The Different Effects of Early and Late Bevacizumab (Avastin) Injection on Inhibiting Corneal Neovascularization and Conjunctivalization in Rabbit Limbal Insufficiency

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PURPOSE. To compare the effects of early, mid, and late subconjunctival injection of bevacizumab on corneal neovascularization (NV) and conjunctivalization in a rabbit limbal insufficiency model.

METHODS. Limbal insufficiency was created surgically, and subconjunctival injections of 2.5 mg bevacizumab twice weekly for 1 month were started immediately (early group), 1 week (mid group), and 1 month after injury (late group). The corneal epithelial alterations, stromal opacity, centricity, extent, and PICS (percentage of involved corneal surface) of the corneal NV were evaluated. The expression of cytokeratins K3, K4, K12, K13, and MUC5 by the corneal surface cells was examined by immunohistochemistry.

RESULTS. Bevacizumab significantly inhibited corneal NV in the early and mid, but not the late, treatment groups at 4 weeks after treatment (PICS: P < 0.01 in the early group, P < 0.01 in the mid group, and P > 0.05 in the late group). Early and mid treatment produced significant inhibition of corneal alteration (P < 0.01 in the early group and P = 0.05 in the mid group) and stromal opacity (P < 0.01 in the early group and P = 0.02 in the mid group) at 4 months after treatment but not in the late group. The immunohistochemistry of cytokeratin on the corneal surface cells at 1 month after treatment was K3(+), K4(+), K12(+), K13(+), and MUC5(−) in the early group; K3(+), K4(+), K12(+), K13(+), and MUC5(−) in the mid group; and K3(+), K4(+), K12(+), K13(+), and MUC5(+) in the late treatment group.

CONCLUSIONS. Early and mid bevacizumab injection inhibited corneal NV, epithelial alteration, and stromal opacity in limbal insufficiency, but late treatment did not. Early treatment with bevacizumab seems to be clinically beneficial in the management of limbal injury such as chemical burn. (Invest Ophthalmol Vis Sci. 2010;51:6277–6285) DOI:10.1167/iovs.09-4571

The formation of corneal neovascularization (NV), a consequence of anterior segment inflammation and injury, is an unwanted pathologic response.1–5 Corneal NV may induce corneal opacity, affect visual acuity, increase the risk of graft rejection after penetrating keratoplasty, and cause bleeding during the creation of corneal flaps in laser in situ keratomileusis (LASIK).1 A variety of situations may induce corneal NV. Among all the predisposing factors, limbal stem cell deficiency (LSCD) is unique, because it not only involves the formation of corneal NV, but also the invasion of conjunctival surface cells onto the corneal surface.5–9 Under normal conditions, stem cells of the corneal epithelium reside in the limbus and serve as continuous suppliers of corneal surface cells.8,9–11 The limbal stem cells are imperative for corneal maintenance, and LSCD leads to the ingrowth of conjunctival cells, neovascularization of the corneal stroma, and, eventually, corneal opacity and visual loss.9–11 Corneal epithelial haziness, recurrent and/or persistent epithelial defects, corneal melting, and chronic inflammation are usually found in LSCD.12–14

The treatment of LSCD is, at present, mostly focused on surgical replacement of limbal stem cells by limbal allograft15–19 or autograft20–23 transplantation, cultivated limbal stem cells,8,24–26 or cultivated oral mucosal epithelial cell sheets.27–29 However, such treatment strategies are not without complications. The use of immunosuppressive agents in transplantation of allogeneic limbal stem cells, the potential damage of healthy limbus in transplantation of autologous limbal stem cell surgery, and the unsatisfactory long-term success rates in all treatment strategies are problems that must be solved. Bevacizumab is a humanized murine monoclonal antibody against all types of VEGF.30,31 Its effects have been validated in treating metastatic colorectal cancer, diabetic retinopathy, choroidal NV in pathologic myopia, exudative age-related macular degeneration (ARMD), and corneal neovascularization.37–42 Recently, we reported that subconjunctival injection of bevacizumab could inhibit the formation of corneal NV in various rabbit corneal NV models. In that preliminary study, injections of bevacizumab, begun immediately after injury, inhibited the formation of corneal NV in rabbit corneas with mechanically induced LSCD. To expand on the findings in that study, we sought to evaluate the influence of the time of onset of treatment on the inhibition of corneal NV and corneal surface conjunctivalization.
in rabbit LSCD. We used digital photography to record the changes in corneal morphology and immunohistochemical staining, to examine the differences in corneal surface cells in early, mid, and late bevacizumab treatment groups. We also used a colony-forming assay to identify the phenotype of the corneal surface cells in the three treatment groups.

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932969/)
**METHODS**

**Chemicals and Antibodies**

Bevacizumab (100 mg/4 mL; Avastin) was purchased from Roche Pharmaceuticals (Welwyn Garden City, UK); Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) from Gibco BRL/Life Technologies (Rockville, MD); newborn calf serum (NCS) from Invitrogen (Carlsbad, CA); Swiss albino 3T3 fibroblasts from ATCC (Manassas, VA); and mitomycin C from Roche (Mannheim, Germany). Monoclonal mouse antibody against human epithelial keratin AE5 (K3) was purchased from MP Biomedicals, Inc. (Aurora, OH); mouse antibody against rabbit MUC5AC from Abcam (Cambridge, UK); mouse monoclonal antibodies against human cytokeratin 4 and anti-human cytokeratin 13 from Novocastra (New Castle, UK); monoclonal mouse antibody against human MUC5AC from Abcam; and polyclonal goat antibody against mouse cytokeratin 12, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody, and fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat secondary antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**Animals**

New Zealand albino rabbits (3.0–3.5 kg, 6 months of age) were used in the study. Use, care, and treatment of all animals adhered to the regulations of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experimental procedures were approved by the Committee for Animal Research of the National Taiwan University Hospital. All procedures were performed with animals under general anesthesia induced by intramuscular injection of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg). The eyes were topically anesthetized with 0.5% proparacaine hydrochloride (Alcaine; Alcon, Fort Worth, TX) before manipulation. The right eye of each animal received the experimental drug; the left eye was not treated. To increase the reproducibility of the surgeries in inducing corneal NV, all procedures were performed in the same manner in all eyes by a single investigator. Six eyes were included in every examination condition in the early, mid, and late treatment groups.

**Limbal Injury–Induced Corneal NV Model**

The limbal injury–induced corneal NV model was produced by surgical removal of the circumferential limbal tissue 2 mm into the cornea and 3 mm into the conjunctiva in both eyes. Superficial (100 μm deep) lamellar keratectomy was also performed, to remove the entire corneal epithelium. This model system has been accepted as a reliable limbal insufficiency model and was used in several studies. The cornea NV created in this model occurred within 1 week after surgery, peaked

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=data/journals/iovs/932969/)
Table 2. The Effect of Bevacizumab Injection on Corneal NV in the Early, Mid, and Late Treatment Groups

<table>
<thead>
<tr>
<th>Weeks after initial limbal injury</th>
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<th>Mid Treatment Group</th>
<th>Late Treatment Group</th>
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<tr>
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<td>54±10</td>
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<td>42±6</td>
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<td>P-value</td>
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*P < 0.05, †P < 0.01 by Student’s t-test.

Figure 3. The effects of Bevacizumab treatment on the healing of corneal epithelial defects. (A) In the early treatment groups, Bevacizumab treatment significantly promoted epithelial healing at 2 weeks after treatment. (B) In the mid treatment groups, Bevacizumab treatment significantly promoted epithelial healing at 2 weeks after treatment. (C) In the late treatment groups, the epithelial defects were mostly healed before the initiation of Bevacizumab injection. There was no significant effect of Bevacizumab on the area of epithelial defects.
Recording of Corneal Epithelial Defect, Epithelial Alteration, Stromal Opacity, and NV Formation

The alterations in the corneal epithelium, stromal opacity, and NV formation were photographed with a surgical microscope (OPMI Pico I; Carl Zeiss Meditec, Jena, Germany) twice a week after the induction of LSCD. Fluorescein staining was used to measure the corneal epithelial defects. Photographs were graded for alteration of the corneal epithelium, stromal opacity, and corneal NV (Table 1) by two ophthalmologists who were blinded to the treatment groups. In this animal model, corneal epithelial alterations (corneal epithelial haze or defects) can be used as an indicator of the severity of corneal surface conjunctivalization, because conjunctival epithelial cells invading the corneal surface are usually hazy and easily damaged. The epithelial alterations were graded from 0 to 4 according to the area of epithelial haze or defect (Table 1). Stromal opacity, another indicator of the severity of LSCD, was graded from 0 to + (Table 1). The percentage of involved corneal surface (PICS) by NV was quantified by image analysis software (ImageJ 1.40v; http://rsb.info.nih.gov/ij/index.html/ Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD). The area of NV was measured in pixels, and its ratio to the entire corneal area was determined as the percentage of corneal NV.

Centricity was defined as the length (in millimeters) of the new vessel growth from the limbus, and the area of NV was measured in pixels, and its ratio to the entire corneal area was determined as the percentage of corneal NV. Centricity was defined as the length (in millimeters) of the new vessel growth from the limbus, and the area of NV was measured in pixels, and its ratio to the entire corneal area was determined as the percentage of corneal NV.

Immunohistochemistry

For immunohistochemical staining, rabbit eyeballs were cryopreserved in OCT embedding medium, and 8-μm cryosections were obtained. The sections were air-dried at room temperature for 30 minutes and fixed in cold acetone for 15 minutes. The sections were then permeabilized with 0.4% Triton X-100 for 10 minutes and were blocked by 1% fetal bovine serum (FBS) at 37°C for 1 hour. After incubation with the primary antibodies against K3, K4, K12, K13, and MUC5, the tissue sections were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 1 hour at 37°C. They were then washed and mounted in medium containing propidium iodide (PI; Vector Laboratories, Burlingame, CA) for visualization of nuclei. The central cornea and both sides of the limbus were examined. The normal corneas without limbal injury and the corneas that received limbal injury and were treated with subconjunctival injections of normal saline were regarded as the control. The staining pattern of the tissue sections was observed by conventional fluorescence microscopy (Eclipse E800 with a VFM Epifluorescence attachment; Nikon, Melville, NY) equipped with a digital camera (Spot, with ver. 1.1 CE software; Diagnostic Instruments, Sterling Heights, MI). All experiments were repeated six times, to ensure consistent results.

Colony-Forming Assay

To evaluate the expression of cytokeratins of corneal surface cells in the different groups, we used 3T3 fibroblast feeder cells in a corneal surface epithelial colony-forming assay. Swiss albino 3T3 fibroblasts, grown in DMEM containing 10% BCS at 80% confluence, were treated with 4 μg/ml mitomycin C for 2 hours and then trypsinized and plated at a density of 2 × 10^4 cells/cm^2 in each well of a six-well plate (Corning, Inc., Corning, NY). Epithelial cells isolated from the corneal surface were seeded at a density of 100 cells/cm^2 in each well of the six-well plates on the 3T3 fibroblast feeder layer in supplemented hormone epithelial medium (SHEM) containing 5% FBS. Cultures were incubated at 37°C in 5% CO2 and 95% humidity, and the medium was changed every 2 to 3 days. Colonies were fixed by acetone/methanol (1:1; Sigma-Aldrich, St. Louis, MO) 7 days later, immunostained with K3 and K4 antibodies, and photographed with an inverted microscope and digital CCD camera (Axiovert 100TV; Carl Zeiss MicroImaging, Thornwood, NY, equipped with a Quantix cam-era: Roper Scientific, Tucson, AZ, and MetaMorph 4.6r5 software; Molecular Devices, Downingtown, PA).

Statistical Analysis

Experimental data were analyzed by Student’s t-test. The results are expressed as the mean ± SEM. P < 0.05 denotes statistical significance.

RESULTS

The Wound-Healing Process of the Limbal Injury Model

In the corneas that underwent limbal injury and superficial lamellar keratectomy, the baseline wound-healing process demonstrated successful creation of limbal insufficiency with the formation of corneal NV and corneal surface conjunctivalization (Figs. 1, 2). At 2 weeks after injury, considerable corneal NV was observed emerging from the limbus (Fig. 2). The extent, centrivity, and PI of corneal NV peaked at 3 to 5 weeks and slowly decreased or remained constant until week 8 (Fig. 2, Table 2). At 1 and 2 months after injury, the corneal and limbal surface epithelial cells demonstrated the expression of conjunctival cytokeratins (K4, K13) but not corneal cytokeratins (K3, K12) in all six eyes. The presence of MUC5(+) cells also demonstrated the existence of goblet cells on the corneal surface at 1 and 2 months after injury in all six eyes (Fig. 1). Both sides of the limbus showed the same results in all eyes.

Effect of Bevacizumab on Epithelial Healing in Corneas Undergoing Limbal Injury

At 2 and 3 weeks after bevacizumab injection, early treatment caused significant promotion of epithelial healing (P < 0.01,
Effects of Early, Mid, and Late Treatments on the Phenotypes of Corneal Surface Epithelial Cells, Evaluated by Immunohistochemistry

In the early treatment group, five (83.3%) of six eyes showed a staining pattern of the corneal surface cells of K3(+), K4(+), K12(+), K13(+), and MUC5(-) at 1 month after treatment (Fig. 5), which is the same as in the normal corneas. One (16.7%) of six eyes showed K3(+), K4(-), K12(+), K13(+), and MUC5(-). In the mid treatment group, four (66.7%) of six of eyes showed the staining pattern of K3(+), K4(+), K12(+), K13(+), and MUC5(-) at 1 month after treatment (Fig. 5), whereas two (33.3%) of six eyes showed K3(+), K4(-), K12(+), K13(-), and MUC5(-). In the late treatment group, four (66.7%) of six eyes showed the staining pattern of K3(+), K4(-), K12(+), K13(-), and MUC5(-).

**FIGURE 5.** Representative images of immunohistochemical staining of K3 (A–C, a–c), K4 (D–F, d–f), K12 (G–I, g–i), K13 (J–L, j–l), and MUC5 (M–O, m–o) on corneal and limbal surface cells in the early, mid, and late treatment groups. 1 M after early treatment: 1 month after the limbal injury, in which subconjunctival injection of bevacizumab (2.5 mg, twice a week for 1 month) was initiated immediately after injury. 1 M after mid treatment: 5 weeks after limbal injury, in which subconjunctival injection of bevacizumab (2.5 mg, twice per week for 1 month) was initiated immediately after injury. 1 M after late treatment: 2 months after the limbal injury, in which subconjunctival injection of bevacizumab (2.5 mg, twice per week for 1 month) was initiated at 1 month after injury. Green: the staining of K3, K4, K12, K13, and MUC5; red: PI nuclear counterstaining; white arrows (O): positive staining of MUC5, which implies the migration of goblet cells onto the corneal surface. NEG, negative controls (P–R, p–r). Scale bar, 100 μm.
K4(+) and K12(−), K13(+), and MUC5(+) at 1 month after treatment (Fig. 5), whereas two (33.3%) of six showed K3(−), K4(+), K12(−), K13(+), and MUC5(+). Both sides of the limbus showed the same results, and the staining pattern was similar to that in the central cornea in all eyes (Fig. 5).

Expression of Cytokeratins in Corneal Surface Cells

Immunohistochemistry of epithelial colonies cocultured with 3T3 feeder cells revealed different expression patterns of K3 and K4 in different treatment groups (Fig. 6). In the normal corneas, six (100%) of six presented K3(+) and K4(−) patterns in the cell colonies cultured from the corneal surface. At 1 and 2 months after limbal injury, six of six and five (83.3%) of six corneas, respectively, presented K3(−)/K4(+) patterns in the corneal surface cellular colonies. At 1 month after early treatment, five (83.3%) of six corneas were K3(+) and K4(−), whereas one (16.7%) of six were K3(−)/K4(−). At 1 month after mid treatment, five (83.3%) of six corneas were K3(+) and K4(−), and the remaining one (16.7%) was K3(−)/K4(−). At 1 month after late treatment, four (66.7%) of six corneas were K3(−)/K4(−), and two (33.3%) were K3(+)/K4(+).

FIGURE 6. Representative images of immunohistochemical staining of K3 and K4 of single corneal epithelial cell colonies cocultured with 3T3 feeder cells. Normal: corneal epithelial cell colonies from rabbit cornea without injury or treatment. 1 M and 2 M after injury: corneal epithelial cells colonies from rabbit corneas at 1 and 2 months after limbal injury without bevacizumab injection. 1 M after early treatment: 1 month after the limbal injury, in which subconjunctival injection of bevacizumab (2.5 mg, twice per week for 1 month) was initiated immediately after injury. 1 M after mid treatment: 5 weeks after the limbal injury, in which subconjunctival injection of bevacizumab (2.5 mg, twice per week for 1 month) was initiated 1 week after injury. 1 M after late treatment: 2 months after the limbal injury, in which subconjunctival injection of bevacizumab (2.5 mg, twice per week for 1 month) was initiated at 1 month after injury. In all photos, green: staining of K3 or K4; blue: DAPI nuclear counterstaining; white arrows: margin of the cell colonies. Scale bar, 200 μm.

DISCUSSION

Since LSCD is a vision-threatening ocular disorder, several treatment strategies have been used to treat this disease. In addition to the various forms of limbal replacement surgeries,9,20,22–24,28,29,47,48 some studies have demonstrated the potential for treating LSCD-induced corneal NV and corneal surface conjunctivalization by inhibition of angiogenesis. Huang et al.57 demonstrated that photochemically induced occlusion of the corneal vessels by intravenous rose bengal injection with subsequent argon laser irradiation can trigger transdifferentiation of conjunctiva into a cornea-like epithelium on vascularized rabbit corneas with chronic limbal insufficiency. Doctor et al.59 and Bock et al.51 demonstrated that treatment with subconjunctival injection or topical bevacizumab could inhibit corneal NV in patients with chronic LSCD caused by Stevens-Johnson syndrome, ocular cicatricial pemphigoid, or chemical burn. Compared with these studies, the present study extended the previous understanding of the treatment of corneal NV and corneal surface conjunctivalization by showing that they can be inhibited when bevacizumab is applied earlier after limbal injury, rather than later. We also found that successful treatment of LSCD by bevacizumab is shown by the simultaneous inhibition of corneal epithelial alteration, stromal opacity, corneal NV, and corneal surface conjunctivalization.

Several mechanisms explain this phenomenon. First, VEGF concentration has been reported to peak within 1 week after limbal injury and to decrease gradually thereafter.5,7 Therefore, it is plausible that VEGF has less influence on maintaining corneal NV in chronic LSCD than in acute LSCD, which could decrease the therapeutic effect of anti-VEGF antibody in patients with chronic LSCD. Second, vessels may mature in chronic NV; and, therefore, pericyte coverage may be recruited around the pathologic NV.52 It is reasonable to hypothesize that such coverage may decrease the effect of bevacizumab aimed at causing the regression of newly formed immature vessels. Third, several cytokines/mediators other than VEGF and different mechanisms may control the formation of corneal NV at different stages after limbal injury. Anti-VEGF antibody alone may not be sufficient to inhibit corneal NV formation at all stages after injury.

In addition to the different antiangiogenic effects, we found that early, mid, and late treatment with bevacizumab may lead to different phenotypes of corneal surface cells. In the early treatment group, the corneal surface cells expressed corneaspecific (K3/K12) but not conjunctiva-specific (K4/K13) cytokeratins at 1 month after treatment. In contrast, corneal surface cells in the late treatment group expressed more conjunctiva-specific cytokeratins (K4/K13 but not K3/K12) at 1 month after treatment (2 months after limbal injury). Both corneal and conjunctival cytokeratins (K3/K4/K12/K13) were expressed in the corneal surface cells in the mid treatment group. Sacchetti et al.46 and Expana et al.55 demonstrated that some patients with clinically diagnosed LSCD may have mixed corneal and conjunctival epithelium, which may imply partial LSCD instead of total LSCD. Our data suggest that earlier treatment with bevacizumab not only inhibits corneal epithelial alteration, stromal haze, and NV formation, but also promotes more cornealike phenotypes of corneal surface cells in eyes with LSCD.

The results of our study provide information of important clinical utility. Patients with LSCD may visit the clinic at different stages after limbal damage. Our study demonstrates the importance of early bevacizumab treatment in patients who have sustained a limbal injury. Such treatment prevented the formation of corneal NV and corneal surface conjunctivalization. Bevacizumab also promoted epithelial healing in acute and mid treatment groups from our current studies. Since no
major systemic or ocular side effects were found in all animals in our current and previous studies.36 we expected bevacizumab to be a safe and efficient treatment strategy in acute limbal injury. However, large-scale human studies are still needed before the treatment can be applied in patients. In addition, late treatment may still provide some beneficial effects in patients with chronic LSCD. Although the results were not statistically significant, late injection of bevacizumab seemed to partially inhibit corneal NV formation, stromal opacity, and epithelial alteration. It also triggered the corneal surface cells to express mixed cornea- and conjunctiva-specific markers instead of conjunctival cytokeratins, which implies progress from total to partial LSCD.

Our study has several drawbacks. First of all, the creation of limbal and corneal surface damage by mechanical methods may not accurately mimic conditions in all patients with LSCD. Although this model system has been widely accepted and may provide more constituent baseline results than does a chemical burn-induced limbal insufficiency model, other methods, such as chemical burns and contact lens wearing, should be investigated to simulate real clinical conditions. Second, the 1-month interval after the creation of limbal injury may not truly represent chronic LSCD, and thus a longer interval between the injury and onset of treatment should be tested. Third, we used a high dose of bevacizumab to create significant therapeutic effects. Such a high dose may not be suitable for use in humans.

In conclusion, our study demonstrated that early treatment with bevacizumab of LSCD induced by mechanical injury (surgery) may lead to better therapeutic effect than mid and late treatment, as indicated by inhibition of corneal NV formation and prevention of corneal surface conjunctivalization.

Acknowledgments

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References


