Mitotic Effect of Autologous Blood Injection and Diode Laser Bleb Revision on Rabbit Filtration Blebs

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Objective: To investigate the effect of intrableb autologous blood injection and diode laser bleb revision on cellular proliferation in filtration blebs of rabbits.

Methods: Bilateral filtration surgery with mitomycin was performed on 19 white 2.5- to 3-kg New Zealand rabbits. Autologous blood injection (n=9) or diode laser bleb revision (n=10) was performed on blebs of right eyes (intervention eyes) on day 14 after filtration surgery. The blebs of the left eyes served as controls. Cellular proliferation was assessed by in vivo incorporation of 5-bromo-2'-deoxyuridine (BrdU), after BrdU, 10 mg/kg, was injected via the ear vein on days 15, 16, and 18. The rabbits were euthanized on day 21. Conjunctival sections were stained with hematoxylin-eosin for total cell counts and with anti-BrdU antibody for counts of proliferating cells. The BrdU labeling index was calculated by dividing the mean proliferating cell count by the mean total cell count.

Results: The BrdU labeling index was significantly increased in the blood-injected eyes as compared with the control eyes (P=.03). The BrdU labeling index was increased in the diode laser–revised eyes as compared with the control eyes, approaching significance (P=.06). Intraocular pressure increased significantly in the eyes that underwent bleb interventions in both groups from baseline and compared with the control eyes.

Conclusions: Intrableb autologous blood injection and diode laser bleb revision stimulate cellular proliferation in the rabbit filtration bleb, which may contribute to the rise in intraocular pressure observed clinically after these interventions.

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BLEBS ASSOCIATED with overfiltration and leakage present a challenging management problem. These filtration blebs are often thin, cystic, and ischemic in appearance and occur more frequently after adjunctive antimetabolite application. In some eyes, the resulting low intraocular pressure (IOP) can result in reduced visual acuity from corneal astigmatism, corneal edema, choroidal effusions, shallow anterior chambers, and hypotony maculopathy. When bleb leaks occur, the risk of bleb infection and endophthalmitis may be increased.

In 1993, Wise introduced injection of autologous blood into overfiltering blebs as a way to manage chronic hypotony. Subsequent larger case studies reporting the results of intrableb and peribleb autologous blood injection have cited success rates of 58% to 66%. In 1996, the revision of overfiltering and leaking blebs using a continuous-wave Nd:YAG laser operating in the thermal mode was reported by Lynch et al. Success rates of 64% for overfiltering, 80% for leaking, and 100% for painful blebs were achieved.

Although autologous blood injection and laser bleb revision have demonstrated modest success in the management of overfiltering or dysfunctional blebs, the mechanism by which these methods work has not been well understood. It has been presumed that cellular proliferation is a likely component. We investigated the effect of intrableb autologous blood injection and diode laser bleb revision on cellular proliferation in filtration blebs of rabbits using in vivo incorporation of 5-bromo-2'-deoxyuridine (BrdU) (Sigma, St Louis, Mo) as a marker for cellular proliferation.

RESULTS

CELL COUNTS

Autologous Blood Injection

One rabbit that died 2 days after filtration surgery for unknown reasons was excluded from further analysis. Analysis of the remaining 8 rabbits is summarized in Table 1. The mean total cell count was not significantly different between the blood-injected eyes and the saline-injected eyes. The mean proliferating cell count was greater, though not significantly, in the blood-injected eyes. The BrdU labeling in-
MATERIALS AND METHODS

Approval for research use of animals was obtained from the University of Washington Animal Care Committee, Seattle. All animals were treated in accordance with the Association for Research in Vision and Ophthalmology statement on the use of animals in ophthalmic and vision research.

PILOT STUDIES

BrdU Feasibility

To test the ability of BrdU to act as a marker for cellular proliferation in our rabbit model, full-thickness filtration surgery was performed in both eyes of two 2-3-kg white New Zealand rabbits. The right eye received a 5-minute exposure of mitomycin (0.4 mg/mL) to the sclerotomy site intraoperatively and the left eye received no antimetabolite. The BrdU was given intravenously on the day prior to euthanasia. The rabbits were euthanized 7 days after surgery. The eyes were fixed and stained in the manner described below. Differences in cellularity of hematoxylin-eosin- and anti-BrdU-stained sections between the eyes that received mitomycin and the eyes that did not were qualitatively compared. The eye that received mitomycin during filtration surgery had qualitatively less hematoxylin-eosin and anti-BrdU staining than the eye that did not receive mitomycin (Figure 1).

Peak Cellular Proliferation

To determine the timing of peak cellular proliferation after autologous blood injection, blood was injected subconjunctivally at various intervals in the right eye of a 1.7-kg white New Zealand rabbit that had not undergone any prior filtration surgery. Autologous blood obtained from an ear vein was injected subconjunctivally in the superotemporal quadrant 3 days prior to euthanasia, in the inferotemporal quadrant 10 days prior to euthanasia, in the inferonasal quadrant 13 days prior to euthanasia, and in the superonasal quadrant 10 days prior to euthanasia. The left eye of each rabbit served as a control. In the control left eye, normal saline was injected subconjunctivally in the superonasal quadrant 13 days prior to euthanasia, in the inferonasal quadrant 10 days prior to euthanasia, in the inferotemporal quadrant 7 days prior to euthanasia, and in the superotemporal quadrant 3 days prior to euthanasia. The BrdU was injected intravenously on the day prior to euthanasia. After euthanasia, the eyes were fixed, sectioned, and stained with hematoxylin-eosin and anti-BrdU antibody. Cells from a total of six 40X fields from each interval were manually counted by 2 independent observers. The mean cell counts from each of the 4 intervals in the blood-injected eye were compared with each other and with those of the saline-injected control eye. The greatest cellular activity occurred during day 7 in the blood-injected eye (Figure 2). The proportion of BrdU incorporated into cells was greater in the blood-injected eyes as compared with the control eyes at all intervals. Based on this result, the rabbits in the bleb intervention study below were euthanized 7 days after bleb intervention (day 21 after mitomycin filtration surgery).

BLEB INTERVENTION STUDY

Surgical Technique

Full-thickness filtration surgery with mitomycin under general anesthesia was performed on both eyes of 19 white 2.4- to 2.6-kg New Zealand rabbits. All surgical procedures were performed by 1 of 2 surgeons, both trained to comply with study protocol. The pericocular area was prepared with 10% povidone-iodine. The eye was draped with sterile sheets and a lid speculum was placed. Under an operating microscope, a superior rectus bridle suture was placed using a 6-0 polyglactin 910 (Vicryl) suture. A limbal-based conjunctival flap was dissected in the superotemporal quadrant. A 2 × 2 × 2-mm piece of cellulose sponge soaked in mitomycin (0.4 mg/mL) was applied to the sclera adjacent to the limbus in the superotemporal quadrant with the conjunctiva and Tenon capsule draped over the sponge for 2.5 minutes. The sponge was discarded and replaced with a second mitomycin-soaked sponge for another 2.5 minutes. The second sponge was then discarded and the area was irrigated with 30 mL of balanced salt solution. A full-thickness sclerotomy was performed by creating a limbal entry into the eye, 3 mm in length, using a No. 11 blade. The slit opening was widened using a scleral punch, followed by cautery to the posterior lip. A peripheral iridectomy was performed. The conjunctiva was closed with a running 10-0 nylon suture. Then, 0.3% gentamicin or 0.3% ofloxacin, 1% atropine, and 1% prednisolone acetate were dropped into each eye. On day 14 after surgery, the right eye of each rabbit underwent 1 of 2 bleb interventions: autologous blood injection (n=9) or diode laser revision (n=10). The left eye of each rabbit served as a control. Intraocular pressure was monitored prior to trabeculectomy (day 0), prior to bleb intervention (day 14), and prior to euthanasia (day 21). All IOP measurements were taken by the same individual and calibrated tonometer (Tonopen; Mentor, Norwell, Mass) at the 5% error level.

Bleb Interventions

Autologous Blood Injection. Each of 9 rabbits was sedated with subcutaneous acepromazine, 0.6 to 0.8 mg/kg, (10 mg/mL) and intramuscular xylazine, 5 to 9 mg/kg, (20 mg/mL) on day 14 after filtration surgery. Both eyes were anesthetized with 1 drop of 0.5% proparacaine hydrochloride. Autologous blood (0.5 mL) was withdrawn from an ear vein bleb revision using lower power (2000 mW), but was present in the eyes of the remaining 6 rabbits that underwent laser bleb revision using higher power (3000 mW). Because the first 4 low-power rabbits also showed no clinical IOP effect after laser revision, we considered them to have undergone insufficient treatment and excluded them from further analysis. Detailed
using a 26-gauge butterfly needle attached to a tuberculin syringe. A sterile 30-gauge needle was then placed onto the tuberculin syringe and 0.1 to 0.2 mL of autologous blood was injected into the filtration bleb of the right eye. The filtration bleb of the left eye was injected similarly with 0.1 to 0.2 mL of isotonic sodium chloride solution. One drop of gentamicin or ofloxacin was instilled into each eye after completion of the procedure.

Diode Laser Bleb Revision. Each of 10 rabbits was sedated as described earlier on day 14 after filtration surgery. The right eye was anesthetized with 1 drop of proparacaine. Using a diode laser (IRIS Medical, Mountainview, Calif) set at 2000 milliwatts in duration, 22 to 34 laser applications were administered throughout the surface of the bleb by holding a G probe perpendicular to and 3 mm above the scleral surface (Figure 3). A lower power setting (2000 mW) was used in the first 4 rabbits. Because of a lack of significant histologic and clinical response in these initial rabbits, a higher power setting (3000 mW) was used in the remaining 6 rabbits. An occasional crinkling of the conjunctival surface was noted, but most applications had no visible bleb surface changes. No bleb interventions were performed in the left eyes.

BrdU Injection
5-Bromo-2’-deoxyuridine is a thymidine analog that is incorporated into cells during the S phase of the cell cycle. It is a marker for cellular proliferation and is nonspecific for cell type. To assess cellular proliferation by in vivo incorporation of BrdU, rabbits were sedated as described earlier and BrdU (10 mg/kg) was injected via the ear vein on days 15, 16, and 18 after filtration surgery. The study timeline is outlined in Figure 4.

Histologic Preparation
The rabbits were sedated with subcutaneous acepromazine, 0.6 to 0.8 mg/kg (10 mg/mL), then euthanized with intravenous sodium pentobarbital (120 mg/kg) on day 21 after surgery (7 days after bleb intervention). Both eyes were enucleated and the superior rectus was tagged with a suture for orientation. The eyes were fixed in 10% neutral buffered formalin (Sigma) for paraffin-embedded sections. Three conjunctival sections from the middle portion of the bleb were stained immunohistochemically for collagen I and IV using goat antihuman collagen I and IV (cross-reactivity with rabbit).

Analysis
Cell Counts. “Total cell counts” were obtained from the hematoxylin-eosin–stained sections. “Proliferating cell counts” were obtained from the anti-BrdU sections. To minimize bias, cell counts were performed using the Olympus CUE-2 image analysis system (Galai Instruments, Lake Placid, NY), a computer-assisted counting program. Cell nuclei were counted in two 20× fields per section, yielding a total of 6 fields from each rabbit for each of the stains. The field chosen was similar in all sections and corresponded to a location just beneath the conjunctival epithelium in the middle of the bleb. The epithelium was excluded from cell counts. In the hematoxylin-eosin– and anti-BrdU–stained slides, cell nuclei were counted. The threshold intensity for cell counting was adjusted to exclude background staining. The proportion of proliferating cells was calculated by dividing the mean proliferating cell counts by the mean total cell counts for each eye, and referred to as the BrdU labeling index. From these samples, mean cell counts and BrdU labeling index were calculated and compared using a 2-tailed Student t test.

Collagen Staining. The intensity of collagen staining of the blood injection and control saline injection sections was judged qualitatively by an independent observer and compared under 20× magnification. The field chosen was similar in location in all sections and corresponded to an area just beneath the conjunctival epithelium in the middle of the bleb.

Intraocular Pressure. To evaluate the clinical success of the filtration procedure, IOP measurements prior to surgery (day 0) were compared with IOP measurements on day 14 after surgery for each of the bleb intervention groups. To evaluate the effect of the bleb intervention on IOP, measurements prior to the bleb intervention (day 14) were compared with IOP measurements on day 21 after surgery (day 7 after bleb intervention) for each of the bleb intervention groups. The right eye and left eye measurements performed on day 21 were also compared. Statistical analysis was performed by comparing mean values using the 2-tailed Student t test.

COLLAGEN STAINING
One rabbit from the blood injection group underwent anti-collagen I and IV staining. The staining was qualitatively more intense in the samples from the blood-injected eye as compared with the control saline-injected eye (Figure 5).

cell counts and statistical analysis of the remaining 6 high-power rabbits is summarized in Table 2. The total cell count and proliferating cell count were significantly greater in the laser-treated eyes as compared with the control eyes (P=.04 and .02, respectively). The BrdU labeling index was greater in the laser-treated eyes, approaching significance (P=.06).
INTRAOCULAR PRESSURE

Results of IOP measurements for each intervention group are summarized in Table 3.

Autologous Blood Injection

In both the intervention and control eyes, the mean IOP was significantly decreased 14 days after filtration surgery (P<.001). In the intervention eyes, mean IOP was significantly increased 7 days after autologous blood in-
Injection (\(P = .03\)). In the control eyes, mean IOP remained unchanged 7 days after saline injection. Seven days after blood injection, the mean IOP was no longer significantly different from prefiltration surgery levels in the intervention eyes, but remained significantly lower in the control eyes (\(P = .007\)). When comparing intervention with control eyes 7 days after blood injection, IOP was significantly higher in intervention eyes (\(P = .01\)).

Diode Laser Revision

In both the intervention and control eyes, the mean IOP was significantly decreased 14 days after filtration surgery (\(P < .001\) and .03, respectively). In the intervention eyes, mean IOP was significantly increased 7 days after diode laser revision (\(P = .02\)). In the control eyes, mean IOP remained unchanged. Seven days after diode laser revision, the mean IOP was no longer significantly different from prefiltration surgery levels in the intervention eyes, but remained significantly lower in the control eyes (\(P = .007\)). When comparing intervention with control eyes 7 days after blood injection, IOP was significantly higher in intervention eyes (\(P = .01\)).

Blood Injection vs Laser Baseline IOPs

The baseline mean IOPs of the blood injection group were compared with the baseline mean IOPs of the laser group. Both the intervention and control eyes of the blood injection group had baseline mean IOPs that were significantly lower than the intervention eyes of the laser group (\(P = .03\) and lower, approaching significance, than the control eyes of the laser group (\(P = .06\) and \(P = .07\), respectively).

Although autologous blood injection and Nd:YAG laser revision have been modestly successful in managing overfiltering blebs in human subjects, the mechanisms of action are not well known. In our rabbit model, BrdU was used as a marker for cellular proliferation and demonstrated that cellular proliferation within the wall of the bleb occurs after autologous blood injection and may play an important role in diode laser bleb revision.

After intrableb autologous blood injection, the rise in IOP likely has different mechanisms in the short term vs in the long term. Initially, the increase in IOP may be caused by a reduction of aqueous flow transconjunctivally from blockage by erythrocytes or fibrin. This initial rise in IOP may also potentiate resorption of existing choroidal effusions, further facilitating an increase in IOP. Eventually, fibrosis of the bleb is presumed to occur. Because overfiltering blebs are more often thin, cystic, and lacking cellular elements, fibroblasts may migrate from adjacent tissue. Although both mitomycin and fluorouracil inhibit cellular proliferation, Yamamoto et al have demonstrated that neither agent altered fibro-
blast migration in tissue culture. The blood clot may serve as a scaffold for fibroblasts to migrate toward the bleb wall, allowing fibrosis to occur.

Doyle et al. studied the histologic and clinical effects of peribleb autologous blood injection on leaking blebs in a rabbit model. Sixteen rabbits underwent mitomycin filtration surgery in 1 eye followed by creation of a bleb leak. The rabbits were randomized to receiving either peribleb autologous blood injection or saline injection. Leaks in the blood-injected eyes resolved with increased peribleb cellularity and collagen deposition on hematoxylin-eosin staining. Our rabbit study differs from this study in that blood was injected intrableb, leaks were not created, and BrdU was used to differentiate those cells that are proliferating.

The mechanism of change in bleb morphology after Nd:YAG laser revision has been even less understood. Lynch et al. noted pigmented spots along the inner conjunctival surface and suggested that this may signify that a new layer of cells released from uveal tissue has lined the inner bleb wall. Alternatively, the induction of inflammation is also likely to play a role. No histologic data have been available on these blebs to date. Use of the diode laser for bleb revision has not been previously described. In our rabbit study, we chose to use the diode laser rather than the continuous-wave Nd:YAG laser because of logistical issues at our institution. The scleral and conjunctival tissues have a high water content with an absorptive wavelength peak close to 3.0 µm. The diode laser (wavelength, 0.81 µm) would be expected to be absorbed less well by these tissues than the Nd:YAG laser (wavelength, 1.06 µm).

The primary focus of this study is represented by our histologic data. However, IOP data were also included to allow a comparison of the clinical result with the histologic result within each treatment group. When comparing mean IOPs of the intervention vs control groups at baseline (day 0), before bleb intervention (day 14), and after bleb intervention (day 21), there were no significant differences within each intervention group. However, when comparing mean IOPs between the intervention groups, the mean baseline IOP of the blood

<table>
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<th>Table 3. Intraocular Pressure Summary*</th>
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<td>Blood injection (N = 8)</td>
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<td>Day 0 (Before Surgery)</td>
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<td>Day 14 (Before Bleb Intervention)</td>
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<td>Day 21 (After Bleb Intervention)</td>
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<td>Laser revision (N = 6)</td>
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<td>Day 14 (Before Bleb Intervention)</td>
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<td>Day 21 (After Bleb Intervention)</td>
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* All data are presented as mean (SD) and as millimeters of mercury.
† Intracocular pressure significantly lower than day 0 (same eye).
‡ Intracocular pressure significantly higher than day 14 (same eye) and day 21 (contralateral eye).
Injection group was significantly lower than that of the laser group. Retrospectively, all methods were reviewed and no obvious reason for this difference was identified. One plausible explanation may be that the blood injection portion of the study was completed first, followed by the laser portion of the study. Baseline (day 0) IOPs were obtained after induction of anesthesia and just before glaucoma surgery. Because the injection group included the first rabbits operated on in the study, the timing of IOP measurement after induction of anesthesia may have been initially delayed in comparison with the laser group as the facility of the anesthesia technique was improved. Day 14 and day 21 measurements may also reflect anesthesia timing differences between the 2 groups. We do not believe the differences in IOPs between treatment groups represent a fundamental defect in either the surgical method or the histologic method. The same 2 surgeons, both experienced in filtration surgery, were involved in all cases. All specimens were processed by 1 individual with extensive prior experience in BrdU labeling and cell counts.

Based on a rather large SD of our absolute cell counts of total cells and proliferating cells, chance interindividual variability may have occurred in our small sample size. The proportion of proliferating cells, represented by the BrdU labeling index, seemed to be a more valid measure of cellular activity than absolute cell counts. We therefore relied on comparisons of the BrdU labeling index to make final judgments about the extent of cellular proliferation.

The autologous blood injection group demonstrated a significantly higher BrdU labeling index compared with the saline controls, confirming that cellular proliferation is an important mechanism with this technique. This also correlated with the increased collagen staining that was studied in 1 rabbit in this group, though no conclusions can be drawn about collagen production from these limited data. Clinically, this histologic response in the blood-injected eyes was associated with a significant rise in IOP. Although study protocols were quite different, these findings are consistent with the findings reported by Doyle et al.8

The diode laser revision group also demonstrated a significant rise in IOP compared with control eyes. The BrdU labeling index was greater in the laser-treated eyes, approaching significance. A dose-response relationship was demonstrated by a lack of response at a lower power (2000 mW) setting compared with a higher power (3000 mW) setting. It is possible that even more power is needed to obtain an optimal tissue response when using the diode laser. Because there was a significant clinical response, there may be important mechanisms other than cellular proliferation in laser-revised blebs. The potential feasibility of using the diode laser for bleb revision was demonstrated.

Although it is likely that the proliferating cells counted in both the blood-injected and laser-revised blebs are mostly fibroblasts, this cannot be concluded from our study. The BrdU is incorporated into all cells undergoing mitosis and is nonspecific for cell type. Although the final common pathways may be similar with both of these bleb interventions, the mechanism by which this is achieved may differ. After the injection of autologous blood, the blood itself likely provides the factors necessary to stimulate cellular proliferation and migration, whereas after laser bleb revision the damaged tissue may be the source for these factors.

Our small sample size does not warrant comparing outcomes of autologous blood injection with those of laser bleb revision. We do not intend to suggest superiority of one technique over the other but simply to suggest a mechanism of action for both involving cellular proliferation. We demonstrated that autologous blood injection after filtration surgery in rabbits significantly stimulates cellular proliferation. Diode laser bleb revision at the power settings used in our study also seems to stimulate cellular proliferation in rabbit filtration blebs, approaching significance. These effects may contribute to the rise in intraocular pressure that is often observed clinically after autologous blood injection and laser bleb revision.

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