Th2-Biased Immune System Promotion of Allogeneic Corneal Epithelial Cell Survival after Orthotopic Limbal Transplantation

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Purpose: The Th2-biased immune system can promote penetrating keratoplasty survival in mice. A series of experiments were performed to determine whether this system could prolong corneal limbal transplant (LT) survival.

Methods: BALB/c (H-2b) mice were immunized with 50 µg of keyhole limpet hemocyanin (KLH) in incomplete Freund's adjuvant. Four weeks later, the corneal epithelium, including the limbal area, was removed, and the mice received LT from B10.D2 (H-2k), C57BL/10 (H-2b), or enhanced green fluorescence protein (EGFP) transgenic (H-2b) donor mice. Immediately thereafter, recipient mice were immunized with 50 µg of KLH or Hanks' balanced salt solution (HBSS; control) in complete Freund's adjuvant. The allograft fates were assessed clinically. Lymphocytes of recipients were examined for donor-specific proliferation and for donor-specific cytokine production in vitro.

Result: The regenerated epithelia of all C57BL/10 (n = 14) and B10.D2 (n = 18) grafts were rejected swiftly in control mice, whereas 66.6% of C57BL/10 grafts (8/12, \( P < 0.001 \)) and 62.8% of B10.D2 grafts (22/35, \( P < 0.001 \)) in the KLH immune group remained significantly clear for 8 weeks. Moreover, EGFP donor epithelial cells were detected from the healthy corneas of KLH-immunized mice. As for the in vitro assay, at 1 week after B10.D2 grafting, lymphocytes from KLH-immunized groups showed neither proliferation nor increased cytokine secretion.

Conclusions: The Th2-biased immune system can support LT prolongation irrespective of donor disparity and can suppress corneal neovascularization. This prolongation is not due to induction of donor-specific regulatory cells, but is presumably at least associated with the suppression of allosensitization.


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anterior chamber hemorrhage during or after surgery, or suture loosening and lenticule separation during the observation period) were excluded from the study. In all sets of graft-surviving experiments, the results of daily performing experiments (5–12 limbal transplants) were combined and compared.

Evaluation and Scoring of Limbal Transplantation

Grafts were evaluated daily by slit lamp biomicroscopy. Methylene blue (0.2%) staining was used to evaluate corneal reepithelialization by donor corneal epithelium proceeding from limbus to central cornea. At each time point grafts were scored for opacification and neovascularization (NV). A previously described scoring system was used to grade the degree of opacification from 0 to 4+: 0, clear cornea; 1, lenticular and regional corneal epithelial edema, opacity, or clearly visible iris vessels; 2, diffuse epithelial edema, corneal opacity or both, obscuring iris vessels; 3, diffuse epithelial edema, corneal opacity or both, iris vessels not visible; 4, anterior chamber invisible due to epithelial edema, corneal opacity, or both. Grafts with an opacity score of 2+ or more were considered rejected (immunologic failure), regardless of opacity score at 4 weeks (since some grafts had only transient opacification). Corneal NV was graded from 0 to 4 in each quadrant and totaled (altogether, 0–16): 0, none; 1, invasion to the limbal graft but not to the graft; 2, invasion to the graft, but not to the central 2 mm; 3, invasion to the central 2 mm, but not reaching the center; and 4, invasion to the central cornea.

Culture Medium

Serum-free medium used for cultures was composed of RPMI 1640 medium, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin (all from BioWhitaker, Walkersville, MD) and 1 × 10−3 M 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), supplemented with 0.1% bovine serum albumin (Sigma-Aldrich), and ITS cell culture supplement (1 μg/mL iron-free transferrin, 10 ng/mL linoleic acid, 0.3 ng/mL Na2Se, and 0.2 μg/mL Fe(NO3)3; Collaborative Biomedical Products, Bedford, MA).

Preparation of Lymphocytes and Cell Culture

We used KLH-immunized and control mice that had clear grafts for 1 week after LT. Spleens and graft-site-draining lymph nodes of three animals in each group were collected and pooled. Cells therefrom were then pressed through nylon mesh to produce a single-cell suspension. Red blood cells were lysed. Lymphocytes were then washed and used for adoptive transfer and for cultures. In cell cultures, the lymphocytes were resuspended at 4 × 10^5 in 96-well plates and stimulated with irradiated (2000 rad) B10.D2 splenocytes. In another set of experiment, spleen cells of KLH in IFA preimmunized recipient were stimulated with 50 μg/mL of KLH antigen. Cells were cultured in serum-free medium at 37°C in an atmosphere of 5% CO2.

Proliferation Assay

Cultures similar to those just described were established and sustained for 120 hours. Reconstituted 20 μL XTT (sodium 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium)-bis (+methoxy-6-nitro)benzene sulfonic acid hydrate; Sigma-Aldrich) was added to each well according to the manufacturer’s instructions. After a 4-hour incubation, absorbance at a wavelength of 450 nm was measured. Absorption spectra of tetrazolium reagents were measured with a scanning spectrophotometer and were expressed as optical density.

IFN-γ, IL-4, and IL-10 Assays

Cultures similar to those in the proliferation assay were established and sustained for 48 or 72 hours. At each time point, supernatants were collected and analyzed for IFN-γ, IL-4, and IL-10 contents using ELISA kits according to the manufacturer’s instructions (PharMingen, San Diego, CA).
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Statistical Methods
We constructed Kaplan-Meier survival curves and used the Breslow-Gehan-Wilcoxon test to compare the probability of allograft survival. Statistical analyses were performed with a Mann-Whitney test used for comparison of NV score, and Student’s t test for proliferation response. P < 0.05 were deemed significant.

RESULTS
Fate of Minor H-Only Incompatible Limbal Transplants in KLH-Immunized Recipients
The mouse PKP model in our previous report18 revealed that minor H-only incompatible corneal allografts display more enhanced survival than do MHC+ minor H disparate allografts in KLH-immunized BALB/c recipients. Initially, the fates of minor H–only incompatible B10.D2 donor limbal grafts were examined and compared between KLH immune (n = 35) and control BALB/c mice (n = 18).

As for recovery from epithelial scraping, the entire corneal surface was reepithelialized at 3 to 4 days after LT in both KLH-immunized and control mice, with no statistically significant (data not shown) difference. These host reepithelialization periods were almost the same as in a previous report.8

The appearances of syngeneic (n = 10) and allogeneic LT were virtually identical at 3 days after LT, whether judged clinically or histologically. In control mice, many epithelia appeared absolutely clear on day 7 (Fig. 2B). Some mice were randomly selected and killed on day 7, and the enucleated eyes were examined histologically. Donor lenticule epithelia of control mice contained copious amounts of infiltrating cells in both the limbal (Fig. 2C) and central areas (Fig. 2E), as did the host cornea subepithelium. In contrast, KLH-immunized epithelium contained few invading lymphocytes, and the epithelial cells were aligned in both the limbal (Fig. 2D) and central areas of the cornea (Fig. 2F).

The rate and frequency of epithelial rejection, shown in Figure 3A, showed that all corneas of control mice were rejected swiftly (n = 18), within 10 days (mean survival time = 7.1 ± 0.4 days). In contrast, KLH-immunized mice showed significantly improved epithelial survival, maintaining clear corneas until 56 days after LT (22/35 corneas, 62.8%, P < 0.001). In 56 days after LT, corneal donor graft and host graft were indistinguishably clear. Clinical observations show less NV invasion into the donor lenticule in eyes of KLH-immunized mice. Because inflammatory corneal NV, which is associated with the new development of corneal lymphatics, leads to accelerated allograft rejection, early development of corneal NV was compared. At 5 days after surgery, significant NV suppression could be discerned in KLH-immunized mice (Fig. 3B). During the observation periods, there was no NV invasion over the limbal graft in KLH-immunized mice. In addition, the fates of clear B10.D2 corneas in KLH-immunized mice were virtually identical with syngeneic LT graft.

Characteristics of Alloreactive T Cells from KLH-Immune Mice That Accepted Limbal Grafts
For these studies, splenocytes of BALB/c mice that received an intraperitoneal injection of KLH (50 μg) in IFA (50 μL) were stimulated in vitro with KLH. The supernatants of these cultures contained significant quantities of IL-4 (1254.5 ± 43.7 pg/mL) and IL-10 (2851.3 ± 126.5 pg/mL), but little IL-2 (31.4 ± 4.3 pg/mL) or IFN-γ (98.4 ± 15.6 pg/mL), indicating that the mice had mounted a Th2-type response to KLH. Our next goal was to determine whether LT-acceptant mice with Th2-biased immune systems respond to immunization with alloantigens by developing alloantigen-specific Th2 responses. One week after LT, ipsilateral cervical lymph nodes and spleens were removed, pressed through nylon mesh to produce a single-cell suspension and pooled. T cells were stimu-
Adoptive Transference of Limbal Allograft Survival

From another standpoint, if rejection-suppressing regulatory cells, including donor-specific Th2 cells, are present in the KLH-immunized recipient, their corneal acceptance can be adoptively transferred.18 Because only KLH-immunized mice, not control mice, could promote a clear graft for more than 2 weeks, we prepared B10.D2-grafted KLH-immunized mice with corneas that had remained perfectly healthy for 2 weeks. The mice were killed, and their cervical lymph nodes and spleens were removed. Furthermore, cervical lymph nodes and spleen cells were mixed, and single cell suspensions were prepared, pooled, and injected intravenously (one donor equivalent per recipient) into naive BALB/c mice. Immediately thereafter, the mice received B10.D2 limbal grafts (n = 7). In control, we used BALB/c recipients that were not immunized with KLH and did not receive spleen or lymph cells of recipient mice (n = 8). The fate of LT (Fig. 5), shows that acceptor lymphocytes did not regulate limbal graft rejection. This result indicates that graft acceptance for 2 weeks in KLH-immunized mice is not due to regulatory cells, unlike the PKP model in the previous report.18

Promotion of LT Graft Survival by a Th2-Biased Immune System

A previous report18 revealed that the Th2-biased immune system promotes B10.D2 PKP allograft survival better than C57BL/6 allograft survival, concluding that the Th2-biased immune system may influence indirect presentation of allosensitization in the PKP model. However, because the presence of donor-derived Langerhans' cells in the B10.D2 limbal graft causes direct alloantigen presentation to host T cells (direct recognition), the Th2-biased immune system may have influenced direct presentation of allosensitization and promoted graft survival in this LT model. We therefore performed total disparate (MHCmH disparate) C57BL/10 LT and observed the results clinically. To our surprise, KLH-immunized mice showed significantly improved epithelial survival, more than half of them maintaining clear corneas until 42 days after LT (n = 12, P < 0.02), though control mice rejected all

In vivo with x-irradiated B10.D2 spleen cells. Naive mice (negative) and mice immunized with 10 million B10.D2 splenocytes subcutaneously 1 week before were used as the control. The results of these experiments with lymph nodes are presented in Figure 4. The proliferation response at 96 hours indicates that recipients with limbal allografts did not prime in KLH-immunized or control mice (Fig. 4A). In contrast, T cells from control mice that received HBSS rather than KLH at the time of LT proliferated more vigorously. Supernatants were removed from 24-, 48-, and 72-hour cultures and assayed for IFN-γ content to evaluate Th1 activation, and IL-4 and -10 content for Th2 (Fig. 4B). T cells stimulated with allogeneic spleen cells produced significant amounts of IFN-γ. IFN-γ production by lymphocytes from both KLH-immunized and control mice was similar. As in Th1 cytokine production, IL-4 and -10 production by lymphocytes from both KLH-immunized and control mice was low. Of importance, donor-specific Th2 cytokine production by lymphocytes of KLH-immunized mice was less than that produced by lymphocytes of control mice. Similar results were observed in donor-specific cytokine production in spleen cells. We conclude from these results that LT graft survival in KLH-immunized mice is not due to donor-specific Th2 cells.
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immunized (n = 12; ○) and control mice (n = 14; ●). Results of daily experiments (n = 3) were combined. Graft rejection was scored clinically. Results are presented as Kaplan-Meier survival curves.

C57BL/10 limbal grafts within 14 days (n = 14; Fig. 6). We conclude that the Th2-immune system can promote LT survival in both minor H only and fully disparate combinations.

Evaluation of Long-Term Donor Epithelial Cell Survival

For observations of the presence of donor-derived epithelial cells in long-term clear corneas of KLH-immunized mice that received allogeneic LT, BALB/c mice received limbal grafts of EGFP transgenic mice (C57BL/6 background; n = 15). At an appropriate time, the mice were killed and their eyes enucleated, frozen, embedded, sectioned, and observed by fluorescein microscopy to detect EGFP-derived donor cells. The grafted eyes with rejected corneas showed no positive fluorescence on day 7 after surgery (n = 2; Fig. 7A). In contrast, donor-derived epithelial cells remained at the central area of the cornea on day 7 (n = 5) (Fig. 7B) and on day 14 (n = 6; Fig. 7C) after surgery in KLH-immunized mice, with the fluorescein intensity being similar to that in normal corneas of EGFP transgenic mice (data not shown). Two sets of experiments were combined, and representative photographs are shown in Figure 7. Positive fluorescence in the epithelial layer was also detected 4 weeks after surgery in KLH-immunized mice (n = 2, data not shown), demonstrating the long-term survival of donor epithelial cells on the ocular surface.

DISCUSSION

Most limbal grafts that fail in both humans and mice do so because of immune-associated rejection, though even the most immunogenetically disparate corneal grafts placed orthotopically can exhibit prolonged, often indefinite, survival. In comparison with LT, the extraordinary success of PKP can be attributed to various features of the normal cornea and anterior segment that in the aggregate account for their immune-privileged state, including the avascularity of the stroma, the absence of corneal lymphatics, and the rarity of indigenous professional antigen-presenting Langerhans’ cells or macro-

genes in the normal graft bed. Because of the factors responsible for ocular immune privilege, it has been found that minor H antigens, rather than antigens encoded within the MHC, are the most important initiators of alloimmunity after PKP. All peptides derived from minor H antigen processing are loaded onto self-MHC molecules on recipient antigen-presenting cells and presented to T cells by the so-called indirect pathway of allorecognition in the PKP model. The Th2-biased immune system was therefore established to induce donor minor H-specific Th2 type response to suppress Th1-mediated immune rejection, because mice that have mounted Th2-type responses to one peptide antigen often display Th2 responses to subsequent immunizations with different antigens. In fact, donor-specific Th2 cells were induced and donor-specific allorejection suppressed in a PKP model mice.

In contrast, less immune privilege is exhibited in LT, which involves the presence of Langerhans’ cells in both donor and recipient limbus. Donor Langerhans’ cells can present alloan
tigens directly to recipient T cells by the so-called direct pathway of allorecognition. In cases of MHC-disparate combination between donor and recipient, MHC alloantigen is presented without the contribution of recipient antigen-presenting cells. In cases of MHC-matched combination, even minor H can be presented directly to recipient T cells by donor Langerhans cells, because both donor and recipient share the same MHC molecule. Theoretically, it is quite natural for minor H antigen presentation in LT to be influenced by the Th2-biased immune system. However, the fact is that neither positive donor-specific Th2 response nor typical donor-specific regulation was detected. Moreover, both MHC disparate and MHC-matched allogeneic LT showed similar graft survival in this system.

The Th2-biased immune system could suppress neovascular invasion at around 5 days after LT, before the initial rejection reaction appeared. Because corneal NV probably plays an important role in facilitating swift antigen presentation and effector elements in the inflamed cornea and is associated with fulminating graft rejection, suppressed initial NV induction should contribute to LT graft survival. Immunologically, alloan
tigen is presented and alloreactive T cells proliferate at around 3 to 5 days in the case of skin transplantation. NV suppression in the Th2-biased immune system is therefore presumably not mediated by alloreactive T cells, but by anti-inflammatory fac-
tors produced in the Th2 response. This NV suppression may be one mechanism to enhance graft survival in this system.

Current prophylactic and therapeutic regimens for LT rejection are associated with significant complications. Moreover, although immunosuppression can mediate clinical allograft survival, it is suggested that donor cells do not survive on the rat ocular surface indefinitely.7 The Th2-biased immune system unexpectedly promoted donor epithelial cell survival for more than 4 weeks. Hence, the Th2-biased immune system or a modified system can be an effective immune therapy in LT.

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References