The normal human cornea is avascular, a situation maintained by expression of anti-angiogenic factors. Inflammatory and angiogenic stimuli allow vascular invasion when the balance between such factors is tilted toward angiogenesis. Wound healing is composed of several overlapping phases and includes the induction of acute inflammation, rapid proliferation of reparative cells, and formation of permanent scars. In the complex microenvironment of a healing corneal wound, in which multiple growth factors are found, vascular endothelial growth factor (VEGF) has an important role. Several mechanisms of pathologic neovascularization (NV) have been described in wound healing. These processes involve the actions of basic fibroblast growth factor (bFGF), prostaglandin, interleukin (IL)-2 and IL-8, platelet-derived growth factor (PDGF), and VEGF.

VEGF is essential for other forms of wound- and inflammation-related neovascularization, but use of anti-VEGF therapies for NV inhibition requires caution because the efficiency of wound healing may be affected.

Bevacizumab (Avastin; Genentech, San Francisco, CA) is a recombinant humanized monoclonal immunoglobulin G1 antibody directed against VEGF. This antibody binds and deactivates VEGF, which can result in inhibition of abnormal blood vessel formation and decreased vascular permeability. Since mid-2005, off-label use of bevacizumab has been reported in ocular systems and the drug has shown promising short-term results in the treatment of intraocular neovascular conditions.

Systemic administration of bevacizumab results in a low incidence of hypertension and thrombosis, but several reports have suggested that small doses delivered topically do not cause serious adverse effects. However, one group reported corneal epithelial change in NV patients after topical application of bevacizumab. Such application resulted not only in vascular suppression but also caused spontaneous loss of epithelial integrity and progressive stromal thinning.

In this study, we evaluated the effects of bevacizumab on corneal wound healing time, cell growth, and expression of Ki67 in scratch assays on the levels of cell surface integrins and collagen, and expression of integrin mRNAs in human corneal epithelial and fibroblast cells.

**MATERIALS AND METHODS**

**In Vivo Rabbit Model of Corneal Epithelial Wound Closure**

New Zealand White rabbits, weighing 2 kg and housed in the animal facilities of Yonsei University, were given rabbit chow and water ad libitum and were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Yonsei University Medical Center.

Twenty rabbits were anesthetized using ketamine (30 mg/kg of body weight, intramuscularly) and xylazine (6 mg/kg, intramuscularly), and proparacaine hydrochloride 0.5% (Alcaine; Alcon, Fort Worth, TX) was applied topically. The corneal epithelium was abraded after a 30 second application of 20% (v/v) ethanol using a #15 surgical blade (Bard-Parker; BD Surgical Systems, Franklin Lakes, NJ) on the central portion of the cornea that had been demarcated with an 8.0 mm trephine. Olofaxcin eye ointment was applied once a day and phosphate buffered saline (PBS) containing 0.5, 0.5, 1.0, or 1.5 mg/mL bevacizumab was applied twice daily after suturing. Corneal epithelial defects were stained with fluorescein and photographed every day after wounding until epithelial defects were completely closed.
To determine the area of corneal neovascularization, the pixels covering the vascularized corneal portion were measured on digitized photographs using Image J 1.31v software (kindly provided by Wayne Rasband of the Research Services Branch, National Institute of Mental Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). The photographs were evaluated by two investigators. Differences in epithelial defect areas were compared using Kruskal-Wallis tests. Commercial software (MedCalc for Windows, version 7.6.0.0; MedCalc Software, Mariakerke, Belgium) was used for statistical analysis.

**Culture of Corneal Epithelial and Fibroblast Cells**

Telomerase (hTERT)-immortalized human corneal fibroblast cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal calf serum (Gibco-BRL), 1% (w/v) glutamate, and 1% (w/v) penicillin-streptomycin at 37°C under 5% (v/v) CO₂, in six-well tissue culture plates. Telomerase-immortalized human corneal epithelial cells were grown in KGM-2 medium (Clonetics-BioWhittaker Inc., Walkersville, MD) supplemented with 200 U/mL penicillin, 200 µg/mL streptomycin, and 0.5 µg/mL amphotericin B (BioWhittaker) at 37°C under 5% (v/v) CO₂, also in six-well tissue culture plates.

**Scratch Assay of Corneal Epithelial and Fibroblast Cells and Ki67 Staining**

Each cell line was cultured with supplemented media for 2 days and monolayers in culture plates were wounded using a small scalpel. Injured cells were cultured with 0, 1.0, 2.5, or 5.0 mg/mL bevacizumab.

**Figure 1.** A rabbit cornea stained with fluorescein to measure the size of epithelial defects after mechanical debridement. Corneal epithelium regenerated by day 3 in the control and 0.5 mg/mL bevacizumab groups. In animals treated with bevacizumab concentrations of 1.0 mg/mL and above, delayed epithelial closure (days 4 to 5 after debridement) was evident.
in the cell culture media for 24 hours. Cells were photographed using a microscope 24 hours after wounding. Each experiment was repeated three times. To determine the distance between the leading edges of growing cells, the pixels were counted on digitized photographs using Image J 1.31v software.

Corneal epithelial and fibroblast cells were cultured in two-well chamber slides and underwent scratch assay and were treated with 0, 1.0, 2.5, or 5.0 mg/mL bevacizumab for 24 hours. For Ki67 immunostaining, cells were fixed with 2% paraformaldehyde (PFA, Wako) and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO). The cells were treated with rabbit polyclonal to Ki67-proliferation marker (Abcam, Cambridge, MA). The cells were then treated with donkey anti-rabbit IgG antibody (Alexa Fluor 594; Invitrogen, Carlsbad, CA).

Identification of Cell Surface Integrin α and β Groups, and Types I and IV Collagen

To identify cell surface integrins and collagen, we used three different assay kits (Alpha Integrin-Mediated Cell Adhesion Array Kit, Beta Integrin-Mediated Cell Adhesion Array Kit, and CytoMatrix Cell Adhesion Strips Human Collagen Type I and IV kit; Chemicon, Temecula, CA), in which 96-well plates were coated with mouse monoclonal antibodies against human adhesion molecules. The plates were used to capture cells expressing specific surface adhesion molecules. Corneal epithelial cells and fibroblasts exposed to 0 or 1.5 mg/mL bevacizumab for 24 hours were prepared as single cell suspensions using non-enzymatic dissociation buffer (PBS with 2 to 5 mM EDTA). After adjusting the cell density to 1 x 10^5 to 1 x 10^7 cells/mL with assay buffer, the mixture was incubated at 37°C under 5% (v/v) CO2 for 45 minutes. After several washes with PBS, cell staining solution (100 μL/well) was added and incubated for 5 minutes. After several washes with PBS, extraction buffer (100 μL/well) was added and the suspension rotated on an orbital shaker for 5 to 10 minutes. Reaction intensities were measured at 570 nm (VERSAmax; Molecular Devices, Sunnyvale, CA). Experiments were performed in triplicate. Differences between groups were compared using the Mann–Whitney U test. Statistical analysis software (SPSS version 12.0; SPSS, Chicago, IL) was used.

**FIGURE 2.** Sizes of epithelial defects measured with Image J (software developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Statistically significant epithelial defect size differences were observed on day 3 and day 4 after debridement. *Statistically significant between groups (Kruskal-Wallis test).**

**FIGURE 3.** Corneal epithelial cells growing and Ki67 expression after wounding with a small scalpel. (A) Twenty-four hours later, the control group and 1 mg/mL bevacizumab treatment group showed covering of the wounded area by proliferating cells. As the concentration of bevacizumab increased, the proliferation of corneal epithelial cells was progressively delayed until 24 hours after injury. (B) The expression of Ki67 positive cell was decreased with increasing bevacizumab concentration. (C) The distance between the leading edge of the growing cell was measured by Image J 1.31v software. Statistically significant differences were observed in the bevacizumab-treated groups. Student-Newman-Keuls test was used for all pairwise comparisons.
Reverse-Transcriptase–Polymerase Chain Reaction Analysis of Integrin α2, α3, α5, β1, and β2, and Fibronectin mRNA Expression

Corneal epithelial cells and fibroblasts exposed to 0 or 1.5 mg/mL bevacizumab for 24 hours were prepared for mRNA extraction. RNA concentration and purity was determined spectrophotometrically (Gene Quant II; Pharmacia Biotech, Cambridge, UK). Total RNA was converted into cDNA using a premix kit (One-Step RT-PCR Pre-Mix Kit; iNtRON, Daejeon, Korea). To measure integrin α1 transcription, an mRNA aliquot was incubated in a thermal controller (Model TPC-100; MJ Research, Watertown, MA) for 1 cycle of reverse transcription at 45°C for 30 minutes and denaturation at 94°C for 5 minutes, followed by 33 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The primers used were 5'-GCT GCT GTG CAT TAG ATA TTA G-3' and 5'-CTG TAA CTT CTG GTG AAA TCC T-3'. Integrin α2 transcription was measured as above but the annealing temperature was 57°C for 1 minute. The primers used were 5'-TAC GTG GG GAT GAC CTA-3' and 5'-TGG GTG GGT GCA GGA TGA AGC T-3'. Integrin α5 transcription was measured identically to the integrin α1 procedure described above. The primers used were 5'-ACT AGG AAA TTC ATT CAT ACT CTG TG-3' and 5'-GCA TAG TTA GGT TTC TTT GTT GG-3'. Integrin β1 transcription was measured identically to the integrin α1 procedure described above. The primers used were 5'-GTT GCT GGA ATT GTG TTC TCT CTT GTT GG-3'. RT-PCR products were analyzed by electrophoresis in 1% (w/v) agarose gel. Amplified products were viewed using an image-documentation system (ImageMaster VDS; Pharmacia Biotech Inc., Uppsala, Sweden). β-actin levels were used to normalize expression levels of each mRNA. Gels were photographed and DNA band densities quantitated using a quanitative imaging system (Fluor-S Multilimager; Bio-Rad, Hercules, CA). Experiments were performed three times.

RESULTS

Delayed Corneal Epithelial Wound Closure by Bevacizumab

In rabbits receiving eyedrops with 0 or 0.5 mg/mL bevacizumab, corneal epithelium regenerated by day 3 after mechanical debridement. However, in animals receiving eyedrops with 1.5, 2.5, or 5.0 mg/mL bevacizumab, delayed epithelial closure was noted, and wounds did not heal until day 4 or day 5 after debridement (Fig. 1). Also, two corneas in the 1.5 mg/mL bevacizumab-treated group and three corneas in the 2.5 and 5.0 mg/mL-treated animals showed detachment of epithelial layers and spontaneous increases in epithelial defect size during wound healing. This indicated that high concentrations of bevacizumab induced not only delayed epithelial regeneration but also weakened the cohesion strength between the corneal epithelium and the damaged stromal bed. The sizes of measured epithelial defects were compared. Statistically significant epithelial defect size differences were observed on day 3 and day 4 after debridement (Fig. 2).

Delayed Covering of Wounded Area of Corneal Epithelial Cells and Fibroblasts and Reduced Expression of Ki67 by Bevacizumab

Twenty-four hours after creating wounds using a small scalpel, control epithelial cells had proliferated to completely cover the wounded areas. As the concentration of bevacizumab increased, covering of corneal epithelial cells was increasingly delayed, and at bevacizumab concentrations of 2.5 and 5 mg/
mL, proliferation did not commence until 24 hours after injury (Fig. 3A). The number of Ki67 positive cells reduced concentration of bevacizumab dependent manner (Fig. 3B).

Corneal fibroblasts growing under 2.5 and 5 mg/mL bevacizumab were just beginning to proliferate 24 hours after wounding (Fig. 4A). Also, expression of Ki67 was decreased in the same group (Fig. 4A).

Expression of Cell Surface Integrins α and β Groups, and Expression of Collagen Types I and IV

Surface expression of integrins α1, α2, α3, α5, β1, β2, and β4 and collagen types I and IV in corneal epithelial cells significantly decreased in the presence of bevacizumab (Fig. 5). With corneal fibroblast cells, similar results were seen, except that expression of collagen IV was minimal even before exposure to bevacizumab (Fig. 6).

Expression of Integrin α2, α3, α5, β1, and β2 mRNA

We evaluated the expression of mRNA encoding integrins α2, α3, α5, β1, and β2 in corneal epithelial and fibroblast cells. In corneal epithelial cells, the expression levels of integrin α3 and α5 mRNA significantly decreased in the presence of bevacizumab (Fig. 7). However, in corneal fibroblast cells, the expression of mRNA encoding integrins α2, α3, α5, β1, and β2 all significantly decreased in the presence of bevacizumab (Fig. 8). Decreases in the expression levels of various integrin mRNAs correlated with cell surface expression of integrins in corneal fibroblast cells. However, levels of mRNAs encoding integrin α2, β1, and β2 in corneal epithelial cells were not affected by bevacizumab, even though the drug decreased surface expression of these proteins (see above).

Discussion

Integrity and transparency of the corneal epithelium are critical to vision. For maintenance of corneal epithelial integrity and clarity, a good understanding of the corneal wound healing process and NV is necessary. The biological effects of VEGF are mediated by at least two tyrosine kinase receptors, VEGF receptors (VEGFR), VEGFR-1 and VEGFR-2.19 The blockade of VEGFR-1 significantly suppressed VEGF-induced corneal inflammation20 and strong upregulation of VEGFR-2 was reported during corneal NV under ischemic as well as inflammatory conditions.21 After injury, a combination of rapid signal transduction events and cell migration are essential for wound healing.22 Such cell migration involves epithelial proliferation, cell migration, cell stratification, and stromal wound healing.23 Several cytokines and other factors are involved in the regulation of this cascade and many of these materials also function to regulate NV.24 Therefore, it is not possible to view corneal wound healing and NV as separate events.

Several studies on the safety profile of bevacizumab in corneal cells have been published.25–27 Using in vitro assays, it has been reported that bevacizumab was not toxic to corneal cells of human origin at doses usually used for corneal NV treatment.23,24 These studies used MTT assays and immunohistochemistry to evaluate cell viability and cytotoxicity. The same researchers reported that five applications of bevacizumab (25 mg/mL) eyedrops in a cornea chemically burnt with NaOH had clear anti-angiogenic effects, anti-fibrotic activity, and maintained corneal transparency without specific toxicity.25

We have clearly demonstrated that bevacizumab inhibits new corneal NV in humane corneas,18 and have also reported significant inhibition by bevacizumab of corneal NV in a rabbit suture model.28 However, during both animal experiments, and when caring for our patients, we have experienced de-
layed corneal epithelial healing, spontaneous epitheliolapthy, and stromal thinning.

During the rabbit corneal epithelial healing study, we observed a clear bevacizumab-caused delay in corneal epithelial wound healing after debridement. The epithelium healed completely by day 3 in control corneas, or in those receiving bevacizumab at 0.5 mg/mL. In the 1.5, 2.5, or 5.0 mg/mL bevacizumab-treated groups, corneal epithelium did not heal completely by day 4 and spontaneous epithelial defects were seen during the prolonged healing period, compromising epithelial adhesion to the corneal stroma. To simulate the animal study, we used cellular proliferation assays (using corneal epithelial and fibroblast cells) after scrape injury. By 24 hours, control cultures of both cell types completely covered the gaps induced by scraping, but covering of the wounded area was delayed and the number of Ki-67 positive cells was decreased by bevacizumab in a concentration-dependent manner. To determine which adhesion molecules might be affected by bevacizumab application, we performed ELISA assays to identify cell surface integrins of groups α and β and collagen types I and IV. The expression of all these factors, in both corneal epithelial and fibroblast cells, was decreased by application of bevacizumab. Corneal wound healing was related to expression of adhesion molecules. Integrins are well-known molecules of this type that bridge the cell to many components of the extracellular matrix (ECM), such as laminins and collagens, and thereby transduce intracellular signals that alter numerous cell properties such as adhesion, migration, proliferation, and survival. Integrin α5β1 is the classic fibronectin receptor, and when expressed with additional integrins that bind to fibronectin, mediates cellular attachment to the ECM. VEGF-R1, which contains the binding determinants for VEGF, is involved in the interaction with α5β1 integrin. The essential role of integrin α5β1 in the cornea as a regulator of corneal NV was suggested. Exposure to culture medium containing 1.5 mg/mL bevacizumab reduced expression of surface integrins and collagens. This means that bevacizumab might inhibit not only corneal NV, but also the adhesion of corneal cells, and other integrin-related events.

When RT-PCR was used to evaluate the effect of bevacizumab on the synthesis of integrin mRNAs in corneal cells, there were differences in the expression of relevant mRNAs between corneal epithelial and fibroblast cells. RT-PCR of mRNA from corneal fibroblast cells showed that the expression levels of mRNAs encoding all integrins α2, α3, α5, β1, and β2 decreased. However, in corneal epithelial cells, inhibition of mRNAs encoding only integrin α3 and α5 was seen. Integrin surface expression on corneal epithelial cells was affected by bevacizumab, but the mRNA expression was not affected at levels of 1.5 mg/mL.

NV is the result of a complex interplay among pro-angiogenic factors, cell adhesion, and matrix remodeling. Blocking of integrin α1β1 reduces inflammation-induced tissue response and promotes cornea allograft survival, and integrin α5 inhibiting small molecules blocks the outgrowth of new lymphatic vessel into the cornea. Induction of angiogenesis by VEGF is associated with selective upregulation of integrin αvβ3. Other members of the integrin family implicated in mediating the angiogenic response include α1β1, α2β1, and α5β1, and these have also been shown to be involved in VEGF-associated angiogenic events.

Therefore, modulation of corneal NV by blocking VEGF function also influences corneal physiology, especially development of the inflammatory state, which is expected to contribute to the wound healing process.

Previous reports have focused on cell viability assays to evaluate corneal safety of bevacizumab. In this study, we measured the effect of bevacizumab on the rate of epithelial wound healing, cell proliferation, and expression of integrins. We found that bevacizumab delayed corneal wound healing and inhibited integrin expression. These data explained our earlier findings of corneal epitheliolapthy after application of bevacizumab-containing topical eyedrops, even though corneal new vessel development was successfully inhibited.

When considering the use of bevacizumab to reduce corneal new vessel development, physicians should remember that the drug might delay wound healing and should be cautious in applying bevacizumab under active inflammatory conditions.

References


