of corneal cells into a 1.5-mL Eppendorf tube (Fisher Scientific, Pittsburgh, PA) and adding 50 μL of sterile PBS to the tube. Anti-sFLT antibody was added to the tube in a 1:200 concentration, and the resultant mixture was incubated overnight at 4°C on a refrigerated shaker. The following day, 400 μL of agarose beads were placed in a separate Eppendorf tube and washed with an equal amount of PBS. For washing, the beads and PBS were inverted several times and spin pulsed in a minicentrifuge. After the supernatant was removed, another 400 μL of PBS was added to the beads, and the procedure was repeated. After three washes, the agarose beads were resuspended in 40 μL of PBS. The washed bead mixture was aliquoted into the Eppendorf tube containing human corneal cells and incubated overnight at 4°C on a refrigerated shaker. The following day, the beads were collected and washed by using the aforementioned procedure. The beads were resuspended in a 50 μL 1:19 mixture of 2-mercaptoethanol (BME):Laemmli buffer. After the beads were boiled for 5 minutes and submerged in ice for 5 minutes immediately thereafter, they were centrifuged at 4°C, 10,000 rpm, for 10 minutes. The supernatant was collected and loaded into 6% SDS-polyacrylamide gels. After transferring these gels, NCP (nitrocellulose) membranes were probed for the VEGF and KDR proteins. NCP membranes were blocked for 1 hour at room temperature with 10% milk in PBS and then incubated for 2 hours in a dilution of 1:1000 VEGF primary antibody (BD-PharMingen):10% milk. The appropriate secondary antibody dilution of 1:5000 (BD-PharMingen) was used to incubate the membranes for 2 hours at room temperature. After they were washed with PBS-Tween 20, the membranes were developed on film (BioMax Light Film; Eastman Kodak, Rochester, NY) using a chemiluminescence kit (ECL; Pierce, Rockford, IL). To probe for KDR, NCP membranes were stripped (Restore Western Blot Stripping Buffer; Pierce) and reprobed in a dilution of 1:250 VEGFR-2 primary antibody (Chemicon)-10% milk and 1:1000 of the appropriate secondary antibody (Santa Cruz Biotechnology).

HMECs (transfected with pCMV.Flt24K, empty pCMV, and PBS) and control and plasmid-injected mouse corneas were subjected to the same Western blot analysis protocol without immunoprecipitation. NCP membranes were probed for XBP-1 protein in a dilution of 1:200 XBP-1 primary antibody (Santa Cruz Biotechnology) 10% milk and 1:1000 of the appropriate secondary antibody (Abcam, Cambridge, MA).

Western blot analysis for caspase-3 on was also performed mouse corneal samples without immunoprecipitation. NCP membranes were probed for caspase-3 in a dilution of 1:1000 caspase-3 primary antibody (Cell Signaling Technology, Danvers, MA)-10% milk and 1:1000 of the appropriate secondary antibody (Abcam).

Apoptosis Assay

The apoptosis assay was performed according to the manufacturer's instructions (Chemicon). Briefly, after harvesting and fixation of mouse corneas, cryosection slides were thawed at room temperature. Slides were fixed in 1% paraformaldehyde at room temperature and postfixed in a precooled 2:1 mixture of ethanol-acetic acid. Fifty-five microliters of working-strength TdT enzyme was pipetted onto the sections, and slides were incubated in a humidified chamber at 37°C for 1 hour. For the negative control, TdT enzyme was replaced with water. Working-strength stop/wash buffer (1 mL stop/wash buffer to 34 mL water) was used to stop the reaction. Seventy-five microliters of working-strength anti-digoxigenin conjugate (40 μL blocking solution and 35 μL antibody) was then applied to sections, and slides were incubated in a humidified chamber for 30 minutes at room temperature in the dark. Slides were then incubated with mouse CD31 antibody (Abcam) at a 1:200 dilution for 2 hours, followed by an Alexa Flour 647 goat anti-mouse IgG secondary antibody (Invitrogen-Molecular Probes, Eugene, OR) at a 1:500 dilution for 1 hour at room temperature. Slides were finally counterstained with propidium iodide at a dilution of 1:2000 and sealed with antifade medium (Vectashield; Vector Laboratories, Burlingame, CA) and a coverslip. Slides were stored at −20°C and protected from light.

Results

VEGF Binding by Flt Intraceptors and Flt24K

Western blot analysis of HMMCs, transfected with pCMV.Flt23K or pCMV.Flt24K, immunoprecipitated with anti-FLT antibody, and probed for VEGF proteins, detected a 50-kDa band (consistent with a VEGF homodimer interacting with the intraceptor) in transfected cells. Western blot analysis for KDR

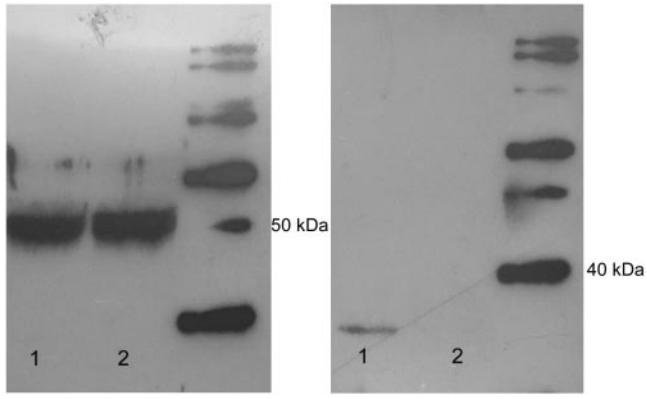

Figure 1. *Lane 1*: pCMV.Flt24K; *lane 2*: pCMV.Flt23K. *Left:* Western blot (anti-VEGF antibody) of HMMCs transfected with intraceptors, grown in hypoxia 48 hours, and immunoprecipitated with anti-FLT antibody. *Right:* Western blot (anti-KDR antibody) of HMMCs transfected with intraceptors, grown in hypoxia 48 hours, and immunoprecipitated with anti-FLT antibody. Control cells (transfected with empty pCMV vector) did not show bands for KDR or VEGF after immunoprecipitation for FLT (blots not shown). β-Actin internal control was equivalent in all lanes (blots not shown). *Right lane*: size marker.

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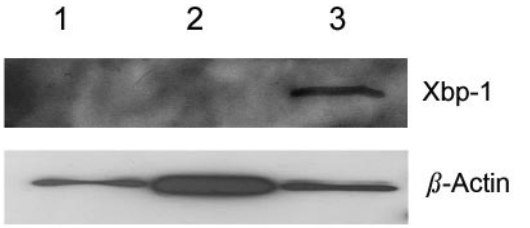

FIGURE 2. *Lane 1*: PBS; *lane 2*: empty pCMV; *lane 3*: pCMV.Flt23K. Western blot with anti-XBP-1 of hypoxic HMECs shows that intraceptors upregulate spliced XBP-1 (molecular mass of visible band in *lane 3* is ~55 kDa). β-Actin internal control was equivalent in all lanes (blots not shown).

after immunoprecipitation with anti-Flt revealed a 30-kDa band present in cells transfected with pCMV.Flt24K but not pCMV.Flt23K (Fig. 1). Based on this result, we concentrated in subsequent experiments on the potential effects of Flt24K on the UPR and apoptosis.

UPR and Apoptosis

The UPR induces conversion of the constitutive 30-kDa form of X-box binding protein (XBP)-1 through alternative splicing to its active 55-kDa form, which induces ER-associated degradation of sequestered proteins. Western blot analysis of HMECs transfected with Flt24K showed that the 55-kDa spliced variant of XBP-1 was upregulated in these cells (Fig. 2). In vivo, we found that Fl24K promoted the conversion of the inactive form of XBP-1 to its active form (Fig. 3). In vitro, we transfected HMECs with pCMV.Flt24K and performed RT-PCR for CHOP 24 hours later (experiments in triplicate). We found that Flt24K induced elevation of CHOP levels (Fig. 4). Activated caspase-3 (19 kDa) levels were also elevated in injured corneas treated with pCMV.Flt24K (Fig. 5).

Apoptosis in Vascular Endothelial Cells in Injured Corneas

Mouse corneas injected with pCMV.Flt24K or empty pCMV vector2 weeks after injury indicated that intraceptor-treated corneas had significantly more apoptotic loci in corneal neovascular endothelium (Fig. 6).

Regression of Injury-Induced Corneal Neovascularization

The mean percentage of neovascularized corneal area 2 weeks after scrape injury of control eyes was 58.3% ± 8.7% and 3 weeks after injury was 67.6% ± 10.5%. The mean percentage area of corneal neovascularization 21 days after corneal injury and 7 days after intrastromal injection was 55.4% ± 2.7% in mice injected with empty pCMV, and 19.3% ± 6.1% in mice

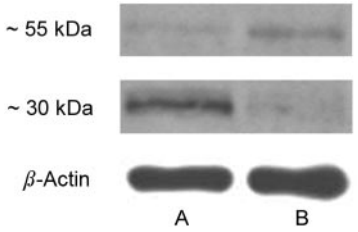

FIGURE 3. *Lane A*: mouse corneas injected with empty pCMV vector; *lane B*: pCMV.Flt23K. Representative photograph of Western blot showing expression of the 30-kDa form of XBP-1 in control corneas and elevation of the active 55-kDa form in corneas injected with plasmids expressing intraceptors.

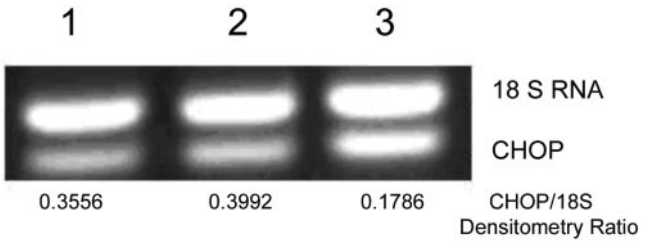

FIGURE 4. *Lane 1*: HMECs in medium; *lane 2*: cells transfected with empty pCMV; *lane 3*: cells transfected with pCMV.Flt23K. RT-PCR for CHOP was performed 24 hours after transfection of HMECs. CHOP mRNA transcript measured by semiquantitative densitometry was elevated 60% by Flt23K and 80% by Flt24K over that in cells transfected with empty pCMV.

injected with pCMV.Flt24K ($P < 0.001$ for comparisons with both empty pCMV vector and control animals; $n = 8$ all groups; representative images in Fig. 7 and quantitative data in Fig. 8).

DISCUSSION

We have demonstrated in a prior study that Flt intraceptors can inhibit hypoxia-induced VEGF secretion by corneal epithelial cells in vitro and injury-induced corneal angiogenesis in vivo.[30] In the current study, we demonstrated that Flt24K could bind VEGFR-2 as well as VEGF, induce factors associated with apoptosis and the UPR, and cause regression of corneal neovascularization via apoptosis. We found that Flt24K induces regression of angiogenesis caused by corneal injury by more than 50%. The detection of a 30-kDa band (consistent with a KDR fragment) in cells transfected with pCMV.Flt24K but not pCMV.Flt23K is consistent with our hypothesis that Flt24K can heterodimerize with both VEGF and KDR. Furthermore, we have found that UPR-associated levels of proapoptotic factors, such as caspase-3, spliced XBP-1, and CHOP, are elevated in vitro and in vivo in the presence of Flt24K. These are likely to have significant impact on the physiology of corneal neovascular endothelium, as we observed apoptosis of these cells in vivo when treated with Flt24K. We believe the UPR can cause apoptosis of endothelial cells by itself or in combination with the downregulation of VEGF.

Previous studies have relied on complexing KDEL with cytokines to generate "intrakines."[31,43–46] Our previous studies have demonstrated that Flt intraceptors can inhibit corneal angiogenesis.[30] By demonstrating the utility of targeting VEGF with ER-specific retention signals in regressing angiogenesis, the results in the present study indicate that possible therapeutic regimens for corneal neovascularization can be based on the utilization of Flt-1 intraceptors for the intracellular sequestration of VEGF. Further, by illuminating the mechanistic

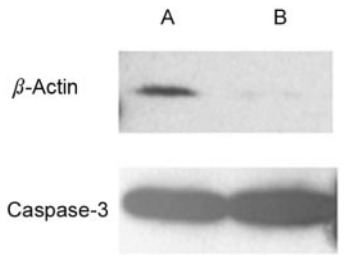

FIGURE 5. Activated caspase-3 levels (19 kDa) were elevated in injured corneas treated with pCMV.Flt24K (**A**) relative to corneas treated with empty pCMV vector (**B**).

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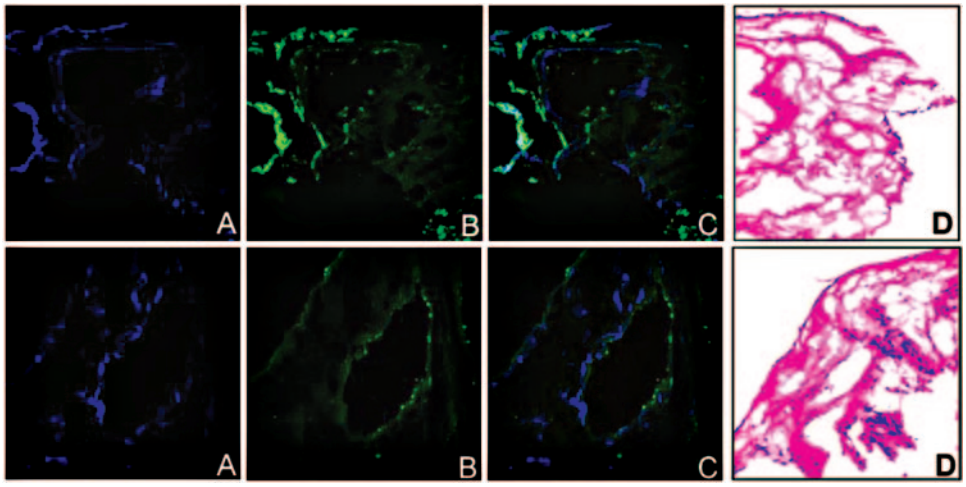

FIGURE 6. Mouse corneas were subjected to mechanical alkali trauma and injected 2 weeks later with either pCMV.Flt24K (*top*) or empty pCMV (*bottom*) vector. One week later, corneas were harvested and subjected to (**A**) immunohistochemistry for anti-CD31 (*blue*; specific for vascular endothelium) and (**B**) TUNEL (*green*) staining. (**C**) Merged images, indicating colocalization of TUNEL staining with anti-CD31 staining in the cornea, confirming that vascular endothelial cells undergo apoptosis after exposure to intraceptors. (**D**) Corresponding hematoxylin-eosin–stained sections. Magnification: (**A–C**) ×20; (**D**) ×10.

events involved in this process, we hope to provide insights into future potential targets in the treatment of angiogenesis.

Disrupting VEGF expression with intraceptors is potentially complementary to extracellular blockade by antibodies or aptamers, as intracellular gene silencing may sabotage intracellular autocrine loops that have been demonstrated for VEGF in cancer and endothelial cells.[27,28,47–53] Intraceptors may also be more effective than alternative gene-silencing approaches relying on RNAi, antisense oligonucleotides or ribozyme,s which can have off-target effects, whereas Flt is highly specific for VEGF. This approach may also be more effective than recently reported approaches to the sequestering of VEGF by using PlGF-KDEL (placental growth factor [PlGF] can heterodimerize with VEGF)[54] or a complex of an anti-VEGF Fab fragment with KDEL,[55] as the FLT receptor which is the basis of our construct has an extremely high affinity—more than would be expected with intracellular antibody fragments or PlGF.

We believe this approach, which is highly efficient and specific, may be synergistic with current approaches, as it targets intracellular mechanisms and thus can prevent intracellular as well as extracellular effects of the genes of interest. By demonstrating the utility of targeting VEGF and VEGFR-2 intra-

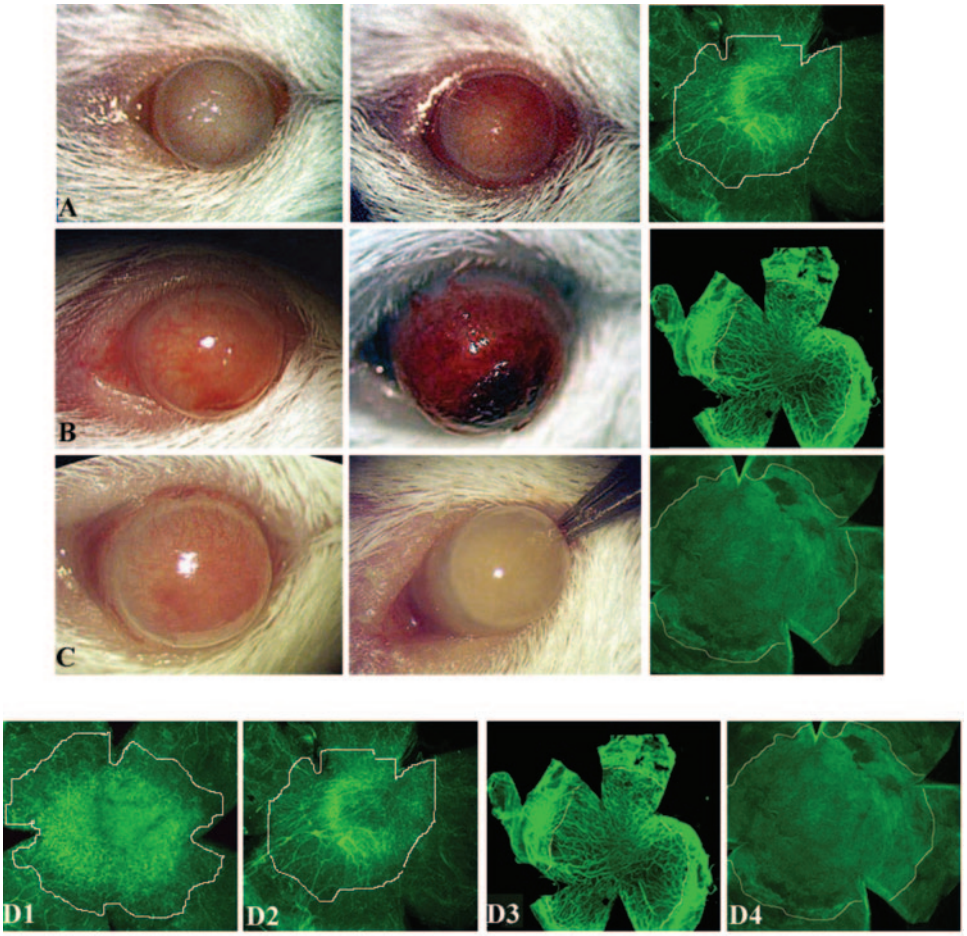

FIGURE 7. (**A**) Control (saline injected); (**B**) empty pCMV; (**C**) Flt24K. Shown are representative photographs of corneas 7 (*left column*) and 14 (*middle*) days after injury. Images in each row in the first two columns are of the same cornea, demonstrating that vessels progressively increased in area in control but regressed in intraceptor-treated corneas. *Right column*: representative photographs of FITC-coupled anti-CD31–stained corneas used for digital fluorescence imaging and quantitation of neovascularization (*white outline*: limbal border). (**D**) Representative photographs of anti-CD31–stained corneas. (**D1**) Untreated cornea at 2 weeks after injury; (**D2**) untreated cornea at 3 weeks after injury; (**D3**) cornea 1 week after injection with empty pCMV vector and 3 weeks after injury; (**D4**) cornea 1 week after injection with Flt24K and 3 weeks after injury.

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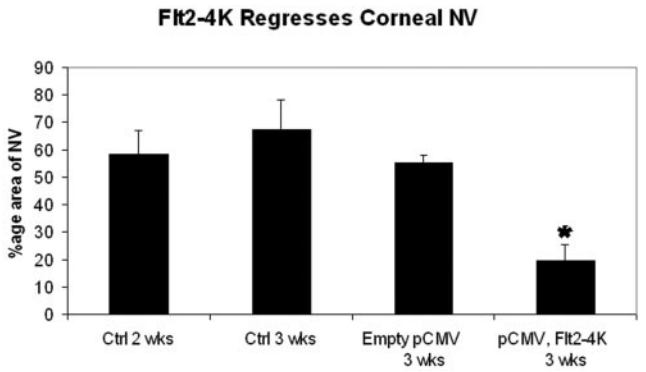

FIGURE 8. pCMV.Flt23K and -.Flt24K induced significant regression of neovascularization relative to control (measurements taken 17 days after injury and 7 days after injection).

cellular autocrine loops, we hope this approach can improve treatment and alleviation of vision loss induced by corneal neovascularization and, potentially, other angiogenic diseases.

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