·Basic Research·

Effect of trapping vascular endothelial growth factor-A in a murine model of dry eye with inflammatory neovascularization

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Abstract

• **AIM:** To evaluate whether trapping vascular endothelial growth factor-A (VEGF-A) would suppress angiogenesis and inflammation in dry eye corneas in a murine corneal suture model.

• **METHODS:** We established two groups of animals, one with non-dry eyes and the other with induced dry eyes. In both groups, a corneal suture model was used to induce inflammation and neovascularization. Each of two groups was again divided into three subgroups according to the treatment; subgroup I (aflibercept), subgroup II (dexamethasone) and subgroup III (phosphate buffered saline, PBS). Corneas were harvested and immunohistochemical staining was performed to compare the extents of neovascularization and CD11b+ cell infiltration. Real-time polymerase chain reaction was performed to quantify the expression of inflammatory cytokines and VEGF-A in the corneas.

• **RESULTS:** Trapping VEGF-A with aflibercept resulted in significantly decreased angiogenesis and inflammation compared with the dexamethasone and PBS treatments in the dry eye corneas (all P <0.05), but with no such effects in non-dry eyes. The anti-inflammatory and anti-angiogenic effects of VEGF-A trapping were stronger than those of dexamethasone in both dry eye and non-dry eye corneas (all P <0.05). The levels of RNA expression of VEGF-A, TNF-alpha, and IL-6 in the aflibercept subgroup were significantly decreased compared with those in the PBS subgroup in the dry eye group.

• **CONCLUSION:** Compared with non-dry eye corneas, dry eye corneas have greater amounts of inflammation and neovascularization and also have a more robust response to anti-inflammatory and anti-angiogenic agents after ocular surface surgery. Trapping VEGF-A is effective in decreasing both angiogenesis and inflammation in dry eye corneas after ocular surface surgery.

• **KEYWORDS:** dry eye; aflibercept; neovascularization; inflammation; dexamethasone

INTRODUCTION

D ry eye can be a pre-angiogenic and pre-inflammatory milieu [1-6]. Therefore, preoperative corneal dryness aggravates injury or surgery-induced neovascularization (NV), lymphangioggenesis (LY) and inflammation.

In patients with dry eye, preoperative suppression of inflammation is considered to be important for fostering a less inflammatory milieu before surgery[4,7-10].

Accordingly, postoperative suppression of inflammation is more important in patients with dry eye compared with patients without dry eye, because dry eye corneas exhibit amplified inflammation and preangiogenic characteristics after surgery.

Many researchers[11-12] have shown that the core mechanism of dry eye disease is inflammation. The treatment with topical or systemic anti-inflammatory drugs improves dry eye disease symptom and signs[12-15].

The inflammatory role is increased according to the severity of dry eye [13-15]. We hypothesized that there may be a greater role of anti-vascular endothelial growth factor-A (VEGF-A) therapy especially in severe dry eye with inflammation, such as preexisting dry eye with ocular surgery.

Figure 1 Schedule of dry eye inductions and subconjunctival injections in the three different subgroups: aflibercept, dexamethasone, and PBS.

Even though the effect of bevacizumab for treating ocular surface inflammation and dry eye has been reported[16-18], there has not been any further research on the role of other anti-VEGF-A agents in dry eye disease with inflammatory NV. Here, we evaluated the effect of trapping VEGF-A, aflibercept as a treatment.

We evaluated whether trapping VEGF-A could suppress postoperative angiogenesis and inflammation in dry eyes and we compared the effects with those achieved in non-dry eyes. We also evaluated whether the effect is comparable to that of well known anti-inflammatory treatment, corticosteroids.

MATERIALS AND METHODS

Experiment Animal Model Mice were assigned to one of 2 groups, the normal eye (non-dry eye, 38 eyes) or the anticholinergic-induced experimental dry eye model (42 eyes). In the dry eye group, we began dry eye induction 2wk before the planned surgical insults. Dry eye was induced in the mice by pharmacologically applying a topical application of 1% atropine sulfate (Alcon Korea, Seoul, South Korea) twice in the first 48h of the experiment [19]. Subcutaneous injections of 0.3 mL of 10 mg/mL scopolamine hydrobromide (Sigma-Aldrich, St. Louis, MO, USA) were also given 2 times a day each day for the entire duration of the experiment (23d)[19].

After two weeks of dry eye induction, two corneal stromal sutures were made to induce angiogenesis and inflammation in both the dry eye and non-dry eye groups.

Effects of Anti –inflammatory and Anti –angiogenic Drugs in Dry Eyes vs Non –dry Eyes with Ocular Surface Surgery Each of the 2 groups was again divided into three subgroups according to the treatment: subgroup I [Eylea® (aflibercept), 5 mg/mL, 15 μL, 12 eyes], subgroup II (dexamethasone, 5 mg/mL, 15 μL, 12 eyes), and subgroup III [phosphate-buffered saline (PBS), 15 μL, 12 eyes], for a total of 6 experimental subgroups (Figure 1).

Aflibercept, dexamethasone, or PBS was injected into the mice in each subgroup as appropriate in both the dry eye and non-dry eye groups on the operation day. Treatments were then given daily until the ninth postoperative day. After harvesting the corneas, immunohistochemical double staining for CD11b and CD31 was performed. Immunostained cornea sections were examined on fluorescence and confocal microscopes as described below.

Immunohistochemical Staining After harvesting the corneas on the ninth postoperative day, we performed immunohistochemicalstaining to assess the levels of NV and inflammatory infiltration. Each cornea was trimmed of any remaining limbus and iris. Immunohistochemical staining for

vascular endothelial cells and inflammatory cells was performed on corneal flat mounts. Fresh corneas were dissected, rinsed in PBS for 30min, and fixed in 100% acetone (Sigma) for 20min. After washing in phosphate buffered saline with Tween® 20 (PBST) (0.1% Tween® 20/PBS), nonspecific binding sites were blocked with 3% bovine serum albumin (BSA)/PBS for 3 nights at 4℃. Corneas were then incubated overnight with fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-mouse CD31 antibodies (1:500; 558738, BD Pharmingen) and Alexa Fluor® 647 rat anti-mouse CD11b antibodies (1:100; 557686, BD Pharmingen) in 3% BSA/PBS at 4℃. After this incubation, the corneas were washed four times with PBST at room temperature and mounted with the anti-fading agent Gelmount.

Fluorescence Microscopy Examination After immunochemical staining for vascular endothelial cells and flat mounting of the corneas (7 to 8 eyes from each group), images of the corneal vasculature were captured using acamera attached to a fluorescence microscope (OLYMPUS BX51, Tokyo, Japan). NV was quantified using ImageJ (National Institutes of Health) as described below. The total area of NV was calculated as follows: total NV (%)= (neovascularized area of total cornea/total cornea area) × 100%.

Confocal Microscopy Examination After harvesting corneas on the ninth postoperative day and performing immunohistochemical staining, we evaluated CD11b+ cell infiltration with a confocal microscope.

Briefly, 3-4 regions from each cornea (3 eyes from each group) were chosen from both the dry eye and non-dry eye groups. A confocal microscope (LSM 510 META, Carl Zeiss, Germany) was used to quantify the area of inflammatory infiltration in each cornea. Horizontal sections (objective magnification ×10) of 17-19 images were obtained from the top surface to the bottom of the cornea at 5-μm intervals and stacked to create a final image stack. In each image stack, inflammatory infiltrationwas quantified by setting a threshold level of fluorescence above which cells were captured and processed using ImageJ (National Institutes of Health). The percentage area of CD11b+ cell infiltration was analyzed in each stack image using the pixel area.

Quantitative Real −time Polymerase Chain Reaction Analysis of Gene Expression in the Mouse Cornea Total RNA was purified with an RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Complementary DNA synthesis was performed with Reverse Transcription Master Premix (Elpis Bio, DaeJeon, South Korea) using SYBRGreen (Roche, Basel, Switzerland) and thermocycling was performed using a LightCycler® 2.0 instrument (Roche). We used published primer sequences for mouse GAPDH[10], mouse VEGF-A[20], mouse TNF-alpha[21],

and mouse IL-6 [22]. Each gene expression level was analyzed by the Ct method, using GAPDH expression as an internal control. The relative expression level of each sample is expressed as a fold change compared to the normal control. At the conclusion of the 23d of treatment [including dry eye induction (0.3 mL of 10 mg/mL of scopolamine hydrobromide, 2 times a day) and subconjunctival injection of each treatment], we compared the RNA expression levels of mouse VEGF-A, mouse TNF-alpha, and mouse IL-6 between the treatment subgroups in the dry and non-dry cornea groups.

Additionally, we compared the expression levels of VEGF-A, TNF-alpha, and IL-6 in unoperated corneas. After 2wk of dry eye induction with scopolamine (0.4 mL of 10 mg/mL of scopolamine hydrobromide, 2 times a day) and topical application of 1% atropine sulfate (Alcon), the RNA expression levels of mouse VEGF-A, mouse TNF-alpha, and mouse IL-6 in unoperated dry corneas and unoperated non-dry corneas (normal corneas) were quantitated and compared.

Statistical Analysis Statistical analysis was performed using SPSS 11.5 (IBM, Armonk, NY, USA). The extents of NV and inflammatory cell infiltration were compared between the two groups using an unpaired two-tailed t-test. Real-time polymerase chain reaction results were compared using ANOVA (analysis of variance) and the unpaired t-test. A P value <0.05 was considered statistically significant.

RESULTS

Comparison of Neovascularization The extents of NV are compared in Figure 2. The non-dry eye group showed less NV than the dry eye group with injection of PBS (P=0.017). In the dry eye group, aflibercept treatment (1.034%±0.188%) resulted in significantly decreased NV compared with the PBS (8.035% ±0.390%) and dexamethasone treatments (2.167%±0.282%) (P=0.000, P=0.049, respectively). In the non-dry eye group, aflibercept (0.163 % ±0.034%) and dexamethasone (0.867%±0.211%) treatments both resulted in decreased NV compared with PBS treatment (3.734% ± 1.668%); however, these decreases didn't reach statistical significance (P=0.108, P=0.130, respectively). Aflibercept treatment resulted in significantly less NV than dexamethasone treatment (P=0.015).

Comparison of CD11b+ Inflammatory Cell Infiltration The levels of CD11b+ inflammatory cell infiltration are compared in Figure 3. The non-dry eye group showed less inflammatory cell infiltration than the dry eye group with injection of PBS (P=0.001). In the dry eye group, aflibercept treatment (6.800% ±1.135%) resulted in significantly decreased inflammatory cell infiltration compared with the dexamethasone (13.259 % ±1.867%) and PBS treatments (22.399%±2.656%) (P=0.012, P=0.000, respectively). In the non-dry eye group, aflibercept (4.199%±1.046%) and
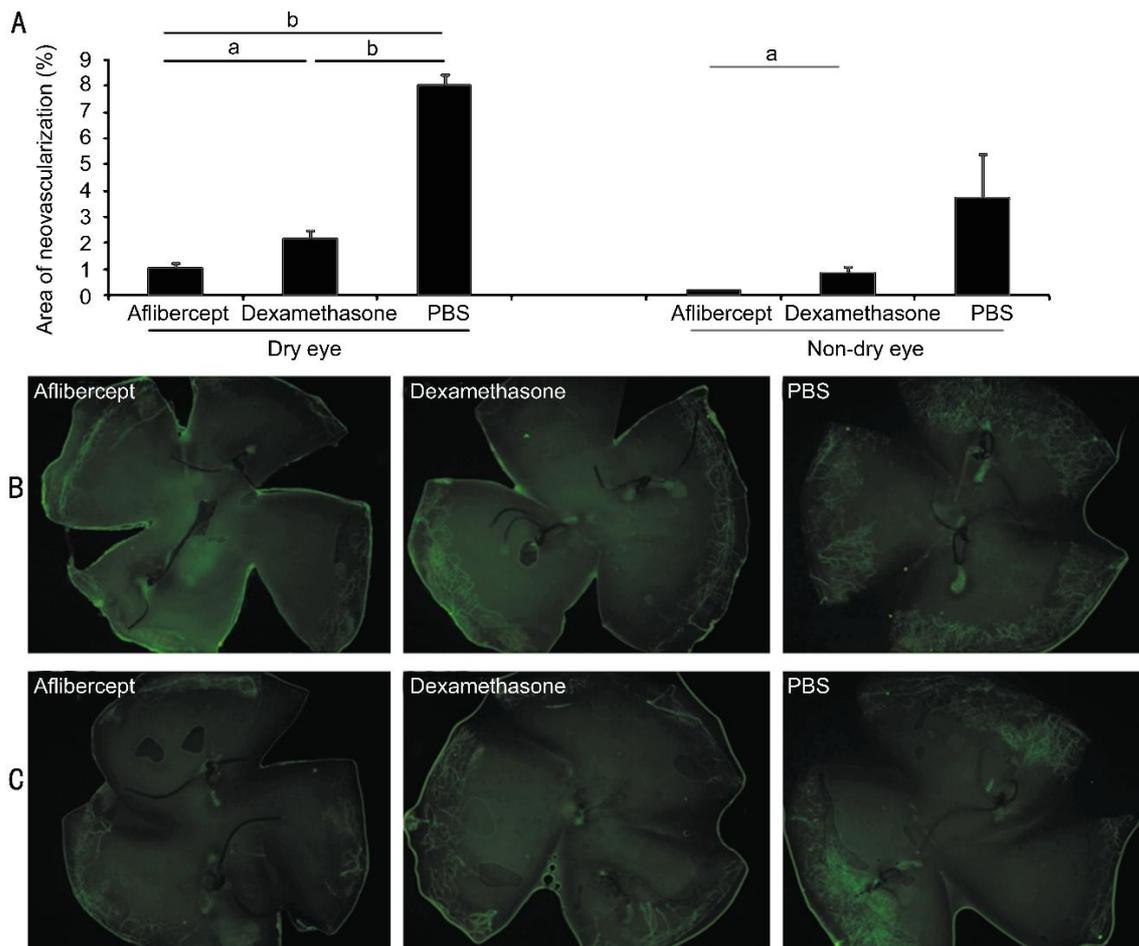
Figure 2 Comparison of neovascularization A: Extent of NV in aflibercept-treated, dexamethasone-treated, and PBS-treated dry eye and non-dry eye corneas; B: Representative pictures of dry eyes; C. Representative pictures of non-dry eyes. ᵃP<0.05, ᵇP<0.01.

dexamethasone (7.415% ±0.914%) treatments resulted in decreased inflammatory cell infiltration compared with the PBS treatment (9.462%±2.027%); however, these decreases didn't reach statistical significance (P=0.062, P=0.318, respectively). Aflibercept treatment was associated with significantly less inflammation than dexamethasone treatment (P=0.045).

Real–time Polymerase Chain Reaction

Comparison in unoperated corneas The mRNA expression levels of VEGF-A, TNF-alpha, and IL-6 in unoperated dry and unoperated non-dry (normal) corneas are shown in Figure 4. The mRNA expression ratios of VEGF-A, TNF-alpha, and IL-6 are expressed normalized to GAPDH (normal cornea=1.0). The levels of VEGF-A were different in dry corneas *vs* non-dry corneas, although this difference was not significant (P=0.057). However, the levels of TNF-alpha and IL-6 were significantly different between the dry and normal corneas. Specifically, the dry eye group showed increased TNF-alpha and IL-6 expression (P=0.000 and P=0.000, respectively).

Comparison in operated corneas The mRNA expression levels of VEGF-A, TNF-alpha, and IL-6 in dry and non-dry corneas with corneal stromal sutures are shown in Figure 5.

Dry Eye Group In the dry eye group, the mRNA

expression ratios of VEGF-A, TNF-alpha, and IL-6 are expressed normalized to GAPDH (dry eye PBS group=1.0) (Figure 5A). The aflibercept group showed significantly reduced VEGF-A expression compared with the PBS group (P =0.001). Similarly, the aflibercept group showed significantly reduced TNF-alpha expression compared with the PBS group (P=0.003). The aflibercept group tended to have reduced IL-6 expression compared with the PBS group, although this difference was not significant (P=0.085).

Non–dry Eye Group In the non-dry eye group, the mRNA expression ratios of VEGF-A, TNF-alpha, and IL-6 are expressed normalized to GAPDH (non-dry PBS group=1.0) (Figure 5B). The expression levels of VEGF-A, TNF-alpha, and IL-6 were not significantly different between any of the treatment subgroups in the non-dry eye group.

DISCUSSION

Dry eye disease is a multifactorial disorder of the tear film and ocular surface and becoming recognized as an immune/inflammation-mediated disorder[3,11-12,15,23].

Dry ocular surfaces present variable degrees of inflammation characterized by an enhanced expression of proinflammatory cytokines; chemokines such as IL-6 and TNF-alpha; and immune cells such as lymphocytes, macrophages, and dendritic cells [15,24]. In severe dry eye disease, there would be
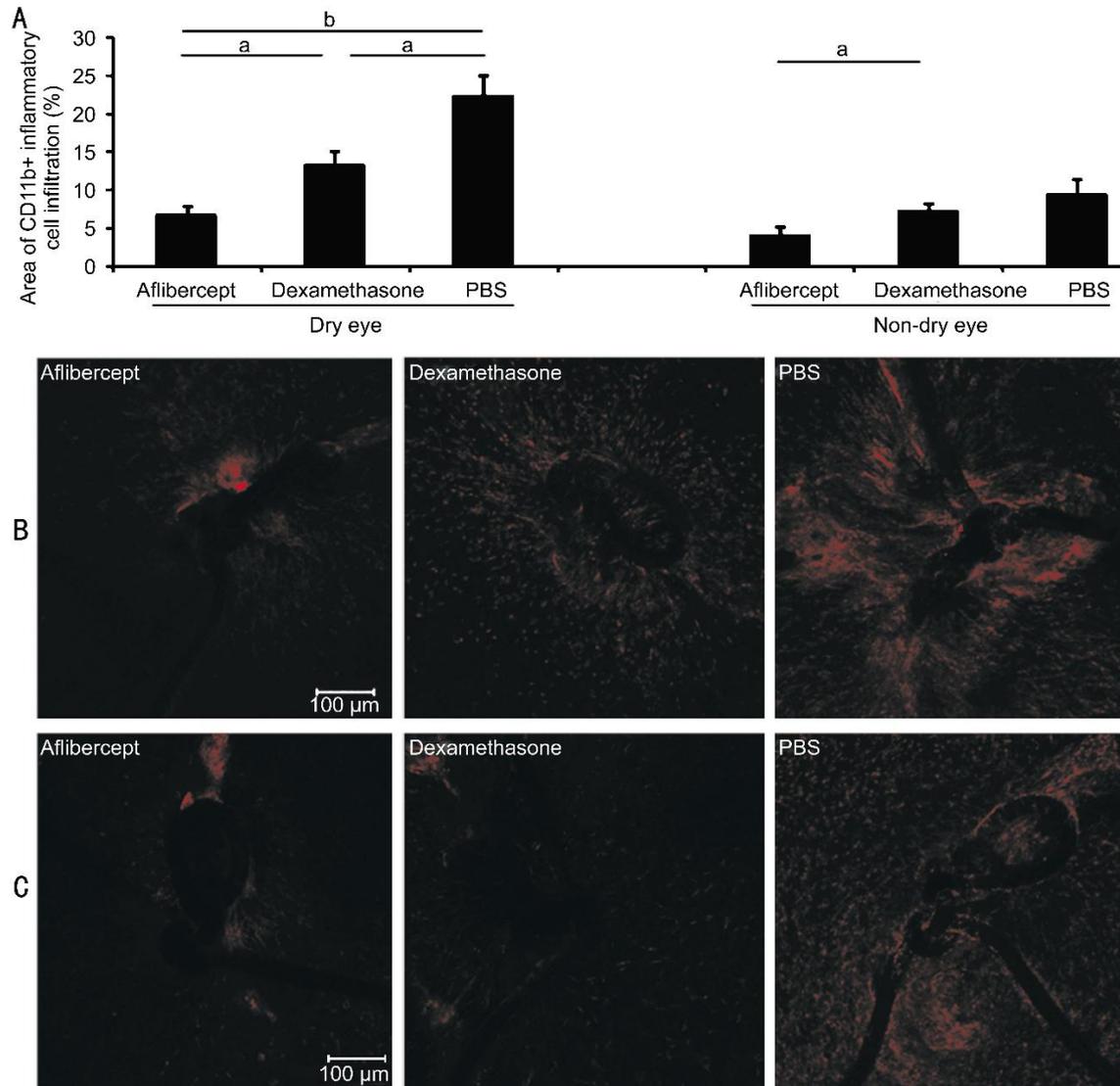
Figure 3 Comparison of inflammatory infiltration A: Extent of inflammatory infiltration in aflibercept-treated, dexamethasone-treated, and PBS-treated dry eye corneas and non-dry eye corneas; B: Representative pictures of dry eyes; C: Representative pictures of non-dry eyes. a P <0.05, b P <0.01.
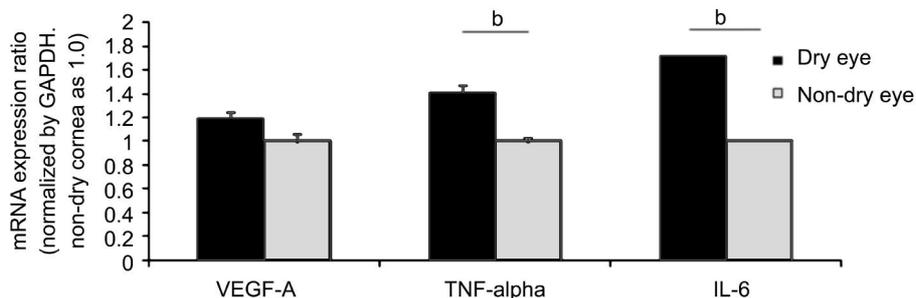

Figure 4 mRNA expression of VEGF−A, TNF−alpha and IL−6 in unoperated dry and unoperated non−dry cornea (normal cornea) The mRNA expression ratio of VEGF-A, TNF-alpha and IL-6 was normalized by GAPDH (normal cornea as 1.0); b P <0.01.

a greater inflammatory role. The concentration of inflammatory cytokines and chemokines is increased according to the severity of dry eye[13-15].

The status of corneas of pre-existing dry eye can have higher potential for postoperative complication[4,8,10]. In these status of severe dry eye with inflammatory NV after ocular surgery, we can expect that there may be a role of anti-VEGF-A therapy.

In our previous reports, postoperative corneal inflammation and lymph(angiognenesis) were significantly increased in dry corneas compared with non-dry corneas [4]. Thus, dry eye can be part of a pre-angiogenic milieu that can switch immediately into amplified angiogenesis with only minimal additional corneal injury.

Based on the inflammatory pathogenesis of dry eye disease [11-12], delay in dry eye disease progression may be
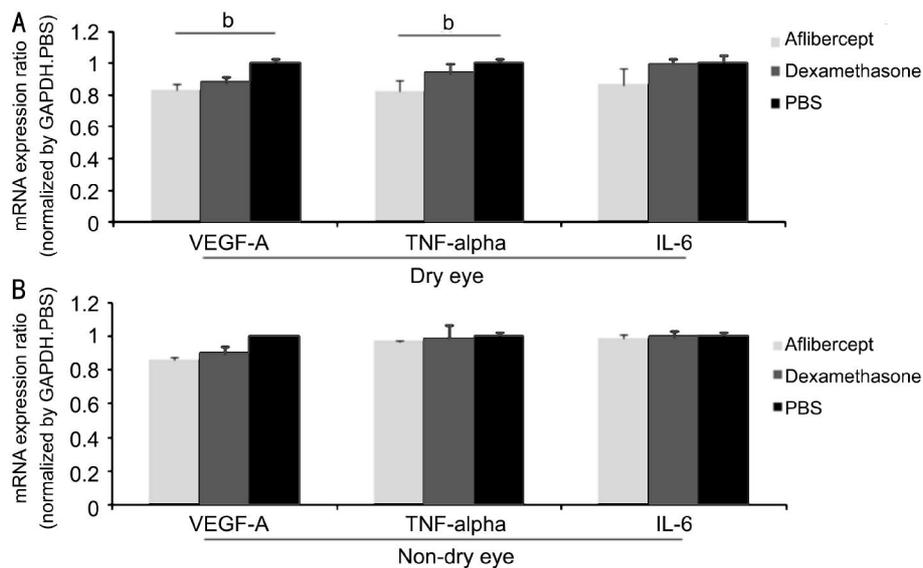
Figure 5 mRNA expression levels of VEGF−A, TNF−alpha and IL−6 in corneas with corneal stromal sutures A: Dry eye. The mRNA expression ratio is expressed normalized to GAPDH (PBS group of dry cornea=1.0); B: Non-dry eye. The mRNA expression ratio is expressed normalized to GAPDH (PBS group of non-dry cornea=1.0). $^b P<0.01$.

achieved by any means that can break the vicious cycle of ocular surface inflammation. Indeed, several reports have already revealed that treatment with topical or systemic anti-inflammatory drugs, such as cyclosporine and steroids, improves dry eye disease symptom and signs[12,15].

Along with inflammation, dry eye disease has increasingly been related to pathologic (lymph)angiogenesis [1-2,15,25]. Moreover, surgical insults on ocular surface can aggrevate dry eye[4,7-9,26].

Our real-time polymerase chain reaction analysis of gene expression levels in unoperated corneas showed that VEGF-A, TNF-alpha, and IL-6 were expressed at higher levels in dry corneas than in non-dry corneas. Similarly, Goyal *et al* [1-2] reported increased VEGF-A, VEGF-C, and VEGF-D levels in dry corneas. Dry corneas have lost ocular surface homeostasis, which also includes the angiogenic privilege of cornea, meaning that the normal transparent cornea is devoid of lymphatic and blood vessels [24,27]. Distruption of the normal immunoregulatory mechanism in dry eye disease can lead to inflammation and pathologic (lymph)angiognesis.

Thus we propose that anti- (lymph)angiogenic agents are a potential therapeutic option for dry eye corneas after surgical insults.

VEGF-C blockage has already been reported to have anti-lymphangiognenic effects in dry eyes [2]. Lymphangiogenesis in dry eye corneas was significantly decreased by blocking VEGF-C.

However, few studies have investigated the effect of trapping VEGF-A in dry eye. Jiang *et al* [16] and Erdurmus and Totan [17] reported the effect of bevacizumab for treating ocular surface inflammation in dry eye.

We hypothesized that VEGF-A trapping is an effective treatment for dry eye because increasing evidence indicates that dry eye is related to lymph(angiogenesis), especially with only minimal additional corneal injury [1-2,24-25]. Here we tested the effect of VEGF-A trapping in dry eye cornea and compared the effects of VEGF-A trapping in dry *vs* non-dry eye corneas after surgical insult.

We also added a corneal suture model to the dry eye model to amplify NV and inflammation. These additions enabled us to better test whether VEGF-A trapping can decrease NV and inflammation as a treatment of dry eye.

Inflammation and NV after ocular surgery are both mediated by VEGF- A[27-28].

Corneal angiogenesis is driven by VEGF-A and plays an important pathogenic role in a variety of ocular surface diseases. Angiogenic responses to VEGF-A are mediated by VEGF receptor 1 (Flt-1) and VEGFR-2 (KDR) [28]. In addition to angiogenesis, vascular endothelial growth factor receptor-1 (VEGFR-1) is involved in VEGF-dependent migration and gene expression of monocytes and macrophages [28-30]. VEGF-A, which acts *via* VEGF receptor 2, is thought to be the main stimulator of angiogenesis and vascular permeability in neovascular disease [20]. Thus blocking the VEGF-VEGF receptor axis can reduce both NV and inflammation[17-18].

Inflammation in dry eye has classically been treated with steroids and cyclosporine[12].

Corticosteroid therapy has been the standard anti-inflammatory and anti-angiogenic treatment for diseases associated with corneal NV and inflammation [31-33]. Corticosteroids exerts their anti-inflammatory effects by inhibitiing chemotaxis and phagocytosis and by blocking the synthesis of prostaglandins. Corticosteroids also inhibit VEGF-A induced angiogenesis and have immune-

inflammatory suppressive action by reducing the number of circulating T lymphocytes and inhibiting macrophages, mast cells, and other cell types that release agents contributing to angiogenesis[31-33].

In our model of induced dry eye corneas and ocular surface surgery, we used aflibercept to trap VEGF-A. Aflibercept has been widely used to trap VEGF-A as an anti-angiogenic treatment. Aflibercept is a fusion protein, combining the key binding domains of VEGF receptors 1 and 2 and the Fc portion of immunoglobulin G [34-36]. Aflibercept binds VEGF-A with higher affinity than bevacizumab and ranibizumab[35,37]. It also binds VEGF-B and PLGF[37].

Aflibercept binds and neutralizes all isoforms of VEGF-A, but does not directly block VEGF-C or D[35,37].

We found that the anti-angiogenic and anti-inflammatory effects of trapping VEGF-A were significantly effective for treating dry eye corneas with corneal surgery. However, this strategy did not have any significant effects in non-dry eyes.

We suggest this difference is due to the preangiogenic milieu in the dry eye. Specifically, VEGF-A and cytokines are expressed at higher levels in postoperative dry eye corneas, which are then more susceptible to anti-angiogenic and anti-inflammatory treatments.

Dry eye corneas have higher levels of angiogenesis and inflammation and also have heightened responses to anti-angiogenic and anti-inflammatory agents.

Our immunohistochemical staining of CD31 and CD11b revealed that trapping VEGF-A was more effective in decreasing NV and inflammation than dexamethasone in both dry and non-dry eye corneas.

In a slightly different approach, Nakao *et al*[38] compared the effects of dexamethasone and bevacizumab and found that dexamethasone had a larger therapeutic window in inflammatory angiogenesis. Moreover, Mirabelli *et al*[39] reported that dexamethasone had a superior effect to anti-VEGF-A in reducing inflammatory angiogenesis.

Dexamethasone is a classic treatment of dry eye and is known to partly act *via* anti-VEGF-A[32].

However, we found that the VEGF trap was superior to dexamethasone as an anti-inflammatory agent and as an anti-angiogenesis agent, irrespective of the dryness of the cornea. We hypothesize that the VEGF trap aflibercept is a stronger anti-angiogenic and anti-inflammatory agent and has a wider spectrum than these other anti-VEGF agents[25,37,40].

Although anti-VEGF agents appear to have beneficial effects, they have also been reported to have some side effects[41-42]. Special care should be taken to use anti-VEGF agents on damaged corneas such as those found in dry eye and ocular surface injury, since anti-VEGF agents can inhibit wound healing.

We should also consider the indication of use of trapping VEGF-A. As seen in our results, the anti-angiogenic and anti-inflammatory effects of trapping VEGF-A were significantly effective for treating dry eye corneas with corneal surgery, however no significant effects in non-dry eyes. The role of anti-VEGF might be greater in severe dry eye with ocular surface inflammation.

Our results indicate that the anti-angiogenic as well as anti-inflammatory effects of VEGF-A trapping make it a potential therapeutic option for dry eye corneas after surgical insults.

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