Bevacizumab Promotes T-Cell–Mediated Collagen Deposition in the Mouse Model of Conjunctival Scarring

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Submitted: July 26, 2017
Accepted: January 9, 2018

PURPOSE. We determine the effects of bevacizumab on collagen production in a mouse model of conjunctival scarring.

METHODS. Experimental surgery was performed as described for the mouse model of conjunctival scarring, and bevacizumab was introduced by conjunctival injection. The capacity of bevacizumab to recognize conjunctival VEGF-A was determined by ELISA. Col1a1 was measured by real-time PCR and immunoblotting. T cells and collagen were visualized by immunofluorescence and picrosirius red staining of bleb cryosections. Conjunctival CD4+ or CD8α+ T cells were counted by flow cytometry. Mouse splenic T cells were cultured with bevacizumab/IgG and their numbers, cell cycle, and collagen production were measured using a cell counter, flow cytometry, and siroil soluble collagen assay, respectively. Reconstitution experiments in severe combined immunodeficiency (SCID) mice were performed by injection of freshly isolated T cells on day 2 postoperatively.

RESULTS. Bevacizumab recognized approximately 20% of endogenous murine VEGF-A. Injection of bevacizumab raised Col1a1 expression in the blebs at mRNA and protein levels. Bevacizumab did not induce collagen in conjunctival fibroblasts, but increased CD4+ and CD8α+ cell numbers as well as collagen production by these cells. Collagen appeared to accumulate in the vicinity of T cells in the bevacizumab-treated blebs. While SCID blebs did not show elevated collagen levels, reconstitution with CD4+ or CD8α+ cells resulted in increased Col1a1 expression at mRNA and protein levels.

CONCLUSIONS. Bevacizumab increased collagen production in the mouse model of conjunctival scarring. This collagen induction was mediated by T cells that were also stimulated by bevacizumab to increase in numbers.

Keywords: bevacizumab, collagen, conjunctiva, T cells

Bevacizumab (Avastin; Genentech/Roche, Basel, Switzerland) is a recombinant full-length humanized monoclonal anti-VEGF-A antibody designed to inhibit all VEGF-A isoforms. It was first approved by the United States Food and Drug Administration in February 2004 for the treatment of metastatic colorectal cancer.1 Bevacizumab has since been found to be efficacious in improving progression-free and/or overall survival in patients with various other advanced cancers.2 In the eye, anti-VEGF-A therapy is central to the treatment of multiple diseases characterized by vascularization. Bevacizumab is used widely off-label for the treatment of choroidal neovascular membranes in exudative age-related macular degeneration (AMD).3–5 In fact, neutralization of VEGF-A activity has become the cornerstone of therapies for AMD and diabetic retinopathy (DR) and has transformed the clinical management and outcome of these eye diseases.6–8

VEGF-A is known to affect multiple components of the wound healing cascade, including angiogenesis, epithelialization, and collagen deposition.9 VEGF-A also was implicated in pathologic wound healing with scar formation.10 Moreover, anti-VEGF gene therapy has been reported to attenuate experimentally-induced lung fibrosis.11 These observations suggest that VEGF-A inhibition may have antifibrotic therapeutic value. It is not surprising then that bevacizumab also has been evaluated as an adjunctive antiscarring therapy following glaucoma filtration surgery (GFS) for the treatment of glaucoma.12 Studies comprising small numbers of patients who underwent GFS with bevacizumab treatment as an adjuvant antifibrotic therapy produced mixed results in relation to surgical outcome.13–20 The human data contrasted with the more promising observations obtained in in vitro studies on conjunctival fibroblasts, the main effector cells implicated in fibrosis of the conjunctiva, and in in vivo studies in rabbit models of GFS.21–23 The in vivo mechanism(s) for the observed antifibrotic property of bevacizumab is unclear; although these laboratory studies implicated the capacity of bevacizumab to...
inhibit fibroblast proliferation as well as reduce expression of profibrotic TGF-β and collagen deposition as reasons behind the improved experimental surgical outcomes.21-27

To add to the confusion, a growing number of reports from clinical trials evaluating the effect of anti-VEGF-A therapies, including bevacizumab, on AMD, DR, as well as myopic choroidal neovascularization, have begun describing an association between this form of treatment and scar formation.28-41 These observations suggest that repeated intravitreal injections of anti-VEGF-A therapies may, in fact, increase the risk of ocular scarring, a major factor leading to sustained loss of visual acuity.42

To address directly the effects of bevacizumab on scarring, we used the mouse model of conjunctival scarring, which closely mimics patients' response to mitomycin C in GFS.43,44 We have shown previously that this mouse model was a reliable system for demonstrating antifibrotic drug effects and mechanisms.45 By measuring increase in type I collagen production as the scarring response,46 we demonstrate in this study that instead of suppressing collagen production, bevacizumab induced the opposite effect in this model and further reveal that T cells mediated this profibrotic response.

MATERIALS AND METHODS

Mouse Model of Conjunctival Scarring

All experiments with animals were approved by the Institutional Animal Care and Use Committee (IACUC) and treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement on the Use of Animals in Ophthalmic and Vision Research. Experimental surgery resulting in conjunctival scarring was performed as described previously.45 NIH:ST3/BL6 and Balb/c mice were obtained from the National University of Singapore Centre for Animal Resources. 129SVK wild-type mice were obtained from Benaroya Research Institute (Seattle, WA, USA) while severe combined immunodeficient (SCID) mice were obtained from the Animal Resources Centre (Canning Vale, Australia). Mice, especially SCIDs, were used in the experimental model at 8 weeks or younger. Bevacizumab was obtained from F. Hoffmann-La Roche Ltd (Basel, Switzerland) in the form of a clinically-ready injectable fluid at a concentration of 25 mg/mL. For day 2 analyses, 5 μL bevacizumab at 25 mg/mL was injected into the operated conjunctiva immediately postoperatively. For day 7 analyses, operated conjunctiva (blebs) were given a second injection on day 2 postoperatively. Human IgG (009-000-003; Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) was used as control and given at the same regimen.

VEGF-A Assay

Experimental surgery was performed on 129SVK wild-type mice and conjunctival tissues were harvested on day 2 postoperatively. The operated bleb tissues from five mice were pooled into each sample, and a total of nine samples were collected (n = 9, 45 mice). The contralateral unoperated conjunctival tissues were pooled similarly for comparison. Tissues were collected and processed as described previously.47 The protein content of each lysate was determined to correct for protein loading. The premixed 32-plex, Milliplex MAP mouse cytokine/chemokine antibody array (Merck Millipore, Billerica, MA, USA) was incubated with the tissue lysates according to instructions by the manufacturers and measured using the Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA, USA).

Binding Capacity of Bevacizumab for Mouse Conjunctival VEGF-A by VEGF Enzyme-Linked Immunosorbent Assay (ELISA)

Experimental surgery was performed on both eyes of 10 mice without the injection of any drugs. The bleb tissues were harvested and processed as described previously.47 The pooled lysate was divided into aliquots of 200 μL each and treated with 10 μL PBS or 100 μL PBS containing 125 or 250 μg IgG or bevacizumab in triplicate together with 50 μL (bed-volume) of protein G-sepharose. The mixtures were incubated at 4°C overnight and spun down the next day. The supernatants were collected and analyzed using the mouse VEGF Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) for the remaining VEGF-A, which was not captured by bevacizumab.

Real-Time Quantitative PCR Analysis (qPCR)

mRNA expression in cultured cells was analyzed as described previously.43,44 For analysis of mRNA expression in bleb tissues, surgery was performed on the left eye of each animal and the eye was injected with either IgG or bevacizumab. Tissues were pooled from three eyes per sample. A total of five samples were collected for each antibody treatment (n = 5), involving a total of 30 mice. Bleb tissues were collected in RNAlater solution (Life Technologies, Carlsbad, CA, USA) and analyzed as described previously.47 The mouse Col1a1 primers used were: forward 5'-CCCACTCCAGCGGCAA GAG-3', reverse 5'-GCCATCGCTGAGGGCGAG-3'. All samples were amplified by qPCR in triplicate. All mRNA levels were measured as Cg threshold levels. The best housekeeping gene (Actb, Rnai851, Gapdh, or Rpl13α) for each experimental condition was determined using the NormFinder software.48 Values were calculated as fold change by the 2^ΔΔCt method.

Immunoblotting

Surgery was performed on the left eye of each animal and the eye was injected with either IgG or bevacizumab. Tissues were pooled from five eyes per IgG or bevacizumab injection in each experimental set. A total of three independent sets of experiment were performed (n = 3), involving a total of 30 mice. Tissues were harvested, processed, and proteins resolved by SDS-polyacrylamide gel electrophoresis followed by immunoblotting as described previously.47 Anti-type I collagen antibodies (1:2000; cat# H00001277-M01) used were from Abnova Corp. (Littleton, CO, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Jackson Immunoresearch Laboratories, Inc. Densitometric analyses, where potential errors in loading were corrected to levels of the housekeeping Actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), were performed as reported previously.47

Immunostaining of Cryosections and Picrosirius Red Polarization Microscopy

Immunostaining and picrosirius red staining combined with polarization microscopy were performed as described previously.43 For immunofluorescent analysis, we used antibodies specific for mouse COL1A1 (Novus Biologicals, Littleton, CO, USA), CD4 (BD Pharmingen, San Diego, CA, USA), and CD8α (BD Pharmingen). Secondary antibodies were conjugated to AlexaFluor-594 (Invitrogen, Eugene, OR, USA). Nuclei were visualized by mounting the sections in 4',6-diamidino-2-phenyl-
Bevacizumab Induces Collagen Production by T Cells

Flow Cytometry

Surgery was performed on both eyes of each animal and the eyes were injected with either IgG or bevacizumab. Tissues were pooled from 20 eyes per IgG or bevacizumab injection in each experimental set. A total of five independent sets of experiment was performed (n = 5), involving a total of 100 mice. Samples were processed and analyzed as described previously. All antibodies used were obtained from BD Biosciences (San Jose, CA, USA). Anti-mouse CD45, CD4, and CD8a antibodies were conjugated to allophycocyanin (APC), BD Horizon V450, and phycoerythrin (PE) respectively. Isotype controls for gating were IgG conjugated to the respective fluorochromes. Staining with 7-AAD (ViaProbe; BD Biosciences) was used to exclude nonviable cells with live cells being defined as 7-AAD–negative. More than 20,000 cells of each sample were acquired using the BD FACScverse flow cytometer (BD Biosciences) and analyzed using FlowJo 7.6.

Cell Culture

Primary mouse conjunctival fibroblasts were cultured as described previously. For primary mouse T-cell cultures, T cells were harvested from the spleens of C57BL/6/J mice. Spleens were macerated through a 70 µm cell strainer in RPMI 1640 containing 2 mM glutamine, 50 µM 2-mercaptoethanol, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal calf serum (FCS). Following the lysis of red blood cells with 140 mM NH4Cl in Tris buffer (pH 7.4), the cells were cultured in triplicates and then passed through a 40 µm cell strainer and counted. Mouse CD4+ cells were further isolated using CD4 (L3T4) microbeads (Miltenyi Biotec, Bergisch Gladbach Germany) while CD8a+ cells were isolated using the CD8a (Ly-2) microbeads (Miltenyi Biotec). T cells were cultured at 1 × 106 cells/well of a 24-well dish, in activating medium composed of RPMI1640 (Gibco Life Technologies, Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM glutamine, 50 µM 2-mercaptoethanol, 100 U/mL penicillin, 100 µg/mL streptomycin, 10% FCS (PAA, Piscataway, NJ, USA), 2.5 µg/mL anti-mouse CD3ε (eBioscience, San Diego, CA, USA) and 2.5 µg/mL anti-CD28 (eBioscience). All experiments described were based on primary T cells cultured for 3 days, unless otherwise indicated. Three independent primary cultures from different batches of mouse spleens were performed for each experiment (n = 5).

T Cell Counts

CD4+ and CD8a+ cells were cultured in triplicates for 3 days. Cell numbers were measured using the ADAM-MC automatic cell counter (Digital BioTechnology Co. Ltd, Gwanak-gu, Seoul Korea) according to manufacturer’s instructions. Number of viable cells was determined based on staining mammalian cell DNA with the fluorescent dye, propidium iodide, as provided in the ADAM-MC kit.

Cell Cycle Analysis

CD4+ and CD8a+ cells were cultured in triplicates and then processed for cell cycle analysis using the Guava cell cycle reagent following manufacturer’s instructions (Guava Technologies, Hayward, CA, USA). A total of 1500 cells from each sample were analyzed. Cell populations were quantified using the Guava EasyCyte Plus flow cytometry system (Guava Technologies) and the data were analyzed using the Guava cell cycle software (Guava Technologies).

Soluble Collagen Assay

Soluble collagen in the conditioned media from T cells in 24-well plates were measured using the Sircol assay (Biodocolor, Ireland). 200 µL each of conditioned media, normal culture medium (as blank), and collagen standards diluted in normal culture media were incubated overnight with 20% volume of isolation and concentration reagent at 4°C. Then, 1 mL of sircol dye reagent was added to all pelleted samples and collagen content was assayed according to the manufacturer’s protocol. The concentration of soluble collagen was determined from the standard curve and results were normalized to total RNA content of the respective cells recovered.

Reconstitution of SCID Mice With T Cells

Experimental surgery was performed in SCID mice and injected with either IgG or bevacizumab immediately postoperatively. On day 2 postoperatively, 2000 freshly isolated splenic CD4+ or CD8a+ cells combined with 125 µg of either IgG or bevacizumab in 5 µL volumes were injected into the operated conjunctiva. The bleb tissues were harvested on day 7 after experimental surgery. Independent experiments involving CD4+ or CD8a+ cells isolated from different batches of spleens were performed for each quantitative interrogation by real-time PCR (n = 5) and immunoblotting (n = 3). The number of tissues required for pooling in each sample for the respective quantitative analyses was as mentioned above.

Statistical Analysis

Data are expressed as mean ± SD. The significance of differences between two conditions was determined by the 2-tailed Student’s t-test using Microsoft Excel 5.0 software, with significance at P < 0.05. Where more than two treatment conditions were compared, the significance of differences between the conditions, corrected by Bonferroni post hoc adjustment, was determined by 1-way ANOVA using SPSS statistics.

RESULTS

Neutralization of Mouse Conjunctival VEGF-A by Bevacizumab

Since bevacizumab was developed against human VEGF-A, we first verified that bevacizumab captured significant endogenous mouse VEGF-A induced in the mouse model of conjunctival scarring. VEGF-A was highly induced (3.82-folds) in bleb tissues 2 days after experimental surgery, which is the inflammatory phase of wound healing (Fig. 1A). Using the day 2 bleb lysates, we demonstrated that 17.6% of endogenous VEGF was captured by 125 µg bevacizumab, while 22.3% of endogenous VEGF was captured by 250 µg bevacizumab (Fig. 1B). The amount of endogenous VEGF captured by 125 µg was not significantly different from that captured by 250 µg bevacizumab. Further, 125 µg bevacizumab is the equivalent of an injection of 5 µL of a clinical preparation of bevacizumab at 25 mg/mL. In subsequent experiments involving conjunctival injection of bevacizumab in the mouse model of conjunctival scarring, bevacizumab was injected in 5 µL volumes at 25 mg/mL. Therefore, approximately 20% of
endogenous VEGF in the conjunctiva may be expected to be neutralized by bevacizumab injection at this dosage.

**Induction of COL1A1 by Bevacizumab in the Mouse Model of Conjunctival Scarring**

Although VEGF-A has an expression profile more similar to proinflammatory than profibrotic markers in the mouse model of conjunctival scarring, we speculated that VEGF-A inhibition may produce long-term effects that manifest in the late phase of wound healing. To determine the effect of bevacizumab on fibrosis, we compared the expression of Col1a1 in the bevacizumab-injected tissues to that in human IgG-injected controls. Since day 7 is the critical time point when fibrosis is established, this was the main time point analyzed for collagen expression in subsequent experiments involving the mouse model in this study. We found that bevacizumab injection induced significant Col1a1 mRNA upregulation when compared to IgG controls (Fig. 2A). Immunoblotting confirmed that COL1A1 expression was elevated in the bevacizumab-injected tissues at the protein level (Fig. 2B). This inductive effect of bevacizumab on Col1a1 expression occurred in the absence of significant alterations in VEGF-A protein levels in the day 7–treated tissues (data not shown), suggesting that diminished VEGF-A activity alone may be sufficient to produce this biological response.

Given that conjunctival fibroblasts are implicated as the effector cells that drive the fibrotic response in GFS by over-producing collagen, we examined the effect of bevacizumab on Col1a1 in primary mouse conjunctival fibroblasts. We failed to observe induction of Col1a1 expression in these cells (Fig. 2C), suggesting that another cellular source was responsible for the increase in Col1a1 expression in the bevacizumab-treated blebs.

**Increased Immunolabeling for T Cells in Bevacizumab-Treated Blebs**

Curiously, immunofluorescence analyses of days 2 and 7 blebs revealed the increased presence of CD4+ (Fig. 3A) and CD8a+ (Fig. 3B) T cells in the bevacizumab-treated blebs. On day 2 postoperatively, CD4+ (Fig. 3A) and CD8a+ (Fig. 3B) cells were
conspicuous in the conjunctival epithelium (CE), which does not usually express collagen. Collagen-negative CE is observed consistently in our previous studies, including the human conjunctiva.45–47,52 Interestingly, this accumulation of CD4+ and CD8a+ cells in the CE of the day 2 bevacizumab-treated bleb is associated with increased collagen deposition observed in and underneath the CE of the picrosirius red-stained day 2 bevacizumab-treated bleb (Fig. 3C, arrow). Immunostaining for COL1A1 confirmed the increased deposition of COL1A1 in and underneath the CE of the day 2 bevacizumab-treated bleb whereas the CE of the IgG-treated bleb remained COL1A1-free (Fig. 3D, arrows).

On day 7 postoperatively, in the fibrotic phase, increased CD4+ and CD8a+ immunostaining continued to be observed in the bevacizumab-treated blebs, but now localized mainly in the conjunctival matrix (Figs. 3A, 3B, arrowheads). Coincidental increased collagen deposition also can be seen in the bevacizumab-treated conjunctival matrix at this time point (Fig. 3C, arrowhead). Immunostaining for COL1A1 further indicated that increased COL1A1 expression in and underneath
Bevacizumab Induces Collagen Production by T Cells

FIGURE 4. Bevacizumab upregulates CD8a+ numbers in the days 2 and 7 blebs. Mice were injected with the indicated antibodies after experimental surgery and %CD45+CD8a+ cells were measured by flow cytometry in days 2 and 7 blebs. Left, %CD45+CD8a+ cells of each of five pooled samples for each treatment on days 2 (top) and 7 (bottom) are shown. Middle, CD45+CD8a+ numbers were calculated by multiplying the percentage of CD45+CD8a+ cells by the total number of live cells counted in each sample. Mean fold increase with bevacizumab treatment and P value comparing bevacizumab to IgG treatments are indicated. Right, representative dot plots showing gating for %CD45+CD8a+ in the top right quadrant with the mean % ± SD of five independent sets of experiments indicated.

To determine whether bevacizumab affects T cell numbers, we proceeded to quantify CD4+ and CD8a+ cells in the days 2 and 7 blebs by flow cytometry. Although CD4+ cells were visualized clearly in the cryosections, %CD4+ cells as measured by flow cytometry was very low, typically less than 1% of live cells in days 2 and 7 samples from five independent sets of experiments. Hence, data for CD4+ cells in the treated blebs are not shown. On the other hand, consistent with the immunofluorescence observations where CD8a+ cells generally were more abundant than CD4+ cells in the treated blebs, CD8a+ cells were present in numbers that were measured more accurately by flow cytometry. We determined that CD45+CD8a+ cells were significantly higher in the bevacizumab-treated blebs compared to the corresponding IgG-treated controls on both days (Fig. 4). Moreover, the increase in CD8a+ cells was greater in the day 7 blebs compared to day 2 blebs upon bevacizumab treatment. These data indicated that bevacizumab treatment has an inductive effect on CD8a+ cell numbers in the operated conjunctiva. The sustained, significant upregulation of these cells in the day 7 fibrotic phase suggested a potential relationship between these T cells and increased collagen deposition in the scarring response following bevacizumab treatment.

Increased CD4+ and CD8a+ Cells in Bevacizumab-Treated Blebs

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Bevacizumab Increases T Cell Numbers and Expression of COL1A1

To determine whether bevacizumab affects T cell numbers, we treated primary mouse splenic CD4+ and CD8a+ cells with bevacizumab or IgG for 1, 3, and 5 days. As can be observed, CD4 and CD8a numbers generally increased rapidly from days 1 to 3 and appeared to plateau or show limited increase up to day 5, likely as a result of nutrient depletion, changes in culture medium conditions, and/or high cell densities (Fig. 5A). Therefore, the cell growth profiles suggested that a 3-day culture period is best suited for analysis of drug effects on these cells. CD4 and CD8a cell numbers were significantly increased in the presence of bevacizumab compared to IgG in 3-day cultures (Fig. 5B). To determine the possible causes for the increase caused by bevacizumab, we examined the viability and cell cycle profiles of these cells. Bevacizumab significantly increased the viability of CD8a cells compared to IgG treatment (Fig. 5C). On the other hand, bevacizumab significantly reduced the number of CD4 cells in the G1 phase, in favor of small, although insignificant, increases in cells in the S and G2/M phases (Fig. 5D). Crucially, bevacizumab induced an increase in Col1a1 mRNA in CD4+ and CD8a+ cells (Fig. 5E), while Vegfa transcript levels were not altered significantly (data not shown). Increased soluble collagen production measured in the culture media of mouse CD4+ and CD8a+ cells confirmed the capacity of bevacizumab to induce collagen production in these cells (Fig. 5F).

T Cells Are Necessary for Increasing COL1A1 in Bevacizumab-Treated Blebs

To determine that T cells are crucial for COL1A1 increase with bevacizumab treatment, SCID mice were subjected to experimental surgery as before. SCID mice, which lack mature T cells,9 did not demonstrate an increased Col1a1 transcript expression when injected with bevacizumab compared to IgG (Fig. 6A). This contrasted with the induction of Col1a1 mRNA in wild-type Balb/c mice, with which SCID mice are congenic to, upon treatment with bevacizumab (Fig. 6A). These data suggested that the presence of T cells is required for the
Bevacizumab increases primary mouse splenic CD4+ and CD8α+ numbers and collagen expression. (A) Cell counts of mouse CD4+ and CD8α+ cells cultured with or without the indicated antibodies for 1, 3, or 5 days. Values are the means of quadruplicates ± SD. (B) Cell counts of mouse CD4+ and CD8α+ cells cultured with or without the indicated antibodies for 3 days. Values are the means of quadruplicates ± SD. (C) Viability of mouse CD4+ and CD8α+ cells cultured with or without the indicated antibodies for 1, 3, or 5 days. Viability is expressed as % viable cells of total cells counted. *P < 0.05 (post hoc Bonferroni-adjusted) comparing bevacizumab- to IgG-treated CD8α cells on day 3 and the mean fold increase is indicated. (D) Cell cycle profiles of mouse CD4+ and CD8α+ cells treated with or without the indicated antibodies for 3 days. Values are the mean of triplicates ± SD. (E) Col1a1 transcripts in mouse CD4+ and CD8α+ cells treated with or without the indicated antibodies for 3 days. (F) Soluble collagen in the conditioned media of mouse CD4+ and CD8α+ cells cultured for 3 days. Values were normalized to total RNA recovered in each sample and calculated as fold change in soluble collagen relative to untreated control. Data shown are the mean fold ± SD of three independent experiments. *P < 0.05 (post hoc Bonferroni-adjusted) comparing bevacizumab- to IgG-treated cells and, where significant, the associated fold changes are indicated.
Figure 6. Bevacizumab requires T cells to induce collagen expression in conjunctival blebs. (A) Col1a1 transcripts in the day 7 blebs of Balb/c or SCID mice. Mice were injected twice on days 0 and 2 after experimental surgery and Col1a1 was measured by real-time PCR analyses. Values shown are calculated as fold changes from the IgG-treated blebs. The mean fold change of five samples and P value comparing IgG to bevacizumab-treated blebs, where significant, are shown. SCID mice, reconstituted with CD4+ (B–D) or CD8a+ (E–G) in combination with either IgG or bevacizumab, were analyzed for mRNA (B, E) and protein (C, F) expression as well as by immunofluorescence analysis (D, G). For Col1a1 mRNA expression in the day 7 blebs of treated SCID mice, five samples of pooled tissues were analyzed by real-time PCR, each sample consisting of three eyes from three mice. For COL1A1 protein expression in the day 7 blebs of treated SCID mice, three independent samples of pooled tissues were analyzed by immunoblotting, each sample consisting of five eyes of five animals. Denstometric analysis, relative to GAPDH expression, is shown below each immunoblot. Colocalization of injected T cells (red fluorescence) in the day 7 SCID blebs with COL1A1 (green fluorescence) was visualized by immunostaining and confocal microscopy. Insets show magnified images of the boxed area coimmunolabeled for the respective T cells and COL1A1. Scale bar: 75 μm.
increase in Col1a1 expression in response to bevacizumab treatment. To confirm the requirement for T cells in eliciting the collagen induction by bevacizumab, we reconstituted the SCID mouse conjunctiva with freshly isolated murine splenic T cells. Experimental surgery was performed on SCID mice as before, and bevacizumab was injected immediately postoperatively. CD4+ cells, in combination with bevacizumab, were then injected into the operated SCID conjunctiva on day 2, during the inflammatory phase when the tissue milieu is likely to support T-cell survival. The bleb tissues were harvested on day 7 and analyzed for collagen production. Parallel experiments were performed with IgG as controls. We detected increase in Col1a1 expression at the transcript (Fig. 6B) and protein (Fig. 6C) levels when CD4+ cells were injected together with bevacizumab as opposed to IgG. Immunofluorescence analysis of the bevacizumab-treated bleb cryosection that was injected with CD4+ cells revealed the association of these cells with COL1A1 (Fig. 6D). Similarly, the conjunctiva of SCID mice subjected to experimental surgery followed by injection with freshly isolated murine splenic CD8α+ cells together with bevacizumab expressed elevated COL1A1 at the mRNA and protein levels (Figs. 6E, 6F). Injected CD8α+ cells in the SCID conjunctiva were also intimately associated with COL1A1, as visualized by immunofluorescence analysis of immunostained sections (Fig. 6G). Collectively, these data demonstrated that the presence of T cells is essential and sufficient to induce collagen expression in the bleb in response to bevacizumab.

**DISCUSSION**

To our knowledge, this is the first study that reveals a potential profibrotic effect of bevacizumab therapy when applied in a scarring model. Furthermore, we demonstrated that, rather than fibroblasts, T cells mediated the increase in collagen production in response to bevacizumab. These intriguing findings are supported by quantitative analyses in the mouse model of GFS and in vitro using primary T cells.

Previous laboratory studies on the effectiveness of bevacizumab as a monotherapeutic antifibrotic drug for GFS commonly suggested positive attributions21,22,24 or at worse, no effects on scarring.23 These studies have mainly relied on qualitative analyses, since reagents for substantive molecular analyses in the rabbit, which is the most commonly used model, is limited. In this backdrop, and incongruous with the predicted effects of anti–VEGF-A therapy, our discovery that bevacizumab triggered the induction of collagen production in the mouse model of conjunctival scarring came as a surprise. Repeated experimentation using various robust quantitative and qualitative methods yielded the same conclusion. The noninvolvement of fibroblasts in collagen induction by bevacizumab directed our attention toward other cell types, in this case, T cells, since these ostensibly were greater in numbers in the bevacizumab-treated blebs. Previous studies have indicated that VEGF suppressed T cell expansion and that anti-VEGFR-2 reversed this effect.54,55 In agreement, we reported that VEGF-A blockade by bevacizumab increased T cell numbers in vitro and in vivo in the conjunctiva. Our data additionally suggested that this T cell effect may be due to either increased T cell viability or cell cycle progression induced by bevacizumab. Moreover, it appears that the measured T-cell responses to bevacizumab were most likely the result of diminished VEGF-A activity, since significant alterations in VEGF-A expression were not detected in vitro or in vivo under the experimental conditions described in this study.

The capacity of T cells to produce collagen is a property that has never been reported before in any other systems. The role of T cells as key regulators of the immune system is well-established, and they are known to participate in fibrosis indirectly, especially in the wounded heart.56 On the other hand, the direct participation of T cells in fibrosis has not been documented before this study. Some parallels perhaps may be drawn with macrophages, cells that are involved in the immune response, but that also possess the capacity to express virtually all known mammalian collagen genes and partake in wound healing by secreting collagen directly.57 Our study suggested that T cells also may participate directly in tissue repair and contribute to fibrosis upon physiologic VEGF-A suppression. We do not exclude the possibility that T-cell interaction with other cell types in the tissue environment may be altered in the presence of bevacizumab, resulting in other cellular sources adding to the increased collagen production. Nonetheless, the regulation of collagen expression in T cells by VEGF-A implies added complexity to the intricate relationships between angiogenesis, inflammation and tissue remodeling following injury.

If bevacizumab promotes scarring, this effect should have been reported in patients given the increasingly widespread use of this drug for various indications. The notable absence of reported fibrosis in cancer patients receiving bevacizumab treatment may be explained by the application of bevacizumab mainly as an adjunctive drug in combination with other potent chemotherapeutic agents that are likely to suppress the profibrotic effect. On the other hand, the application of bevacizumab for treatment of AMD and DR involves use of this drug as the sole therapeutic agent in a localized fashion, a situation similar to the study described here. Indeed, subretinal fibrosis following repeated systemic or intravitreal bevacizumab treatment has been observed and reported increasingly, not only in AMD29–36 but also in proliferative DR in clinical trials.57–59 T cells may be implicated in these pathologies in response to bevacizumab treatment since emerging data suggest that T cells are key to the manifestation of AMD-like pathology in the retina.58,59 Intriguingly, choroidal neovascularization (CNV) was indicated specifically as an increased risk for scarring with anti-VEGF treatment,53 and T cells have been described to accumulate in CNV in AMD.60 T cells also are known to infiltrate the vitreous in proliferative diabetic retinopathy.61 Therefore, the involvement of T cells in these diseases may set the stage for the greater risk of scarring following anti–VEGF-A therapy. Our findings, thus, provide a plausible explanation for the clinically observed subretinal scarring in AMD and DR eyes on long-term anti–VEGF-A treatment.

Collectively, this study suggested that monotherapeutic bevacizumab intervention will not be effective in suppressing collagen production where T cells are present. Therefore, our data may explain the mixed observations in various experimental and clinical studies of bevacizumab as anti-fibrotic adjunctive therapy for GFS. Hence, the notion of using bevacizumab and other anti-VEGF-A agents as anti-fibrotic therapeutics warrants reconsideration, particularly where the target tissue environment features T cell accumulation. Finally, the profibrogenic activity of VEGF-A-suppressed T cells could potentially undermine the therapeutic benefits of VEGF-A-targeted drug modalities. Therefore, this pro-scarring potential of bevacizumab or anti–VEGF-A therapeutics may need to be restrained to sustain therapeutic success in long-term treatment of diseases characterized by vascularization.

**Acknowledgments**

Supported by the Singapore National Research Foundation under its Translational and Clinical Research (TCR) Programme (NMRC/
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