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Award Number: DAMD17-01-2-0032

TITLE: Low Vision Research @ The Schepens Eye Research Institute

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CONTRACTING ORGANIZATION: The Schepens Eye Research Institute
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REPORT DATE: July 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Final (1 Jul 2001 - 30 Jun 2003)

4. TITLE AND SUBTITLE
Low Vision Research @ The Schepens Eye Research Institute

5. FUNDING NUMBERS
DAMD17-01-2-0032

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

This research proposal, Low Vision at the Schepens Eye Research Institute, is a collaborative effort on the part of four Investigators at the Institute whose goal is to advance the studies on low vision. Project 1 focuses on the promotion of corneal transplant longevity using targeted delivery of anti-apoptotic genes and has successfully delivered the desirable genes into corneal explants. Project 2 involves studies on biodegradable polymers and CNS stem cells, and has been able to incorporate CNTF and GDNF in slow release biopolymers which they have tested in mouse models. Project 3 encompasses research on photoprotection by macular pigment and tests the hypothesis that macular pigment protects the retina from damage leading to degradation of macular function. Project 4 is a continuation of the remote diagnosis of retinal damage; the initial instrument design for the testing of human subjects is completed. Project 5 is also a continuation of the identification of factors that can predict dry eye complications of LASIK surgery. Patient enrollment is proceeding well and early analyses are complete.

14. SUBJECT TERMS
No Subject Terms Provided.

15. NUMBER OF PAGES
55

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Once again, in Year 2, The Schepens Eye Research Institute has mounted a multi-disciplinary approach in its research effort aimed at advancements in low vision research. The proposal, "Low Vision Research at The Schepens Eye Research Institute," maintains its military relevance in the areas of instrumentation, tactical advantage, and personnel readiness. This proposal encompasses four individual research projects led by one Principal Investigator. In Year 2, the research includes studies on corneal trauma, infection, repairing the diseased retina, macular disease, and remote diagnosis of retinal damage. Recent events in this country have stepped up the need for low vision research, especially within our military personnel. This proposal is timely and each individual project addresses the impact on our armed services.

Project 1. Promotion of Corneal Transplant Longevity Using Targeted Delivery of Anti-Apoptotic Genes.
Investigator: **Dr. Reza Dana**

Body:

Severe corneal trauma and infection may lead to corneal scarring, with resultant need for corneal transplantation. Unfortunately, many of these grafts need to be maintained on toxic medications to prevent rejection; others simply succumb to the host immune system. The main aim of this project has been to deliver specific anti-apoptotic ("anti-death") genes to the corneal endothelium using viral vectors to promote transplant longevity. We have had significant progress in this project. Our team has successfully delivered desirable (reporter) genes to the corneal endothelium under conditions that closely simulate processing of eye bank eyes. We have transplanted these grafts and have shown longterm retention of transfected gene product over many weeks, clearly establishing the feasibility of using this or similar technologies to modify the cornea. In addition to survival factors (which have been the aim of this grant), other factors that could modulate immunity, suppress scarring, or increase host defense, can likewise be delivered to the cornea.

Key Research Accomplishments:

- Successful delivery of desirable genes into corneal explants;
- Demonstration of in-vivo retention of transfected gene product (protein) after transplantation;
- Demonstration of the intimate link between apoptosis and transplant failure;
- Better understanding of the molecular mechanisms that regulate apoptosis/cell death in the cornea (and hence cause scarring)

Reportable Outcomes:

Two manuscripts: one completed and submitted (provided in the appendix); the other in preparation.
Two scientific presentations at the Annual Association for Research in Vision and Ophthalmology (2002 and 2003): see listed references below
One patent: Promotion of corneal graft survival by anti-apoptotic gene therapy

Conclusions:

Corneal trauma and infection are the most common eye problems faced by active military personnel. These problems often lead to scarring. When scarring is severe, corneal transplantation may be

indicated. However, apoptosis (or non-necrotic cell death) is a cellular process that complicates all of these outcomes: (a) it can lead to scarring due to irregular tissue remodeling (from injury); (b) it can potentiate inflammation; (c) it can lead to corneal graft cell loss while the tissue is being stored (before transplantation); (d) it can lead to graft failure/decompensation after transplantation. In our experiments, we have:

a. demonstrated the feasibility of inserting (through transfection) desirable genes into the corneal endothelium
b. performed 'a' in a setting that closely resembles the manner by which corneas are handled in eye banks throughout the country
c. demonstrated retention and functioning of adenoviral-based gene therapy in the cornea in vivo
d. demonstrated the close relationship between specific apoptotic (capase-dependent) mechanisms and graft loss
e. identified specific targets to modulate these molecular processes- for example, by showing that a bcl-xl transgenic cornea is retained in the eye for a significantly longer period than wild-type controls

Unfortunately, our work has been complicated by the toxicity of adenoviral vectors we have used to insert genes into the cornea. We intend to address this issue by using retroviral or non-viral (e.g., liposome-based) vectors in our future work.

This work is highly significant because:
-it directly addresses one of the most pressing issues in eye disease in the military.
-it opens the door to virtually many other molecular targeted strategies in that we can modify many corneal responses by similar strategies.

References:

Abstracts

Qian Y, Leong F, Kazalauskas A, Dana MR. Ex vivo adenovirus-mediated gene transfer to corneal graft endothelial cells in mice. *ARVO abstract* 2002; 3193: 129.

Beauregard C, Dana MR, Capase Activation and Apoptosis in the Inflamed and Transplanted Cornea. *ARVO abstract* 2003: 4282.

Papers

Qian Y, Leong F, Kazlauskas A, Dana MR. Ex Vivo Adenovirus-Mediated Gene Transfer to Corneal Graft Endothelial Cells in Mice. *Invest Ophthalmol and Vis Sci*; (Submitted).

Beauregard C, Huq S, Barabino S, Zhang Q, Kazlauskas A, Dana MR, Capase Role of Apoptosis in corneal transplant failure and inflammation (In preparation).

Patents

Dana MR, Kazlauskas A. Promotion of corneal graft survival by anti-apoptotic gene therapy. US Patent Application 60/304,975 filed July 2001.

Appendices: (attached separately)

(i) Manuscript: Qian et al
(ii) Scientific presentation at the May 2003 annual ARVO Meeting
(iii) CV of Principal Investigator

Project 2. Biodegradable Polymers and CNS Stem Cells
Investigator: **Michael J. Young, Ph.D.**

Body:

We have made significant progress in our efforts to use biodegradable polymers as part of a larger effort aimed at retinal tissue engineering. We have developed biodegradable polymers that release growth and survival factors slowly, locally, and over an extended period [1]. We have also developed techniques for constructing biodegradable polymer/ progenitor cell composites [2], and tested these for biocompatibility in mouse [3] and pig models [4]. We have also been successful in devising strategies to induce appropriate progenitor cell differentiation using polymers and specific growth factors [5].

Key Research Accomplishments:

- Incorporated CNTF and GDNF in slow release biopolymers
- Tested these in mouse models
- Developed biodegradable polymer/ progenitor cell composites
- Tested these in mouse and pig models
- Used biodegradable polymers to control differentiation of progenitor cells

Reportable Outcomes:

Abstracts presented at 2003 Association for Research in Vision and Ophthalmology conference:

a. Zahir, T., *et al., Differentiation of retinal progenitor cells into specific cell types.* Invest Ophthalmol Vis Sci, 2003. 44: E-Abstract 1666.
b. Khoobehi, A., *et al., Biodegradable Microspheres with Long-term Release of Survival Factors in the DBA/2J Mouse Model of Glaucoma.* Invest Ophthalmol Vis Sci, 2003. 44: E-Abstract 126.
c. Kiilgaard, J.F., *et al., Transplantation of xenogeneic retinal stem cells to pig subretinal space.* Invest Ophthalmol Vis Sci, 2003. 44: E-Abstract 487.
d. Klassen, H., *et al., Human retinal progenitors can be cultured from cadaveric tissue and express GD2 ganglioside and other known surface markers.* Invest Ophthalmol Vis Sci, 2003. 44: E-Abstract 1691.
e. Lavik, E., *et al., Polymer scaffolds provide support and guidance for retinal stem cells in retinal degeneration models.* Invest Ophthalmol Vis Sci, 2003. 44: E-Abstract 508.
f. Ng, T., *et al., Immunogenicity of Retinal Progenitor Cells Seeded on Biodegradable Polymers.* Invest Ophthalmol Vis Sci, 2003. 44: E-Abstract1679.
g. Shatos, M., *et al., Isolation, characterization, and expansion of porcine retinal progenitor cells.* Invest Ophthalmol Vis Sci, 2003. 44: E-Abstract 1694.
h. Warfvinge, K., *et al., Survival, Integration and Differentiation of Retinal Progenitor Cells from GFP Transgenic Mice Transplanted Subretinally to Adult, Normal Pigs.* Invest Ophthalmol Vis Sci, 2003. 44: E-Abstract 483.

Conclusions:

Our results thus far provide important data for our ongoing work aimed at repairing the diseased retina. This complex task requires a broad approach, and we submit that the application of tissue engineering strategies offer great promise for someday restoring vision to the blinded eye. Our future work will build upon our accomplishments, combining slow-release biomaterials with biodegradable polymer/ progenitor cell composites, such that a new outer retina can be constructed.

References:

1. Khoobehi, A., *et al., Biodegradable Microspheres with Long-term Release of Survival Factors in the DBA/2J Mouse Model of Glaucoma.* Invest Ophthalmol Vis Sci, 2003. **44**: p. E-Abstract 126.
2. Lavik, E., *et al., Polymer scaffolds provide support and guidance for retinal stem cells in retinal degeneration models.* Invest Ophthalmol Vis Sci, 2003. **44**: p. E-Abstract 508.
3. Ng, T., *et al., Immunogenicity of Retinal Progenitor Cells Seeded on Biodegradable Polymers.* Invest Ophthalmol Vis Sci, 2003. **44**: p. E-Abstract1679.
4. Warfvinge, K., *et al., Survival, Integration and Differentiation of Retinal Progenitor Cells from GFP Transgenic Mice Transplanted Subretinally to Adult, Normal Pigs.* Invest Ophthalmol Vis Sci, 2003. **44**: p. E-Abstract 483.
5. Zahir, T., *et al., Differentiation of retinal progenitor cells into specific cell types.* Invest Ophthalmol Vis Sci, 2003. **44**: p. E-Abstract 1666.

Appendices:

Khoobehi, A., *et al., Biodegradable Microspheres with Long-term Release of Survival Factors in the DBA/2J Mouse Model of Glaucoma.* Invest Ophthalmol Vis Sci, 2003. **44**: p. E-Abstract 126.
Purpose: Neurotrophic growth factors have been shown to enhance retinal ganglion cell (RGC) survival when given as intravitreous bolus injections. Microspheres fabricated from biodegradable polymers can allow for a more gradual, physiological release of these factors. The DBA/2J mice have been shown to develop pigment dispersion glaucoma with symptoms beginning as early as four months. These mice provide a suitable model for testing glaucoma therapeutics. We wish to promote RGC survival in the DBA/2J mice using biodegradable polymers releasing retinal survival factors.

Methods: Microspheres with GDNF, CNTF, and BDNF were formed using a spontaneous emulsification technique in which a solution of the polymer, AOT, and drug of interest were vortexed then added to a rapidly stirring solution of polyvinyl alcohol in water to precipitate the polymer/AOT/drug solution forming poly(lactic-co-glycolic acid) (PLGA) spheres of controlled size. Rhodamine labeled microspheres were created for in vivo imaging and histological analysis. Release studies were performed in vitro in PBS for 60 days and release was quantified using ELISA assays for the individual growth factors. Two-month-old DBA/2J mice were anesthetized and injected with 1 É l of concentrated microspheres (2% rhodamine microspheres) into the vitreous. Evaluation was performed by in vivo imaging using a flourescent microscope and by histological sections.

Results: ELISA assays showed an insignificant initial burst of 2 to 4 ng of growth factor per mg, which plateaued for ~25 days, followed by a phase of gradual release lasting approximately 30 days. Release curves differed significantly depending on microsphere size and the survival factor in question. In vivo flourescent images show rhodamine-labeled microspheres immediately post-

operatively and 7 days afterwards. Histological sections demonstrate microspheres in the vitreous cavity. No signs of uveitis or inflammation were noted.

Discussion: We have fabricated and characterized microspheres to deliver GDNF and other factors in a controlled manner for up to 60 days with minimal initial burst. The DBA/2J mouse tolerates injections of these polymers, and it is a promising model for studying such therapeutics.

Lavik, E., *et al.*, *Polymer scaffolds provide support and guidance for retinal stem cells in retinal degeneration models.* Invest Ophthalmol Vis Sci, 2003. **44**: p. E-Abstract 508.

Purpose: Retinal stem cells (RSCs) show great promise in studies of retinal development, and may someday be used to treat retinal degeneration. RSCs are self-renewing cells capable of differentiating into the different retinal cell types, including photoreceptors. We hypothesized that a biodegradable polymer scaffold could deliver these cells to the subretinal space in a more organized manner than bolus injections, while also providing the graft with laminar organization and structural guidance channels.

Methods: Highly porous scaffolds were fabricated from blends of poly(L-lactic acid) and poly(lactic-co-glycolic acid) using a freeze-drying technique which produces pores oriented normal to the plane of the scaffold. RSCs (originally isolated from neonatal GFP-transgenic mice) were seeded on the polymers and cultured for 14 days. Seeded scaffolds were then either co-cultured with degenerating mouse retinas or transplanted to the healthy or injured retinas of juvenile pigs.

Results: Co-culture of the seeded scaffolds with explanted degenerating retina was associated with substantial migration of RSCs from the scaffold into the explant. Remaining RSCs maintained a radial orientation within the polymer, in some cases exhibiting morphologies suggestive of photoreceptors and expressing photoreceptor markers. Such morphologies have not been seen in culture without polymer substrates. Mouse RSCs transplanted to the porcine model survived, oriented and remained within the polymer scaffolds for 14 days. However, no transplanted cells were found integrating in the retina, and in HE-sections a giant cell reaction was observed. Pore size increased over this period, a sign the scaffolds were degrading.

Conclusions: Degradable polymer scaffolds provide support and laminar organization for grafted RSCs, as well as physical guidance to the cells. Donor cell morphology appears to benefit from this strategy, particularly in culture. Scaffold substrates provide a useful tool for delivery of stem cells to the retina as part of a tissue engineering strategy.

Ng, T., *et al.*, *Immunogenicity of Retinal Progenitor Cells Seeded on Biodegradable Polymers.* Invest Ophthalmol Vis Sci, 2003. **44**: p. E-Abstract1679.

Purpose: To study the immunobiology of the retinal progenitor cells seeded on biodegradable polymers by transplantation into the kidney subcapsular space in mice. Methods: Retinal progenitor cells (RPCs) harvested from the retina of postnatal day 1 EGFP (Enhanced Green Fluorescence Protein) mice (C57BL/6 background) were isolated and maintained in culture. RPCs were seeded on biodegradable PLGA polymers (50:50 glycolic acid: lactic acid; 40kDa) and were maintained in vitro for 1 week. A 1 mm2 piece of the RPCs / polymer composite was transplanted into the kidney subcapsular space in either syngeneic (C57BL/6) or allogeneic

(BALB/c) mice. The animals were sacrificed at 1, 2, 4, 6 and 8 weeks post- transplantation (wpt). The kidney receiving the composite graft was evaluated for EGFP fluorescence and then fixed in 4% paraformaldehyde and cryosectioned for immunostaining. GS lectin and antibodies against MHC II

(IAb for C57BL/6 and IAd for BALB/c) mice were used to detect infiltration of recipient derived immune cells. Antibodies against neurofilament, GFAP, nestin and recoverin were used to determine the differentiation status of the RPCs in the polymer. Some animals at 4 wpt were used for the detection of delayed hypersensitivity by challenge with either allogeneic spleen cells (C57BL/6) or RPCs.

Results: EGFP fluorescence was detected in RPCs / polymer in all grafts up to 8 wpt. The polymer became increasingly translucent and disappeared at 8 wpt. Cells were found positive to neurofilament and GFAP at all time points. Recoverin+ cells in the polymer were only found at 2 and 4 wpt. GS lectin and MHC II+ cells were found at 2 wpt and onward. However, there is no delayed hypersensitivity specific to either allogeneic antigens or to RPC antigens.

Conclusions: Biodegradable polymers are effectively degraded and absorbed by the host kidney over time without any apparent immune response. RPCs possess immune privilege properties. Biodegradable polymers provide an alternate method for delivery of progenitor cell populations, and may be useful in retinal transplantation.

Warfvinge, K., *et al.*, *Survival, Integration and Differentiation of Retinal Progenitor Cells from GFP Transgenic Mice Transplanted Subretinally to Adult, Normal Pigs.* Invest Ophthalmol Vis Sci, 2003. 44: p. E-Abstract 483.

Purpose: To investigate the survival, integration and differentiation of retinal progenitor cells from GFP transgenic mice after subretinal transplantation to adult normal pigs.

Methods: Retinal progenitor cells derived from postnatal day 1 GFP-transgenic mice were transplanted subretinally either as single cell suspension, as spheres or as a biodegradable polymer/progenitor composite to 20 normal non-immunosuppressed adult pigs. Prior to transplantation, small areas of the retina of 10 pigs were damaged with either laser treatment or by mechanical scraping of the neuroretina. The pigs were sacrificed at different intervals ranging from 30 minutes to five weeks post-transplantation. The eyes were immunohistochemically examined for various progenitor-, retina-, RPE or glial specific antibodies.

Results: The GFP expressing mice cells survived well up to 14 days post-transplantation. After 5 weeks, only a few GFP cells were found in 3 out of 6 pigs. The cells integrated mainly into the RPE in the non-damaged pig retinas transplanted with cell suspension. In the animals grafted with spheres or polymer membranes, no integrating cells were found, although cells survived in the subretinal space either as spheres or within the membrane. In the pigs receiving laser treatment prior to grafting of cell suspension, GFP cells were found integrating into all layers of the retina. Cells co-expressing PCNA or Ki67 and GFP were only found in the pig sacrificed after 30 minutes. Although the grafted cells ceased proliferation and in some cases differentiated into cells morphologically similar to mature retinal neurons, only nestin and GFAP co-localized with GFP among all the various progenitor-, retina-, RPE or glial specific antibodies used.

Conclusions: Our results show that retinal progenitor cells from GFP transgenic mice can survive, integrate and differentiate in the xenogeneic pig retina. However, the cells do not survive for extended periods, and do not differentiate into mature RPE or retinal cells. The cells integrate into the RPE and retina only when delivered as a cell suspension. The retinal integration is markedly enhanced by laser treatment prior grafting. Allogeneic progenitor cells will likely be better tolerated, and therefore more useful for studying cell replacement strategies in large animal models.

Zahir, T., *et al.*, *Differentiation of retinal progenitor cells into specific cell types.* Invest Ophthalmol Vis Sci, 2003. **44**: p. E-Abstract 1666

Purpose: To differentiate retinal progenitor cells (RPCs) into specific cell types (bipolar and photoreceptor cells) using exogenous factors, to enhance survival and integration of transplanted cells.

Methods: The RPCs used in these studies were isolated from the neuroretina of postnatal day one GFP transgenic mice. They were then treated with several reagents including BDNF, CNTF, GDNF, KCl, retinoic acid, RPE cell conditioned medium, sodium butyrate, succinylated concanavalin A and taurine for up to two weeks. RPCs were also seeded onto biodegradable poly (lactic-co-glycolic acid) polymers and treated with CNTF for up to 14 days. These cells were then added to retinal explants from C3H (retinal degeneration) mice to determine if the cells would preferentially integrate into the inner nuclear layer and express the appropriate bipolar cell markers.

Results: In vitro studies showed that CNTF (20ng/ml) treatment resulted in changes in cellular morphology and was able to increase the number of cells expressing bipolar markers (PKC and MGluR6). Retinoic acid (500 nM) and sodium butyrate (4 mM), in combination, induced changes in cellular morphology and the differentiated cells stained positive for photoreceptor marker (Recoverin), albeit with higher rates of cell death. Surviving RPCs differentiated into bipolar (>90%) and photoreceptor (>90%) cell types upon treatment with CNTF and retinoic acid and sodium butyrate in combination, respectively. In addition, the RPCs treated with CNTF prior to explantation, integrated into the inner nuclear layer and expressed bipolar cell markers.

Conclusion: Exogenous treatments, and biodegradable polymer substrates offer great promise for manipulating the differentiation of stem cell populations prior to grafting. Future studies will combine these two approaches, with RPCs being seeded onto polymer substrates into which specific molecules have been incorporated. This will allow controlled release of the molecule, which may in turn induce differentiation and integration of RPCs.

Project 3. Photoprotection by Macular Pigment
Investigator: **Max Snodderly, Ph.D.**

Body:

The proposal for this project had the following hypothesis and technical objectives.

HYPOTHESIS

We are investigating the hypothesis that macular pigment (MP) protects the retina from damage leading to degradation of macular function and risk for macular disease, especially age-related macular degeneration (AMD). A subsidiary hypothesis is that the photostress test could be used to identify subjects who would benefit from intervention. At the same time, measurements of macular pigment (MP) could be used to track the effectiveness of interventions attempting to increase the accumulation of carotenoids in the retina. We also propose to organize and publish data on nonhuman primate foveas that will be the best models for human retinas. This work will facilitate study of the biological mechanisms causing retinal damage by light and the establishment of methods for preventing loss of retinal function and progression to retinal disease.

TECHNICAL OBJECTIVES

1. Determine whether MP is related to the time necessary to recover from exposure to a bright light.

This is termed photostress recovery, and we will measure it as the time necessary for a subject to recover the ability to read small letters after being exposed to a bright light. Such situations arise, for example, when entering a dark tunnel or a dimly lit room on a bright sunny day. We are particularly interested in the possibility that older subjects with low MP may find it especially difficult to recover from light adaptation. To assess this possibility, older and younger subjects with a wide range of MP densities will be studied.

2. Evaluate potential animal models for studying the mechanisms of protection by MP.

Our goal is to summarize foveal data on MP density, retinal thickness, and the foveal avascular zone from three primate species in a single paper so that investigators will have a convenient source of information when choosing their study species or evaluating experimental data from different species. To meet this objective, we propose to augment our database of retinal parameters of different species of monkeys with additional measurements on rhesus monkey retinas so that definitive comparisons can be made.

REPORT

Objective 1

To accomplish the first objective, 24 volunteer subjects from 61 to 75 y (mean ± SD: 67.9 ± 4.6) were recruited from the local area. Their macular pigment density was measured at Schepens in two separate visits. For 11 of the 24 subjects, the measures at 30 min eccentricity were within 0.1 OD, and the average of the data for the two visits was taken as their value. For the other 13 subjects, a third measure was taken, and the results from the two visits that were most similar were averaged to derive the best estimate of their macular pigment. The profile of the pigment density was measured by circular spots with radii of 0.25, 0.5 degrees and ring targets with radii of 1 and 1.75 degrees. On the same day that macular pigment was measured, macular photostress recovery times were measured on the same subjects at the Massachusetts Eye and Ear infirmary. Since two subjects had unreliable visual acuities during the multiple visits, their data were excluded from further analysis. Thus data from 22 subjects were used for comparisons between macular pigment density and photostress recovery time. The average recovery time was 23.2 ± 9.2 s. The possible association between macular pigment and photostress recovery was assessed by Pearson correlation. Results are tabulated below:

Correlation between photostress recovery time and ...	Macular pigment density at radii			
	0.25 degree	0.5 degree	1 degree	1.75 degree
Correlation coefficient, r	0.245	0.297	0.271	-0.229
P value	0.272	0.179	0.223	0.306

These results indicate that there is no significant relationship between macular pigment density and photostress recovery time ($P > 0.1$) for this group of older subjects. This outcome did not confirm our initial hypothesis that macular pigment would be negatively correlated with photostress recovery time for older subjects. For that reason we did not repeat the measures in young subjects as initially planned.

Objective 2

Rhesus monkey retinas have been studied to measure the macular pigment of the fovea and the foveal avascular zone in retinal whole mounts. The data are summarized in two pages of graphs in the Appendix. The results indicate that rhesus differ from two other common species of laboratory primates in having relatively low macular pigment and relatively small foveal avascular zones. The mean area in mm sq of the foveal avascular zone of rhesus (0.22) is smaller than the foveal avascular zone of squirrel monkeys (0.35) and cynomolgous monkeys (0.33, $p < 0.01$). Similarly, the macular pigment density at 460 nm is lower at the central peak for rhesus (mean 0.12) than for squirrel monkeys (0.23) and cynomolgous (0.30, $p < 0.01$). The individual differences among the monkeys also mirror the wide individual variations that occur in humans. It is especially interesting that macular pigment profiles of monkeys can be unimodal or trimodal just as they can in humans. These differences may be related to differences in foveal shape that are linked to other retinal properties with prognostic importance for risk of disease. Because some rhesus monkeys have virtually no macular pigment, even when fed a normal diet, they may be useful models for examining risk factors for aging and disease processes.

Key Research Accomplishments:

- Macular pigment density and photostress recovery have been studied in the same group of human subjects. They were not correlated.
- Foveal avascular zones have been measured for rhesus monkeys as models for humans. They are smaller than the avascular zones of two other common species of laboratory monkeys.
- Macular pigment profiles have been measured for rhesus monkeys. Their macular pigment density was found to be lower than two other common species of laboratory monkeys. They may consequently be useful models for humans with low macular pigment.

Reportable Outcomes:

The comparative studies of monkey retinas will be summarized for one, or possibly two, journal articles. This information will be useful for researchers choosing animal models for human macular disease.

Conclusions:

The human studies of macular pigment and photostress recovery were done on a group of subjects with relatively high amounts of macular pigment (mean 0.5 OD at 30 min eccentricity). Thus it is possible that we did not sample enough individuals with low macular pigment to find an effect. It would be worth repeating these measurements with a group that was pre-selected for large numbers of individuals with low macular pigment.

The studies of monkey retinas show that rhesus monkeys are quite different from other species commonly used in vision laboratories. Investigators choosing animal models for human disease will want to evaluate whether these monkeys are suitable for their experiments. Also, the interpretation

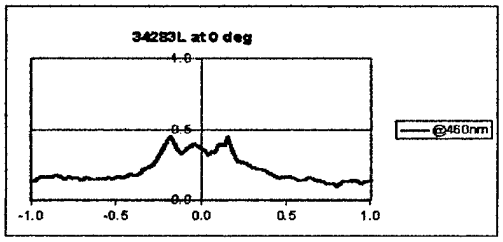
of some experiments in visual neuroscience hinges on the contribution of the macular pigment to color selectivity of some neural pathways. These data indicate that the contribution may be smaller than previously thought, and hence they may encourage re-interpretation of some experimental data.

References:

None.

Appendices:

Macular pigment density at 460 nm measured along the horizontal meridian in whole mounts of 8 rhesus monkey (Macaca mulatta) retinas. The vertical scale is from 0 to 1.0 OD. Note that several animals had nearly flat profiles indicating essentially no macular pigment.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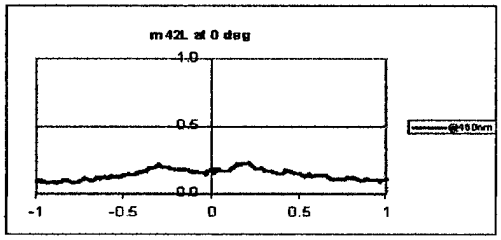
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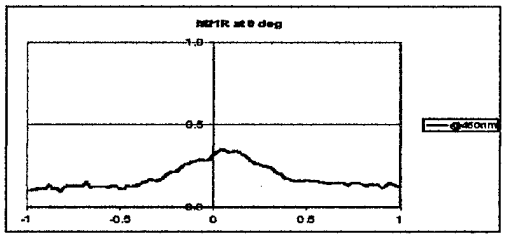
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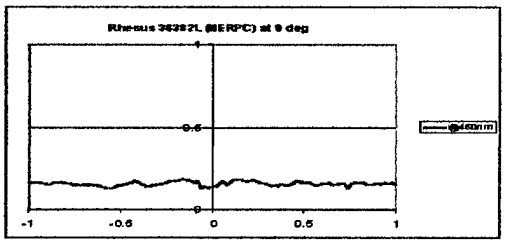
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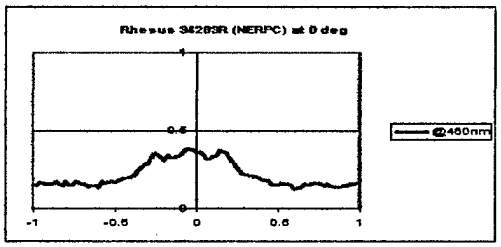
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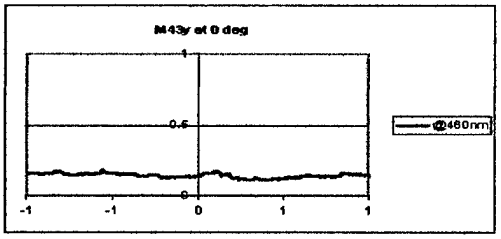
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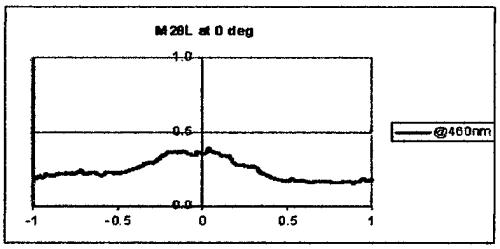
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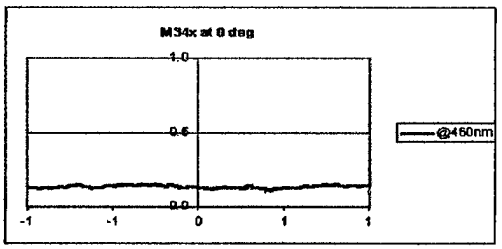
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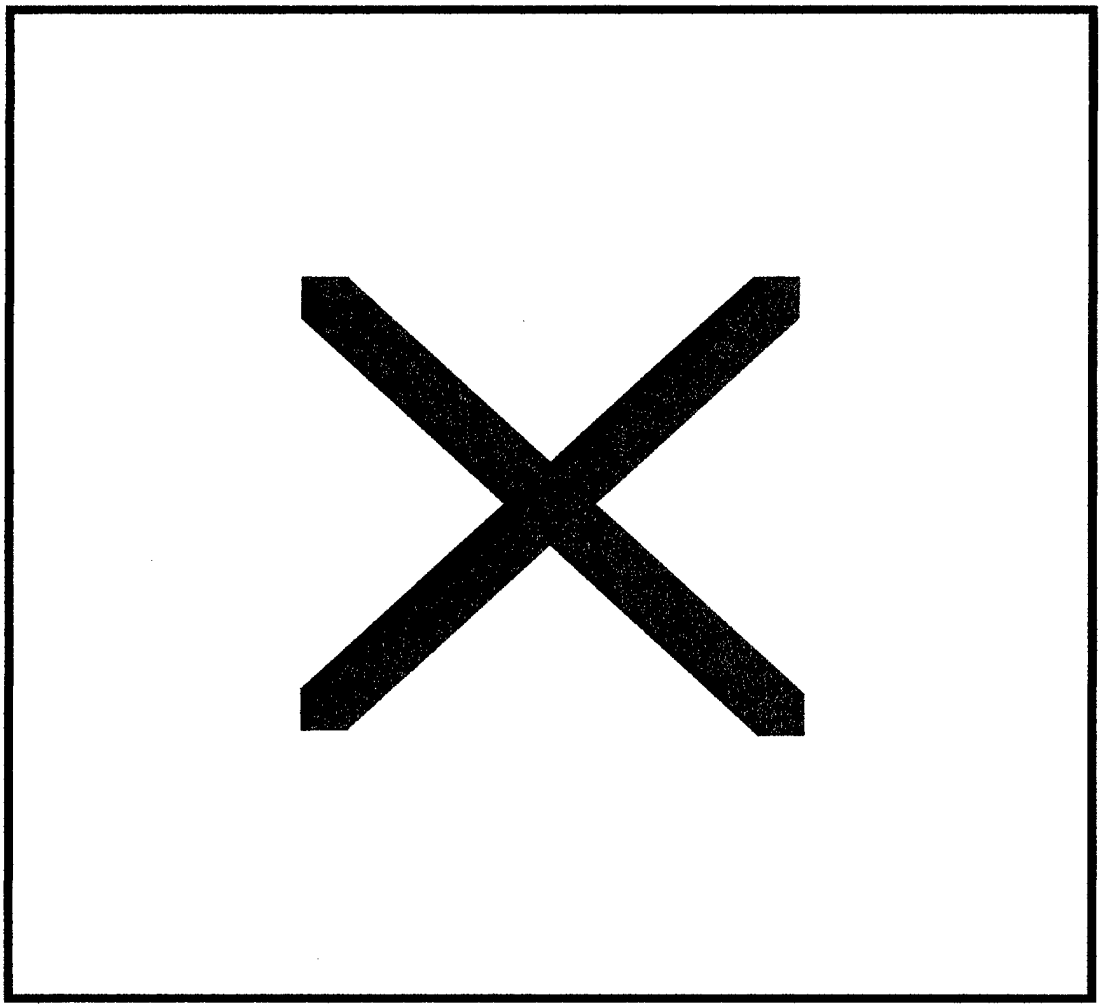
Comparison of Foveal Avascular Zones of Three Primate Species



This figure shows boxplots of measurements of the foveal avascular zones of three primate species. The line near the center of the box is the mean of the distribution. The boundary of each box is determined by the 25th and 75th percentiles. The whiskers are determined by the 5th and 95th percentiles.

Data were recorded for 23 squirrel monkeys, *Saimiri sciureus* (SS), 16 cynomolgous monkeys, *Macaca fascicularis* (MF), and 11 rhesus monkeys, *Macaca mulatta* (MM).

Project 4. Remote Diagnosis of Retinal Damage
Investigator: **Ann E. Elsner, Ph.D.**

Body:

The progress in the fiscal year beginning 7/1/02 was extensive, featuring digital image acquisition in a battery operated mode and obtaining our first images of a living human eye on 9/27/03 with a safe and low cost system built primarily from off the shelf parts. Specifically, we created and produced optical designs 5 – 10, and tested all with a model eye, obtaining high contrast images when illuminated with a low enough laser power as to be safe for human use. We obtained images of

living human eyes without dilating drops once human subjects approval was finalized. Technical progress continued to be steady. We introduced an inexpensive Vertical Cavity Surface Emitting Laser into our system as an illumination source, including designing and building driver circuitry. We studied optical designs 7 – 10 in detail, obtaining our smallest footprint to date with 8, but determining an unacceptable loss of light. We developed software for optical modeling to locate and correct the design flaw, and we now use this concept as a key design constraint. We have now moved this work into ZEMAX to provide a detailed analysis of our system constraints and the spot diagrams at key locations. We designed all circuitry to run on DC power supplies, and obtained images from this system using only battery power and a battery-operated laptop and novel image capture card, thereby assuring portability to locations without 60 Hz, 110 V power standards. We collected a considerable amount of data with Design 9, which we implemented on a breadboard with head positioning and a focusing lens: Design 10. We obtained final human subjects approval for Task 2, the testing of laboratory normal subjects, and obtained images of living human eyes. We needed less than half the laser power that we specified for this configuration, an amount that is comfortable and safe for viewing even if there is failure of the scanning mechanism. We have carried out initial experiments with a series of apertures and other configuration changes, and plan more for next year. For further safety and power savings, and to assist in alignment, we designed and built the initial interlock system. We continue to optimize the instrument for these subjects, as we are not yet permitted to test patients, even though it has always been our laboratory practice to optimize instrumentation work throughout the development process on the actual target population, i.e. patients. This forces us to still rely on archived images from other instruments, which may have some important differences, as the gold standard. We have written a patent, which is now published in the foreign arena, and described the device and goals to interested companies. The U.S. patent application is still being examined. We have put together a full system plan, and have specified an embedded image acquisition system. This has been submitted for presentation at the Optical Society of American Annual Meeting in 2003. We will order the necessary parts for the full electronics control and begin implementing this design upgrade using carry-over funds. These funds are substantial enough to support our continuing into next fiscal year, as we had a delay in beginning the work until we received initial Task 1 approval, and then also Task 2 approval to test our design. We examined the differences between the most portable as opposed to the most low cost system. We then designed a very user friendly system, described in a 5 year grant proposal, in response to an RFA for low costs systems to benefit health care worldwide. As venture capital seems to be much less plentiful that when we made or initial proposal, this type of grant seemed an ideal next step to fund the development beyond the laboratory prototype stage. However, we plan to continue interaction with companies that are interested in eventual production and distribution of this device. We have developed extensive software for the acquisition, display, and analysis of images, including standard measures of image quality. We have developed novel algorithm to analyze features seen on the human retina in a more objective manner, taking into consideration that many important features in the human eye do not fall on a straight line, or are horizontal or vertical. Our images are of sufficient quality at present to merit quantitative assessment, and in fact demand this to determine if further design changes or a choice of optical configurations lead to actual improvement.

Key Research Accomplishments:

- completed initial instrument design for the testing of human subjects
- achieved Direct Current, battery operation necessary for portability
- obtained images of from undilated, volunteer subjects
- performed patent search, wrote and submitted patent, and have foreign patent publication

Reportable Outcomes:

Scientific Presentations:
Elsner AE, Stewart JB, Schwarz RA. Elsner AE, Stewart JB, Schwarz RA. Towards a portable digital retinal imager. Optical Society of America Annual Meeting, Orlando, FL, 2002.
Elsner A.E. Remote Diagnosis of Retinal Damage. Product Line Review for ophthalmology and vision grants, November 18, 2002.
Elsner AE, Stewart JB, Schwarz R, Zavriyev A, Cheney MC. Towards a Digital Retinal Imaging Device for Remote Diagnosis. The "Ocular Challenges for the 21st Century Military" symposium posters at Schepens Eye Research Institute, Harvard Medical School, 9/19/02.
Elsner AE, Cheney MC, Smithwick QYJ. A Portable, Easy Use, Near IR Retinal Imaging Device. In Vivo Microscopy Sumposium and Poster Session. Wellman Laboratories, Harvard Medical School, 3/17/2003.
Patents Published:
Elsner AE. Device for digital retinal imaging. WO 03/039332 A2 Foreign publication 15, May, 2003.
Follow-on research funding applications to date:
"Low Cost Device for Digitial Retinal Imaging," submitted 3/03 in response to RFA EB-03-006 by the NIH.

Conclusions:

A digital retinal imaging device is being built that can be made to be low cost, portable, and user friendly. These three goals are not always compatible, i.e. the head positioning system that is easiest to use is neither low cost nor lightweight; the lowest cost device lacks some features that help the end user; the most portable system has to be more rugged than the lowest cost. Once a portable prototype is finished and approved for patient testing, then the best data for these three goals can be obtained.

References:

Elsner AE, Stewart JB, Schwarz RA. Towards a portable digital retinal imager. Optical Society of America Annual Meeting, Orlando, FL, 2002.

Elsner AE. Device for digital retinal imaging. WO 03/039332 A2 Foreign publication 15, May, 2003.

Appendices:

Towards a portable digital retinal imager

Ann E. Elsner, Jason B. Stewart

A portable, hand-held scanner is being developed to provide high image contrast, yet operate in a manner similar to an everyday digital camera. Additional goals include battery operation and non-invasive, non-mydriatic image acquisition for remote diagnosis of retinal trauma and disease.

Wellman Lab InVivo Microscopy Symposium Poster 3/17/2003

Laser Scanning Digital Camera for Retinal Diagnosis
Quinn Y.J. Smithwick, Michael C. Cheney, Ann E. Elsner

Schepens Eye Research Institute, Harvard Medical School

The goal is to build a low cost digital device for retinal diagnosis, based on laser scanning to provide high contrast images. The device differs from most commercial retinal imaging devices in that it uses a scanning slit and a 2D detector array. The main illumination source is an 850 nm Vertical Cavity Surface Emitting Laser, which is stable, inexpensive, and extremely small. The scanning is provided by a DC motor that rotates a mirror cube. Confocal apertures conjugate to the retinal plane in both the illumination and detection pathways further improve image contrast. Descanning to produce a slit is performed by the same mirror cube, and rescanning, again via the same mirror cube, returns the slit to a 2D configuration that focuses onto the 2D array. Illumination and detection pathways are separate throughout. The present 2D detector array is a consumer grade CCD with VGA resolution. The entire device can be operated via 12 volt batteries. The current image capture can also be performed with a battery-operated a laptop and image capture card.

Sponsored by DAMD 17-01-2-0032

Project 5. Identification of Factors that Can Predict Dry Eye Complications of LASIK Surgery
Investigators: **Drs. Dimitri Azar and Darlene A Dartt**

Body:

Correction of vision through laser refractive surgery gives the military a tremendous advantage on the battlefield. The initial military experience demonstrated great success in garrison and the training environment. The early reports from recent combat operations have shown enthusiastic support for the role of refractive surgery as a force multiplier (through decreased down time to lost optical devices and decreased incidence of contact lens-related complications) and mission-enhancing procedure. While the military is intrigued by the rapid visual recovery and minimal incidence of postoperative haze with LASIK, the possibility of two of the major complications associated with LASIK, the eye's susceptibility to trauma and the occurrence of dry eye syndrome are concerning. The consequences of minor ocular trauma are exaggerated after LASIK primarily due the presence of the LASIK flap and the LASIK-induced dry eyes. The latter is the "silent enemy" in LASIK whose role was greatly underestimated or underappreciated until only recently. Upon questioning, more than half of patients report dry eye symptoms and up to 90% of patients exhibit signs of ocular surface disease. This complication occurs because during the surgery a flap is cut in the cornea through the stroma and the flap lifted to allow laser induced remodeling of the corneal curvature. The stromal incision severs about 70% of the sensory innervation to the central cornea. Dennervation of the cornea leads to dry eye as activation of corneal sensory nerves drives tear secretion by stimulating the lacrimal gland, corneal and conjunctival epithelia, and the conjunctival goblet cell to secrete tears. A decrease in the amount or composition of tears is well known to cause dry eye. For this project we have designed a clinical trial involving 40 patients undergoing LASIK surgery and performed a variety of tests on tears and the ocular surface to determine which of these measurements is predictive of dry eye complications after LASIK surgery. We have recruited 20 patients (10 males and 10 females) who have entered this study. One patient has completed the entire 9-month study. All patients to date have completed all visits; at one visit two pieces of data are missing from one patient. All tests for each visit have been completed, dry eye questionnaires and conjunctival impression cytology samples are currently being analyzed. Recruitment of the remaining 20 subjects is currently progressing. The study will finish one year after the last patient has been recruited.

Key Research Acccomplishments:

- Twenty patients (10 males and 10 females) have entered the study.
- The average age of all patients is 33 years (36 years for males and 31 years for females).
- The average spherical equivalent for all patients is –1.98 and varies from –6.6 to +5.62. Males and females have the same average value.
- All patient visits have been completed; tow pieces of data are missing for one patient visit.

Reportable Outcomes:

There will be no reportable outcomes until the study is completed.

Conclusions:

We should be able to recruit the remaining subjects for this project over the next year and complete the study one year after the final patient has been recruited. We believe that we will be able to identify a test of the tear film and occur surface that will indicate that an individual is predisposed to dry eye following LASIK surgery.

References:

None.

Appendices:

None.

APPENDIX
(R. Dana)

Ex Vivo Adenovirus-Mediated Gene Transfer to Corneal Graft Endothelial Cells in Mice

Short Title: Adenovirus-mediated gene transfer to corneal grafts

Authors: Y. Qian, M.D, Ph.D., F. L. Leong, Ph.D., A. Kazlauskas, Ph.D., M. R. Dana, M.D., M.P.H.

Institution: The Schepens Eye Research Institute, Department of Ophthalmology, Harvard Medical School, Boston, MA

Word Count: 3277

Number of Figures: 6

Section Code: Cornea

Keywords: Corneal transplantation, adenoviral vector, GFP, gene transfer, gene expression, endothelium, graft survival

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Grant Support: Roche Organ Transplantation Research Fund; Eye Bank Association of America; and the U.S. Department of Defense and the Aging Eye Center, Schepens Eye Research Institute. Andrews Kazlauskas is a Research to Prevent Blindness Olga Keith Weiss Scholar.

Proprietary Interest: None

ABSTRACT

Purpose: Genetic modulation of donor tissue prior to corneal transplantation may have the potential to modulate alloimmunity and/or to prevent corneal endothelial cell death. This study was conducted to optimize adenovirus-mediated gene transfer to donor corneal endothelium and to delineate the kinetics of marker gene expression in syngeneic and allogeneic corneal grafts.

Methods: BALB/c mouse corneas were incubated with replication-deficient adenovirus encoding GFP or empty vector ex vivo at a dose of 6×10^7 PFU or 6×10^6 PFU at temperatures of 4°C or 37°C. Following ex vivo infection, the donor corneas were transplanted orthotopically to BALB/c or C57BL/6 recipients. After transplantation, localization of GFP in the grafts was determined in cryosections of enucleated eyes, and GFP expression in the grafts was visualized in vivo by using epifluorescent microscopy over 12 weeks. All grafts were evaluated clinically by slit-lamp biomicroscopy.

Results: GFP expression was found to be restricted to the corneal endothelium. In vivo expression of GFP in syngeneic corneal grafts was demonstrated for up to 12 weeks. Syngeneic grafts incubated with the vector at 4°C exhibited more extensive and longer duration of expression of green fluorescence than grafts incubated at 37°C. Moreover, the syngeneic grafts infected at 4°C maintained their transparency, whereas those infected at 37°C displayed high degree of opacity. Corneal allogeneic grafts infected with a low dose of the vector displayed longer GFP expression and graft survival than the allogeneic grafts infected with a high dose of the viral vector.

Conclusions: Adenoviral vector can selectively and efficiently deliver exogenous gene(s) to the endothelium of corneal grafts during hypothermic organ preservation. Gene expression is retained in vivo in corneal syngeneic grafts for longer periods as compared to allogeneic grafts.

INTRODUCTION

Corneal transplantation, also known as penetrating keratoplasty, involves the replacement of diseased cornea by a graft of homologous tissue. It is the major treatment available to reverse the loss of sight caused by damage to the cornea which represents the second most common cause of blindness worldwide after cataract. It is estimated that well over 10 million people are corneally blind in the world.[1] While corneal transplantation is the most common and arguably one of the most successful form of solid tissue transplantation, graft failure is still not uncommon. Loss of the endothelial cell of the transplanted cornea is a major factor contributing to the failure of corneal transplants. A number of factors including allograft rejection, endothelial contact with an intraocular lens or vitreous, uncontrolled inflammation, additional ocular surgery, eye trauma, and increased intraocular pressure, have been shown to cause a rapid decline in endothelial cell numbers leading directly to graft failure.[2] Hence, while immune rejection targeted at the endothelium is the leading cause of graft failure, endothelial cell loss likely represents a common denominator of graft edema and failure.[3]

The cornea is a particularly suitable candidate tissue for gene-based approaches in transplantation for several reasons: (a) Unlike some other solid organs, it can be preserved for periods of several weeks, allowing time for ex vivo genetic alteration *before* transplantation; (b) the transparency of the cornea allows for direct visualization of any significant consequences of gene transfer; (c) maintenance of corneal transparency is so dependent on the normal activity of corneal endothelial cells that they make for an attractive target for gene therapy strategy since alterations in this one cell layer can have profound effects on the remainder of the tissue; (d) corneal endothelial cells are readily accessible (as they are in direct contact with the storage medium) and hence amenable to gene transfer; and finally, (e) the corneal endothelium has a

distinct advantage as a target for gene therapy. Transgene expression in most organs is transient and is limited by a cellular immune responses directed against transduced cells, viral proteins, and foreign transgene proteins.[4, 5] In contrast, prolonged and persistent adenovirus-mediated transgene expression is observed in immune privileged sites such as the eye and brain.[6] Because the anterior chamber of the eye is an immune privileged site, the anatomic location of the corneal endothelium makes it an optimal site for gene transfer.

We have a well-established and validated mouse model of murine corneal transplantation that faithfully reflects the experience with keratoplasty in humans.[7, 8] While several groups have had some success in gene transfer to the corneal endothelium of human,[9] rabbit,[10, 11] rat,[11] and sheep,[12] their techniques cannot be applied directly to our mouse model of transplantation because different animal models and genes have been tested, and the gene transfer was conducted at 37°C in all those studies—an unacceptably high temperature for long-term storage of the donor corneal tissue in the standard storage media in use worldwide. In addition, all of these studies examined ex vivo gene expression and none has reported the kinetics of adenovirus-mediated gene transferred in the endothelium of corneal grafts in vivo.

In this study, we used adenoviral vector encoding GFP to infect mouse donor corneas, and transplanted these donor corneas orthotopically to either syngeneic or allogeneic recipients. We aimed to examine the localization and kinetics of GFP expression in corneal grafts in vivo, and the effect of such a gene transfer on the fate of the corneal grafts.

METHODS

Animals

BALB/c mice (Taconic farm, NY) aged 8 to 10 weeks were used as corneal recipients; BALB/c and C57BL/6 mice of the same age were used as corneal donors. Prior to all surgical procedures, each animal was deeply anesthetized by intraperitoneal injection of 3 – 4 mg of ketamine and 0.1 mg of xylazine. All animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Infection of Donor Corneas Ex Vivo

The vector used in this study was an E1- and E3-deleted serotype 5 adenoviral vector carrying the GFP gene (ad-GFP) under the transcriptional control of the cytomegalovirus promoter that was constructed, propagated in 293 cells, purified by density gradient method, and stored at -70°C until use. Both GFP adenoviral and control ('empty') vector that contains no GFP insert were provided by the Harvard Gene Therapy Initiative. Based on our preliminary experiments regarding optimal gene transfer conditions, freshly harvested BALB/c cornea (N = 4 per group) was incubated at 4°C or 37°C in 5% CO_2 culture incubator for 2 hours with 200 μl of RPMI 1640 medium (Bio Whittaker) supplemented with 10% FBS-(Hyclone, Utah) containing ad-GFP. Different virus titers (10^7 plaque-forming units [PFU], 10^6 PFU, or no virus as control) were tested for optimal efficacy of gene transfer. After the 2-hour incubation, the infected corneas were washed with PBS three times and immediately used as donors for corneal transplantation (Figure 1).

Orthotopic Penetrating Corneal Transplantation

Orthotopic corneal transplantation was performed as described previously.[7] Briefly, after induction of mydriasis, the recipient cornea is marked with a 2.0-mm trephine (Storz Instrument Co., St. Louis, MO) and excised with microscissors. The donor corneas infected with GFP adenoviral vector, control empty vector, or no vector was transplanted into host corneal bed with 8 interrupted 11-0 nylon sutures (Sharpoint, Vanguard, TX). The corneal sutures are removed 7 days after surgery.

Localization and Assessment of GFP Gene Expression in Corneal Grafts

BALB/c eyes (N = 4) bearing ad-GFP infected corneal syngeneic grafts were enucleated and embedded in OCT on dry ice 1 week after ex vivo gene transfer and corneal transplantation. The embedded tissues were cut into 7 μm cryosections and examined using epifluorescent microscopy.

The expression GFP in the corneal grafts was followed in vivo for 12 weeks by using epifluorescent microscopy once daily for a week and then twice weekly after ex vivo gene transfer and corneal transplantation. At each time point, mice bearing infected corneal grafts were anaesthetized and then GFP expression was observed non-invasively by using an epifluorescent microscope. PBS was applied to the eyes to prevent corneal dryness during observation and ophthalmic antibiotic ointment was applied afterwards. The images of expressed green fluorescence in the corneal grafts were captured digitally by using Spot Camera, and the intensity and size of the GFP-expressing area within the corneal grafts in each eye was analyzed by NIH Image.

Clinical Evaluation of Corneal Grafts

Grafts were evaluated for 12 weeks by using slit-lamp biomicroscopy two times a week. At each time point grafts were scored for opacification using a previously described and standardized scoring system which grades corneal grafts' opacification between 0 – 5+. Grafts with an opacity score of 2+ or greater after 3 weeks were considered failed.[13] Kaplan-Meier analysis was employed to assess the effect of transgene expression on graft survival.

RESULTS

Adenoviral vector-mediated GFP gene expression in the endothelium of corneal syngeneic grafts

The cornea is composed of the following major layers: (a) an outer stratified squamous nonkeratinized epithelium, (b) an inner dense connective tissue stroma with its resident cells, and (c) a monolayered cuboidal endothelium bordering the anterior chamber (Figure 2A). To evaluate whether adenoviral vector can deliver gene to the corneal endothelium of the grafts, we examined GFP expression in cryosections of corneal grafts 1 week after adeno-GFP mediated gene transfer and corneal transplantation. GFP expression was found to be virtually entirely restricted to the corneal endothelial layer, with only trace to absent expression in the stroma (Figure 2B). There was no GFP expression noted in the corneal epithelium. No GFP expression was seen in corneal grafts infected with empty adenoviral vector (Figure 2C). The results indicate that adenoviral vector is an efficient transfer vehicle to deliver exogeneous gene specifically to corneal endothelial cells.

Kinetics of GFP expression in adenoviral vector-infected *syngeneic* corneal grafts

To determine the life span of GFP in grafted corneas in the absence of alloimmunity, we used syngeneic corneal transplants from BALB/c to BALB/c mice. The BALB/c donor corneas were infected with $6x10^7$ pfu of adeno-GFP for 2 hours ex vivo at either 37°C or 4°C; the latter is a temperature commonly used for human donor cornea storage. With an ex vivo infection temperature of 4°C, the syngeneic corneal grafts maintained their transparency (Figure 3A), and in vivo expression of GFP in these syngeneic corneal grafts was demonstrated for up to 12 weeks with peak expression from day 3 to week 5 (Figure 3B). With an ex vivo infection temperature of 37°C, GFP expression in the corneal grafts lasted less than 2 weeks (Figure 4A). Syngeneic grafts incubated with the vector at 4°C exhibited a larger area and longer expression of green fluorescence than grafts incubated at 37°C (Figure 4A and 4B). These results demonstrated that 4°C incubation temperature was a better infection condition to prolong GFP expression in the corneal transplants in vivo than 37°C. Thus, under optimal infection conditions GFP gene expression could be retained in vivo, as demonstrated for syngeneic grafts, for prolonged periods of time.

Clinical outcome of adeno-GFP infected corneal *syngeneic* grafts.

During the first week after transplantation, grafts infected with adeno-GFP at 4°C exhibited an opacity score of 2.6 ± 0.4 (Figure 4C), representing a typical postoperative response seen routinely in our murine corneal transplant model even without any viral vector infection. After removal of corneal sutures at week 1, the opacity score decreased dramatically and the grafts became clear and maintained their transparency up to week 4. Between weeks 4 and 8, many grafts developed central exposure epitheliopathy without any stromal edema, most likely due to repeated anesthesia and exposure to slitlamp biomicroscopy; in such cases the

epitheliopathy spontaneously healed and left a light scar that diminished with time. Similar to the grafts infected at 4°C, grafts infected ex vivo at 37°C also exhibited an early postoperative opacity during the first week after transplantation. However, the opacity scores remained higher than those for 4°C. After suture removal at week 1, the grafts remained opaque with opacity scores ranging from 2.6 ± 0.5 to 3.2 ± 0.71 (Figure 4C). Thus, both in terms of GFP expression as well as clinical endpoints, the data indicate that 4°C incubation with viral vector is a preferred infection condition than 37°C.

Adenoviral vector-mediated delivery of green fluorescent protein (GFP) marker gene to *allogeneic* corneal grafts

Corneal allogeneic grafts infected with a high dose ($6x10^7$ PFU) of the viral vector at 4°C exhibited transient GFP expression which lasted less than a week. Considering that the cumulative effect of higher dose of adenoviral vector in addition to allogeneic challenge of the host may be overly toxic to the corneal grafts, we reduced the vector dose to $6x\ 10^6$ PFU. Infected with this lower dose of vector, the grafts displayed continued clarity and longer GFP expression up to 3.5 weeks (Figure 5). However, even at this lower dose, the relatively longer GFP expression afforded did not extend appreciably beyond 4 weeks – the time when graft rejection usually occurs in our murine model of corneal transplantation.[14] Indeed, 100% corneal allograft infected with the adenoviral vector at the high dose of 6×10^7 PFU failed at week 1, whereas the grafts infected with the adenoviral vector at a dose of 6×10^6 PFU survived until week 3.5 ($p = 0.0082$). The survival rates of the grafts infected with no vector or empty vector were 50% and 25%, respectively (Figure 6); in both cases higher than allografts infected with

adeno-GFP ($P < 0.05$), suggesting GFP toxicity above and beyond graft damage by adenoviral toxicity and alloimmunity.

DISCUSSION

Several previous studies using lac Z as a reporter gene have shown that transgene expression mediated by adenoviral vector specifically localized to the endothelial layer of cultured corneas.[9-12] Consistent with these ex vivo studies, our results demonstrate that adenoviral vector mediated GFP expression was confined within the endothelium of corneal grafts in vivo. However, it remains unclear why the expression of the gene product(s) is seen only in the endothelium. Wild-type adenovirus infection, as well as adenoviral vector-mediated gene transfer, depends on virus interaction with the Coxsackie adenovirus receptor (CAR) mediating virus attachment to the cell surface, and on interaction with $alpha_v beta_3$ and $alpha_v beta_5$ integrins mediating virus entry into the cell.[15, 16] Both the epithelium and endothelium of the normal cornea express the integrin $alpha_v beta_5$;[17, 18] however, there is no report regarding the presence of CAR in the cornea in the literature. A study on the heart reveals that there is no correlation between the adenoviral vector expression patterns and the virus receptor patterns,[19] suggesting that adenovirus receptor expression is necessary but not sufficient for vector transfer. Other factors, such as anatomical barriers[19] and stages of cell cycle,[20] may also play a role.

Our study is the first report regarding the fate of transgene expression mediated by adenoviral vector in corneal grafts in vivo. This is relevant because transfer of desirable genes to corneal grafts is a natural application of gene therapy strategies to the ocular anterior segment, among others. We used GFP as a marker gene as opposed to enzyme markers, such as β-gal,

because GFP is readily visible by the epifluorescent microscope,[21] and therefore its expression can be observed in vivo directly and prospectively without any invasive procedures, allowing correlation of gene transfer with physiological parameters of the corneal graft. We here report that prolonged and persistent adenovirus-mediated transgene expression up to 12 weeks (last observation point) was observed in our syngeneic corneal grafts infected at 4°C. This finding is consistent with the concept that the cornea is an immune privileged tissue and the anterior chamber in which the corneal endothelium is located is an immune privileged site.[22-24] Studies have shown that intracameral injection of adenoviral vector is capable of transferring exogenous genes to the corneal endothelium.[25-27] However, gene transfer by this approach induces significant inflammation and reversible corneal abnormalities.[26, 27] In our model the viral vector is used ex vivo, which greatly attenuates the side-effects of viral infection with in vivo administration. The technology of ex vivo transgene delivery to the endothelium of syngeneic grafts may have potential clinical applications for inherited diseases of the corneal endothelium such as Fuchs' dystrophy in which the endothelium undergoes apoptosis with subsequent development of stromal edema.[28] For example, transferring appropriate functional genes to the diseased corneas ex vivo, followed by corneal autograft transplantation, could offer one way of restoring endothelial viability and avoiding allogeneic graft rejection.

While gene transfer was conducted at 37°C in all previous studies,[9-12] we are more interested in infecting donor corneas at 4°C rather than at higher temperature because human donor corneas are stored at 4°C in eye banks. Indeed, our findings demonstrate that 4°C is feasible and superior to 37°C as an ex vivo infection temperature for corneas. Interestingly, ex vivo GFP expression was stronger in cultured corneas infected at 37°C than at 4°C with the same dose of ad vector (data not shown). However, once the infected corneas were transplanted to

recipients, the in vivo GFP expression in corneal grafts infected at 37°C became much less intense than that in those infected at 4°C, and the grafts infected at the higher temperature prior to transplantation remained edematous and opaque throughout the observation period, suggesting that the function of graft endothelium became compromised at higher incubation temperatures. Interestingly, corneas incubated with adenoviral vector at 4°C for 2 hours displayed no GFP expression ex vivo if the corneas were continuously incubated at 4°C. However, 12 hours after transferring these corneas to 37°C, GFP expression could be detected ex vivo (data not shown). In the aggregate, these results imply that adenoviral vector is able to attach to, or penetrate into, the corneal endothelium at 4°C but requires higher temperatures to express its encoded gene.

Our findings provide relevant information for delivery of exogenous gene(s) during corneal storage of eye bank tissue similar to the effective gene transfer that has been shown during cold organ preservation for other solid organ transplants, such as liver and heart.[29, 30] In sharp contrast to our syngeneic data, our data show that GFP gene expression mediated by high-dose adenovirus in the setting of corneal allotransplantation is short-term, and toxic to grafts in vivo. Reduction in viral dose prolongs the transgene product expression and delays the onset of vector induced graft failure. However, the desired gene expression can be retained in vivo in corneal allogeneic grafts for only short periods of time compared to that in syngeneic grafts. Additionally, allografts infected with low viral doses survived for shorter periods of time than did empty adenoviral vector-infected allografts, and allografts infected with empty adenoviral vector showed shorter survival than did non-infected corneal allografts. These data imply that both GFP and adenoviral vector products can have deleterious side-effects in the setting of allotransplantation. Indeed, the transient loss of ACAID in the allografted eyes,[31] may be relevant in explaining, at least in part, the observed deleterious effects of adenoviral vector on corneal

allografts. Several studies have attempted to prolong corneal allograft survival by delivering functional genes, such as TNF receptor,[32] IL-4,[33] and IL-10,[34] to donor corneas. While the majority of these studies show minimal to no improvement in allograft survival,[32, 33] one study reports significant prolongation of sheep allogeneic corneal grafts infected with ad-IL-10.[34] However, the kinetics of the functional transgene expression in vivo are not reported in these studies. In summary, our syngeneic data reflect the promise of prolonged expression of transgene products specifically at the level of the corneal endothelium. However, based on our data derived from allogeneic grafts, caution must be exercised in interpreting the results of gene transfer strategies to corneal grafts using adenoviral vector, since it is quite possible that the real beneficial effects of the transgene product are outweighed by the deleterious effects of the adenoviral vector. The use of less immunogenic "gutless" adenoviral vector may prove useful in circumventing some of the observed shortcomings of adenoviral vectors in the allogeneic setting.[35]

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FIGURE LEGEND

Figure 1. Flow diagram of our experimental methods. A: Infection of donor corneas ex vivo with adenoviral vector; B: Orthotopic penetrating corneal transplantation; C: Examination of the infected grafts using slit lamp biomicroscopy and epifluorescent microscopy.

Figure 2. Localization of GFP in the endothelium of corneal syngeneic grafts. A: Normal mouse cornea is histologically composed of the epithelium (*), the stroma (**), and the endothelium (arrow); B: Expression of GFP in the endothelium (arrow) of corneal syngeneic grafts; C: No GFP expression is observed in empty adenoviral vector infected corneal grafts.

Figure 3. GFP expression in *syngeneic* corneal grafts infected with adeno-GFP vector. A: Biomicroscopic appearance of an adeno-GFP infected syngeneic corneal graft at week 4; B: GFP expression in a representative syngeneic corneal graft infected with the adeno-GFP vector ex vivo at 4°C at different time points.

Figure 4. Variance in GFP expression and opacity scores in corneal *syngeneic* grafts infected with adeno-GFP vector at 4°C or 37°C. A: Kinetics of GFP expression in adeno-GFP infected grafts; B: Area of GFP expressed in adeno-GFP infected grafts; C: Opacity scores of adeno-GFP infected grafts.

Figure 5. GFP expression in *allogeneic* corneal grafts infected with adeno-GFP vector at dose of $6x10^6$ PFU. A: Biomicroscopic appearance of an adeno-GFP infected allogeneic corneal graft at

week 1; B: GFP expression in a representative allogeneic corneal graft infected with adeno-GFP vector ex vivo at 4°C at different time points.

Figure 6. Kaplan-Meier survival curves for *allogeneic* corneal grafts infected with adeno-GFP vector at dose of $6x10^6$ PFU or $6x10^7$ PFU. Grafts infected with empty adenoviral vector or no vector served as controls. There is a positive correlative between dose of adenoviral vector and graft failure.

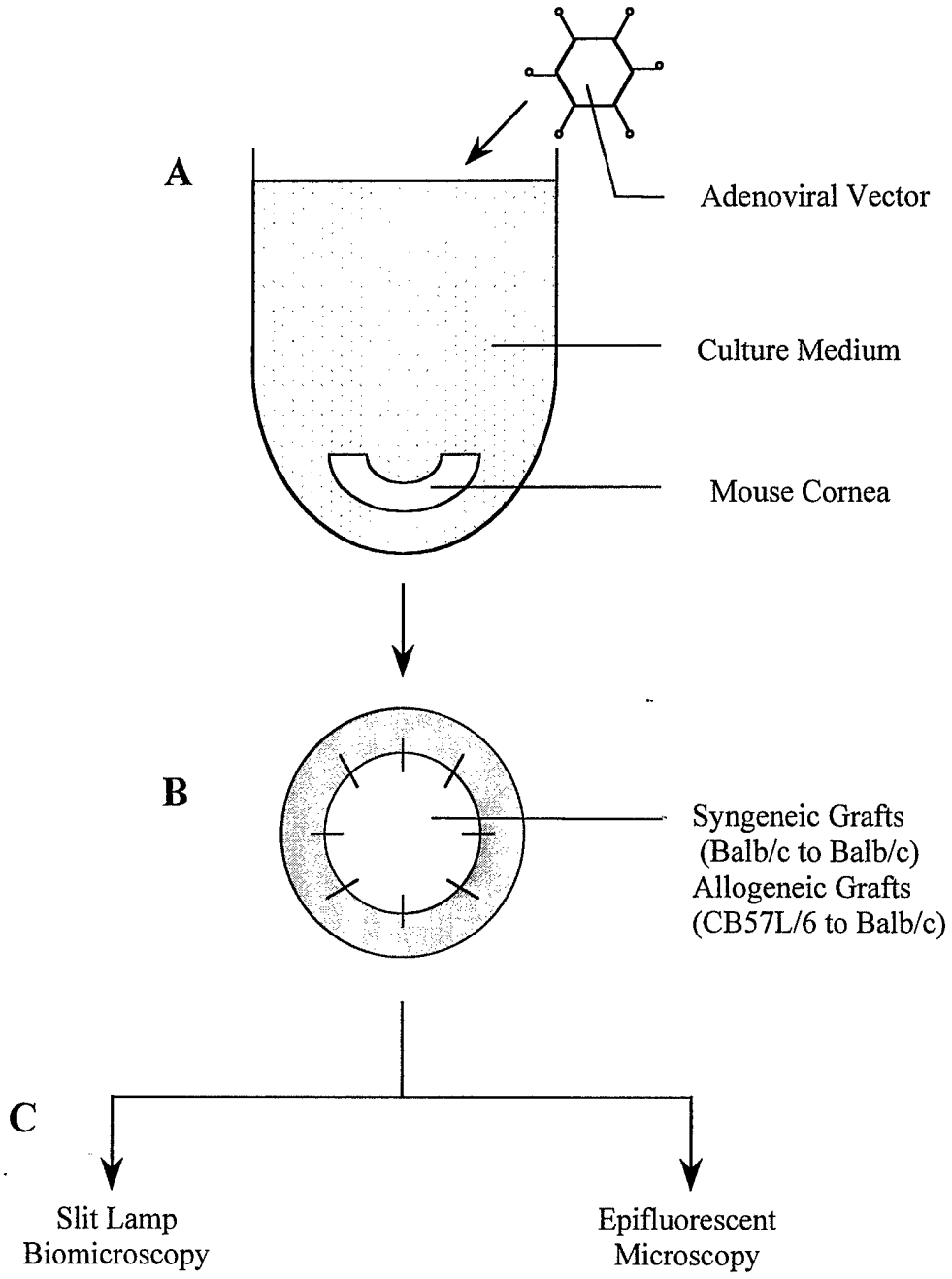

A
Adenoviral Vector
Culture Medium
Mouse Cornea
B
Syngeneic Grafts
(Balb/c to Balb/c)
Allogeneic Grafts
(CB57L/6 to Balb/c)
C
Slit Lamp
Biomicroscopy
Epifluorescent
Microscopy

Figure 1

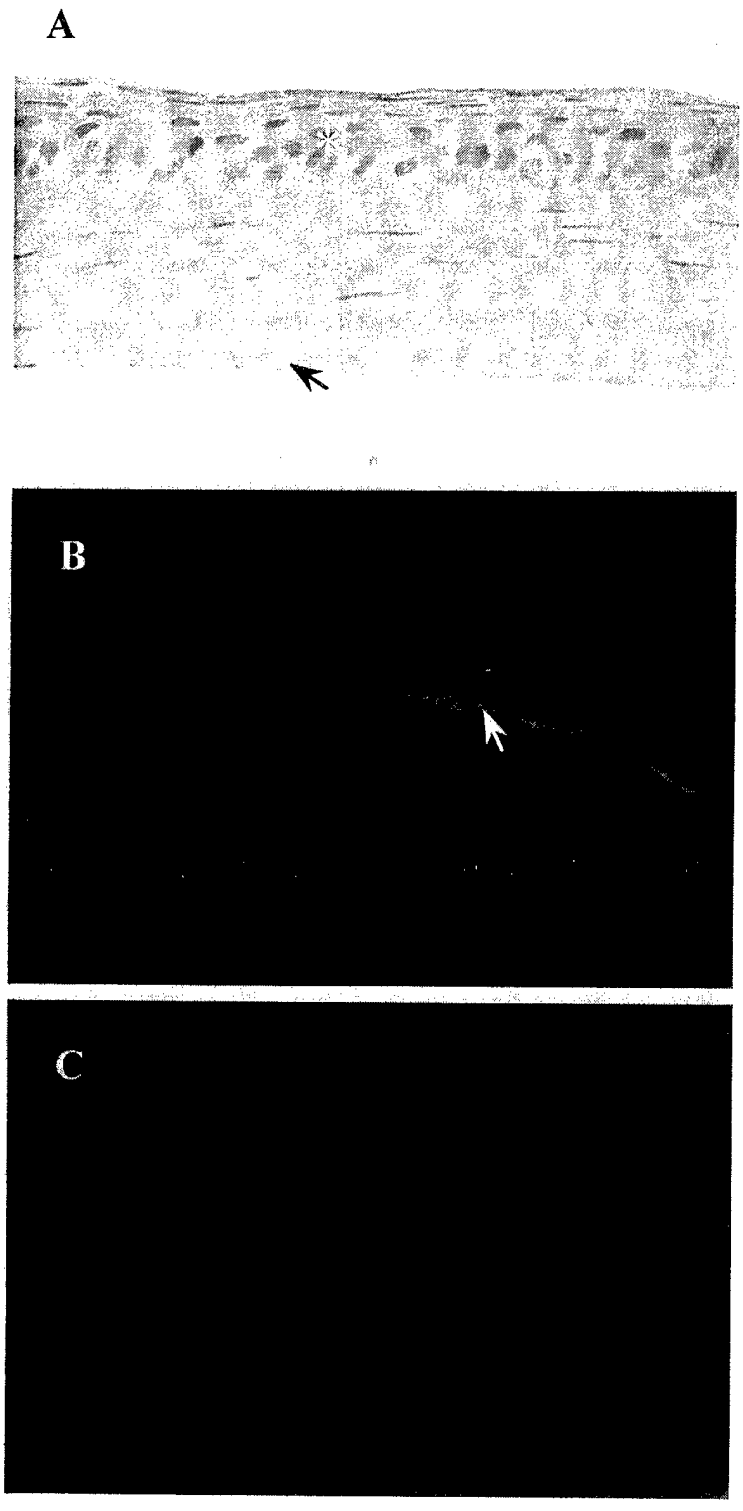


Figure 2

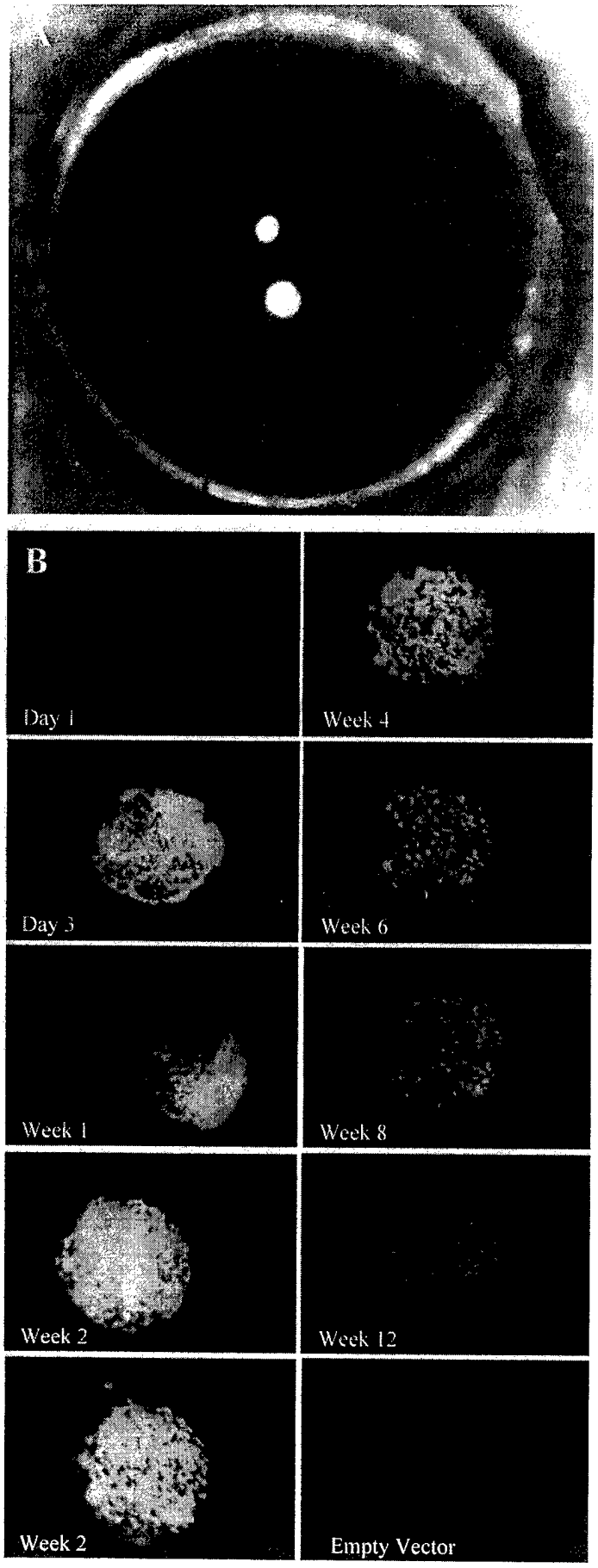

B
Day 1
Week 4
Day 3
Week 6
Week 1
Week 8
Week 2
Week 12
Week 2
Empty Vector

Figure 3

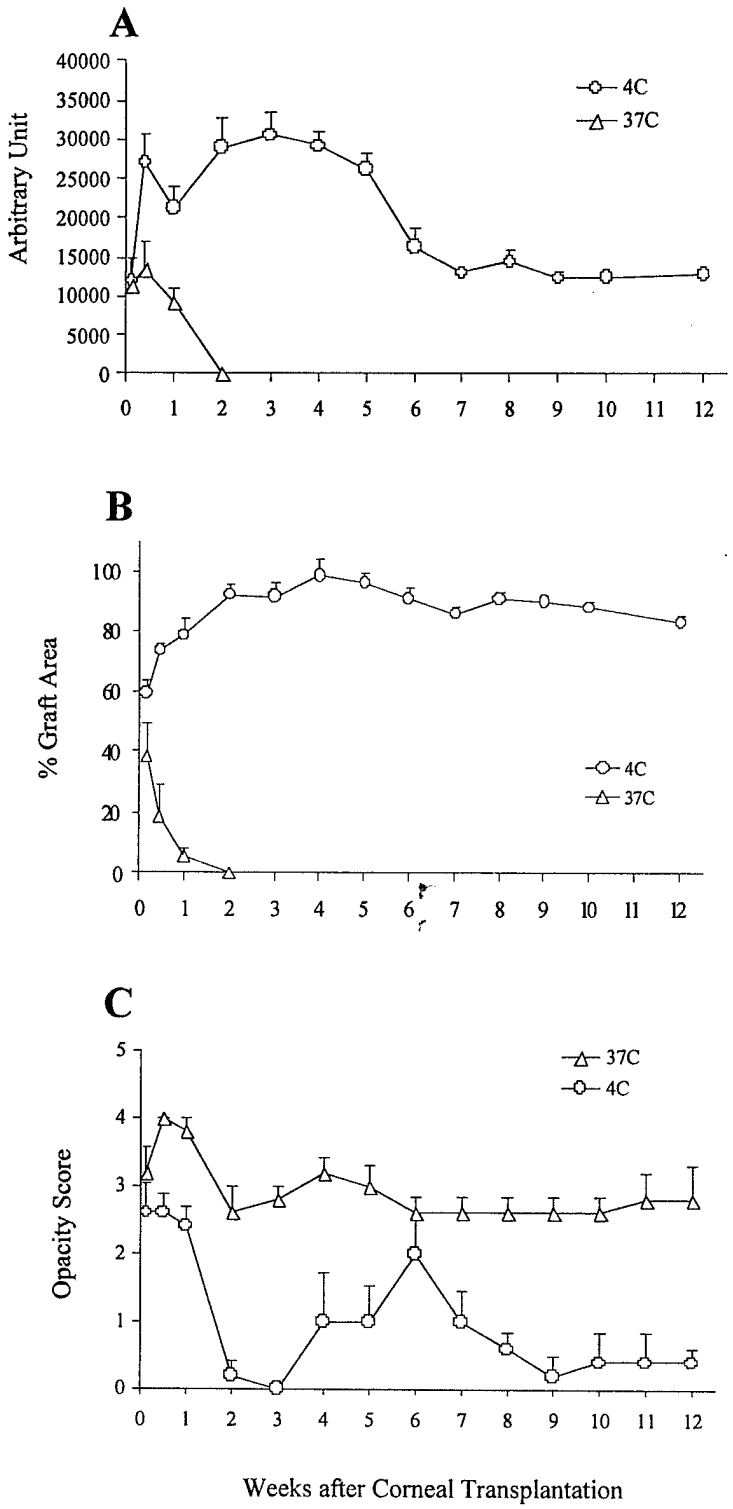

A
Arbitrary Unit
40000
35000
30000
25000
20000
15000
10000
5000
0
4C
37C
0 1 2 3 4 5 6 7 8 9 10 11 12
B
% Graft Area
100
80
60
40
20
0
4C
37C
0 1 2 3 4 5 6 7 8 9 10 11 12
C
Opacity Score
5
4
3
2
1
0
37C
4C
0 1 2 3 4 5 6 7 8 9 10 11 12
Weeks after Corneal Transplantation

Figure 4

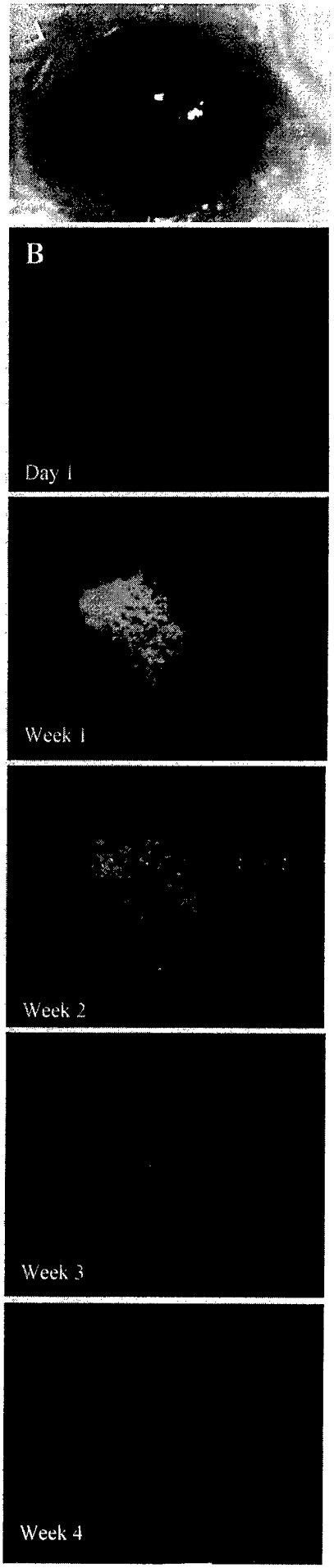


Figure 5

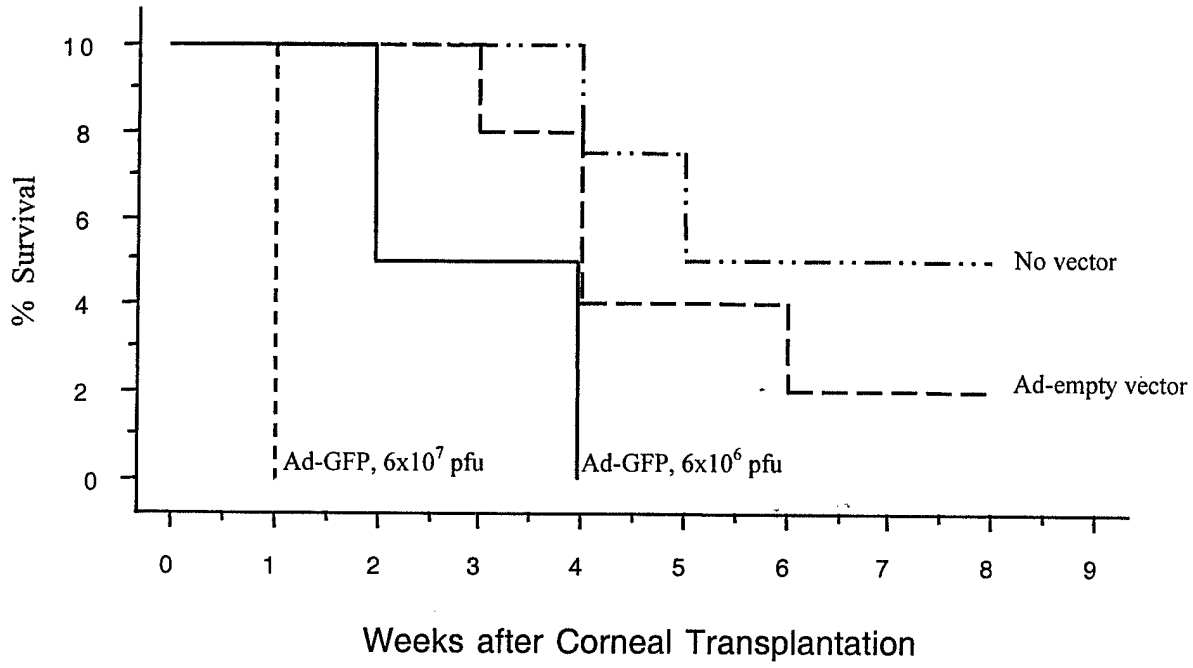
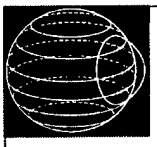

% Survival
10
8
6
4
2
0
0
1
2
3
4
5
6
7
8
9
Weeks after Corneal Transplantation
No vector
Ad-empty vector
Ad-GFP, 6x10^7 pfu
Ad-GFP, 6x10^6 pfu

Figure 6



Caspase Activation and Apoptosis in the Inflamed and Transplanted Cornea

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Department of Ophthalmology
Harvard Medical School

Corneal Transplantation

- Most common solid tissue graft
- Failure rates (C57BL/6 to BALB/c model):
  - Low-risk (no inflammation): up to 50%
  - High-risk (inflamed host bed): over 95%
- Main reason for graft failure:
  - Immune-mediated destruction with 2° corneal edema

Apoptosis in Transplantation

- Thought that apoptosis promotes corneal graft survival by killing alloreactive T cells
  - Not known whether apoptosis of resident graft cells also occurs
- Whereas graft destruction is mediated by DTH, the mechanisms by which this response leads to graft failure are unknown

Hypothesis

We hypothesize that 'bystander' apoptosis, triggered by infiltrating alloreactive cells, contributes to graft failure by killing resident corneal graft cells

Questions

I. Does apoptosis occur in the cornea following transplantation and/or inflammation?

II. Which cell types undergo apoptosis?

- Immune- or non-immune cells?

III. Which mediators are responsible for triggering apoptosis in corneal cells?

I. Apoptosis in cornea following transplantation and/or inflammation

Mouse models:

- Corneal allograft (B6 to BALB/c)
  - High-risk
  - Low-risk
- Corneal syngeneic graft (B6 to B6)
- Corneal inflammation
  - Intrastromal sutures

Apoptosis Detection

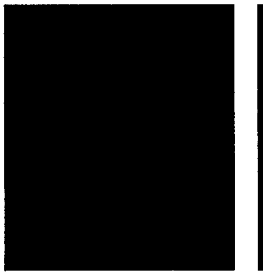
1. TUNEL assay
   - Labels 3'-OH ends of fragmented DNA that occur in apoptotic cells

2. Active Caspase-3
   - Effector/executioner caspase
   - Antibody to cleaved active form (not inactive precursor form)
   - Western blot detection

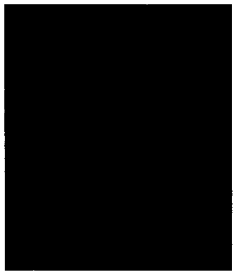
Normal Corneas

TUNEL

BALB/c

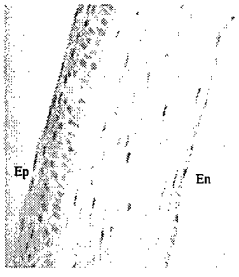


C57BL/6



H&E

BALB/c

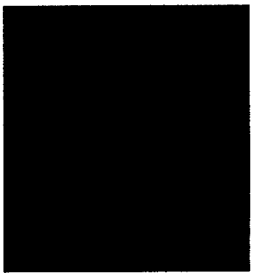


400X

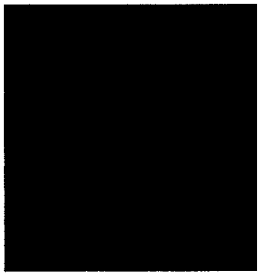
Ungrafted, Inflamed Corneas

TUNEL

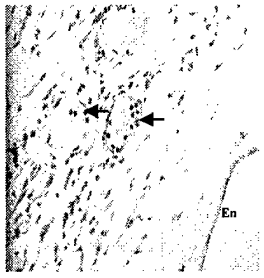
3 days



14 days



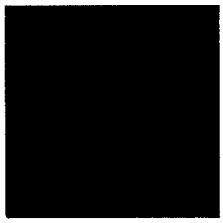
H&E



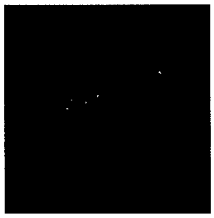
400X

Corneal Grafts

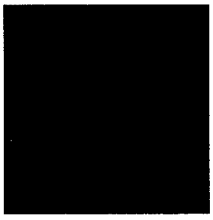
Low-Risk, Accepted



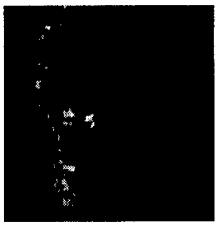
Low-Risk, Rejected



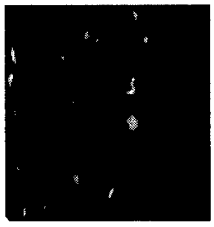
Syngeneic Graft



High-Risk, Accepted



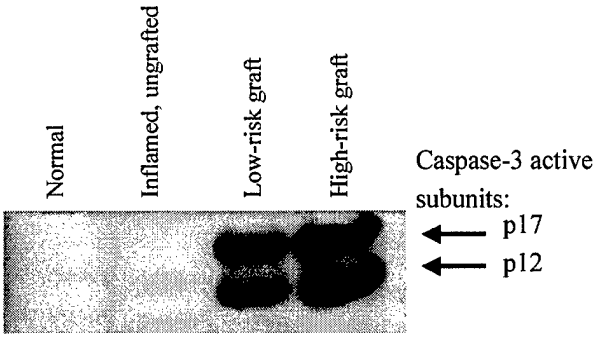
High-Risk, Rejected



400X

Active Caspase-3 Western Blot

Homogenized corneal tissue



Results

- Alloreactive infiltration of the cornea is correlated with apoptosis
- High-risk > Low-risk
- Rejected > Accepted

II. Cell types are undergoing apoptosis

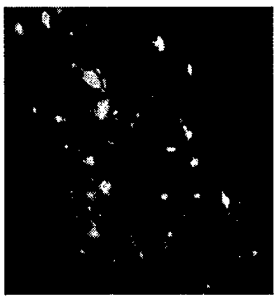
Double immunostaining:

TUNEL

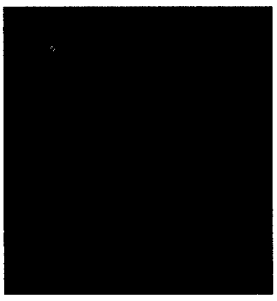
anti-CD45 (pan-leukocyte marker)

TUNEL (green) + CD45 (red)

High-Risk Graft



Inflamed, Ungrafted



400X

Results

- Only non-hematopoetic cells of the cornea undergo apoptosis
- Apoptosis correlates with alloimmunity, not with non-specific, suture-induced inflammation

III. Apoptotic Mediators in Cornea

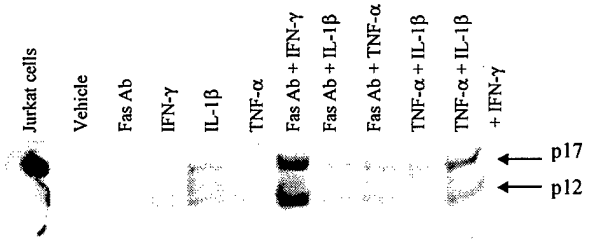
Cell lines:

- Mouse corneal endothelial cells
- Mouse keratocytes

Methods:

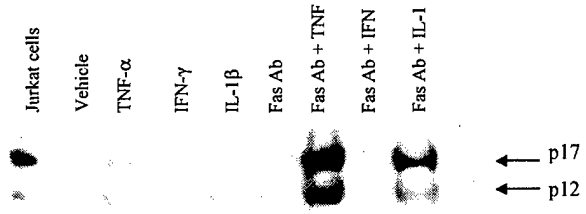
- Agonistic Fas MAb (JO-1), TNF-α, IL-1β, and IFN-γ added to cells
- Western blot for active caspase-3

Corneal Endothelial Cells



•24 hr incubations

Keratocytes
(MK/T-1 cell line)



•24 hr incubations

Results

- Neither cell line undergoes apoptosis in response to Fas MAb alone
- Synergistic effect of Fas MAb with pro-inflammatory cytokines in promoting apoptosis
- Differential response of the cell lines to pro-apoptotic factors

Summary

- Apoptosis occurs in allogeneic corneal grafts and is correlated with graft failure
- Only $CD45^-$ cells undergo apoptosis
- In vitro, there appears to be synergistic pro-apoptotic effect of Fas ligation and pro-inflammatory cytokines