

An Anti-VEGF-B Antibody Fragment Induces Regression of Pre-Existing Blood Vessels in the Rat Cornea

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PURPOSE. We tested the ability of an antibody fragment with specificity for vascular endothelial growth factor-B (VEGF-B) to regress nascent and established corneal blood vessels in the rat.

METHODS. A single chain variable antibody fragment (scFv) with specificity for VEGF-B was engineered from the 2H10 hybridoma. Binding to rat, mouse, and human VEGF-B was confirmed by surface plasmon resonance. Activity of the anti-VEGF-B scFv on developing and established corneal blood vessels was assessed following unilateral superficial cautery in male and female outbred Sprague Dawley rats. Groups (untreated, control scFv-treated, or anti-VEGF-B scFv-treated) comprised 6 to 22 rats. Treatment consisted of 5 μ L scFv, 1 mg/mL, applied topically five times per day for 14 days, or two subconjunctival injections, 50 μ g scFv each, applied 7 days apart, or combined topical and subconjunctival treatment. Corneal vessel area was quantified on hematoxylin-stained corneal flat-mounts, and groups were compared using the Mann-Whitney *U* test, with post hoc Bonferroni correction. Immunohistochemistry for cleaved caspase-3 was performed.

RESULTS. Topical anti-VEGF-B scFv therapy alone did not regress corneal blood vessels significantly ($P > 0.05$). Subconjunctival injection and combined treatment regressed 14-day established corneal blood vessels (25% reduction in vessel area [$P = 0.04$] and 37% reduction in vessel area [$P < 0.001$], respectively, compared to results in untreated controls). Cleaved caspase-3 was identified in vascular endothelial cells of anti-VEGF-B scFv-treated corneas. In scFv-treated rats, corneal endothelial cell function was maintained to 12 weeks after treatment and a normal blink reflex was present.

CONCLUSIONS. The anti-VEGF-B scFv significantly regressed established but not developing corneal blood vessels in rats.

Keywords: VEGF-B, antibody fragment, corneal neovascularization, eye drops, subconjunctival injection

Avascularity helps to maintain normal human corneal transparency and immune privilege.^{1–3} Infection, chemical or mechanical insult, and inflammation can disrupt the fine balance between proangiogenic and antiangiogenic factors and lead to unregulated growth of new corneal blood vessels.³ The prevalence of corneal neovascularization, a potentially sight-threatening condition, is estimated to be 1.4 million people per year in the United States alone.³ As well as causing visual impairment, neovascularization is a significant risk factor for the failure of a subsequent corneal graft.⁴

Vascular endothelial growth factor-A (VEGF-A) is a major proangiogenic growth factor that promotes neovascularization *in vitro*⁵ and *in vivo*,^{6,7} and promotes survival of newly-formed vessels.⁸ Intraocular levels of VEGF-A are elevated in many pathologic conditions, including corneal neovascularization.^{9,10} Anti-VEGF-A therapy for corneal neovascularization has shown some benefit in regressing newly-formed vessels,^{11–13} but regression of established vessels has not been observed to our knowledge.¹¹

VEGF-B has structural similarities to VEGF-A,¹⁴ but is not strongly proangiogenic and does not increase blood vessel permeability.^{15,16} Recent evidence suggests a major role of VEGF-B in trans-endothelial fatty acid transport,¹⁷ but it also has been demonstrated to be a potent survival factor (as distinct from a growth factor) for vascular endothelial cells, pericytes, and smooth muscle cells.¹⁸ In the eye, VEGF-B deficiency leads to increased apoptosis of endothelial cells and, therefore, poorer survival of blood vessels in the posterior segment.¹⁸ Furthermore, VEGF-B overexpression augments pathologic new vessel survival in animal models of choroidal and retinal neovascularization.¹⁹ VEGF-B also acts as a survival factor for different types of neurons, including cortical neurons in the brain,²⁰ retinal neurons in the eye,²⁰ and motor neurons in the spinal cord.²¹ Taken together, these findings suggest that VEGF-B might be a potential target for therapy directed at established blood vessels.

Whole antibody therapeutics designed for use in the anterior segment of the eye are generally administered by



TABLE. Treatment Regimen to Detect the Antineovascular Activity of the Anti-VEGF-B scFv

Treatment		Mode of Delivery	Total scFv, μg
Immediate - commencing day after cautery			
Topical	Untreated	Monitored only, no treatment	0
	Control scFv	5 μL eye drop 1 mg/mL 5 \times daily from days 1-14 after cautery	350
	Anti-VEGF-B scFv	5 μL eye drop 1 mg/mL 5 \times daily from days 1-14 after cautery	350
Combined topical and subconjunctival injection	Untreated	Monitored only, no treatment	0
	Control scFv	5 μL eye drop 1 mg/mL 5 \times daily from days 1-14 after cautery + 10 μL subconjunctival injection 5 mg/mL in 20% pluronic F127 on days 1 and 8 after cautery	450
	Anti-VEGF-B scFv	5 μL eye drop 1 mg/mL 5 \times daily from days 1-14 after cautery + 10 μL subconjunctival injection 5 mg/mL in 20% pluronic F127 on days 1 and 8 after cautery	450
Delayed - commencing 15 days after cautery			
Topical	Untreated	Monitored only, no treatment	0
	Control scFv	5 μL eye drop 1 mg/mL 5 \times daily from days 15-28 after cautery	350
	Anti-VEGF-B scFv	5 μL eye drop 1 mg/mL 5 \times daily from days 15-28 after cautery	350
Subconjunctival injection	Untreated	Monitored only, no treatment	0
	Control scFv	10 μL subconjunctival injection 5 mg/mL in 20% pluronic F127 on days 15 and 22 after cautery	100
	Anti-VEGF-B scFv	10 μL subconjunctival injection 5 mg/mL in 20% pluronic F127 on days 15 and 22 after cautery	100
Combined topical and subconjunctival injection	Untreated	Monitored only, no treatment	0
	Control scFv	5 μL eye drop 1 mg/mL 5 \times daily from days 15-28 after cautery + 10 μL subconjunctival injection 5 mg/mL in 20% pluronic F127 on days 15 and 22 after cautery	450
	Anti-VEGF-B scFv	5 μL eye drop 1 mg/mL 5 \times daily from day 15-28 after cautery + 10 μL subconjunctival injection 5 mg/mL in 20% pluronic F127 on days 15 and 22 after cautery	450

intracameral or subconjunctival injection, as they penetrate the cornea poorly following topical application.^{22,23} However, antibody fragments, such as the single chain antibody fragment (scFv), have been shown to penetrate through the cornea on topical application.²³ We describe the generation and characterization of an anti-VEGF-B scFv with specificity for human, mouse, and rat VEGF-B.²⁴ We formulated the anti-VEGF-B scFv for local delivery to the eye and evaluated its effect on growing, as well as established, vessels in an alkali-induced model of corneal neovascularization in the rat.

METHODS

Engineering of Anti-VEGF-B scFv

Antibody variable heavy (V_H) and variable light (V_L) chain genes were amplified from cDNA isolated from an anti-VEGF-B murine hybridoma, 2H10,²⁴ with custom-designed primers 2H10VHfor, 2H10VHback, 2H10VLfor and 2H10VLback (GeneWorks, Adelaide, Australia; Supplementary Table S1), using a previously described protocol.²⁵ The V_H and V_L were joined using splice-by-overlap-extension polymerase chain reaction (PCR) to generate a scFv as described previously.²³ The sequence of the scFv was confirmed using an ABI BigDye Terminator sequencing kit on an ABI PRISM Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Existing scFvs with activity against *Acanthamoeba*,²⁶ anti-VEGF-A,²⁷ and influenza virus coat protein (CSL Ltd, Parkville, Australia), prepared as described above, were used as negative controls.

Production and Purification of Anti-VEGF-A and Anti-VEGF-B scFv

For in vitro use, a well-characterized anti-human VEGF-A scFv²⁷ and the newly-constructed anti-VEGF-B scFv were produced in *Escherichia coli* and purified by immobilized metal ion chromatography as described previously.²⁷ Visualization of the components of protein solutions containing scFv was achieved by gel electrophoresis. A 5 μg protein sample was mixed with 4 \times loading buffer (BioRad, Hercules, CA, USA) with 100 mM dithiothreitol and incubated at 95°C for 2 minutes. Samples were then loaded into wells of a 4% to 20% BioRad TGX stain free precast gel in a Criterion gel tank. A voltage of 300 V was applied for 20 minutes and the gel was imaged using the BioRad gel-Doc EZ imager. For in vivo experiments, the anti-VEGF-B scFv cDNA sequences were codon-optimized for eukaryotic expression, transiently expressed in FreeStyle 293 cells (Thermo Fisher Scientific, Waltham, MA, USA) and purified from conditioned medium by immobilized metal affinity chromatography followed by preparative size exclusion chromatography into mouse-tonicity PBS.

Binding of Anti-VEGF-B scFv to Human VEGF-B, Human and Rat VEGF-A

A direct ELISA was used to detect binding of the anti-VEGF-B scFv to human VEGF-B and human and rat VEGF-A. ELISA plates were coated with 1 $\mu\text{g}/\text{mL}$ recombinant human VEGF-B (CSL Ltd), human or rat VEGF-A (ProspecTany, Rehovot, Israel) overnight at 4°C in a humidified box, then blocked with 5% wt/vol skim milk (Fonterra, North Adelaide,

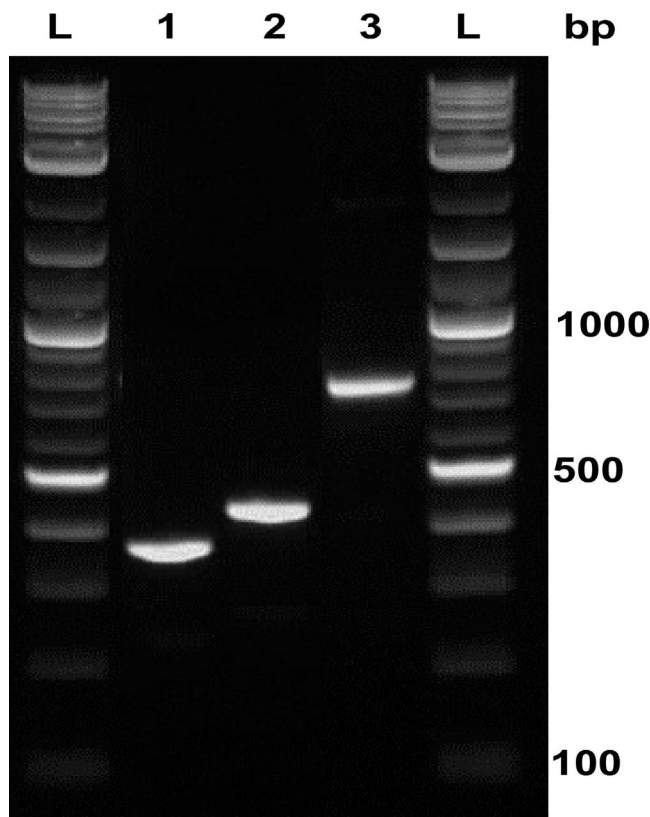


FIGURE 1. Generation of an anti-VEGF-B scFv gene from the 2H10 hybridoma. The V_L (lane 1) and V_H (lane 2) regions were amplified from 2H10 hybridoma cDNA, and assembled into an scFv gene (lane 3) by splice-by-overlap-extension (SOE) PCR. The expected product sizes for the V_L , V_H , and SOE product were 387, 407, and 771 bp, respectively. The observed bands corresponded to expected product sizes. A 500 ng sample of the 2 Log ladder size-marker was run in lanes marked L. All fragment lengths are represented in bp.

Australia) in Dulbecco's A PBS pH 7.2 for 1.5 hours. ScFv then was added for 1.5 hours at room temperature. Diluent (0.1% skim milk in PBS) was added as an additional negative control. Bound scFv was detected with 1:2000 diluted anti-HIS antibody (Sigma-Aldrich Corp., St. Louis, MO, USA) and 1:1000 horseradish peroxidase conjugated anti-mouse antibody (Millipore, Billerica, MA, USA) for 1.5 hours each at room temperature. Tetramethylbenzidine (TMB; BD, Franklin Lakes, NJ, USA) was used as the substrate and absorbance was measured at 450 nm.

Affinity of 2H10 scFv for Human, Mouse and Rat VEGF-B

The affinity of the 2H10 scFv for human, mouse, and rat VEGF-B was assessed by surface plasmon resonance. Species-specific VEGF-B (human, mouse, or rat; CSL Ltd) was immobilized separately on a CM5 chip (GE Lifesciences, Pittsburgh, PA, USA) to approximately 100 RU. 2H10 scFv was injected at 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0 nM in duplicate and random order. Association was monitored for 200 seconds and dissociation was monitored for 300 seconds. Regeneration was accomplished by two 30-second injections of 6 M guanidine-HCl. Running buffer was HBS-EP (GE Lifesciences) with 0.1% wt/vol bovine serum albumin (BSA)

pH 7.3. Analysis temperature was 37°C with a flow rate of 100 μ L/minute.

Functional Neutralization of Human VEGF-B by Anti-VEGF-B scFv

Neutralization of the functional effects of VEGF-B by the anti-VEGF-B scFv was assessed using a VEGFR1/EpoR/BaF3 cell-based assay as described previously.^{16,28}

Formulation of scFv for In Vivo Experiments

ScFvs were formulated for topical delivery as an eye drop to a final concentration of 1 mg/mL purified protein in 0.9% NaCl (Chem-Supply, Adelaide, Australia), 0.5% capric acid (Sigma-Aldrich Corp.) and 1.5% hypromellose (DOW, Midland, MI, USA), as described previously.²³ ScFvs were formulated for subconjunctival injection at 5 mg/mL with 20% wt/vol Pluronic F-127 (Sigma-Aldrich Corp.) in normal saline. Aseptic techniques and endotoxin-low glassware was used to prepare all scFv for in vivo use. Endotoxin levels were measured by the Limulus amoebocyte lysate assay (Associates of Cape Cod, East Falmouth, MA, USA) and were below 3 EU/mL.

Rats and Anesthesia

All experiments involving animals adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the institutional Animal Welfare Committee. Adult (>12 weeks) Sprague-Dawley rats were allowed unlimited access to water and rat chow, and were exposed to a 12-hour light-dark cycle. Room temperature was 24°C and ambient humidity was maintained at between 40% and 55%. Approximately equal numbers of male and female rats were used. Anesthesia was induced by inhaled isoflurane (Bomac, Auckland, New Zealand) in a glass chamber and maintained with 2% isoflurane in oxygen, 2 L per minute, delivered through a nose cone.

Induction of Corneal Neovascularization by Silver Nitrate Caутery

Topical anesthetic agent (0.5% proxymetacaine eye drops; Alcon, Fort Worth, TX, USA) was applied to the right eye. After 2 minutes, the right central cornea was cauterized using a silver nitrate potassium nitrate applicator (Graham Field, Atlanta, GA, USA) for 5 seconds. The eye was washed with sterile ophthalmic balanced salt solution (BSS; Alcon), chloramphenicol ointment (Aspen Pharma, Sydney, Australia) was applied, and the eyelid was sutured shut for 24 hours.

Treatment With Anti-VEGF-B scFv

The treatment regimen used to assess the antineovascular activity of the anti-VEGF-B scFv on nascent and established rat corneal blood vessels in study rats compared to controls is described in the Table.

Quantification of Corneal Neovascularization

A flat-mount-based method was used for the quantification of corneal neovascularization. Rats were injected with 35 units of heparin (Hospira, Lake Forest, IL, USA) per 100 g body weight by intraperitoneal injection and killed by an overdose of inhaled isoflurane after 30 minutes. The thoracic and abdominal cavities were opened and the inferior vena cava and descending aorta were clamped close to the heart. An incision was made in the right auricle and a 21-gauge winged

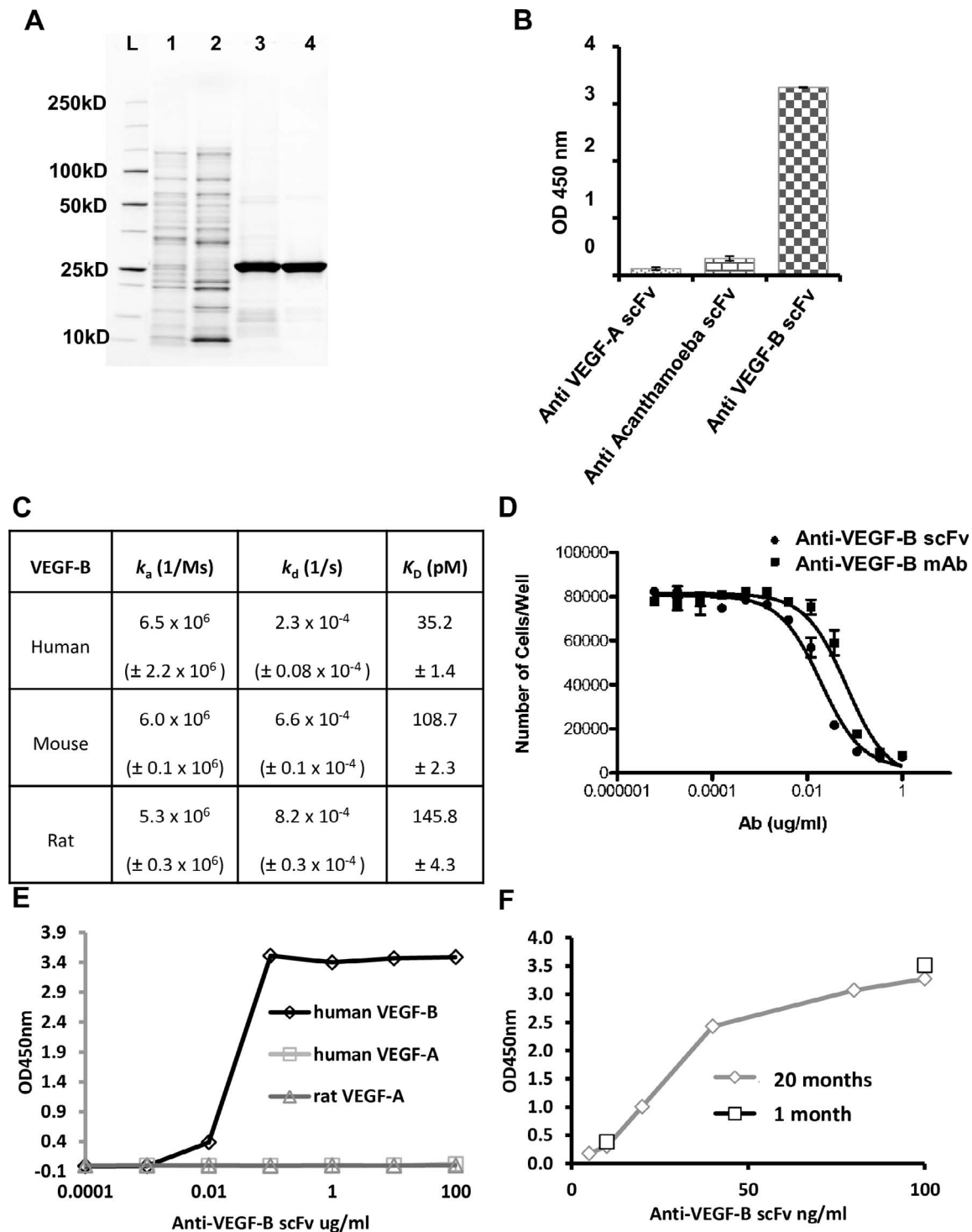


FIGURE 2. Characterization of the anti-VEGF-B scFv. **(A)** Purification of the anti-VEGF-B scFv by immobilized metal ion chromatography (IMAC). Aliquots of the crude bacterial lysate (*lane 1*), flow-through from the IMAC column (*lane 2*), purified pooled fractions (*lane 3*), and purified dialyzed scFv (*lane 4*) were separated on a 4% to 20% gradient polyacrylamide gel. After purification, the predominant band was approximately 25 kDa, the expected size of the anti-VEGF-B scFv. Samples of 5 μ g protein were loaded in each lane and 5 μ L of Precision Plus Protein unstained standard (BioRad) was run in *lane L*. **(B)** The anti-VEGF-B scFv demonstrated strong binding to VEGF-B in a direct ELISA. Neither anti-VEGF-A nor anti-*Acanthamoeba* scFvs bound to VEGF-B. The bars represent the mean optical density at 450 nm of three technical replicates. Error bars: standard deviation. **(C)** Parameters for anti-VEGF-B scFv binding to human, and rat VEGF-B, measured by surface plasmon resonance. **(D)** The ability of the anti-VEGF-B scFv and the parental mAb to neutralize the biological activity of VEGF-B was assessed in a cell-based assay. The anti-VEGF-B mAb and scFv inhibited VEGF-B-induced proliferation in the cell-based assay in a concentration-dependent manner. **(E)** The binding of the anti-VEGF-B scFv to human VEGF-B and human and rat VEGF-A was assayed by ELISA. The scFv bound to VEGF-B but not to human or rat VEGF-A. **(F)** Anti-VEGF-B scFv was assayed for binding to human VEGF-B by direct ELISA, then stored at 4°C for 20 months before binding was retested. Binding activity of the scFv was maintained during storage.

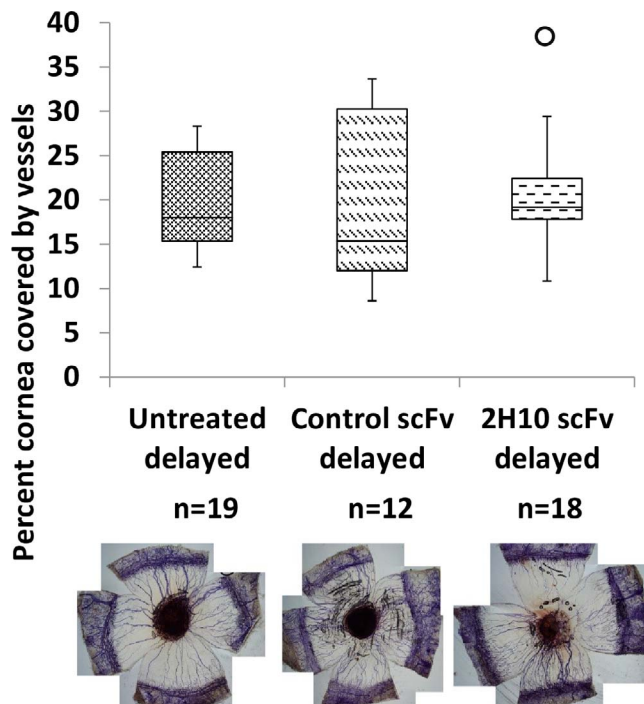


FIGURE 3. Effect of topical anti-VEGF-B scFv on 14-day established corneal neovascularization in the rat. There was no significant difference in the percentage of the cornea covered by blood vessels in untreated, control scFv-treated, and anti-VEGF-B scFv-treated animals ($P = 0.87$, Kruskal-Wallis test), after topical scFv treatment of established corneal blood vessels. The *box* outlines the middle two quartiles, the *line* is the median, and the *whiskers* delineate the minimum and maximum values, the *empty circle* represents an outlier. n = number of rats in each group. Representative corneal flat-mounts are displayed under each group.

infusion set (Terumo, Tokyo, Japan) was inserted into the cavity of the left ventricle. The rat was perfused with 50 mL warm heparinized saline, followed by 50 mL of 1:3 hematoxylin:PBS, pH 6.0, containing 0.32 mL/l papavarine HCL (Hospira), at 120 mm Hg through the winged infusion set. Both eyes were enucleated and placed in buffered formalin for 15 minutes at room temperature and then placed in PBS. The cornea was dissected and flat-mounted. Flat-mounted corneas were imaged and the corneal neovascular area was quantified in a blinded fashion using ImageJ software (National Institutes of Health [NIH], Bethesda, MD, USA) as described in Supplementary Figure S1.

Assessment of Corneal Nerve Function

The blink reflex was used as a surrogate for corneal nerve function. The effect of anti-VEGF-B scFv and the control scFv on corneal nerve function was assessed using a 6-0 nylon suture (Ethicon, Somerville, NJ, USA) as described previously.²⁹ The blink reflex was tested in additional groups of animals (4–18 rats/group) that underwent treatment as described above, after which they were monitored for an additional 12 weeks, to assess long-term function.

Endpoint Histology and Immunohistochemistry for Cleaved Caspase-3

Whole eyes were removed from rats at the end of treatment, formalin fixed, embedded in paraffin wax, sectioned, and

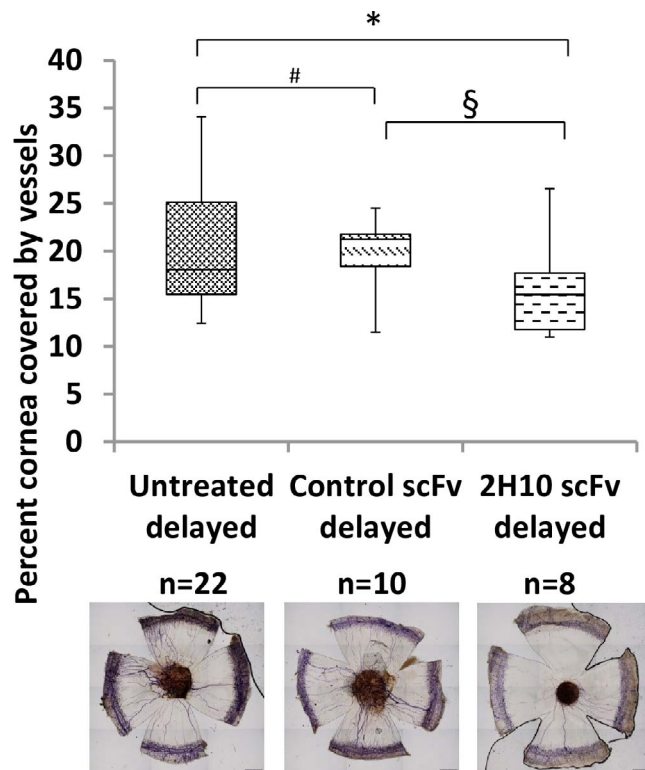


FIGURE 4. Effect of subconjunctival anti-VEGF-B scFv on 14-day established corneal neovascularization in the rat. Treatment of established corneal vessels with anti-VEGF-B scFv, delivered by subconjunctival injection, resulted in significantly less percentage of the cornea covered by vessels when compared to results in untreated ($*P = 0.04$) or control scFv ($\S P = 0.009$)-treated animals (Mann-Whitney U test). There was no significant difference between untreated and control scFv-treated groups ($\#P = 0.73$). The *box* outlines the middle two quartiles, the *line* is the median, and the *whiskers* delineate the minimum and maximum values. n = number of rats in each group. Representative corneal flat-mounts are displayed under each group.

stained with hematoxylin and eosin as described previously.³⁰ All sections were examined by a qualified pathologist (SK).

Apoptotic cells were detected by immunohistochemistry for cleaved caspase-3 according to the manufacturer's instructions (Rabbit anti-cleaved caspase-3 polyclonal #9661; Cell Signaling Technology, Danvers, MA, USA). Antibody was diluted to 1/5000 and positive labeling was detected using the Novolink polymer detection system (Leica, Wetzlar, Germany) according to the manufacturer's instructions. Normal rabbit serum diluted 1/10 in PBS was used as a negative control. Rat thymus was used as a control tissue.

Statistical Analysis

Statistical analysis was performed using SPSS 22 software (IBM, Armonk, NY, USA). As data were not normally distributed (Shapiro-Wilk test), nonparametric tests were used to determine statistical significance. Two groups were compared using the Mann-Whitney U test; three or more groups were compared using the Kruskal-Wallis test. Comparisons among subsets of data were performed with the Mann-Whitney U test with post hoc Bonferroni correction for multiple comparisons. The significance level (α) was set at 0.05.

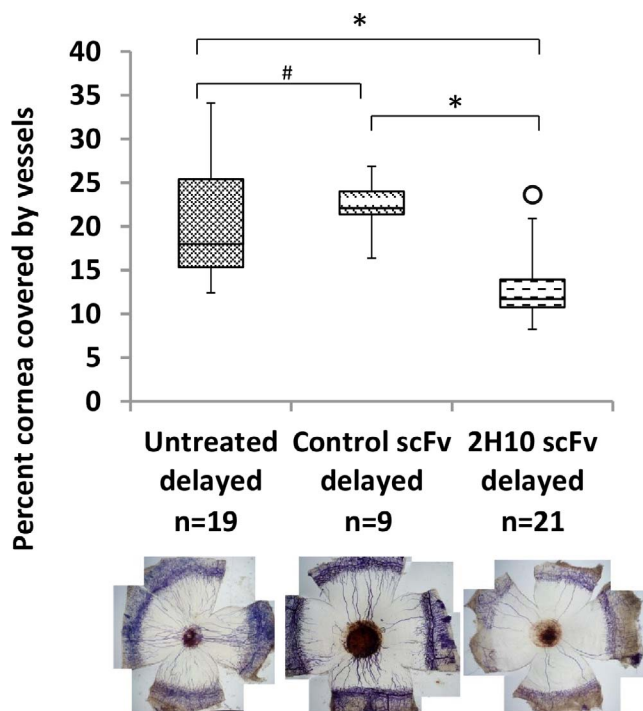


FIGURE 5. Effect of combined topical and subconjunctival anti-VEGF-B scFv on 14-day established corneal neovascularization in the rat. Combined topical and subconjunctival scFv caused significant reduction corneal blood vessel area in anti-VEGF-B scFv-treated animals when compared to results in either untreated or control scFv-treated groups ($*P < 0.001$, Mann-Whitney U test). There was no significant difference between untreated and control scFv-treated animals, $\#P = 0.66$. The box outlines the middle two quartiles, the line is the median, and the whiskers delineate the minimum and maximum values, the empty circle represents an outlier. n = number of rats in each group. Representative corneal flat-mounts are displayed under each group.

RESULTS

Generation and Characterization of an Anti-VEGF-B scFv

Products amplified from 2H10 hybridoma cDNA corresponded to the expected sizes of the V_L and the V_H genes (387 and 407 base pairs [bp], respectively, Fig. 1). A scFv gene then was generated using an assembly PCR protocol. The expected size of the product was 771 bp, and the predominant band obtained ran slightly below the 800 bp marker (Fig. 1). The sequence of the scFv gene was confirmed by Sanger sequencing to match the sequence of the 2H10 hybridoma V_L and V_H chain genes (Supplementary Fig. S2). The scFv gene then was expressed in bacteria, and scFv protein purified for further characterization.

Purified scFv consisted predominantly of a single species of approximately 25 kDa when separated on a polyacrylamide gel (Fig. 2A). Binding of the anti-VEGF-B scFv to human VEGF-B was assayed by direct ELISA. A strong positive signal was obtained with the anti-VEGF-B scFv (Fig. 2B; Supplementary Fig. S3), indicating binding to human VEGF-B. Control scFvs with specificity for human VEGF-A²⁷ and *Acanthamoeba*²⁶ returned negligible readings. The anti-VEGF-B scFv bound to human, mouse, and rat VEGF-B with high affinity (Fig. 2C) and the data fitted well to a 1:1 kinetics model (Supplementary Fig. S4). The binding affinity of the anti-VEGF-B scFv to VEGF-B was highest for human and approximately 3- and 4-fold lower for the mouse and rat VEGF-B, respectively. The anti-VEGF-B

scFv blocked VEGF-B-induced proliferation of VEGFR1/EpoR/BaF3 cells, which were engineered to proliferate in the presence of VEGF-B, with comparable potency to the parental 2H10 monoclonal antibody (mAb; Fig. 2D). However, the anti-VEGF-B scFv did not bind to human or rat VEGF-A (Fig. 2E). The anti-VEGF-B scFv maintained binding to human VEGF-B after storage at 4°C for up to 20 months (Fig. 2F). Following formulation for ocular delivery, the scFv maintained binding to human VEGF-B, immediately and after 3 months of storage at 4°C (Supplementary Fig. S5).

Effects of Anti-VEGF-B scFv Treatment on Corneal Vessels

Unilateral corneal neovascularization was induced in Sprague-Dawley rats by superficial cautery with silver nitrate. Neovessels sprouted from the limbal plexus 3 to 4 days after cautery and reached the site of cautery within 7 to 10 days. At 14 to 28 days after cautery remodeling of the vessels continued (Supplementary Fig. S6).

The effect of topical treatment with anti-VEGF-B scFv eye drops, or subconjunctival injection of anti-VEGF-B scFv, or topical treatment combined with subconjunctival injection on developing as well as established corneal vessels was assessed by image analysis on corneal flat-mounts, following euthanasia of the rats. Anti-VEGF-B scFv, irrespective of treatment regimen, had no effect on growing vessels (Supplementary Fig. S7). Furthermore, topical anti-VEGF-B scFv treatment did not cause regression of established vessels (Fig. 3, $P = 0.87$). However, subconjunctival injection of anti-VEGF-B scFv resulted in a 26% reduction in established corneal vessel area when compared to results in untreated controls (Fig. 4, $P = 0.04$). Combined topical and subconjunctival injection reduced corneal blood vessel area by 37% when compared to that in the untreated group (Fig. 5, $P < 0.001$). There were no significant differences between untreated and control scFv-treated groups ($P = 0.66$).

The data for combined scFv therapy were separated by sex (male and female rats) and reanalyzed. Male rats treated with anti-VEGF-B scFv showed significantly less corneal area covered with vessels than did untreated or control scFv-treated males, $P = 0.003$ and $P = 0.009$, respectively. There was no significant difference between untreated and control scFv-treated males, $P = 0.53$. A similar result was observed in the females. Female rats treated with anti-VEGF-B scFv showed significantly less corneal area covered with vessels than did untreated or control scFv-treated females, $P = 0.006$ and $P = 0.048$, respectively. There was no significant difference between untreated and control scFv-treated females, $P = 0.11$. These data indicated that the anti-VEGF-B scFv regressed pre-existing corneal blood vessels in male and female rats.

Immunohistochemistry for cleaved caspase-3 was performed to detect apoptotic cells in the corneas of rats treated with anti-VEGF-B scFv. Blood vessel endothelial cells in the cornea of anti-VEGF-B scFv-treated rats labeled positive for cleaved caspase-3 (Fig. 6). Corneal blood vessels in control scFv-treated rats were negative for cleaved caspase-3. These data suggested that anti-VEGF-B scFv induced apoptosis in corneal blood vessels.

Assessment of Side Effects of Treatment

Mild corneal edema was observed after cautery, but resolved spontaneously. Administration of a scFv formulated in eye drop form or as a subconjunctival injection was well tolerated, with no overt signs of inflammation apparent (Supplementary Fig. S6). The cornea remained clear after administration of scFv (topical and/or subconjunctival injection) for up to 14 days,

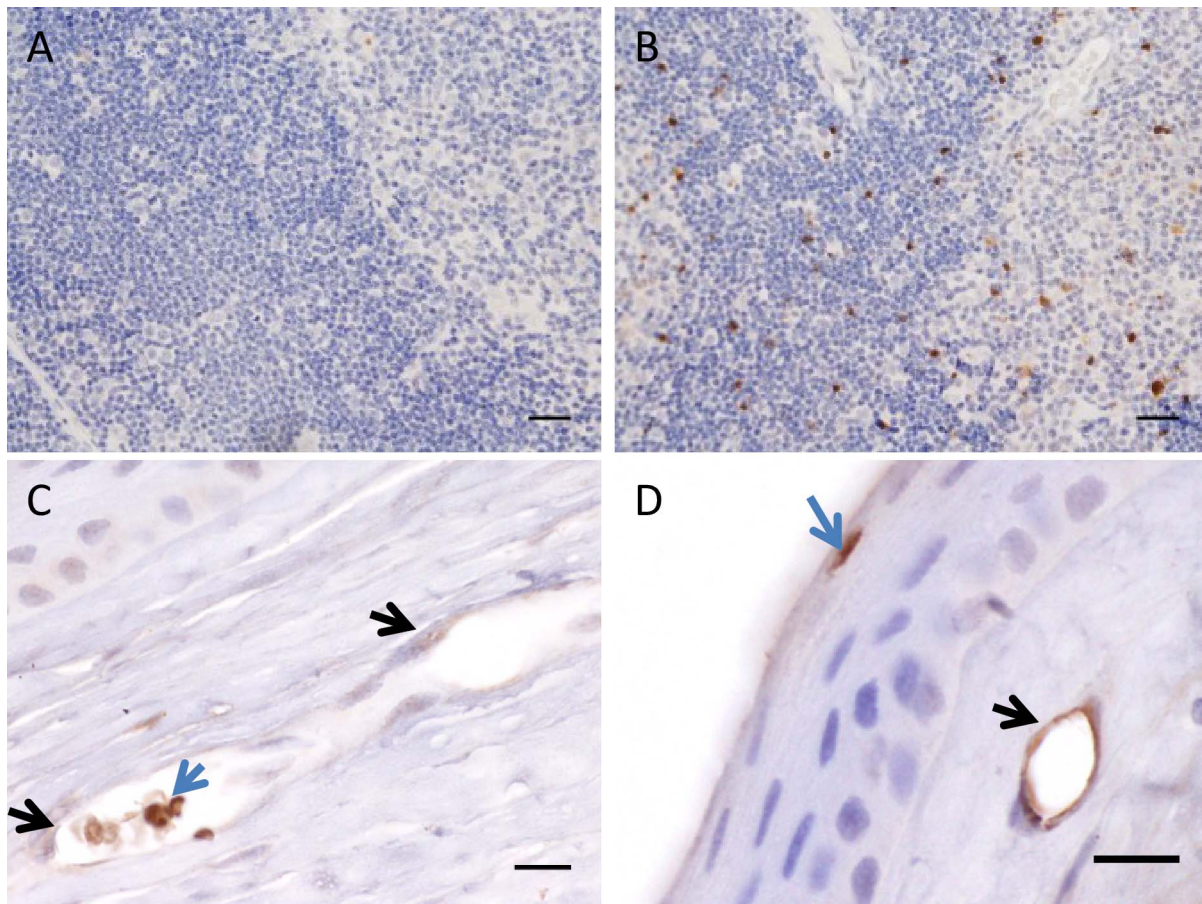


FIGURE 6. Immunohistochemistry for cleaved caspase-3. Corneal sections from control and anti-VEGF-B scFv-treated rats were labeled with cleaved caspase-3 antibody to detect apoptotic cells. (A) Rat thymus labeled with normal rabbit serum was used as a negative control. (B) Positive labeling for cleaved caspase-3 in apoptotic cells in the rat thymus. (C) Blood vessels in the cornea of rats treated with the control scFv were negative for cleaved caspase-3 (black arrows). Red blood cells in the lumen of blood vessels (blue arrow). (D) Blood vessel endothelial cells labeled positive for cleaved caspase-3 in corneas treated with anti-VEGF-B scFv (black arrow). Rare corneal epithelial cell labeling positive for cleaved caspase-3 served as an internal positive control (blue arrow). Scale bars: 20 μ m.

indicating that corneal endothelial cell function was not adversely affected by the drug treatment or the vehicle.

Assessment of Blink Reflex as a Surrogate for Nerve Function. A normal blink reflex was observed in animals from all groups (untreated, control scFv, anti-VEGF-B scFv; Supplementary Table S2). The blink reflex was unaffected up to 12 weeks after cessation of treatment.

Endpoint Histology. Corneas treated with anti-VEGF-B scFv or control scFv appeared morphologically normal at 12 weeks after cautery (Figs. 7A–C). The corneal epithelium showed no indication of thinning (Fig. 7D). Furthermore, treatment with the anti-VEGF-B scFv had no detrimental effect on the integrity of the corneal endothelial cell monolayer (Fig. 7E).

DISCUSSION

A major therapeutic target for the treatment of corneal neovascularization is VEGF-A, which stimulates new blood vessel growth into the cornea.⁶ A meta-analysis of published human case studies concluded that subconjunctival and topical bevacizumab (an anti-VEGF-A antibody) can reduce corneal neovascular area.³¹ Further, a randomized controlled trial by Petsoglou et al.¹³ demonstrated that subconjunctival injection of bevacizumab was able to reduce corneal vessel area by 36%

in patients with recent neovascularization, when compared to results in controls. Thus, anti-VEGF-A therapy is somewhat effective in treating newly-formed corneal vessels, but treatment of established vessels has hitherto proven more difficult.³²

VEGF-B is closely related to VEGF-A^{14,33} and, although it does not induce angiogenesis,^{15,16} it can act as a potent survival factor for blood vessels.^{18,34} VEGF-B-deficient endothelial cells undergo apoptosis in response to oxidative stress or serum starvation in vitro.¹⁸ Moreover, VEGF-B increases blood vessel density in cardiac ischemia models in the mouse,^{35,36} pig, and rabbit.³⁷ These studies indicate that depletion of VEGF-B is detrimental to blood vessel survival, while its addition augments blood vessel survival. Furthermore, VEGF-B has been shown to be expressed endogenously in the mouse cornea³⁸ and in human aqueous humor,³⁹ and its levels increased after epithelial debridement.³⁸ Thus, VEGF-B is an attractive target for the treatment of unwanted, established blood vessels.

The 2H10 hybridoma produces an antibody with activity against human, mouse, and rat VEGF-B.²⁴ The cross-species reactivity of the 2H10 antibody is an attractive property, as it allows in vivo testing in murine models of disease that can be translated into human clinical studies with the humanized form of the antibody.⁴⁰ The 2H10 antibody has already been shown to modulate blood vessels at the back of the eye: in a mouse

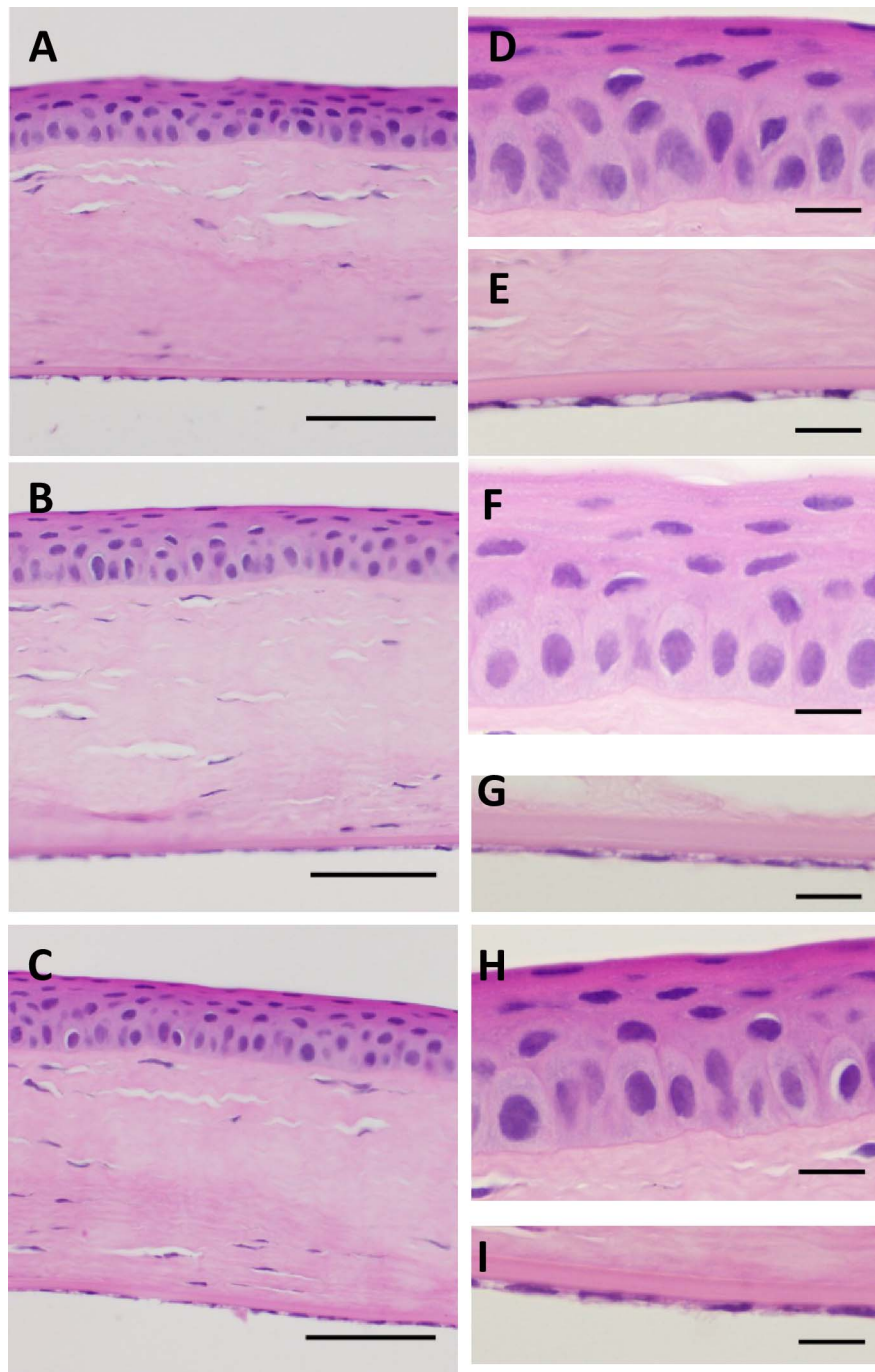


FIGURE 7. Endpoint histology was performed at 12 weeks following cessation of therapy, to assess toxicity of the treatment. Hematoxylin and eosin-stained section of a cornea treated with (A) anti-VEGF-B scFv (B) control scFv or (C) untreated. High magnification images of epithelium (D, E, H) and endothelium (E, G, I). A morphologically normal epithelium was observed in all groups up to 3 months after therapy. Furthermore, a monolayer of corneal endothelial cells was present. *Scale bars:* (A–C) 50 μ m, (D–I) 10 μ m.

model of oxygen-induced retinopathy, intravitreal injection of the antibody promoted blood vessel regression and reduced the number of neovascular tufts.¹⁸ This effect likely resulted from increased endothelial cell apoptosis, as evidenced by colocalization of TUNEL⁺ cells with isolectin IB4-stained endothelial cells.¹⁸ These data suggested that the 2H10 antibody or a smaller fragment, such as the scFv, might be useful for the treatment of established corneal vessels. Antibody fragments, such as scFv's, are particularly attractive as they lack the Fc portion of whole antibodies, which has

been shown to be responsible, in part, for off-target effects of antibody biologics.⁴¹

The anti-VEGF-B scFv we constructed maintained the binding specificity of the 2H10 antibody to VEGF-B, and was functionally active in vitro and demonstrated high affinity binding to human, mouse, and rat VEGF-B, and was stable on storage. Topical or local application is the preferred option for the delivery of ophthalmic drugs, to reduce the likelihood of systemic side effects. Subconjunctival injection is an established route for administration of drugs to the anterior segment

of the eye, and has been used to deliver anti-inflammatory agents, such as triamcinolone,⁴² and antibiotics.⁴³ Clinically, bevacizumab and ranibizumab already have been delivered by subconjunctival injection for the treatment of nascent corneal neovascularization.^{13,32,44}

The local administration of anti-VEGF-B scFv did not prevent the ingrowth of new vessels in a rat model of corneal neovascularization, when compared to administration in either untreated or control scFv-treated groups. This result was not entirely surprising, as transgenic overexpression of VEGF-B induces neither angiogenesis nor lymphangiogenesis.^{15,16,45} Furthermore, VEGF-B protein does not induce endothelial cell proliferation or migration in vitro,⁴⁶ nor angiogenesis in vivo in the brain²¹ or eye.²⁰

Thus, we examined local delivery of anti-VEGF-B scFv for the treatment of established corneal vessels in the rat. Topical therapy alone did not lead to regression of corneal vessels. However, anti-VEGF-B scFv delivered by subconjunctival injection reduced blood vessels by 25% when compared to results in a control scFv group. Furthermore, combined treatment with anti-VEGF-B scFv eye drops and subconjunctival injection proved more effective than subconjunctival injection alone. Complete regression of the blood vessels was not observed at the concentration of scFv and time-point tested. However, a reduction of 37% was achieved compared to results in controls, which was comparable with the results observed in a randomized trial of bevacizumab in newly-developing corneal neovascularization in humans.¹³ Furthermore, we demonstrated positive labeling for cleaved caspase-3 in the corneal blood vessels of anti-VEGF-B scFv-treated animals, suggesting that the anti-VEGF-B scFv induced apoptosis in endothelial cells. Previous studies using the parental 2H10 antibody, from which the anti-VEGF-B scFv was derived, observed a similar mechanism of action in an oxygen-induced retinopathy model in the mouse.¹⁸

No adverse side effects associated with the anti-VEGF-B scFv therapy were observed. However, as VEGF-B is a survival factor for multiple cell types, including neurons, further assessment of corneal nerves and the long-term effects on corneal endothelium must be performed to address safety, ahead of any clinical trial.

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