EXTENDED REPORT

Soluble vascular endothelial growth factor receptor-1 contributes to the corneal antiangiogenic barrier

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6 December 2006**Purpose:** Pathological neovascularisation within the normally avascular cornea is a serious event that can interfere with normal vision. Upregulation of vascular endothelial growth factor (VEGF) has been associated with neovascularisation in the eye, suggesting that maintaining low levels of VEGF is important for corneal avascularity and intact vision. This study aims to determine the expression profile and possible contribution of sVEGFR-1 to the corneal avascular barrier.**Design:** Experimental case series investigating VEGF and soluble fms-like tyrosine kinase (sFlt) levels in normal and neovascularised human corneas.**Participants:** Four normal human corneas, five human corneas with alkali burns, three human corneas with aniridia, one with ocular cicatricial pemphigoid and two with interstitial keratitis were examined.**Methods:** Western blot and immunohistochemical analyses were performed to determine sFlt and VEGF levels in normal and neovascularised human corneas. Immunoprecipitation was utilised to demonstrate sFlt–VEGF binding.**Results:** Normal human corneas strongly express sFlt in the corneal epithelium and weakly in the corneal stroma close to the limbus. VEGF is bound by sFlt in the normal human cornea. Neovascularised human corneas have greatly reduced expression of sFlt and significantly less VEGF bound by sFlt.**Conclusions:** sFlt is highly expressed in the human cornea and normally sequesters VEGF.

It has long been unclear how the normal cornea remains avascular. Many studies have linked various antiangiogenic factors such as angiostatin, endostatin, thrombospondin, interleukins 4 and 13, and other proteolytic fragments of extracellular matrix components to corneal avascularity.¹ Vascular endothelial growth factor (VEGF) has been determined to be a key mediator of angiogenesis in many models, including the cornea.² Many antiangiogenic factors are linked with downregulating or counteracting VEGF, but the responsible factors remain elusive.³

We have recently shown that soluble fms-like tyrosine kinase (sFlt) is essential to corneal avascularity in a variety of animal models.⁴ Our present study investigates sFlt and its presence in the normal and diseased states in humans to further characterise its potential role in anti-angiogenesis. sFlt has been shown to be antiangiogenic in several models by acting as a decoy receptor for secreted VEGF and also inactivating membrane-bound VEGF receptors 1 and 2 by heterodimerisation.^{4–7}

sFlt consists of the first six domains of membrane-bound VEGF receptor 1, and a unique 31 amino acid tail, the purpose of which remains unknown, but which is highly conserved in the animal kingdom.⁸ sFlt, which lacks the membrane-proximal immunoglobulin-like domain, the transmembrane spanning region and the intracellular tyrosine-kinase domain, is generated by alternative splicing.⁹

Herein, we describe our observations that sFlt is highly expressed in the cornea and normally sequesters VEGF in a consecutive series of corneal specimens from people with normal corneas and patients with neovascular corneal disease.

METHODS

All experiments and procedures involved were conducted in accordance with the Declaration of Helsinki and approved by the Institutional Human Assurance Committee. We have

analysed human corneas using techniques of immunohistochemistry, immunoprecipitation and Western blotting. Four normal human corneoscleral specimens (normal limbus and sclera derived after use of central cornea for transplantation) and three normal central corneas (derived after use of limbus for limbal stem cell transplant tissue) were used from the Georgia Eye Bank (Atlanta, Georgia, USA). A consecutive series of five patients with alkali burns, three patients with aniridia, two patients with remote history of interstitial keratitis and one with ocular cicatricial pemphigoid were also examined; all of these had corneal neovascularisation involving more than two quadrants of the cornea (the patients with prior interstitial keratitis had regressed or "ghost" neovessels).

Table 1 Demographic data of the patients

Age (years)	Race	Sex	Diagnosis
35	Black	Female	Alkali burn
52	Black	Female	Alkali burn
75	White	Female	OCF
42	White	Male	Aniridia
37	White	Male	Alkali burn
26	White	Male	Alkali burn
65	Black	Male	Interstitial keratitis
19	White	Female	Aniridia
42	White	Male	Alkali burn
23	White	Female	Aniridia
58	White	Male	Interstitial keratitis

OCF, ocular cicatricial pemphigoid.

Abbreviations: PBS, phosphate-buffered saline; sFlt, soluble fms-like tyrosine kinase; VCAM, vascular cell adhesion molecule; VEGF, vascular

Figure 1 Soluble fms-like tyrosine kinase (sFlt) staining of the normal human cornea. (A, B) Normal human cornea shows heavy, brown sFlt staining in corneal epithelium and lighter staining in stromal keratocytes. (C, D) Negative controls using non-specific IgG show no staining (A and C magnification = $\times 20$, B and D magnification = $\times 40$).

IMMUNOHISTOCHEMISTRY

Human corneal specimens were placed in neutral buffered formalin and then paraffinised. Sections 4 μ m thick were cut from paraffin blocks and mounted on treated slides (Superfrost plus, VWR Scientific Products, Suwanee, Georgia, USA). Slides were air dried overnight, then placed in a 60°C oven for 30 min. Slides were then deparaffinised in two changes of xylene for 7 min and run through two changes of absolute ethanol for 2 min each, two changes of 95% ethanol for 2 min, 80% ethanol for 2 min, 70% ethanol for 2 min and finally with distilled water. Slides were pretreated, if required, with primary antibody with Target Retrieval Solution (pH 6) (Dako, Carpinteria, California, USA) using a steamer (Black & Decker rice steamer, Black & Decker, Miramar, FL, USA) and then rinsed in distilled water. Endogenous peroxidase was quenched with 0.3% H_2O_2 in distilled water for 5 min, followed by distilled water for 2 min. Slides were then incubated in Power Block (Biogenex, San Ramon, California, USA), rinsed in distilled water and placed in 1 $\times$ phosphate-buffered saline (PBS) for 5 min. Slides were then incubated with primary antibody (rabbit anti-C-terminal of sFlt, provided by Dr Angela Orecchia, IDI, Rome, Italy) and vascular cell adhesion molecule (VCAM) antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) in a 1:1000 concentration for 2 h at room temperature, followed by rinsing in two changes of 1 $\times$ PBS. Incubation with secondary biotin-labelled affinity isolated goat anti-rabbit immunoglobulins (LSAB2 - HRP kit, Dako, Japan, Kyoto and Carpinteria, California, USA) followed for 10 min, and the slides were again rinsed in two changes of 1 $\times$ PBS. Slides were then incubated in streptavidin-HRP (LSAB2, Dako, Carpinteria, California, USA) for 10 min and rinsed in two changes of 1 $\times$ PBS. Bound antibody was detected with the 3,3'-diaminobenzidine substrate kit (Dako-DAB substrate kit for peroxidase-HRP). Slides were then counterstained with haematoxylin (Richard-Allan Scientific, Kalamazoo, Michigan, USA).

CORNEAL MICROSCOPY

Images of human cornea were captured using a Spot 1.3 Cooled Color Digital camera (Spot Diagnostic Instruments, Michigan, USA) attached to an Olympus AX 70 True Research System microscope (Olympus America, Melville, New York, USA)

Figure 2 The normal human limbus is shown. (A) Staining for soluble fms-like tyrosine kinase (sFlt) only (brown) with corneal epithelial and weak stromal staining. (B) Double immunostaining for sFlt (brown) and vascular cell adhesion molecule (VCAM-1) (red). VCAM-1 staining indicates blood vessels in the sclera (S) approaching the limbus, but sparing the cornea (C). Negative controls using non-specific IgG not shown.

controlled by Spot 3.4 software. General morphology images were captured with $\times 20$ magnification; endothelial and stromal images were captured at $\times 40$ and $\times 100$ oil immersion magnifications. All images required 9 V bright field. The images were fed into a Sony Trinitron MultiScan 500PS computer (Dell Computer Corporation, Round Rock, Texas, USA) and saved as joint photographic experts group (jpeg) files.

IMMUNOPRECIPITATION AND WESTERN BLOT

Human corneas were harvested, freeze-fractured with a mortar and pestle, and placed in 200 μ l radioimmunoprecipitation assay buffer (Tris-HCl, NaCl, NP-40, Na-deoxycholate and protease inhibitors). After incubation for 1 h in radioimmunoprecipitation assay buffer, tissue samples were sonicated on ice four times at 15 s intervals at level 4 intensity. Immunoprecipitation of corneal cells with anti-sFlt antibody (provided by Dr Angela Orecchia, IDI, Rome, Italy) began with pipetting 50 μ l of corneal cells into a 1.5 ml Eppendorf tube (Fisher Scientific, Pittsburgh, Pennsylvania, USA) and adding 50 μ l of sterile PBS to the tube. Anti-sFlt antibody was added to the tube in 1:200 concentration and the resulting mixture was incubated overnight at 4°C on a refrigerated shaker. The next day, 400 μ l of agarose beads were placed in a separate Eppendorf tube and washed with an equal amount of PBS. To wash, the beads and PBS were inverted several times and spin pulsed in a mini-centrifuge. After removing the supernatant, another 400 μ l of PBS was added to the beads and the procedure was repeated. After three washes, the agarose beads were finally resuspended in 40 μ l 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 next day, the beads were collected and washed using the aforementioned procedure. The beads were

Figure 3 Western blot demonstrating relative expression of soluble fms-like tyrosine kinase (sFlt) in the human cornea and sclera. In humans, sFlt is highly expressed in the limbal cornea (lane 1), considerably expressed in the central cornea (lane 2) and expressed to a lesser degree in the sclera (lane 3); M is marker. β -Actin serves as internal control.

resuspended in 50 μ l of a 1:19 mixture of 2-mercaptoethanol:Laemmli buffer. After boiling the beads for 5 min and submerging them in ice for 5 min immediately thereafter, the beads were centrifuged at 4°C, 10 000 rpm, for 10 min. The supernatant was collected and loaded into 6% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels. After transferring these gels, nitrocellulose paper membranes were probed for VEGF protein. Nitrocellulose paper membranes were blocked for 1 h at room temperature with 10% milk in PBS and then incubated overnight at 4°C in a concentration of 1:1000 VEGF primary antibody (BD Bioscience/Pharmingen, San Diego, California, USA). The appropriate secondary antibody concentration of 1:5000 (BD Bioscience/Pharmingen) was used to incubate the membranes for 2 h at room temperature. After washing with PBS-Tween 20, the membranes were developed on film (Kodak BioMax Light Film, Kodak, Rochester, New York, USA) using a chemiluminescence kit (ECL, Pierce, Rockford, Illinois, USA).

RESULTS

Table 1 lists the demographic data of the patients.

All normal tissue was rated as good-plus or excellent by the eye bank. Normal human corneas express sFlt strongly in the corneal epithelium. There is also weak expression by stromal keratocytes; this expression is preferentially exhibited closer to the limbus. No endothelial expression of sFlt was observed. The presence of vasculature (VCAM positivity) is mainly observed in the sclera sparing the cornea. Figures 1 and 2 show representative images of normal human corneas.

The levels of sFlt expression vary according to the corneal-scleral region examined. Western blot demonstrates that the limbal and central corneal regions have higher levels of sFlt compared with the scleral region. Also, after immunoprecipitation of the human cornea with anti-sFlt antibody and subsequent western blotting for VEGF, we were able to show that VEGF is bound by sFlt in the normal human cornea. Figures 2–4 show representative images of western blots of normal human corneas. The slight variation in band size for the sFlt+ bands in fig 3 may represent variable differences in glycosylation of sFlt in the microenvironments of the cornea, sclera and limbus.

Figure 5 Relative expression of soluble fms-like tyrosine kinase (sFlt) in normal versus neovascularised human cornea. Lane 1: sFlt expression in the normal human cornea. Lane 2: diminished human corneal sFlt expression in the neovascularised cornea. β -Actin serves as internal control.

Figure 6 A representative image showing decreased expression of soluble fms-like tyrosine kinase (sFlt) in a neovascularised cornea from alkali burn. (A) In a patient with neovascularised cornea (see vessel in stroma) from alkali burn. (B) sFlt staining is diminished in the corneal epithelium relative to normal (shown in fig 1). Negative controls using non-specific immunoglobulin G (IgG) not shown. Magnification = $\times 40$.

In specimens of neovascularised human corneas (eg, from alkali burn or aniridia), sFlt expression is greatly reduced, as was demonstrated by western blotting (fig 5) and immunostaining techniques (figs 6 and 7). VCAM staining confirms the presence of blood vessels in neovascularised specimens (fig 7).

As noted above, immunoprecipitation in the normal human cornea demonstrates VEGF binding. In the neovascularised cornea, significantly less VEGF is bound by sFlt (representative image shown in fig 8).

In patients with a remote history of interstitial keratitis, a condition in which the cornea undergoes neovascularisation in the teenage years with spontaneous resolution leaving ghost vessels, immunohistochemical analysis shows sFlt expression in the corneal epithelium and ghost vessels in the corneal stroma (confirmed by collagen IV positivity and VCAM negativity) (representative photograph shown in fig 9).

DISCUSSION

Our findings in a series of corneal specimens that sFlt is expressed highly in normal corneal tissue with a preferential gradient are consistent with a limbal antiangiogenic barrier. Further, sFlt appears to be decreased in conditions of corneal neovascularisation, and immunoprecipitation studies reveal desequstration of VEGF correlated with decreased sFlt in such conditions. Intriguingly, restoration of corneal epithelial sFlt expression is associated with regression of neovessels into ghost vessels in interstitial keratitis.

VEGF receptor 1 is a transmembrane receptor that binds VEGF with high affinity, initiating intracellular signalling. Alternative splicing of VEGF receptor 1 mRNA results in the production of a soluble form of VEGF receptor—that is, sFlt. This soluble receptor is an endogenous inhibitor of VEGF

Figure 7 A representative image demonstrating decreased expression of soluble fms-like tyrosine kinase (sFlt) in neovascularised cornea from aniridia. In a patient with neovascularised cornea from aniridia, vascular cell adhesion molecule (VCAM) staining (red) is prominent in stroma in vessels, but minimal sFlt staining (brown) is present in corneal epithelium (A: $\times 10$; B: $\times 20$). Negative controls using non-specific IgG not shown. Magnification = $\times 40$.

Figure 8 Western blot showing comparison of vascular endothelial growth factor (VEGF) levels in normal versus neovascularised cornea. Lane 1: western blot for VEGF after immunoprecipitation of normal human corneal lysate for soluble fms-like tyrosine kinase (sFlt) shows considerable binding of VEGF. Lane 2: neovascularised human cornea (patient with ocular cicatricial pemphigoid) with less VEGF binding. Lane 3: marker-VEGF is at 25 kDa.

signalling that acts by sequestering VEGF and possibly by forming dimers with full-length VEGF receptor 1, resulting in dominant-negative effects.¹⁰

This endogenous inhibitor of angiogenesis has proven to be an effective therapeutic tool when administered exogenously, as it has been shown effective in the experimental treatment of several neovascular diseases including arthritis,¹¹ retinal neovascularisation,^{12, 13} and cancer of the thyroid and pancreas.^{14, 15} In each of these instances, sFlt is linked to significant decreases in pathologic neovascularisation, with Rota *et al*¹³ showing a 97.5% decrease in pathologic retinal neovascularisation without affecting pre-existing retinal vessels.

In summary, we show that sFlt is present in the normal human cornea and is found in higher quantities throughout the epithelium and in the stroma close to the limbus. We also show that VEGF that is normally sequestered by sFlt in the cornea is released in the presence of lower sFlt levels in neovascularised human corneas. This comports with our recent observations of the critical role of sFlt in the evolutionary basis of corneal avascularity, and the findings herein provide a powerful human correlate.⁴ Statistical analysis was not performed in this study, owing to the small sample size and the fact that immunohistochemistry is by its nature qualitative and difficult to quantify. However, the authors are confident in the reliability and reproducibility of the methods employed. Future studies will need to conclusively determine whether sFlt is critical for avascularity by development of knockout animal models. These studies are under way.

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Figure 9 Representative image of corneal specimen from patients with remote history of interstitial keratitis. Soluble fms-like tyrosine kinase (sFlt) expression is strongly present in corneal epithelium (brown staining; black arrow). Ghost vessels (collagen IV + (red), vascular cell adhesion molecule (VCAM) negative) are present in the corneal stroma (white arrows). Magnification = ×40.

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