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

Alicia How, Jocelyn Leng Leng Chua, Amanda Charlton, Roseline Su, Marcus Lim, Rajesh S. Kumar, Jonathon G. Crowston, and Tina T. Wong

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 Ophthal Vis Sci. 2010;51:928–932) DOI:10.1167/iovs.09-3949

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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.\(^\text{19}\) 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 dose 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 that was 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.\(^\text{20}\) 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 \(\mu\)m sections were cut. The sections were stained for the presence of scar tissue formation (Sirius red F3BA; 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.\(^\text{21}\) The specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were cut and stained for the presence of scar tissue formation (Sirius red F3BA; 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.\(^\text{21}\)

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

Quantification of collagen I and fibronectin expression was performed using realtime PCR. Total mRNA from the sectioned conjunctival bleb tissues (delineated 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, microplate-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 <0.05 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% vs. 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).

**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’-CACGGCTTCCATTACGAC-3’&lt;br&gt;Reverse: 5’-TTTTGTAATTGAAGCTTCCTGCCC-3’</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Forward: 5’-ACC AAC CTT AAT CCG GGC AC-3’&lt;br&gt;Reverse: 5’-TCA GAA ACT GTG CTT TGC TG-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5’-AGAGACGGCTATCTTCTTGTT-3’&lt;br&gt;Reverse: 5’-CTTGGGTCGGTGGTAGAGTCTAT-3’</td>
</tr>
</tbody>
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\(^{\text{19}}\) Combined bevacizumab and 5-FU for Subconjunctival Fibrosis 929
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). Bars: (A, B) 200 μm; (C, D) 500 μm. ‘b’ (A–D) indicates the subconjunctival space (bleb). (*P < 0.05).

mRNA Expression of Collagen I and Fibronectin 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 to reduce conjunctival fibrosis and vascularization postoperatively.

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

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Day</th>
<th>Mean Difference (A – B) (Std. Error)</th>
<th>Significance</th>
<th>Confidence Interval for Difference†</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>21</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>1.857 (0.348)</td>
<td>0.001†</td>
<td>1.004 2.711</td>
</tr>
<tr>
<td>5-FU</td>
<td>21</td>
<td>0.875 (0.201)</td>
<td>0.003†</td>
<td>0.372 1.378</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0.875 (0.237)</td>
<td></td>
<td>0.301 1.449</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>21</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>–0.143 (0.297)</td>
<td>1.000</td>
<td>–0.87 0.584</td>
</tr>
<tr>
<td>Bevacizumab/5-FU</td>
<td>21</td>
<td>1.750 (0.257)</td>
<td>0.001†</td>
<td>0.982 2.518</td>
</tr>
<tr>
<td></td>
<td>28</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.

† Adjustment for multiple comparisons by Bonferroni.
Angiogenesis constitutes an important element of wound repair. 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, relatively little attention has previously been given to the reported anti-fibrotic effect of VEGF-A. Combining and 5-FU as an effective antifibrotic combination for the treatment of scarring after trabeculectomy.

VEGF inhibition has been shown to attenuate fibrosis in a murine model of allergic airway disease through downregulation of transforming growth factor β-1 expression and the phosphoinositide 3-kinase/Akt pathway signaling. 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. In addition to the expected elevated levels of VEGF-A present in ocular fluids patients with diabetic retinopathy and other retinal disorders, raised VEGF-A levels in aqueous humor from non-neovascular glaucoma patients has also been reported. 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.
References


