was easy to conclude that no dystrophin was expressed in cone cells. However, in the current immunofluorescent preparations, two distinct immunostaining patterns were recognized: a small dot (~1 μm) was in the rod sphere and a large aggregation (~5 μm) was in the cone pedicle. It has been known that a cone pedicle has two kinds of synapses—flat and invaginated. In the immunoelectron micrographs, retinal dystrophin was localized at both synapses in cone pedicles. The flat synapse has no component of horizontal cells; therefore retinal dystrophin seems to be much less associated with horizontal cells than with bipolar cells. Why do no abnormal ERG responses arise in cone cells of patients with DMD? It may be because of the diversity of localizations and functions of dystrophin isoforms. However, very little is known about whether their localizations and functions are different in photoreceptor cells. Further study is needed to clarify the functional significance of the dystrophin localized in cone cells.

In summary, it is clear that retinal dystrophin is localized in cone pedicles as well as in rod spheres. It is a pivotal component in photoreceptor cells and may be closely associated with signal transmission from photoreceptor cells to bipolar cells.

**Key Words**

bipolar cell, cone pedicle, dystrophin, electron microscopy, immunocytochemistry

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**Treatment of Bleb Leaks With Transforming Growth Factor-β in the Rabbit Model**

**J. William Doyle, M. Fran Smith, J. Alfredo Garcia, Gregory Schultz, and Mark B. Sherwood**

**Purpose.** The mechanism through which peribleb injection of autologous blood results in resolution of bleb leak in the rabbit model remains unclear. This study evaluates the clinical and histologic effects of peribleb injection of transforming growth factor-β (TGF-β) after leak induction in mitomycin-C-treated blebs.

**References**


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**Method.** Posterior lip sclerectomies treated with mitomycin-C were created in New Zealand White rabbits. On postoperative day 7, a standardized stab incision was performed on all blebs, and the eyes were randomized to receive a peribleb injection of either TGF-β or of a balanced salt solution.

**Results.** Injection of TGF-β was associated with the resolution of bleb leak and maintenance of a functioning bleb in 50% (4 of 8) of treated eyes. The remaining TGF-β-treated eyes and control eyes demonstrated continued bleb leaks or bleb failures with intraocular pressure returning to preoperative levels. Histologic examination revealed increased peribleb cellularity and denser collagen deposition in the TGF-β-treated eyes compared with that observed in control eyes.

**Conclusions.** Peribleb TGF-β injections may contribute to healing bleb leaks, but the injections do not appear in this model to be as useful as whole-blood injections. *Invest Ophthalmol Vis Sci.* 1997;38:1630–1634.

We have reported our experience using peribleb injections of autologous blood to treat refractory bleb
leaks in humans\(^1\) and in the rabbit model.\(^2\) In the rabbit model, eight out of eight such treated leaks resolved with bleb function maintained. Histology was remarkable for significantly increased fibroblasts and denser collagen deposition. However, it was unclear which component(s) of whole blood were most vital to the healing process.

Transforming growth factor-\(\beta\) (TGF-\(\beta\)) is a protein growth factor derived from whole-blood plasma.\(^3,4\) It is a potent chemoattractant for corneal epithelial cells, stromal fibroblasts, and endothelial cells in tissue culture,\(^5\) and it enhances collagen synthesis in healing dermal incisions.\(^6,7\) This study, using rabbit eyes with induced bleb leaks after filtering surgery with mitomycin-C, was designed to evaluate whether the increased number of fibroblasts and dense collagen deposition noted with peribleb injection of autologous whole blood was related to this single blood component.

**MATERIALS AND METHODS.** A prospective, randomized study was performed using New Zealand White rabbits weighing between 2 and 4 kg. Prior approval of the protocol was obtained from the University of Florida’s Institutional Animal Care and Use Committee. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

A standard posterior lip sclerectomy\(^8\) was performed on 18 right eyes of 18 different rabbits by the same surgeon (JWD). A superior limbal-based conjunctival flap was raised. Intraoperative mitomycin-C (0.4 mg/ml) soaked in a cellulose sponge was applied between the sclera and conjunctiva for 5 minutes. This concentration of mitomycin-C was chosen on the basis of our previous experience, which demonstrated long-lasting blebs and reduction of intraocular pressure (IOP) in the rabbit model with this dose.\(^8\) A linear, 2-mm incision was made and the anterior chamber entered. A 1.5-mm cross-section scleral punch was used to remove a standard posterior block of tissue. A peripheral iridectomy was made. The conjunctival incision was closed with a running 8-0 polyglactin suture on a BV 130-4 needle (Ethicon, Somerville, NJ). A 30-gauge paracentesis was made and balanced salt solution (BSS) injected into the anterior chamber to confirm the presence of a patent sclerostomy and to inflate the bleb. The conjunctival incision and bleb were inspected to rule out any leaks. Maxitrol ointment was applied at the end of the surgery.

The postoperative examinations were done under ketamine and xylazine anesthesia. The intraocular pressure was measured using a Tonopen tonometer (Mentor, Norwell, MA), the status of the bleb was assessed on a 0 (none) to 3 (high) scale, the anterior chamber depth was estimated, and fluorescein was used to determine the presence of any bleb leaks.

On postoperative day 7, all eyes were examined as above, to insure that the blebs were functioning and that no leaks were present. After the examination, all blebs were punctured with a 75-Beaver blade to yield a standard 2-mm incision. All incisions were inspected to assure that they were Seidel positive. Maxitrol ointment was applied to all eyes after the incision in the bleb was made.

All eyes were reexamined on postoperative day 8 and were randomized to receive peribleb subconjunctival injections of either TGF-\(\beta\), 2 \(\mu\)g in 0.2 ml bovine serum albumin (BSA; \(n = 8\)), 0.2 ml balanced salt solution \((n = 8)\), or 0.2 ml BSA alone \((n = 2)\). The concentration of TGF-\(\beta\) was selected as that which produced maximal chemotactic effects in tissue culture.\(^5\) Injections were made around but not into the bleb, using a 30-gauge needle. Maxitrol ointment was applied after all injections.

On postoperative day 11 (3 days after peribleb injections), all eyes were reexamined with attention to bleb status, IOP, and anterior chamber depth. All rabbits were euthanized by an intracardiac injection of pentobarbital and the eyes removed. Care was taken not to disturb the conjunctiva over the bleb during the enucleation.

Two sample Student’s \(t\) tests and Wilcoxon’s rank sum tests were used to compare clinical variables of IOP and bleb status (bleb height and presence or absence of bleb leak) pre- and postinjection, in treated and control eyes. Failure was described at postoperative day 11 as either persistent bleb leak or final IOP elevated to within 3 mm Hg of preoperative IOP. Fisher’s exact tests were used to determine differences between control and treated eyes. A \(P\) value of less than 0.05 was considered statistically significant.

The enucleated eyes were fixed in a 10% formalin solution (Buffered Formalde-Fresh; Fisher Scientific, Pittsburgh, PA). After 48 hours in fixative, the bleb was dissected from the surrounding tissues and processed to paraffin. Once embedded in the paraffin blocks, sections were cut at 6-\(\mu\)m intervals. Sections were placed on slides and stained with Harris’ hematoxylin–eosin–phloxine.

The slides were examined using an Olympus BH Binocular microscope, (Olympus Optical, Tokyo, Japan) and photomicrographs were obtained. Cell counts were obtained from six sites on two separate slides prepared from each eye. The examiner of the slides was unaware of whether the eye had received peribleb TGF-\(\beta\) or BSS. The six sites counted were as follows: right side of the bleb area, just under the conjunctiva (site 1); middle of the collagen–fibroblast response, right side of the bleb (site 2); directly under the bleb, not including underlying sclera (site 3); in the middle of the collagen–fibroblast response, left side of the bleb (site 4); at the left side of the bleb...
area, just under the conjunctiva (site 5); and in the subconjunctival space as far as possible from the bleb area (site 6). The grid used was 0.2 × 0.2 mm, and all cell nuclei within the grid were counted. The exact areas were located under scanning power, then the magnification, was increased to ×200 for the count. Multivariate analysis of variance was used to test for treatment, location, and slide number effects and interactions.

RESULTS. Clinical Results. Preoperative mean values for IOP averaged 16 mm Hg (range, 15 to 18 mm Hg). All 18 posterior lip sclerectomies were functioning on postoperative day 7, with a mean IOP of 6.8 mm Hg (range, 4–9 mm Hg). The average bleb height was 2.75 (range, 2 to 3) on a scale of 0 to 3 (as noted in Materials and Methods).

After the blebs were punctured with a 75-Beaver blade, all were found to be Seidel-positive. Examination 24 hours after puncture on postoperative day 8 revealed all blebs still to be leaking, with a mean IOP of 5.9 mm Hg (range, 3 to 7 mm Hg). All blebs remained high (2.75). Anterior chamber depth varied from less than half deep to fully deep, with a mean depth of approximately 50%. At this time, eyes were randomized and received subconjunctival injections of BSA (n = 4), BSS (n = 8), or TGF-β (n = 8).

On postoperative day 11, the two control eyes that received subconjunctival injections of BSA demonstrated failed, Seidel-negative blebs and a mean IOP of 16 mm Hg. The eight control eyes, which received peribleb injection of BSS, all met a failure criterion. Fifty percent (n = 4) of the eyes had a persistent bleb leak associated with shallow anterior chambers or low IOP (<4 mm Hg), or both, and the other 50% (n = 8) demonstrated failed, Seidel-negative blebs and IOPs returning to preoperative levels (average 13 mm Hg; range, 12 to 15 mm Hg). The final average IOP of the entire control group was 8.25 mm Hg (range, 2 to 15 mm Hg). On postoperative day 11, 50% of the eyes (n = 4) receiving TGF-β demonstrated healed bleb leaks, high blebs, and lowered IOP (mean 7.25 mm Hg; range, 6 to 9 mm Hg); and the remaining 50% demonstrated persistent bleb leaks with shallow anterior chambers, or low IOP (mean, 4.25 mm Hg; range, 3 to 6 mm Hg). The final mean IOP of the entire TGF-β-treated group was 5.75 mm Hg (range, 3 to 9 mm Hg).

Failure of the bleb, either by elevation of IOP or by persistence of the leak occurred in 100% of the control eyes (n = 8) and in 50% in the TGF-β-treated eyes (n = 4). This did not reach statistical significance (P = 0.0769; Fisher’s two-sided exact test), although it was suggestive of improvement. There was also no significant difference between the final IOPs in the two groups. However, in that failure was described by either elevation of IOP (flat bleb) or depression of IOP (persistent leak), the final mean values for IOP may not allow accurate assessment of success.

Histologic Results. There were differences in histologic appearance between eyes that had received peribleb injections of BSA or BSS and eyes that had received peribleb injections of TGF-β. Sections from eyes that had received TGF-β showed greater collagen deposition and an increased cellular response adjacent to the bleb. Most of these cells had polygon-shaped nuclei, a finding most consistent with fibroblast proliferation. There was no difference noted in the histologic appearance of those eyes injected with BSA compared with that of control eyes receiving BSS (assessed to determine whether the BSA alone caused an increased inflammatory response). Sections from either BSA-treated or control eyes revealed only a mild cellular, collagenous response filling the bleb space.

Photomicrographs of control and treated eyes (Figs. 1, 2) demonstrate the difference between the two groups in cellularity and density of eosinophilic material. To assess the difference in cellularity, the number of fibroblasts from two slides from each eye were counted as described in Methods. The cell counts were averaged for eyes from each animal. The average number of fibroblast nuclei at each site for control- and TGF-β-treated eyes is shown in Table 1. The total number of fibroblasts counted was significantly higher in the TGF-β-treated eyes than the total number in the control-treated eyes (22 versus 16; P < 0.05; Table 1). There was also a statistically significant increase in the number of fibroblasts at three specific sites adjacent to the bleb (sites 1, 2, and 4; Table 1). The density of the eosinophilic material, consistent with collagen deposition, was noted to be greater in the eyes treated with TGF-β.

DISCUSSION. Clearly, a more effective way is needed to deal with persistent bleb leaks after trabeculectomy with adjunctive antimetabolite. In humans 50% to 60% of such leaks resolve after peribleb injection of whole blood. If the mechanism of action behind such successful treatment could be discerned, perhaps the therapy could be better adjusted and made yet more efficacious.

In results of a prospective, randomized study of bleb leaks in the rabbit model, we found a 100% resolution rate after injection of whole blood. Knowing that the histologic analysis demonstrated probable fibroblast proliferation with collagen deposition both around and within the bleb of the blood group, we hypothesized that a whole-blood injection provided a source of trophic factors. These factors could induce migration and proliferation of fibroblasts from the adjacent areas not treated with the antimetabolite,
FIGURE 1. Photomicrographs of a hematoxylin-eosin-stained blebs on postoperative day 11 after peribleb injection of (A) transforming growth factor-β (original magnification, ×100) and (B) a balanced salt solution (original magnification, ×100). Note the increased number of fibroblast nuclei and the slightly denser eosinophilic material (consistent with collagen deposition) after injection of transforming growth factor-β.

with resulting promotion of healing around and within the bleb.

As was noted above, the same degree of success seen in the rabbit model after whole-blood injection is not seen in humans. Perhaps this is because of a greater propensity for healing (given the proper stimulus) in rabbits. In any event, we chose to continue our work in the rabbit model, believing that if we could identify the blood components associated with 100% resolution of bleb leaks in rabbits, such isolated components might be used with greater efficacy in man.

The TGF-β family of growth factors reversibly inhibits growth of cells derived from ectoderm (keratinocytes and leukocytes) but can stimulate cells derived from mesoderm (fibroblasts). In fact, TGF-β is a potent stimulator of inflammatory cells and may also stimulate synthesis of the extracellular matrix. Therefore, when our experiments with peribleb injection of whole blood showed statistically significant increases in these very same entities (fibroblasts and extracellular collagen), we turned our attention to the TGF-β isolate alone to see if it was the blood component responsible for fibroblast proliferation and the resulting leak seal.

Peribleb injection of TGF-β, at concentrations known to stimulate fibroblasts maximally, caused a significant increase in fibroblast nuclei at several sites adjacent to the bleb. Some clinical success in healing leaks and in preserving bleb function (but not quite to levels of statistical significance) was noted. The low numbers of subjects may have prevented the results...
from reaching convincing statistical significance. However, marked statistical significance was seen with the same number of subjects after whole blood was injected. In that study there was 100% clinical success in eyes that had persistent leakage demonstrated failed healing of bleb leak. It is unclear why none of the four TGF-β-treated eyes that had persistent leakage demonstrated failed blebs and elevated IOPs similar to those seen in four of the control eyes. Perhaps the application of TGF-β resulted in sufficient leak closure to allow some continued preservation of the bleb. If these eyes had been observed for a longer time, some of them may have either healed the leak, preserving the bleb, or gone on to total bleb failure with elevated IOP.

Continued work in this model will involve looking into whether more than one factor in the healing cascade is involved in sealing bleb leaks after injection of whole blood. Does plasma protein diffusion to the area of a leak, with subsequent cross-linking of factors, contribute to leak seal? Additionally, the role, if any, of red blood cells is unclear. The next step in our work involves separating blood into plasma and cellular components, obtaining other independent trophic factors, and proceeding with injections as before.

**Key Words**

antiproliferative effects, bleb leak, glaucoma surgery, rabbit, transforming growth factor-β

**References**


