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PURPOSE. To compare the effects of post-penetrating keratoplasty (PK) and post-keratoprosthesis (KPro) surgery-related inflammation on the posterior segment of the eye and to assess inhibition of tumor necrosis factor alpha (TNFα) and interleukin-1 beta (IL-1β) on these effects.

METHODS. BALB/C (syngeneic) or C57BL/6 (allogeneic) corneas were transplanted onto BALB/C host beds as part of PK or miniature KPro (m-KPro) implantation. Intraocular pressure (IOP) was measured via an intracameral pressure sensor; tissues were harvested and analyzed 8 weeks after surgery. Expression of TNFα and IL-1β in the retina was analyzed using real-time quantitative (q)PCR. Optic nerve degeneration (axon count, circularity, and area) was assessed quantitatively using ImageJ software. After m-KPro implantation, mice were treated with saline, anti-TNFα, or anti-IL-1β antibody, and axonal loss was assessed after 10 weeks.

RESULTS. Mean IOP was within normal limits in the operated and fellow eyes in all groups. The mRNA expression of TNFα and IL-1β was highest in m-KPro groups with either syngeneic or an allogeneic carrier. We observed optic nerve degeneration in both allogeneic PK and m-KPro implanted eyes with an allogeneic carrier. However, TNFα blockade significantly reduced axonal loss by 35%.

CONCLUSIONS. Allogeneic PK and m-KPro implants with an allogeneic carrier lead to chronic inflammation in the posterior segment of the eye, resulting in optic nerve degeneration. In addition, blockade of TNFα prevents axonal degeneration in this preclinical model of allogeneic m-KPro (alloKPro) implantation.

Keywords: Boston keratoprosthesis, keratoplasty, chronic inflammation, optic neuropathy
blockade of TNFα or IL-1β can reduce optic nerve degeneration. For the first time, we shed light on potential mechanisms of posterior segment complications after PK and KPro implantation.

**METHODS**

**Animals**

A total of 125 male mice (8–10 weeks old; Jackson Laboratory, Bar Harbor, ME, USA; 82 BALB/C as recipients; 10 BALB/C and 28 C57BL/6 as donors; and 11 as naïve) were used in this study. Mice were housed in a climate-controlled animal facility at the Schepens Eye Research Institute, Massachusetts Eye and Ear, Harvard Medical School (Boston, MA, USA), and kept under cyclic light conditions (12 hours on/off). All animal experiments were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

For the first part of the study, mice were divided into five groups (10 mice/group): (1) naïve, (2) syngeneic penetrating keratoplasty (synPK) (BALB/C donor cornea transplanted to BALB/C recipient), (3) allogeneic penetrating keratoplasty (alloPK) (C57BL/6 donor cornea transplanted to BALB/C recipient), (4) syngeneic m-KPro implantation (synKPro) (BALB/C carrier corneal graft with assembled m-KPro transplanted to BALB/C recipient), and (5) allogeneic m-KPro implantation (alloKPro) (C57BL/6 carrier corneal graft with assembled m-KPro transplanted to BALB/C recipient).

Second, to analyze the effect of cytokine inhibition, alloKPro implantation was performed. After implantation, mice were divided into four groups and treated with saline, anti-TNFα or anti-HL-1β antibody or kept untreated. Intrapetoraline saline, anti-TNFα antibody (10 mg/kg), or anti-HL-1β antibody (10 mg/kg) was administered once a week starting at day 0 through week 10. The mice were analyzed 10 weeks after the procedure.

**Penetrating Keratoplasty**

Surgeries were performed under a surgical microscope (Zeiss, Jena, Germany). Procedures were performed by two investigators (MO and AC). After euthanasia of the donor mice, corneas were marked with a 2-mm-diameter biopsy punch (Millex, Plainsboro, NJ, USA). To maintain a deep anterior chamber, viscoelastic (Viscoat; Alcon, Irvine, CA, USA) was injected through the groove with a 30-gauge cannula (Rumex, Clearwater, FL, USA). Corneas were excised with Vannas scissors (Storz Instruments Company, San Damis, CA, USA) and placed in phosphate-buffered saline (PBS) until the host corneal bed had been prepared.

Recipient mice were anesthetized by intraperitoneal injection of ketamine (120 mg/kg) and xylazine (20 mg/kg). A drop of 1% tropicamide (Bausch & Lomb, New York, NY, USA) and a drop of 0.5% phenylephrine hydrochloride (Altaire Pharmaceuticals, New York, NY, USA) were applied immediately after the procedure and mice were euthanized 10 weeks after the procedure; the rest of the postoperative treatment was identical to that for the first part.

**Miniature KPro Implantation**

A miniature keratoprosthesis (m-KPro) (JG Machine, Wilming- ton, MA, USA), minimized, but otherwise identical to the B-KPro device used in humans, was used. It consists of a PMMA front plate with central stem and a titanium (Ti) back plate with eight peripheral holes. The diameters of the front and back plate were 1.0 and 1.3 mm, respectively. The design description and the exact dimensions of the m-KPro are described elsewhere. Disth surgical procedure was performed similarly to B-KPro implantation in humans and has been described previously.

Briefly, to prepare for the assembly of the m-KPro device, excised donor corneas were trephined centrally with a 0.5-mm-diameter punch (Harris UniCore punch, Ted Pella, Inc.) and then slid over the stem of the PMMA front plate. The titanium back plate was positioned and locked on the stem such that the donor cornea became securely sandwiched between the front and back-plates. The donor–device complex was then placed in PBS until the host corneal bed had been prepared as described above. After preparation, the donor cornea–m-KPro complex was placed in the recipient bed and secured with eight interrupted 11-0 nylon sutures (Sharpoint, Angiotech Pharmaceuticals) in standard PK fashion.

**Postoperative Treatment**

In the first part of the study, postoperative treatment was identical after PK and m-KPro implantation. One drop of 1% corticosteroid (Pred Forte; Allergan, Irvine, CA, USA) and antibiotic ointment (AK-POLY-BAC; Akorn, Lake Forest, IL, USA) were applied immediately after the procedure, and a tarsorrhaphy was performed using 8-0 nylon sutures (Sharpoint, Angiotech Pharmaceuticals). Corticosteroid was administered once a day for 2 weeks and then every other day for 2 weeks. The tarsorrhaphy was removed 48 hours after the surgery, and corneal sutures were removed 1 week following surgery. Per standard institutional animal care and use protocols, 0.1 mg/kg buprenorphine (Reckitt Benckiser Healthcare Ltd., Hull, England) was applied subcutaneously after the procedure and every 12 hours for 48 hours postoperatively. The mice were euthanized 8 weeks after the procedure for further analysis. Eight weeks in mice corresponds to around 6 years in humans.

In the second part of the study, corticosteroids were not used and mice were euthanized 10 weeks after the procedure; the rest of the postoperative treatment was identical to that for the first part.

**Intraocular Pressure (IOP) Measurement**

To evaluate IOP after syngeneic or allogeneic PK and m-KPro implantation, direct manometry was used. Intracameral IOP measurements were performed in anesthetized mice using a custom-designed 32-gauge needle electromechanical pressure sensor. Measurements were performed by inserting the needle in the anterior chamber of the eye through a clear corneal puncture. Intraocular pressure readings were stored in a personal computer for further analysis.
Reverse Transcription and Real-Time PCR

Retinas were harvested at 8 weeks post surgery. RNA was isolated with the RNeasy Micro Kit (Qiagen, Valencia, CA, USA) and reverse transcribed using Superscript III Kit (Invitrogen, Grand Island, NY, USA). Real-time quantitative (q)PCR was performed using Taqman Universal PCR Mastermix and preformulated primers for murine glyceraldehyde 3-phosphate dehydrogenase (GAPDH), tumor necrosis factor alpha (TNFα), and interleukin-1 beta (IL-1β) (Applied Biosystems, Foster City, CA, USA). The results were normalized to the expression level in naïve mice and were analyzed by the comparative threshold cycle method, using GAPDH as an internal control. Real-time PCR was repeated twice for each cytokine. Retinas of three mice were pooled and duplicates for each group were analyzed.

Optic Nerve Axon Counts, Circularity, and Area

Optic nerves were analyzed as described before.14 Briefly, optic nerves were fixed with half-strength Karnovsky fixative and cut into 1-μm cross sections. Pictures of five cross sections were taken with ×20 magnification under a light microscope to determine optic nerve perimeter. Five pictures of each cross section were taken with ×100 magnification to quantify axon numbers per mm² and axon size. The pictures were systematically analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA). Circularity was defined as $4\pi \times \frac{\text{Area}}{\text{Perimeter}^2}$ with a value of 1.0 indicating a perfect circle. As the value approaches 0.0, it indicates an increasingly noncircular shape.

Statistics

Student’s t-test was used to analyze the data. $P \leq 0.05$ was considered statistically significant. Results are presented as the mean ± standard error of the mean (SEM).

RESULTS

Intraocular Pressure After Syngeneic or Allogeneic PK and m-KPro Implantation

We measured IOP in the operated and fellow eyes of mice after syngeneic or allogeneic PK and m-KPro implantation and in the eyes of naïve mice. All groups showed a similar mean IOP within normal limits (14–19 mm Hg). There was no statistically significant difference between any of the experimental groups (Fig. 1).

Inflammatory Cytokine Expression in the Retina After Syngeneic or Allogeneic PK and m-KPro

To evaluate inflammation in the retina following PK and m-KPro implantation, we quantified mRNA expression of the proinflammatory cytokines TNFα and IL-1β at week 8 after surgery. We detected an increase in TNFα expression in all operated groups compared to naïve mice; however, a significant increase was seen only in the synKPro ($P < 0.01$) and alloKPro ($P < 0.05$) groups (Fig. 2A). The expression of IL-1β was higher in syngeneic and allogeneic m-KPro groups compared to syngeneic and allogeneic PK groups, respectively (Fig. 2B). The alloKPro group showed significantly higher expression of IL-1β compared to naïve mice ($P < 0.05$; Fig. 2B).

Optic Nerve Axon Count, Size, and Circularity After Syngeneic or Allogeneic PK and m-KPro Implantation

To determine the effect of PK and m-KPro implantation on optic nerve changes, we analyzed optic nerve images from week 8 after surgery with ImageJ (Figs. 3A, 3B). The number of axons remained similar in the synPK and synKPro groups compared to the fellow eye. However, we observed axon loss of 13 ± 8% and 19 ± 8% in the alloPK and alloKPro groups, respectively (Fig. 3C). We additionally analyzed axon size and circularity. Average axon size increased in all operated groups compared to naïve mice (Fig. 3D). Axon circularity was reduced in all four groups compared to naïve mice and significantly reduced in the alloKPro group (Fig. 3E).

FIGURE 1. Intraocular pressure measurements in mice. Intraocular pressure was measured in the operated and fellow eyes using manometry 8 weeks post surgery. N, naïve; synPK, syngeneic PK; synKPro, syngeneic m-KPro; alloPK, allogeneic PK; and alloKPro, allogeneic m-KPro. $n = 5$/group.

FIGURE 2. Quantification of proinflammatory cytokines in the retina. Transplant recipients from all groups (syngeneic [syn] and allogeneic [allo] penetrating keratoplasty [PK] and miniature-Boston keratoprosthesis [m-KPro]) were euthanized at 8 weeks post surgery and retinas were collected and analyzed for the expression of (A) TNFα and (B) IL-1β by real-time qPCR. $n = 6$/group; *$P = 0.05$; **$P < 0.05$. Data from one representative experiment of two is shown. N, naïve; synPK, syngeneic PK; synKPro, syngeneic m-KPro; alloPK, allogeneic PK; and alloKPro, allogeneic m-KPro.
TNFα Blockade Protects the Optic Nerve After Allogeneic m-KPro

To determine the protective effect of TNFα and IL-1β blockade on optic nerve degeneration, anti-TNFα or anti-IL-1β antibody was administered after alloKPro implantation. We quantified optic nerve axon numbers 10 weeks after the surgery. Control mice that had m-KPro implantation (treated only with saline) had a reduction of 29 ± 4% in axon numbers compared to unoperated eyes. Similarly, m-KPro mice treated with anti-IL-1β antibody had an associated axon loss of 29 ± 1% compared to unoperated mice, indistinguishable from the saline control group (Fig. 4; P = 0.96). In contrast, m-KPro mice that were treated with anti-TNFα antibody experienced axon loss of only 19 ± 1%, a 35% reduction in axon loss compared to the saline-treated m-KPro implanted mice (P = 0.03).

DISCUSSION

The etiologies of chronic complications after PK and B-KPro surgery such as late endothelial failure, optic neuropathy, epiretinal membrane, macular edema, and retinal detachment remain largely unknown. And, for undetermined reasons, unusually rapid progression of glaucoma can be observed in PK15 as well as in KPro patients.16,17 It is known that anterior segment injuries and procedures like corneal burn or lens removal can induce posterior segment inflammation and damage.18,19 This is most likely caused by posterior diffusion of inflammatory cytokines secreted in the injured anterior segment.20 We have recently shown increased TNFα and IL-1β expression in the cornea after allogeneic PK and m-KPro implantation with allogeneic carrier.10 Increased TNFα pro-
Degenration has been also associated with high IOP and with optic nerve degeneration in murine models of glaucoma. In our study, mean IOP was within normal limits, although some eyes had high IOP and peak values of IOP might not have been detected by a one-time measurement.

With this in mind, we analyzed the extent of retinal inflammation and optic nerve damage following PK and m-KPro surgery in order to elucidate a mechanism by which postoperative complications arise after such procedures. We observed increased retinal inflammation by measuring proinflammatory cytokine expression. We detected increased mRNA TNF expression in the retina after alloPK as well as after synPK and alloKPro compared to naive and synPK. The mRNA expression of IL-1β was higher after synKPro and alloKPro compared with the synPK and alloPK groups, respectively, a pattern that is consistent with the expression of inflammatory cytokines seen in our previous study. By comparing retinal inflammation after syngeneic and allogeneic PK and m-KPro implantation, we found that both the allogeneic carrier graft and the m-KPro device contribute to chronic inflammation of the posterior segment.

We further analyzed optic nerve degeneration and found similar axon numbers in operated eyes of both syngeneic groups (synPK and synKPro) compared with the fellow, nonoperated eye, but we observed axon loss in the alloPK and the alloKPro groups compared to fellow eyes. During degeneration, axons first swell and bead, then decrease in their caliber and lose circularity. While TNFα antagonists could prove a new strategy to protect against chronic damage to the posterior segment after KPro implantation or repeated transplants, we would caution against any generalization derived from our data in regard to this treatment strategy. Rather, we believe that controlled studies evaluating the efficacy and toxicity/safety of anti-TNFα strategies for suppressing optic neuropathy in highly inflamed eyes that undergo keratoplasty or KPro surgery are warranted before any recommendations are made in relation to specific therapeutic approaches.

In summary, this study provides the first insights into the mechanisms by which KPro implantation may lead to optic nerve damage, and supports the anecdotal reports of rapid and progressive optic neuropathy after KPro implantation. Our data further suggest that corneal allograft and the KPro device itself both contribute to the chronic inflammation, affect the posterior segment of the eye, and affect the resultant optic neuropathy. The validation of reducing carrier tissue allogenicity (e.g., via gamma irradiation), or cytokine blockade to reduce optic neuropathy after KPro will require clinical trial confirmation.

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