# Flt-1 Intraceptors Inhibit Hypoxia-Induced VEGF Expression In Vitro and Corneal Neovascularization In Vivo

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**Purpose.** To determine whether subunits of VEGF receptor-1 coupled with an endoplasmic reticulum retention signal can block hypoxia-induced upregulation of VEGF secretion in corneal epithelial cells and block murine corneal angiogenesis induced by corneal injury.

**Methods.** Human corneal epithelial cells, transfected with plasmids encoding Flt23K or Flt24K (the VEGF-binding domains of the Flt-1 receptor coupled with the endoplasmic reticulum retention peptide KDEL), were subjected 2 days after transfection to 5% hypoxia for 24 hours. Supernatant was sampled at 24 hours and assayed for VEGF by ELISA. For in vivo models, mouse corneas underwent intrastromal injections of plasmids encoding Flt23K or Flt24K, and 2 days later, sustained injury induced by topical NaOH and mechanical scraping. Corneas were assessed 2 days later for VEGF ELISA and leukocyte counting or 1 week later for quantification of neovascularization.

**Results.** Hypoxia induced VEGF by human corneal epithelial cells was sequestered by both Flt23K and Flt24K; Flt-1 23K suppressed VEGF secretion as well. Intrastromal delivery of plasmid Flt23K suppressed VEGF by 40.4% ($P = 0.009$), leukocytes by 49.4% ($P < 0.001$), and neovascularization by 66.8% ($P = 0.001$). Flt24K suppressed VEGF expression by 30.8% ($P = 0.042$), leukocytes by 25.8% ($P < 0.001$), and neovascularization by 49.5% ($P = 0.015$).

**Conclusions.** Flt-1 intraceptors, which are endoplasmic reticulum retention signal-coupled VEGF receptors, significantly suppress hypoxia-induced VEGF secretion by corneal epithelial cells in vitro. In vivo, delivery of naked plasmids expressing these intraceptors inhibits injury-induced upregulation of VEGF, leukocyte infiltration, and corneal neovascularization.

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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, 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 clarity and thus vision. Corneal neovascularization is a central feature in the pathogenesis of many blinding corneal disorders, and 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 diminishing or completely preventing corneal neovascularization are greatly needed.

Vascular endothelial growth factor (VEGF) has been demonstrated to be a key mediator of angiogenesis in many models.[2–13] In the cornea, the angiogenic process has been shown to be driven by increased secretion of VEGF.[2] 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,[3,14–16] 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.[16–18]

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. Cancer cells producing VEGF and VEGFR-2 include prostate carcinoma, leukemia, pancreatic carcinoma, melanoma, Kaposi's sarcoma, and osteosarcoma.[27–34] VEGF autocrine loops have also been demonstrated in endothelial cells,[35,36] including hypoxic human umbilical vascular endothelial cells (HUVECs). Further, VEGF can upregulate its own receptor VEGFR-2.[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.

This study introduces a method of disrupting VEGF secretion intracellularly by the use of "intraceptors," receptor subunits that are coupled with the endoplasmic reticulum (ER)

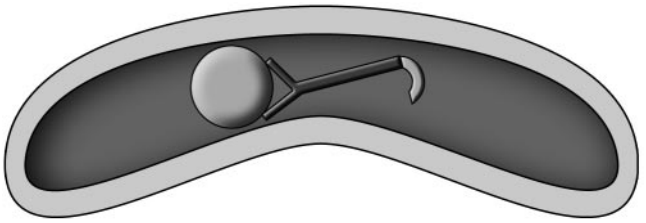


**FIGURE 1.** Cells expressing VEGF (shown as a *circle*) are transfected to express Flt23K or Flt24K (the KDEL sequence is a C-terminal tag). Transfection binds VEGF before it is released and prevents VEGF secretion. It is thought that the KDEL tag would ensure retention and ultimate degradation within the ER (the lumen of which is depicted).

retention-signaling sequence KDEL, as a viable system for the disruption of VEGF signaling. KDEL is a peptide retention signal with the amino acid sequence Lys-Asp-Glu-Leu that binds ER retention receptors,[37] preventing secretion of endogenous ER proteins coupled to KDEL. Linkage of KDEL to various chemokines (termed intrakines) has enabled decreased expression of cognate receptors with significant roles in various diseases processes.[38,39] Coupling of stromal-derived factor (SDF)-1 with KDEL has been demonstrated to block cell surface expression of the SDF receptor CXCR-4. Similar efforts have been used to downregulate cell surface expression of CCR-5 and interleukin-4 receptor.[39–41]

In this study, domain-specific intraceptors consisting of a subunits of Flt-1 coupled with KDEL were designed to downregulate VEGF expression significantly. The domain-specific design of the intraceptors is targeted at maximizing VEGF affinity, while minimizing membrane appurtenance. We hypothesized that coupling domains 2 and 3 (and in a second construct, domain 4 as well) of Flt-1 with KDEL would substantially bind VEGF in the ER and prevent its secretion (as just noted, domain 1 is a secretion signal, domains 2 and 3 are the critical domains for VEGF binding, and domain 4 may also enhance binding), and thus reduce angiogenic events in vitro and in vivo (Fig. 1). We first tested the efficacy of these intraceptors in an in vitro model of hypoxia-induced VEGF upregulation, then in a murine model of corneal injury which induces VEGF production and leukocyte infiltration, which are driving events in the development of corneal angiogenesis.[42–44]

## 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

Separate vectors were constructed containing domains 2 and 3 or 2, 3, and 4 with the ER retention signal tag linked to the end of both sequences. Human Flt-1 cDNA was used as 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 flt2-3(+) (5′-TAG GAT CCA TGG ATA CAG GTA GAC CTT TCG TAG AG-3′) and flt2-3(−) (5′-TAG AAT TCT ATT ACA GCT CGT CCT TTT TTC GAT GTT TCA CAG TGA-3′) were used to amplify flt2-3/KDEL. Primers flt2-4(+) (see flt2-3(+) and flt2-4(−) (5′-TAG AAT TCT ATT ACA GCT CGT CCT TGG CCT TTT CGT AAA TCT GG-3′) were used to amplify flt2-4/KDEL. Both products were digested with *Eco*RI/*Bam*HI and cloned into a pCMV vector (Stratagene, La Jolla, CA). The pCMV vectors containing the modified flt-1 clones were transfected into competent *Escherchia coli* (DH1α) cells and selected for using kanamycin antibiotics. Desired colonies were cultured under selective pressure in Luria's broth, and miniprepped (Eppendorf, Westbury, NY), with plasmid DNA suspended in buffer. Puc19 was used throughout transformations as a positive control.

### Cell Cultures and Hypoxia

Corneal epithelial cells (CRL-11,515; 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; Cohesion, Palo Alto, CA) in keratinocyte-serum free medium (ATCC) with 5 ng/mL human recombinant endothelial growth factor (EGF), 0.05 mg/mL bovine pituitary extract (both from Invitrogen-Gibco, Carlsbad, CA), 0.005 mg/mL insulin, and 500 ng/mL hydrocortisone (both from Sigma-Aldrich). After passage 3, cells were used for experiments at 30% confluence.

For hypoxia experiments, 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.[45,46] Cell culture experiments were performed in triplicate.

### Transfection of Corneal Epithelial Cells

Corneal epithelial cells at 30% confluence were incubated with pCMV.Flt23K or pCMV.Flt24K and transfection reagent (siPORT; Ambion, Austin, TX). Forty-eight hours after transfection, cells were placed in hypoxic conditions (5% $O_2$) in a hypoxia chamber (Coy Laboratory Products, Inc.). Three transfections were made per experiment. Nontransfected cells and cells transfected with empty pCMV vector served as control cultures. The former were placed in hypoxia 48 hours after reaching 30% confluence, although the latter were placed in hypoxia 48 hours after transfection, on schedule with the cells transfected with pCMV.Flt23K or pCMV.Flt24K.

### Model of Corneal Neovascularization

As previously described,[47–49] topical proparacaine and 2 μL of 0.15 M NaOH is applied to one cornea of each mouse. The corneal and limbal epithelia were fully removed with a corneal knife (Tooke; Katena, Denville, NJ) in a rotary motion parallel to the limbus. Erythromycin ophthalmic ointment was instilled immediately after epithelial denudation. Seven animals per subgroup were used.

### Corneal Intrastromal Injection

Effective transfection of plasmid delivery to the cornea has been described.[50] A 30-gauge needle was used to nick the corneal stroma, a 33-gauge needle on a syringe (Hamilton; Reno, NV) was passed through the nick to the center, and 1 μg plasmid in 2 μL of solution (or 2 μL of PBS) was injected. Mice were injected with PBS, empty pCMV vector, pCMV.Flt23K, or pCMV.Flt24K. This procedure was performed by an investigator who did not perform the corneal injury.

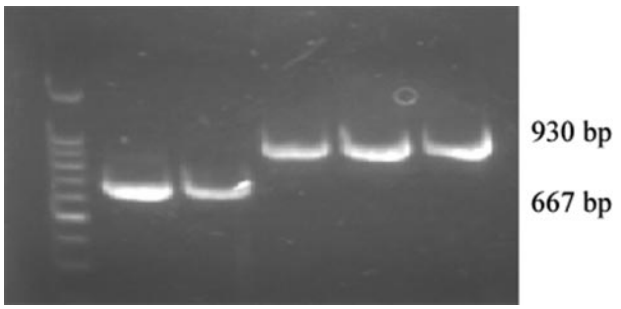
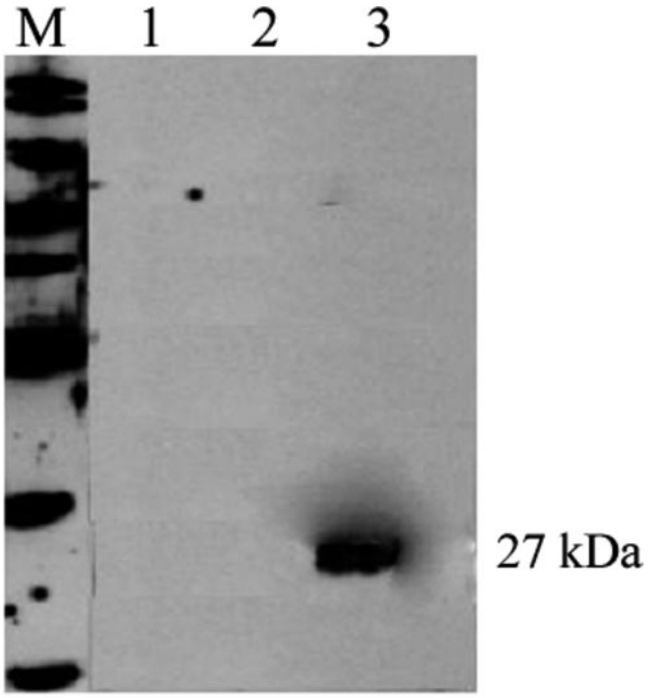



**FIGURE 2.** An image of PCR of Flt23K and Flt24K constructs is shown. Molecular weight marker (*lane 1*). Flt23K was 667 bp in size (*lanes 2* and *3*), and Flt24K was 930 bp (*lanes 4–6*).



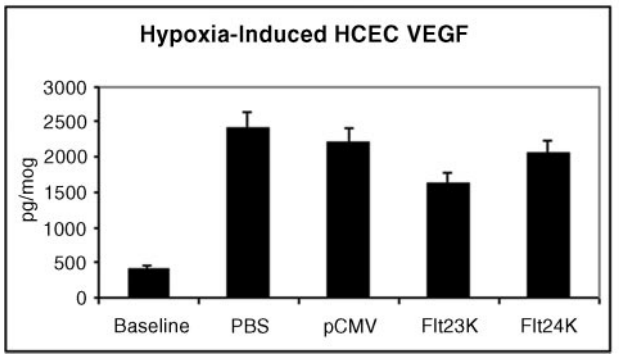




**FIGURE 3.** *Top*: Western blot analysis of human corneal epithelial cell lysates. Analysis (anti-VEGF; 1:200) of human corneal epithelial cells transfected with pCMV.Flt23K (*lane 1*), Flt24K (*lane 2*), or control (*lane 3*). Only the control lane displays free VEGF protein. *Lane M*: standard molecular weight ladder. *Bottom*: Flt23K suppressed hypoxia-induced VEGF upregulation in human corneal epithelial cells in triplicate experiments 56 hours after hypoxia.

## Labeling of Corneal Neovascularization

As previously described,[47–49] immunohistochemical staining for vascular endothelial cells was performed on corneal flatmounts by a masked investigator. Fresh corneas were dissected, rinsed in PBS for 30 minutes, and fixed in 100% acetone (Sigma-Aldrich) for 20 minutes. After the corneas were washed in PBS, nonspecific binding was blocked with 0.1 M PBS, 2% albumin (Sigma-Aldrich) for 1 hour at room temperature (RT). 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 followed by subsequent washes in PBS at RT. Corneas are mounted with an antifading agent (Gelmount; Biomeda, Inc, San Francisco, CA) and visualized with a fluorescence microscope.

## Quantification of Corneal Neovascularization

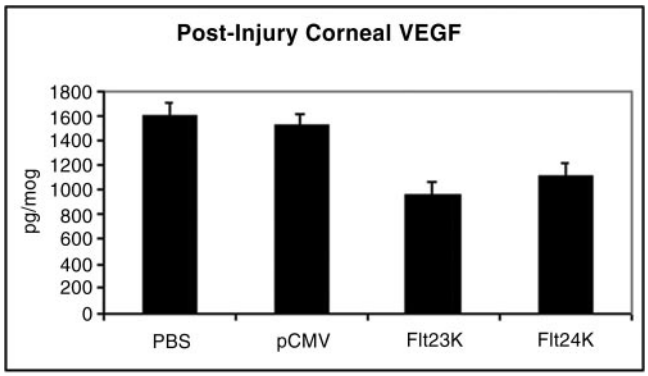
Digital quantification of corneal neovascularization has been described.[51] Images of the corneal vasculature were captured with a CD-330 charge-coupled device (CCD) camera attached to a fluorescence microscope. The images were analyzed (LSM-5 Image Examiner; Carl Zeiss Meditec, Jena, Germany), resolved at 624 × 480 pixels, and converted to tagged information file format (TIFF) files. The 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, using the innermost vessel of the limbal (rim of the cornea) arcade as the border. The total area of neovascularization was then normalized to the total corneal area.

## Harvest for ELISA

Culture medium or corneas harvested for ELISA were placed in 60 μL lysis buffer (20 mM imidazole hydrochloride, 10 mM potassium chloride, 1 mM magnesium chloride, 10 mM EGTA, 1% Triton X-100, 10 mM sodium fluoride, 1 mM sodium molybdate, and 1 mM EDTA [pH 6.8]), supplemented with protease inhibitor (Sigma-Aldrich), followed by homogenization. The lysate was cleared of debris by centrifugation at 14,000 rpm for 15 minutes (4°C), and the supernatant was collected. Total protein was determined with a Bradford protein assay (Bio-Rad, Hercules, CA).

## VEGF ELISA

VEGF was determined by a commercially available ELISA kit (R&D Systems, Minneapolis, MN) which recognizes the unbound 164-amino-acid splice variant of mouse VEGF. The assay was performed according to the manufacturer's instructions. Briefly, standards, cell culture medium, or tissue lysate samples (50 μL) were pipetted into an antibody-coated, 96-well plate containing 50 μL assay diluent and incubated for 2 hours at RT on a shaker. The wells were then washed five times with



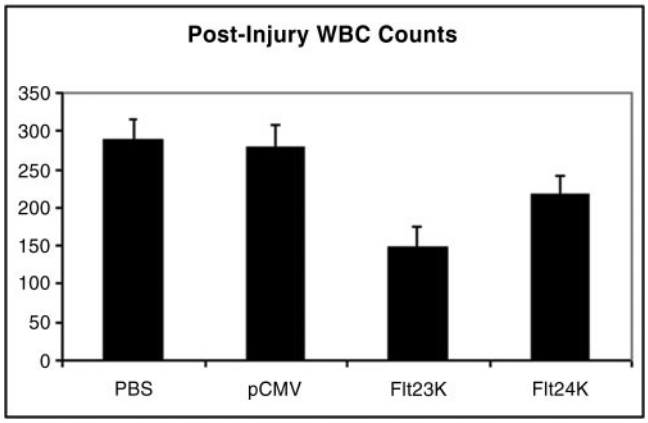




**FIGURE 4.** Flt23K and Flt24K significantly suppressed VEGF expression and leukocyte infiltration 2 days after corneal injury (mean of $n$ = 7 per subgroup).

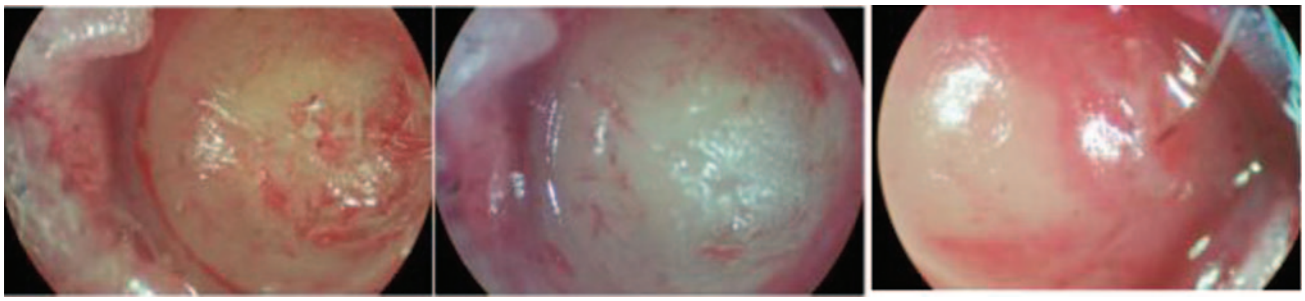


**FIGURE 5.** Representative color photographs of mouse corneas 1 week after injury and 9 days after injection with the empty pCMV vector (*left*), pCMV.Flt23K (*middle*), or pCMV.Flt24K (*right*). Zones of inhibition of neovascularization were visible in corneas injected with plasmids expressing Flt23K or Flt24K.

wash buffer, 100 μL of VEGF conjugate was added, and the samples were again incubated for 2 hours at RT. Samples were washed five times, 100 μL substrate buffer was added, the samples were incubated for 30 minutes at RT, the reaction was stopped, and the absorption was measured with an ELISA reader (Emax; Molecular Devices, Sunnyvale, CA) at 450 nm with λ correction at 570 nm. All measurements were performed in duplicate. The lower limit of ELISA was 3.0 pg/mL. The tissue sample concentration was calculated from the standard curve and corrected for total protein.

### Leukocyte Counts

Two days after corneal injury, corneas were embedded in optimal cutting temperature compound, frozen in liquid nitrogen, and cut into 7-μm-thick sections. After fixation with ice-cold acetone and blocking with normal goat serum, sections were stained with monoclonal rat anti-mouse CD45 (leukocyte common antigen; BD PharMingen), followed by 3,3-diaminobenzidine (DABE)-conjugated anti-rat IgG. Cells were visualized by light microscopy and counted in a masked fashion at ×40. Eight consecutive serial sections were studied.

### Western Blot

Corneal cell and matrix was harvested and placed in 150 μL RIPA buffer (Tris-HCl, NaCl, NP-40, Na-deoxycholate, and protease inhibitors). Immediately afterward, tissue samples were sonicated on ice four times at 15-second intervals, each at level-7 intensity. After centrifugation, samples were loaded onto a 10% SDS-polyacrylamide gel, transferred, and probed for VEGF protein. Membranes were blocked for 1 hour at room temperature with 5% milk in PBST, followed by overnight incubation at 4°C in a concentration of 1:1000 VEGF primary antibody (BD PharMingen), which detects unbound VEGF. The appropriate secondary antibody concentration of 1:5000 (BD PharMingen) was used to incubate the membrane for 2 hours at RT, after which the membrane was washed in PBST and developed on film using a chemiluminescence kit (ECL; Pierce, Rockford, IL).

### Statistics

Data analysis was performed on computer (Excel; Microsoft, Redmond, WA; and SPSS for Windows; SPSS Science, Chicago, IL). Statistical significance was assessed with Student's *t*-test. Data are expressed as the mean ± SEM.

## RESULTS

### Plasmid Synthesis

CDNA inserts of Flt23K and Flt24K were generated and verified to be of the predicted size (Fig. 2). Sequencing verified proper sequence (data not shown).

### Flt23K and Flt24K Sequestered VEGF Intracellularly, and Flt23K Suppressed Hypoxia-Induced VEGF Upregulation in Corneal Epithelial Cells

Western blot analysis showed that cells transfected with Flt23K and Flt24K lost the free VEGF band (Fig. 3). Baseline levels in culture medium of control cells were 405.0 ± 120.1 pg/μg of total protein. There was no significant difference in baseline VEGF expression among the different groups ($P > 0.05$ for all). After 56 hours of hypoxia, VEGF concentration was 2405.3 ± 346.6 pg/μg of total protein in untransfected cells, 2196.7 ± 288.4 pg/μg in cells transfected with empty pCMV vector, 1618.8 ± 89.5 pg/μg in cells transfected with pCMV.Flt23K

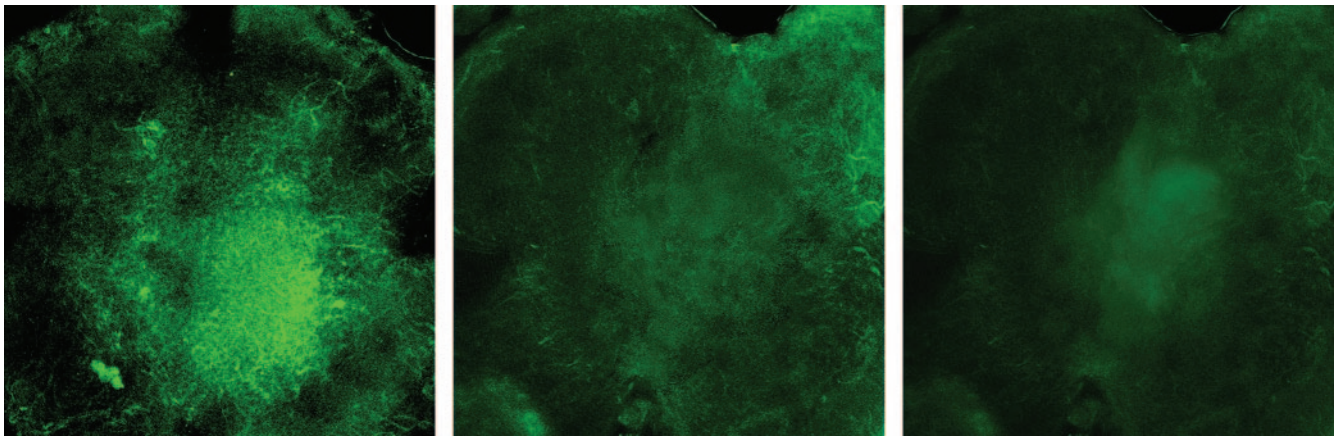


**FIGURE 6.** Representative micrographs of corneal flatmounts of mouse eyes 1 week after injury and 9 days after injection with the empty pCMV vector (*left*), pCMV.Flt23K (*middle*), or pCMV.Flt24K (*right*). Stained with FITC-coupled monoclonal murine anti-CD31 antibody. Magnification, ×40.

($P = 0.03$), and 2037.8 ± 515.0 pg/μg in cells transfected with pCMV.Flt24K ($P = 0.665$; Fig. 3).

### Effect of Flt23K and Flt24K on Injury-Induced Corneal VEGF Expression, Leukocyte Infiltration, and Neovascularization

Mice were injected with PBS, pCMV, pCMV.Flt23K, or pCMV.Flt24K and subjected to injury 2 days later. Corneas were harvested to determine VEGF level or count leukocytes 2 days after injury or for imaging of corneal vessels 1 week after injury. Corneal VEGF concentration was 1595.8 ± 102.9 pg/μg of total protein in mice injected with PBS, 1518.6 ± 65.8 pg/μg in mice injected with the empty pCMV vector, 952.2 ± 186.0 pg/μg in mice injected with pCMV.Flt23K ($P = 0.009$), and 1119.5 ± 152.1 pg/μg in mice injected with pCMV.Flt24K ($P = 0.042$; Fig. 4). Leukocyte counts per section were 288.0 ± 26.9 in mice injected with PBS, 280.0 ± 27.2 in mice injected with the pCMV vector, 148.6 ± 27.0 in mice injected with pCMV.Flt23K ($P < 0.001$), and 216.5 ± 27.4 in mice injected with pCMV.Flt24K ($P < 0.001$; Fig. 4). The mean percentage ± SEM of corneal neovascularization 7 days after corneal injury was 57.7% ± 6.9% in mice injected with PBS, 58.7% ± 7.7% in mice injected with empty pCMV ($P > 0.05$), 19.5% ± 6.4% in mice injected with pCMV.Flt23K ($P = 0.001$), and 30.3% ± 7.4% in mice injected with pCMV.Flt24K ($P = 0.015$). Representative photographs are shown in Figures 5 and 6.

## Discussion

This study demonstrated that intraceptors consisting of the VEGF binding domains of Flt-1 combined with the ER retention signal KDEL can decrease hypoxia-induced VEGF expression in vitro and markedly inhibit injury-induced VEGF expression, leukocyte infiltration, and corneal neovascularization in vivo. We found that the presence of free VEGF, as detected by Western blot within hypoxic cells, was suppressed by both intraceptors, whereas secretion of VEGF as detected by ELISA was suppressed by Flt23K. Flt23K and Flt24K were able to inhibit corneal neovascularization by 66.8% and 49.5%, respectively.

It is unclear why the larger intraceptor, Flt24K, which consisted of domains 2, 3, and 4 of VEGF receptor 1 coupled with KDEL, was not as effective in suppressing hypoxia-induced VEGF upregulation in vitro, injury-induced corneal VEGF expression in vivo, or leukocyte infiltration in vivo and hence was not as effective in suppressing corneal angiogenesis. It is possible that its particular protein configuration impaired its binding to VEGF relative to Flt23K.

To our knowledge, this is the first demonstration of the utility of complexing receptor subunits with KDEL. Previous studies have relied on complexing KDEL with cytokines to generate "intrakines."[37–41] By demonstrating the utility of targeting VEGF with ER-specific retention signals, the results in this study indicated that the "intraceptor" approach can cause significant downregulation in the secretion of VEGF from human corneal epithelial cells and in corneal tissue after injury. In vivo experimentation also showed that neovascularization as a result of corneal insult can in fact be significantly diminished. Thus, possible therapeutic regimens for corneal neovascularization can be based on the utilization of Flt-1 intraceptors for the intracellular sequestration of VEGF.

We believe the intraceptor approach, which is highly efficient and specific, may be superior to current molecular interventions such as antibodies or aptamers, as it targets intracellular mechanisms and thus can prevent intracellular and extracellular effects of the genes of interest. In conclusion, intraceptors may also be significantly more effective than alternative gene-silencing approaches and show great promise as an investigational and therapeutic tool. Future studies should determine whether this approach can reduce corneal neovascularization or be used to curtail angiogenesis in other disorders.

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