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Systemic soluble Tie2 expression inhibits and regresses corneal neovascularization ☆

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Abstract

This study was designed to determine if soluble Tie2 (sTie2) expression inhibits and regresses corneal neovascularization, and if VEGF contributes to its effect. The corneas of BALB/c mice were scraped and the mice were injected with either an adenovirus expressing soluble Tie2 (Ad.sTie2) or an empty adenoviral vector. When injected at the inhibition timepoint (one day prior to corneal injury), the mean percentage of neovascularized corneal area two weeks later in Ad.sTie2-treated mice vs. controls was $56.37 \pm 9.15\%$ vs. $85.79 \pm 3.55\%$ ($p = 0.04$). At the regression timepoint (4 weeks after corneal scrape), the mean area of corneal neovascularization in Ad.sTie2-treated mice was $42.89 \pm 4.74\%$ vs. $75.01 \pm 3.22\%$ in the control group ($p = 0.007$). VEGF expression was significantly higher in Ad.sTie2-treated mice at the inhibition timepoint and there was no significant difference at the regression timepoint. These findings suggest that sTie2 inhibits and regresses corneal neovascularization in a VEGF-independent manner.

Keywords: Angiogenesis; Neovascularization; Cornea; Tie2; Inhibition; Angiopoietin; Regression

Neovascularization is the growth of new blood vessels from preexisting vasculature, and generally does not occur in adults except during tissue repair and in the female reproductive system. In the eye, this process is central to visual damage caused by diabetes mellitus, macular degeneration, rejection of corneal transplants, chemical burns, trachoma, Stevens–Johnson syndrome, and other disorders. The cornea is normally avascular to permit optimal visual clarity. However, in pathological conditions, neovascularization can occur, compromising clarity and thus vision.

Disorders of ocular neovascularization are major contributors to world blindness. The conventional approaches to treating corneal and retinal neovascularization, namely photocoagulation, cryotherapy, photodynamic therapy, laser photocoagulation, fine needle diathermy, and photodynamic therapy leave much to be desired. Multiple steps are required for the formation of functional blood vessels, and numerous factors have been identified that regulate this complex process. Of the long list of factors involved in the angiogenic process, VEGF, the angiopoietins, and their cognate receptors are considered key mediators of angiogenesis. While several strategies have been developed to inhibit the effects of VEGF [1–5], recent studies have also reported a major role for the angiopoietin-Tie2 system in postnatal pathologic angiogenesis [6–18].

During early angiogenesis, endothelial cells proliferate, migrate, and form tubular structures. In subsequent

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stages of angiogenesis, new vessels undergo maturation, a process that appears to be regulated by the endothelial transmembrane tyrosine kinase receptor, Tie2 [7,19]. The ligands for Tie2, the angiopoietins (Ang-1 and Ang-2), participate in angiogenesis. Ang-1 is an activating ligand that induces phosphorylation of Tie2 and promotes endothelial cell survival, migration, and vascular impermeability [9,20] while Ang-2 plays a destabilizing role that can lead to vessel regression or growth [14]. While VEGF has been extensively studied [1,21–26], blockade of angiopoietin-mediated signal transduction as a method to control angiogenesis is still emerging.

In one tumor model, a soluble domain of Tie2 has been shown to control tumor vessel growth substantially [27]. Tie2 blockade has been shown to impair tumor angiogenesis, growth, and metastases [13,20,28,29]. Soluble Tie2 has been shown to inhibit Tie2 in vitro in retinal angiogenesis induced by hypoxia-conditioned medium and in a murine model of retinal ischemia [6,30]. Corneal angiogenesis has been stimulated by angiopoietin-1 and -2 in conjunction with VEGF [6], while it has been inhibited by an aptamer sequestering angiopoietin-2 [31,32].

Considering that treatment of human conditions would require rapid and long-term expression of anti-angiogenic molecules, adenovirus-mediated gene delivery might offer a suitable approach as transgenes are expressed for several weeks in animal models [33–37]. We therefore sought to determine whether systemic delivery of an adenovirus directing expression of soluble Tie2 receptor (sTie2) could inhibit and/or regress corneal neovascularization. Furthermore, we sought to determine whether the sTie2 effects were associated with VEGF suppression, the apoptotic mediator Fas ligand, or the vasculogenic mediator stromal derived factor-1 (SDF-1).

Methods

All animal experiments were approved by the Medical College of Georgia Human Assurance Committee, and Institutional Animal Care and Use Committee and conformed to the Helsinki Declaration on Human research and the Association for Research in Vision and Ophthalmology guidelines for animal use. All adenovirus-related work was performed in a Biosafety Level-2 facility and conformed to NIH guidelines.

Experimental design. Two experimental groups were designed to test the effects of the adenovirus encoding soluble Tie2 (Ad.sTie2) on four-week-old male BALB/c mice (Harlan, Indianapolis, IN). Each group consisted of the corresponding timepoints: injection of adenovirus one day prior to corneal injury to test for inhibition of neovascularization, and injection of adenovirus 4 weeks post-injury to test for regression of neovascularization. Fifty microliters of Ad.sTie2 (1×10^{10} PFU per animal) was injected via tail vein, which is known to result in rapid and sustained systemic expression [13–27]. Corneas were harvested 2 weeks post-injection and either processed for neovascular staining ($n = 5$ mice, per subset) or ELISA testing ($n = 4$ mice, per subset). Control mice received injections of an empty adenoviral vector (Ad.Empty) or normal saline.

Recombinant adenovirus. The transgene expression of Ad.sTie2 is under the control of cytomegalovirus immediate early promoter (CMV). Ad.Empty contains a CMV promoter but encodes no gene product and is used as negative control of Ad.sTie2.

Model of corneal neovascularization. As previously described [38], topical proparacaine and 2 mL of 1.0 M NaOH were applied to the right cornea of each mouse. The corneal and limbal epithelia were removed using a Tooke corneal knife (Arista Surgical Supply, New York, NY) in a rotary motion parallel to the limbus. Erythromycin ophthalmic ointment was instilled immediately following epithelial denudation.

Quantification of corneal neovascularization. Digital quantification of corneal neovascularization has been previously described [39]. The quantification of the neovascularization was performed in blinded fashion. Images of the corneal vasculature were captured using a CD-330 charge-coupled device (CCD) camera attached to a fluorescent microscope. The images were analyzed using LSM-5 Image Examiner (Zeiss; 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 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.

Labeling of corneal neovascularization. After sacrifice, mouse eyes were enucleated. As previously described [38], immunohistochemical staining for vascular endothelial cells was performed on corneal flat mounts by a blinded investigator. Fresh corneas were dissected, rinsed in PBS for 30 min, and fixed in 100% acetone (Sigma) for 30 min. After washing four times in PBS, nonspecific binding was blocked with 10% goat serum, 0.1 M PBS, and 2% albumin (Sigma, St. Louis MO) for 1 h at room temperature. Then the corneas were incubated 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, which was followed by subsequent washes in PBS at room temperature. The slides were washed four times with 0.1 M PBS at room temperature. Corneas were mounted with an antifading agent (Gelmount; Biomeda, San Francisco, CA) and visualized with a fluorescent microscope by a blinded investigator.

Harvest for ELISA. Culture medium or corneas harvested for ELISA were placed in 60 μL RIPA buffer (50 mM Tris–HCl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, and 1 mM PMSF), supplemented with 1 μg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM Na orthovanadate, and 1 mM NaF followed by homogenization. The lysate was cleared of debris by centrifugation at 14,000 rpm for 10 min (4 °C), and the supernatant was collected. Total protein was determined with a BCA 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 164-amino-acid splice variant of mouse VEGF. The assay was performed according to the manufacturer's instructions. Briefly, standards or tissue lysate samples (50 μL) were pipetted into an antibody-coated 96-well plate containing 50 μL of assay diluent and incubated for 2 h at RT on a shaker. The wells were then washed five times with wash buffer, 100 μL of VEGF conjugate was added, and the samples were again incubated for 2 h at RT. Samples were washed five times, 100 μL of substrate buffer was added, the samples were incubated for 30 min at RT, the reaction was stopped, and the absorption was measured with an ELISA reader (Emax; Molecular Devices, Sunnyvale, CA) at 450 nm with lambda 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 concentration.

RT-PCR. Harvested corneas were put into RNA lysis buffer and sonicated (Fisher Scientific, Pittsburgh, PA) four times each for 30 s at 1 min intervals on ice. RNA was isolated using an RNEasy mini kit (Qiagen, Valencia, CA), and afterward, cDNA was synthesized from 1 μg total RNA using reverse transcriptase and oligo(dT) primers (Qiagen). The presence of Fas and FasL in corneas was determined by PCR amplification of cDNA using FasL and Fas primer sets (Sigma, St. Louis, MO). The presence of SDF was determined using 5′-TTAA GCTTCGCGCCATGAACGCCAAGGTC-3′ forward primer and 5′-TTTGCGGCCGCTTACTTGTTTAAAGCCTTCTCCAGCT-3′ reverse primers (IDT, Coralville, IA). The presence and quality of RNA was confirmed for all samples by positive amplification of an 18S housekeeping gene (IDT), and target RNA was amplified within 50 μL reaction mixture using an Eppendorf thermal cycler (Fisher Scientific) programmed for 35 cycles. Amplified products were run on a 2% agarose gel.

Statistics. Data were analyzed by independent sample Student's *t* test (SPSS, Chicago, IL). Type I error not exceeding 0.05 was deemed significant.

Results

The systemic adenovirus injections did not lead to any kind of toxic or significant inflammatory immune response in the Ad.sTie2-treated mice in both inhibition and regression groups.

Fraction of corneal area covered by neovascularization

At the inhibition timepoint, the mean area ± SEM of corneal neovascularization in Ad.sTie2-treated mice was 56.37 ± 9.15% vs. 85.79 ± 3.55% in the control group ($p = 0.04$). At the regression timepoint, the mean area of corneal neovascularization in Ad.sTie2-treated mice was 42.89 ± 4.74% vs. 75.01 ± 3.22% in the control group ($p = 0.007$). Representative images are shown in Fig. 1.

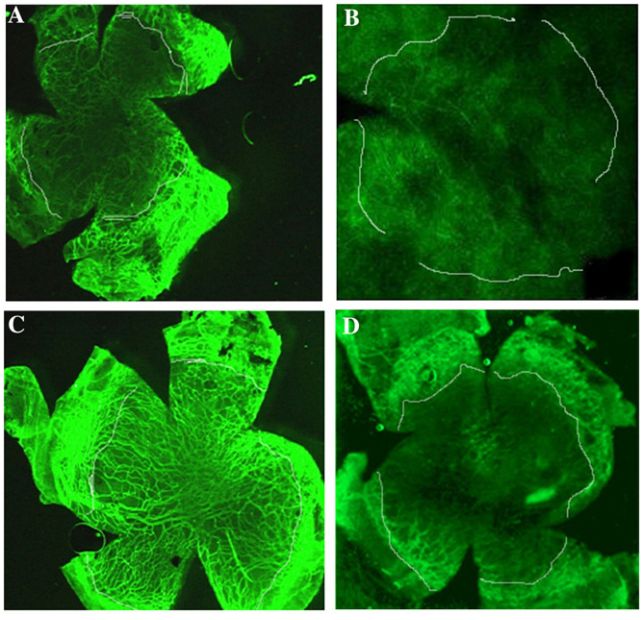

Fig. 1. Representative photographs depicting corneal neovascularization. (A) Control (inhibition timepoint); (B) Ad.sTie2 treated (inhibition timepoint); (C) control (regression timepoint); and (D) treated (regression timepoint).

VEGF expression

VEGF expression was significantly higher in Ad.sTie2-treated mice as compared to the controls at the inhibition timepoint (187.27 ± 10.87 pg/mcg total protein vs. 27.44 ± 1.34 pg/mcg, $p = 0.042$). In contrast, at the regression timepoint, there was no significant difference between VEGF levels in the Ad.sTie2-treated mice and the controls (47.34 ± 17.57 pg/mcg total protein vs. 46.23 ± 16.49 pg/mcg, $p = 0.898$).

Fas and FasL expression

Fas and FasL were undetectable in the corneas of both treated and control groups in the inhibition group, whereas both Fas and FasL were upregulated in Ad.sTie2-treated mice but undetectable in the controls in the regression group. Representative images are shown in Fig. 2.

SDF-1 expression

The stromal-derived factor (SDF) expression was detectable in control mice in both regression and inhibi-

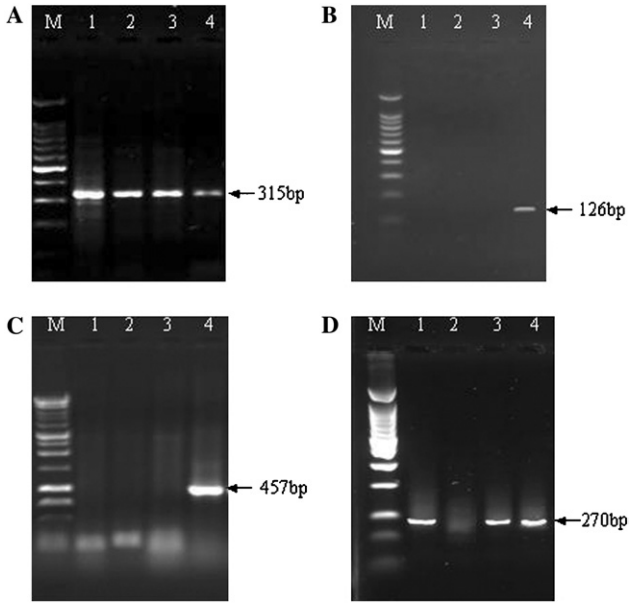

Fig. 2. DNA gel electrophoresis showing expression of 18S RNA (A) and FAS (B), FASL (C), and SDF (D) in injured mouse corneas. In all figures, layout is as follows: Lane M, marker; lane 1, inhibition-control (inhibition timepoint); lane 2, Ad.sTie2 treated (inhibition timepoint); lane 3, control (regression timepoint); and lane 4: treated (regression timepoint).

tion groups, but in Ad.sTie2-treated mice it was detectable only in the regression timepoint, i.e., it was not present at the inhibition timepoint in the treated mice. Representative images are shown in Fig. 2.

Discussion

In this study, we demonstrate that adenovirus-directed systemic expression of soluble Tie2 seems to impede corneal neovascularization. The use of a clinically relevant model (chemical–mechanical trauma) and a clinically relevant endpoint (regression of vessels, not merely inhibition) adds to the import of these findings. Further, we show that this anti-angiogenic effect seems to be VEGF-independent, and that regression of corneal neovascularization is associated with higher levels of Fas ligand and SDF-1.

While inhibition of Tie2 signaling was associated with reduced neovascularization, VEGF was elevated in the inhibition subgroup. This surprising finding may suggest that physiological Tie2 signaling suppresses VEGF expression or that the injured cornea may be secreting more VEGF to recruit blood vessels as a response to angiogenic blockade caused by the inhibition of Tie2 signaling. VEGF is known to induce angiopoietin-2 [15,18], and hence our finding of VEGF elevation may be due to disruption of a feedback loop. It would be intriguing to speculate that blockade of both Tie2 and VEGF pathways would result in a synergistic inhibition of corneal angiogenesis, as has been reported in a murine retinal model [6]. Notably, in spite of elevated VEGF levels in the Ad.sTie2-treated inhibition group, neovascularization was nonetheless reduced compared to control animals. This finding supports previous work demonstrating that angiopoietin/Tie2 signaling critically modulates VEGF-mediated angiogenesis and vascular remodeling, and in some cases can predominate [11,72].

As sTie2 disrupts the effects of both angiopoietin-1 and -2, this study cannot elucidate whether disrupting one or the other is more responsible for the observed reduction in angiogenesis. Both of them may well be involved, as angiopoietin-1 suppresses apoptosis through PI3-kinase and AKT [40], while angiopoietin-2 increases vessel growth in the presence of VEGF [14]. However, a recent study suggests that the effects of sTie2 may be primarily mediated through inhibition of angiopoietin-2. An aptamer targeting angiopoietin-2 was able to reduce bFGF-induced corneal neovascularization by slightly less than 30% [31]. In the present study, soluble Tie2 receptor expression was able to reduce corneal neovascularization by 36–43% depending on the timepoint.

Our finding that Fas and Fas ligand were upregulated in the mice expressing soluble Tie2 expression in the regression subgroup is consistent with our hypothesis that Tie2 blockade may induce regression of vessels through apoptosis. Recently, it has been demonstrated that FasL expressed in the retina controls new vessel growth beneath the retina by inducing apoptosis in vascular endothelial cells, which are known to express Fas antigen [41,42]. VEGF and Tie2 signaling activate AKT and PI3-kinase, known to be anti-apoptotic factors [40,43–49], while pharmacologic agents blocking PI3-kinase and AKT also upregulate FasL [50]. FasL is known to mediate antiangiogenic effects in the cornea [51,52] and apoptosis in endothelial cells [41,53]. A significant increase has been shown in cornea neovascularization in FasL-deficient mice and new vessel growth is inhibited in these mice by anti-Fas antibody [52]. The absence of Fas ligand also is associated with increased neovascularization [54]. Inhibition of PI3-kinase signaling seems to facilitate Fas ligand-induced apoptosis in endothelial and other cell types [55–60]. Fas and Fas ligand are present in normal cornea [52]. They seem to be suppressed in the early injury phase as we could detect their expression neither in the treated nor the control group. The addition of soluble Tie2 at 4 weeks after injury may be able to relieve this suppression after the acute injury phase has passed, while in the early injury phase its effect may be overwhelmed by the effect of the injury.

As formation of blood vessels involves communication between vascular endothelial cells and stromal cells, we also investigated whether SDF levels may be affected in our model. Stromal-derived factor (SDF) is a chemokine involved in vasculogenesis through the recruitment of endothelial progenitor cells [61–65]. SDF is known to be produced by corneal fibroblasts [66] and is upregulated by hypoxia [67]. Endothelial progenitor cells have been found to be present in corneal neovascularization [68]. Anti-angiogenic substances have been found to cause apoptosis in endothelial progenitor cells [69] and reduce tumor angiogenesis [70]. The Tie2 pathway has been found to be important in crosstalk between endothelial cells and stromal cells: Tie2 knockout mice have abnormal vasculogenesis and reduced pericyte encapsulation of vessels [7,19]. We found that Ad.sTie2 administration suppressed SDF expression when given just before corneal injury but not when given 4 weeks after corneal injury. These results support a role for sTie2-mediated suppression of endothelial progenitor cell recruitment to the cornea in the early injury phase. Tie2 blockade may be successful at blocking SDF expression in the early injury phase but not late after injury, as perhaps once SDF expression is established, it may not be susceptible to disruption in Tie2 signaling.

We have previously demonstrated angiostatin to be the first biological agent to induce regression of corneal neovascularization [38]. Angiostatin has been shown to activate Fas ligand-mediated apoptosis in endothelial cells [71]. Here we demonstrate that inhibition of Tie2 signaling by adenoviral-mediated systemic expression of soluble Tie2 can inhibit and regress corneal neovascular-

ization. These effects were not mediated by suppression of VEGF. The regression of corneal neovascularization in this model was associated with elevated Fas/Fas ligand expression, consistent with apoptosis being involved in regression, while the inhibition of corneal neovascularization was associated with suppression of SDF expression, possibly indicating reduced endothelial progenitor cell recruitment to the cornea. This effect most likely represents true regression as pericyte coverage of corneal neovascularization has been demonstrated to largely occur within 2 weeks of injury [73].

This study supports the development of approaches to target multiple mediators, including the Tie2 pathway to suppress disorders of neovascularization. Future investigation should clarify the effects of corneal injury on angiopoietin levels in the cornea, determine the effects of Tie2 blockade on lymphangiogenesis, confirm the presence of endothelial apoptosis in vascularized corneas undergoing vascular regression, and evaluate the fraction of corneal vessels seeded by endothelial progenitor cells.

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