Combined Treatment with Bevacizumab and 5-Fluorouracil Attenuates the Postoperative Scarring Response after Experimental Glaucoma Filtration Surgery

Alicia Hou,1 Jocelyn Leng Leng Chua,1 Amanda Charlton,2 Roseline Su,3 Marcus Lim,1 Rajesb S. Kumar,3 Jonathan G. Crowston,4 and Tina T. Wong1,3

PURPOSE. This study evaluated the use of combined bevacizumab with 5-fluorouracil (5-FU) on postoperative scarring and bleb survival after experimental glaucoma filtration surgery in comparison to the agents alone.

METHODS. Filtration surgery was performed on 26 female New Zealand White rabbits. The rabbits were allocated to one of four treatments: 5-FU combined with bevacizumab, 5-FU alone, bevacizumab alone, or phosphate buffered saline (PBS). The subconjunctival injections were administered immediately postoperatively and weekly for 3 weeks. Clinical assessment and bleb photography were performed. Histologic staining determined the presence of subconjunctival fibrosis and mRNA expression of collagen I and fibronectin in the tissue was quantified.

RESULTS. Bevacizumab in combination with 5-FU resulted in a greater antifibrotic effect compared with monotherapy with 5-FU or bevacizumab alone, as evidenced by the attenuation in fibronectin and mature collagen I expression and deposition (P < 0.05). In addition, this was associated with a 100% bleb survival at day 28 in the combined treatment group compared with monotherapy (50% bevacizumab [P < 0.05] and 25% 5-FU [P < 0.001]). Conjunctival vascularity significantly reduced with bevacizumab treatment both alone and in combination with 5-FU.

CONCLUSIONS. The results provide compelling evidence that combined bevacizumab and 5-FU offers superior antifibrotic effect over monotherapy in a model of glaucoma filtration surgery, while prolonging bleb survival at the same time. A synergistic effect is suggested to be present. (Invest Ophthalmol Vis Sci. 2010;51:928–932) DOI:10.1167/iovs.09-3949

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Aberrant healing and fibrosis are major causes of mortality and morbidity. Vascular endothelial growth factor (VEGF) is a potent endothelial cell specific cytokine that enhances microvascular permeability and vascular endothelial cell proliferation and plays a pivotal role in angiogenesis.1 The therapeutic effects of VEGF blockade in treating solid tumors and age-related macular degeneration (ARMD) have resulted in unprecedented efficacy in clinical trials, alone or in combination with cytotoxic agents.2–3 However, the role of VEGF in wound healing is less clear. VEGF is generally thought to drive fibrosis mainly through promoting angiogenesis,4–5 but there is also evidence to show that VEGF therapy attenuates renal fibrosis, thereby improving kidney function.6 Bevacizumab (Avastin; Genetech, Inc., San Francisco, CA) is a full-length humanized monoclonal antibody directed against all isoforms of VEGF-A and is FDA approved for the treatment of metastatic colorectal cancer.7 Increased rates of wound dehiscence at the colorectal anastomosis have been reported after the administration of intravenous bevacizumab for the treatment of metastatic colorectal cancer.8 This complication may occur several months to years after the original surgical anastomoses were formed, suggesting that bevacizumab may induce long-term inhibition of wound healing.

Ocular scar formation underlies the cause of vision loss or cause of treatment failure for most blinding conditions. Excess scar formation within the filtration tissues after glaucoma filtering surgery obstructs aqueous humor outflow and is the major cause of inadequate intraocular pressure (IOP) lowering and surgical failure. Fibroblasts are the cells chiefly responsible for generating scar tissue after trabeculectomy in the eye.9 The Tenon’s fibroblast is therefore the target of current antifibrosis regimes that are in common use. Intra- or postoperative applications of mitomycin-C (MMC) or 5-fluorouracil (5-FU) have been shown in large prospective randomized trials to inhibit postoperative scarring and improve surgical outcomes.10–12 However, in higher risk eyes a single injection is often met with limited success with the need for several injections to rescue a poorly functioning bleb. As a result of multiple 5-FU injections, the development of sight-threatening complications is a not uncommon association.13,14 Increased vascularity at the bleb site after glaucoma filtration surgery is one of the principal markers used to guide antifibrosis treatment postoperatively. A recent study has demonstrated elevated VEGF levels in aqueous humor in humans after glaucoma surgery and VEGF receptor expression in human Tenon’s capsule fibroblasts (Li LZ, et al. IOVS 2008;49: ARVO E-Abstract 5669). The use of subconjunctival injections of bevacizumab to rescue failing blebs has been described in a limited number of small case reports.15–17 A recent study has reported on the reduction of subconjunctival scarring in vivo with the use of bevacizumab.18 However, following on from this report, we present evidence that the antifibrotic effect of...
bevacizumab in combination with 5-FU is superior to bevacizumab alone in the clinical management of postoperative scarring after glaucoma filtration surgery in the rabbit.

**MATERIALS AND METHODS**

**Experimental Design: In Vivo Model of Subconjunctival Scarring**

An established rabbit model of glaucoma filtration surgery was used, as previously described by Cordeiro et al. All studies were approved by the SingHealth Institute Animal Care and Use Committee and procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Modified filtration surgery was performed only on the left eye of 26 female New Zealand White rabbits by the same masked individual (AH) and at the same site in each animal. In brief, the modified filtration surgery involved inserting a 25 gauge cannula from the limbus into the anterior chamber (after conjunctival dissection), which was secured with 10/0 nylon to allow efflux of aqueous humor into the subconjunctival space. The rabbits were allocated to one of four subconjunctival injections: 0.1 mL of 50 mg/mL 5-FU (ABIC, Netanya, Israel) combined with 0.1 mL of 25 mg/mL bevacizumab (Avastin; F. Hoffmann-La Roche, Basel, Switzerland) (n = 7), or 0.1 mL of 50 mg/mL 5-FU (n = 4), or 0.1 mL of 25 mg/mL bevacizumab (n = 8), or 0.1 mL of phosphate buffered saline (PBS) as the vehicle control (n = 7). The dose of bevacizumab was chosen to replicate as close to the current off-label clinical dosing and regime used post trabeculectomy as possible. The injections were administered posterior to the cannula, under the conjunctiva. The subconjunctival injections into the bleb were administered using a 27 gauge needle immediately postoperatively after the conjunctival closure and weekly for the next 3 weeks by an individual masked to the drug to be administered after each masked clinical assessment and photography.

**Clinical Examination**

This was performed using a standard portable slit lamp. Bleb survival was used as the primary efficacy endpoint and was based on the clinical appearance and masked grading of the bleb. Bleb vascularity and morphology were graded according to the Moorfields bleb grading system. Each rabbit bleb was graded by two masked independent medical personnel (TTW, JLLC). Bleb grading was performed by slit lamp examination and digital photography.

**Histologic Staining**

The specimens were fixed in 4% paraformaldehyde and embedded in paraffin before 5 μm sections were cut. The sections were stained for the presence of scar tissue formation (Sirius red F38A; Sigma, St. Louis, MO). Polarization microscopy of stained collagen fibers was performed to reveal gross collagen bundling patterns and graded using a modified semiquantitative grading system previously described by Shah et al. Any inter-observer variability was then assessed. Reproducibility of the masked readers (TTW, AC) was assessed before grading the slides. No significant between-reader grading differences were found (data not shown).

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis**

Quantification of collagen I and fibronectin expression was performed using real-time PCR. Total mRNA from the sectioned conjunctival bleb tissues (delinated with India ink before fixation) were extracted and purified using a kit (High Pure RNA Paraffin Kit; F. Hoffmann-La Roche, Basel, Switzerland) according to manufacturer’s protocol. cDNA was then synthesized using reverse transcriptase (SuperScript III; Invitrogen, Carlsbad, CA). This was followed by PCR amplification (Power SYBR Green PCR Master Mix; Applied Biosystems, Inc., Foster City, CA). The cycling conditions were as follows: denaturation at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 50°C for 2 minutes and 60°C for 1 minute in a high-throughput, microwell plate-based cycler platform (LightCycler 480; F. Hoffmann-La Roche). The fluorescent threshold was calculated using the system software and results analyzed using the comparative cycle threshold method. The primer sequences designed are shown in Table 1. Within the linear range of amplification, at least three values of each amplification product were normalized to the starting mRNA volume and compared with the corresponding GAPDH values.

**Statistical Analysis**

For the in vivo study, statistical analysis was performed to determine the differences between the four treatment groups. Kaplan-Meier survival analysis was performed for bleb failure using the Mantel-Cox log rank test. Differences in vascularity between treatment groups and controls were compared using a linear mixed model for repeated measures. Bland Altman test was used to evaluate inter-observer variability. Fixed effects variables were taken into account for drug groups, time, and interactions between the two. Changes in vascularity were compared preoperatively and postoperatively using Wilcoxon-Sign rank tests. Histologic analysis was performed with a graphic display of mean values for the groups and 95% confidence intervals (CI). Differences in mRNA expression levels between groups for qPCR analysis was analyzed with ANOVA, with a P value of <.05 considered as significant.

**RESULTS**

**Bleb Survival**

Treatment with bevacizumab lead to a significant improvement in bleb survival as illustrated in the Kaplan Meier survival graph of Figure 1A (37.5%, n = 8, P < 0.05). Combined bevacizumab and 5-FU resulted in 100% bleb survival at day 28 (n = 7, P < 0.001) compared with vehicle PBS treatment. In contrast, none of the PBS-treated (n = 8) and only 25% (n = 4) of the 5-FU–treated blebs survived to day 28.

**Bleb Vascularity**

Masked clinical assessment of conjunctival vascularity before and after surgery revealed no significant differences between the preoperative (baseline) vascularity and at day 28 with bevacizumab treatment alone or in combination with 5-FU compared with PBS and 5-FU treatments (Table 2). The bleb vascularity between the four treatment groups is illustrated in Figure 1B–E. No complications such as the development of cystic avascular blebs or corneal epithelial toxicity were observed in the rabbits with bevacizumab alone or in combination with 5-FU throughout the duration of the study period.

The Bland Altman test showed an excellent agreement of 96.2% for clinical bleb grading of morphology and vascularity performed by the two masked independent observers (95% CI, 94.8–98.4).

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**Table 1. Primers Used in Real-Time PCR**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I</td>
<td>Forward: 5'-GAGCCGTTGACCTACGG-3’&lt;br&gt;Reverse: 5'-TTTGGTATGGAACTGCTTGCC-3’&lt;br&gt;Forward: 5'-GAC ACC CTT AAT CCT GGC AC-3’&lt;br&gt;Reverse: 5'-TCA GAA ACT GTG CTT GTC TGG-3’&lt;br&gt;Forward: 5'-AGAGACGCGGATCCTTCTTG-3’&lt;br&gt;Reverse: 5'-CTTGGCGTTGGTAGAGATCAT-3’</td>
</tr>
</tbody>
</table>

**TABLE 1.** Primers Used in Real-Time PCR
Histologic Markers of Fibrosis

Histologic staining confirmed that bevacizumab lead to an inhibitory effect on subconjunctival scar formation. Sirius red polarizing microscopy of collagen fibers revealed a predominance of densely packed mature collagen I (orange/red) in the control and 5-FU–treated eyes (Figs. 2A, 2B, respectively) compared with bevacizumab and combined bevacizumab and 5-FU treatments (C, D), which demonstrated a more loose arrangement of collagen fibers in the subconjunctival space (yellow/green).

mRNA Expression of Collagen I and Fibronection in Blebs

A significant reduction in transcript levels of collagen I (Fig. 3A) and fibronectin (Fig. 3B) mRNA were found in both the bevacizumab and combined bevacizumab and 5-FU treatments compared with PBS and 5-FU–only treated eyes (*P < 0.05).

DISCUSSION

We present data demonstrating that the anti-VEGF-A monoclonal antibody, bevacizumab, confers potent antifibrosis activity in an established animal model of glaucoma surgery. Our data support the potential clinical benefit in the use of bevacizumab.

TABLE 2. Comparison of Within Group Conjunctival Vascularity Pre- and Post-surgery

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Time (A)</th>
<th>Time (B)</th>
<th>Mean Difference (A − B) (Std. Error)</th>
<th>Significance</th>
<th>Confidence Interval for Difference*</th>
</tr>
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<tbody>
<tr>
<td>PBS</td>
<td>21</td>
<td>0</td>
<td>1.429 (0.307)</td>
<td>0.001†</td>
<td>0.648 - 2.209</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0</td>
<td>1.857 (0.348)</td>
<td>0.000†</td>
<td>1.004 - 2.711</td>
</tr>
<tr>
<td>5-FU</td>
<td>21</td>
<td>0</td>
<td>0.875 (0.201)</td>
<td>0.001†</td>
<td>0.372 - 1.378</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0</td>
<td>0.875 (0.237)</td>
<td>0.003†</td>
<td>0.301 - 1.449</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>21</td>
<td>0</td>
<td>−0.286 (0.259)</td>
<td>0.582</td>
<td>−0.947 - 0.376</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0</td>
<td>−0.143 (0.297)</td>
<td>1.000</td>
<td>−0.87 - 0.584</td>
</tr>
<tr>
<td>Bevacizumab/5-FU</td>
<td>21</td>
<td>0</td>
<td>1.750 (0.257)</td>
<td>0.001†</td>
<td>0.982 - 2.518</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0</td>
<td>0.25 (0.282)</td>
<td>0.797</td>
<td>−0.508 - 1.008</td>
</tr>
</tbody>
</table>

Comparison of mean conjunctival vascularity preoperatively (day 0) and postoperatively (days 21 and 28) showed no significant measured difference in conjunctival vascularity between before surgery and 28 days post surgery in eyes treated with bevacizumab and combined bevacizumab and 5-FU compared with PBS and 5-FU treatments. 5-FU, 5-fluorouracil; PBS, phosphate-buffered saline.

* P < 0.05.
† Adjustment for multiple comparisons by Bonferroni.

FIGURE 1. Bevacizumab treatment prolongs bleb survival and reduces conjunctival vascularity postoperatively. (A) Kaplan Meier Survival curve showing that increased bleb survival was associated with bevacizumab treatment (n = 8) in comparison with PBS (n = 7) and 5-FU (n = 4) treatments. Bleb survival was magnified with the combined treatment of bevacizumab and 5-FU (n = 7), (**P < 0.001, Log Rank). (B–E) Representative clinical appearances in vascularity of filtration blebs at day 28 in a rabbit model of subconjunctival scarring. Treatment with bevacizumab alone and in combination with 5-FU (D, E) resulted in a return to near-normal conjunctival vascularity compared with PBS and 5-FU treatments (B, C).

FIGURE 2. Subconjunctival fibrosis is reduced with bevacizumab treatment in vivo. (A–D) Sirius red polarization microscopy revealed densely packed collagen residing in the subconjunctival space in PBS and 5-FU treatment (A, B) but less mature fibers (yellow-green) in bevacizumab and combined bevacizumab and 5-FU treatments (C, D). Bars: (A, B) 200 μm; (C, D) 500 μm. ![Image](image-url) ('A–D) indicates the subconjunctival space (bleb). (*P < 0.05).
and 5-FU as an effective antifibrotic combination for the treatment of scarring after trabeculectomy.

Angiogenesis constitutes an important element of wound repair.\(^1\) Vascular remodeling occurs due to the carefully balanced interplay of pro-angiogenic and anti-angiogenic factors. In addition to assisting in the removal of cellular debris, angiogenesis facilitates the beginning of wound closure by providing the vascular scaffold for granulation tissue formation. Both angiogenic agonists and antagonists have been identified at various stages of wound repair with either vessel growth or regression occurring depending on the overall stimulus at any one stage in the repair process. Although it is now widely established that VEGF-A is responsible for normal vasculogenesis, hemangiogenesis, and lymphangiogenesis,\(^{22-25}\) relatively little attention has previously been given to the reported anti-fibrotic effects of VEGF-A.

VEGF inhibition has been shown to attenuate fibrosis in a murine model of allergic airway disease through downregulation of transforming growth factor \(\beta\)-1 expression and the phosphoinositide 3-kinase/Akt pathway signaling.\(^{26}\) VEGF also induces a profibrogenic gene expression profile in glomerular endothelial cell line, which was accompanied with upregulation of VEGFR-2 phosphorylation and mRNA expression.\(^{27}\)

In addition to the expected elevated levels of VEGF-A present in ocular fluids patients with diabetic retinopathy and other retinal disorders,\(^{28}\) raised VEGF-A levels in aqueous humor from non-neovascular glaucoma patients has also been reported.\(^{29}\) The significance of this finding is that taken together with the hypoxic insult after surgical manipulation of tissue, which leads to an increase VEGF-A expression by resident fibroblasts and other inflammatory cells, targeting the VEGF-A molecule would appear to be a plausible method of reducing the postoperative scarring response after glaucoma filtration surgery.

The results from the animal study directly support the notion that VEGF-A is an important component in the course of events in postoperative wound repair. Pharmacological neutralization of VEGF-A with administration of bevacizumab postoperatively not only clinically improved bleb survival and reduced conjunctival vascularity over conventional treatment with 5-FU, but also significantly attenuated the fibrotic response, which was corroborated with a reduction in fibronectin and collagen I mRNA expression. Finally, the combined delivery of bevacizumab and 5-FU magnified the antifibrotic effect compared to the two agents separately. Taken together, these findings provide compelling evidence that VEGF-A is a key mediator for the development of conjunctival vascularization and plays a role in the development of subconjunctival fibrosis. Furthermore, it is also important to note that bevacizumab and 5-FU are likely to be working synergistically to induce a more profound effect on fibrosis. It is proposed that the use of 5-FU together with bevacizumab would improve the postoperative wound healing response.

The amount of scar tissue generated in the conjunctival tissues after glaucoma filtration surgery is a critical determinant of surgical outcome, as it determines the resistance to aqueous humor outflow and the degree of intraocular pressure lowering. As a result, the wound-healing post-glaucoma filtering surgery has been studied extensively and a number of well-established experimental models are available.\(^{19,20,50}\) Intra-operative MMC and 5-FU are currently clinically used to inhibit fibrosis and improve surgical outcomes.\(^{52-54}\) These agents are titrated against risk factors for excess scar formation but despite their use a significant failure rate persists.

Recent reports have also explored the use of bevacizumab after glaucoma filtration surgery. These non-randomized, non-controlled case series of 1 to 12 patients have indicated that the agent can be administered safely at the time of surgery or in the postoperative period.\(^{16,17,35}\) In this study, we provide the first data that bevacizumab in combination with 5-FU has a greater anti-scarring effect than monotherapy in vivo. Our study does not establish whether or not a single injection of bevacizumab confers significant anti-scarring activity, although it is likely that repeat administration of the drug alone or in combination with 5-FU would be needed to attenuate the scarring response in patients long-term. A further limitation here is the absence of IOP measurements in this model. However, a reduction in subconjunctival scarring would certainly exert a positive effect on bleb function. A sample size of over 300 patients would be required to determine an antifibrosis effect of similar magnitude to that derived from postoperative administration of 5-FU.\(^{13}\)

To date, anti-VEGF treatments have primarily targeted pathologic angiogenesis for both systemic and ocular neovascular disorders. As fibrosis is the most significant clinical problem that both hinders surgical success and is a principal cause of morbidity, mortality, and blindness, our findings in this study suggest that adjunctive treatment with bevacizumab used in conjunction with 5-FU has the potential to further improve surgical outcomes. Further clinical studies are now needed to establish the role of bevacizumab in glaucoma surgical management.
References


