

Posterior Segment Approach for Subretinal Transplantation or Injection in the Canine Model

Maria E. Verdugo,* Julie Alling,* Eliot S. Lazar,† Manuel del Cerro,†‡ Jharna Ray,* and Gustavo Aguirre*

*James A. Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853
Departments of †Ophthalmology and ‡Neurobiology and Anatomy, University of Rochester School of Medicine, Rochester, NY 14642

A posterior segment approach for cell transplantation or injection into the subretinal space of the dog has been developed. Controlled penetration to the subretinal space was achieved using a 29-gauge injection cannula, either blunted or with a 30° sharpened bevel, and partially ensheathed with moveable plastic tubing. Depending on the injection volume used, the retina detached, and the fluid was reabsorbed within 1–3 weeks, although for smaller volumes the retina reattached within a matter of days. The optimal injection volume used was between 100 and 150 μ l, or two injections of 55 μ l each. By ophthalmoscopy following the surgery, it was possible to serially monitor the injection site and retinal bleb through fundus photography. Light microscopy demonstrates the distribution of stable, viable RPE cells in the subretinal space up to 6 months. The transplantation technique developed for the dog is atraumatic and free from any major surgical or clinical complications. It can be readily used to deliver cells or fluids to localized regions of the subretinal space.

Key words: Animal model; Dog; Posterior segment; Retinal pigment epithelial transplantation; Subretinal injection; Retinitis pigmentosa

INTRODUCTION

Intraocular transplantation of reginal pigment epithelial (RPE) cells provides the prospect of restoring normal function to this cell layer in individuals having primary RPE dysfunction or degeneration, or where there is a photoreceptor abnormality secondary to an RPE defect. It also can be used to transplant genetically modified cells to either replace the function of a mutant gene or to deliver trophic factors to the interphotoreceptor space, which can promote the survival of diseased visual cells. The general surgical techniques for injection or transplantation into the subretinal space that are directed towards the treatment of retinal degenerative diseases have been undergoing constant evolution. During the last two decades, numerous investigators have transplanted RPE cells into the subretinal space of normal eyes, as well as into the eyes of experimental animals with retinal degenerations (10,18,22). The RPE transplantation has been done in several animal models such as rat (18), rabbit (21), monkey (10), and miniature pig (14), including xenografting of human RPE cells into rat

(19), monkey (10), and rabbit (12). Typically, these studies have involved delivering dissociated cell suspensions, sheets, or patches of RPE into the subretinal space.

The transplantation studies in the experimental models have, as their ultimate application, the use of similar treatment strategies in human patients. Primary diseases of the RPE in humans with secondary photoreceptor dysfunction or degeneration would be amenable to transplantation of normal cells or direct in situ delivery of vectors to transfer the normal cDNA and correct the underlying genetic defect. Dysfunction of RPE cells has been implicated in many, frequently untreatable, retinal diseases including age-related macular degeneration. Additionally, recently described mutations in genes expressed exclusively in the RPE of humans and animals [e.g., RPE65 (1,23), cellular retinaldehyde binding protein (CRALBP) (24) and retinol dehydrogenase (27)] make the specific treatment of this cell layer a therapeutic imperative.

Experimental studies have demonstrated that photoreceptor degeneration can be prevented or delayed in sev-

eral ways. For example, transplantation of healthy RPE cells into the subretinal space of RCS rats with photoreceptor degeneration secondary to a primary RPE defect resulted in the functional survival of the host photoreceptors (15,20). Similarly, intraocular injection of basic fibroblast growth factor or other cytokines delayed the photoreceptor degeneration in dystrophic RCS rats, and rescued photoreceptors in the light-damage rat model (8,16). Injection into the subretinal space of genetically modified cells to produce the desired trophic factors, recombinant viral vectors, or recombinant proteins could ensure prolonged availability of the particular substance lacking or deficient in the host photoreceptors or RPE cells. Delivery of the appropriate factors to diseased visual cells may thus provide a therapeutic benefit in a variety of currently untreatable diseases.

For surgical intervention of the subretinal space, three different approaches have been used previously: anterior trans-vitreous (7,14,21,26), posterior trans-scleral (18,26), and open sky (10). Lazar and del Cerro (17) developed a posterior injection technique in the rat that is complication free, allows controlled penetration of the subretinal space, and the placement of the graft is directly visualized through the pupil. This approach appeared of value for use in experimental animals such as the dog because it reduced the need for expensive and extensive instrumentation required by the anterior approach and, as well, avoided potential traumatic injury to the lens. In this study we report the modification of the posterior segment approach for use in dogs to deliver dissociated cells or soluble products to the subretinal space. These modifications were required because in the dog the globe is placed deeply in the orbit, surgical exposure is limited, and the sclera, conjunctiva, and periocular tunics are much thicker than in the rat.

MATERIALS AND METHODS

Experimental Animals and Anesthesia

A total of 15 healthy normal adult dogs, either beagles or crossbred, were used in the studies. All experimental procedures adhered to the guidelines of the ARVO Resolution on Use of Animals in Ophthalmic and Vision Research. The dogs were sedated by intramuscular injection of acepromazine maleate (0.1 mg/kg) administered with atropine sulfate (0.04 mg/kg), and anesthetized by the intravenous injection of ketamine hydrochloride (2 mg/kg) and diazepam (0.2 mg/kg). To maintain anesthesia, the dogs were intubated and kept at a surgical plane of anesthesia with halothane (1.5–2.5%); the dose of halothane was adjusted depending on the size of the dog, tidal volume, and frequency of respiration. Three dogs had bilateral subretinal injections of India ink and 12 dogs had subretinal injections of cultured and dissociated cells. Depending on the study,

injection volumes ranged between 10 and 200 μ l; these volumes were well within the limits tolerated by the eye as the vitreous volume of the normal adult dog eye ranges between 2.5 and 3.2 ml (4). Of the group receiving injections of cultured and dissociated cells, two dogs had RPE autografts from cells collected from the fellow enucleated eye and 10 received allografts (RPE cells or choroidal fibroblasts) of cells obtained from various canine donors; eight dogs received bilateral injections and two received unilateral injections. Note that with the exception of one dog that received subretinal injection of RPE cells in one eye and choroidal fibroblasts in the other eye (Table 1, animal #7), all of the remaining dogs had injections of RPE cells. No attempts were made to tissue type the donors and recipients to optimize the match and avoid potential host vs. graft reaction. Details of the experimental animals and protocols are summarized in Table 1.

Donor Cell Harvest and Preparation of RPE for Transplantation

Eyes from normal dogs were obtained from other investigators at the end of their studies and were used as donors for the allografts. All eyes were collected in ice-chilled $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Puck's saline F (0.127 M NaCl, 3.82 mM KCl, 1.261 mM Na_2HPO_4 , 0.61 mM KH_2PO_4 , 0.1 mM EDTA) containing antibiotics (penicillin, streptomycin, and gentamycin). For autologous transplantation surgery, RPE cells were harvested from one enucleated eye, processed in the same manner as allograft cells, and transplanted into the fellow eye. The RPE cells from donors eyes (allograft or autograft) were harvested and cultured using the method of Ray et al. (25). Cells were dissociated by repeated trypsinization, rinsed twice with 15% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA) containing Dulbecco's modified Eagle medium [DMEM, with high glucose (4500 mg/L): L-glutamine, pyridoxine hydrochloride without sodium pyruvate or sodium bicarbonate] (Life Technologies, Gaithersburg, MD), and plated at a seeding density of $2\text{--}3 \times 10^5$ cells/35-mm dish. Cells were grown in DMEM containing 15% FBS, 2.5% glutamine, and 1% amphotericin B (Life Technologies) and maintained in a humidified atmosphere at 37°C in the presence of 95% air/5% CO_2 . At confluence cells were passaged (1:3 ratio), and first passage cells (P1) were grown until they reached to approximately 70% confluency.

For transplantation, the 70% confluent P1 cells were dissociated with 0.25% trypsin, washed two times with DMEM, and resuspended in DMEM for injection. Cell viability was determined using the trypan blue (Sigma, St. Louis, MO) exclusion test (5). Briefly, 10 μ l of cell suspension was mixed with 40 μ l of 0.4% trypan blue, kept at room temperature for 2–5 min, then a 12 μ l ali-

quot was placed in a Neubauer Hemocytometer slide chamber and counted for the blue and the colorless cells. Colorless cells, which excluded trypan blue, were indicative of live cells, while blue cells were dead; in general, the percentage of dead cells was between 7% and 8%. The numbers of cells and subretinal volumes injected are detailed in Table 1.

Delivery System and Transplantation Procedure

Prior to anesthesia and surgery, the pupils were dilated with 1% tropicamide (Alcon Laboratories, Fort Worth, TX), 1% atropine sulfate (Optopics Laboratories, Fairton, NJ), and 2.5% phenylephrine hydrochloride (Henry Schein, Florham Park, NJ). To obtain the best possible visualization during surgery, the dog's head was fixed with a vacuum pack immobilizer and positioned under a high-resolution operating microscope. Throughout the surgery aseptic conditions were followed.

The delivery system consisted of a custom made 29-gauge cannula (Popper & Sons Inc., New Hyde Park, NY), partially ensheathed with a tight plastic tubing, and connected to a Kloeohn microsyringe (Kloeohn Ltd., Brea, CA) by a 0.38-mm-diameter polyethylene tubing (Becton Dickinson and Company, Sparks, MD), and a 29-gauge blunt adapter needle (Kloeohn Ltd., Brea, CA). For injection volumes less than 50 μ l, we used a 50- μ l syringe; for greater volumes a 200- μ l syringe was used. The sheath used on the cannula was made from the same 0.38-mm polyethylene tubing, and exposed the 3–4 mm of the tip, thus limiting the depth of penetration. The length of the sheath was adjusted on the basis of the dog's age and estimated scleral thickness to allow the cannula to reach subretinal space without penetrating the retina. Such penetration could cause damage to the retina and vitreous, forming holes or retinal tears that can leak transplanted cells or injected fluids into the vitreous cavity. In the first seven surgeries, the 30° sharpened beveled end of the cannula was used; in subsequent surgeries, the rounded blunt end was used to avoid accidental penetration of the retina. Figure 1 is a schematic representation of the injection instruments and placement of the cannula in the subretinal space.

To expose the transplantation site, a Barraquer wire eyelid retractor was used followed by a lateral canthotomy and a fornix-based conjunctival peritomy. The eye was rotated ventrally, exposing the superior rectus muscle, and a 4-0 Dermalon suture (Davis-Geck, Danbury, CT) was placed around the muscle for stabilization. A focal conjunctival peritomy was made 15–17 mm behind the corneo-scleral junction, and bleeding was controlled by pinpoint cautery. A one third scleral flap was dissected with a 45° K-Sharp knife (Katena Products Inc., Denville, NJ) to provide a window at the

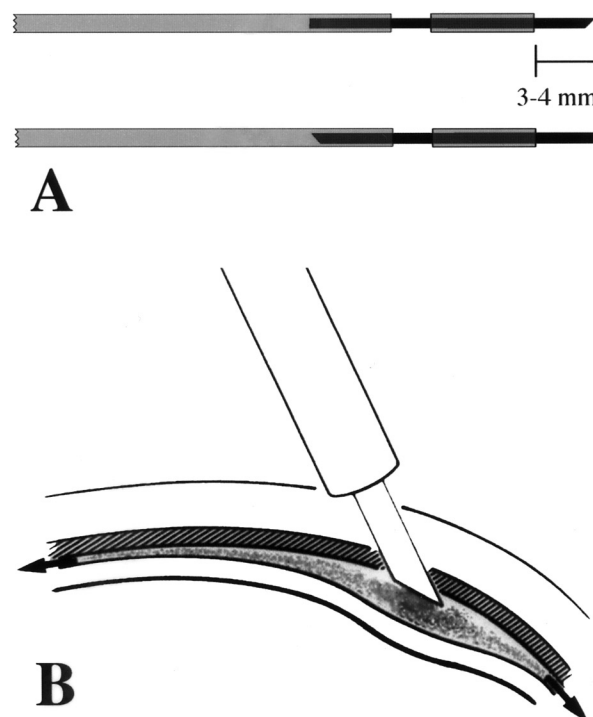


Figure 1. (A) Illustration of the delivery system used for subretinal injections. The 29-gauge cannula has a polyethylene tubing sheath near the tip to control the depth of penetration. A 0.38-mm-diameter polyethylene tube connects the cannula to the injection syringe adapter (not shown). The beveled and blunt orientations of the cannula are shown. (B) Placement of the beveled point of the injection cannula in the subretinal space. Depth of penetration is controlled by the plastic sheath around the cannula, and the direction of fluid flow is illustrated by the arrows.

transplantation site. The thin floor of the flap formed a translucent membrane through which the choroid could be visualized. The blunt tipped or beveled cannula was manually inserted through the floor of the scleral flap and gently rotated in order to penetrate to the desired depth, and then the injection was carried out. In general, resistance to the needle penetration was present primarily at the scleral surface and, to a much lesser extent, at the RPE/Bruch's membrane interface. Successful penetration of this latter layer allowed the easy release of the injection volume with gentle manual pressure of the syringe plunger. Depending of the study, the volume injected varied between 10 and 200 μ l/injection site, and there was minimal reflux if the injection rate was slow, gradual, and volumes less than 150 μ l were used. The superior half of the eye was favored because in the dog this area has a nonpigmented RPE overlying the reflective tapetum lucidum. As a result, it is easier to examine and photograph the injection site(s). However, we have done the same procedure in the inferior retinal quadrants

with no variation in the method or in the results (see Table 1 for details).

Following the injection, the scleral flap was sutured with nonabsorbable 6-0 Dermalon (Davis-Geck, Danbury, CT) to identify the external site of injection once the eye was enucleated, and the canthotomy was closed with 6-0 Dermalon nonabsorbable sutures in a simple interrupted pattern. Immediately following surgery, 2 mg of triamcinolone acetonide (Kenalog-10; Bristol-Myers Squibb Company, Princeton, NJ) was injected subconjunctivally one time to prevent postoperative inflammation. The postoperative treatment consisted of oral Clavamox (11 mg/kg), topical ophthalmic antibiotic/steroid ointment and solution (neomycin, polymyxin B sulfate, and dexamethasone, Altana Inc., Melville, NY), administered 2 times a day for 1 week. Other topical medications (e.g., atropine sulfate 1%, gentamicin, or prednisolone acetate 1%) were used in the postoperative period if there was ocular inflammation (atropine and prednisolone acetate) or if conjunctivitis was unresponsive to the topical antibiotic/steroid medications used.

Clinical Evaluations

The injection site was evaluated immediately after surgery using the operating microscope and a noncontact Super Field fundus lens (Volk, Mentor, OH). Following surgical intervention, animals were routinely examined at several points during the course of the study. In general, dogs were examined during the first week, weekly-biweekly for the first 4–6 weeks, and monthly thereafter. In each case, the clinical exam included evaluation of the anterior segment and a complete funduscopic examination using a Keeler binocular indirect ophthalmoscope (Keeler Instruments, Broomall, PA) and a Volk 2.2 pan-retinal fundus lens. Direct ophthalmoscopy and biomicroscopy were used when necessary. Fundus photography was performed using Kodachrome 25 film (Eastman Kodak Company, Rochester, NY) and a Kowa RC-2 fundus camera (Kowa Company, Ltd., Japan) was used directly or indirectly through the Volk 2.2 or Nikon 20 diopter lenses.

Histological Procedures

The dogs were euthanatized with an overdose of sodium pentobarbital administered intravenously, and the eyes were immediately enucleated and all adhering extraocular muscles removed. The anterior segment was removed with a single-edge razor blade that hemisected the eye behind the lens, approximately 4–5 mm behind the limbus. The eyecup was fixed with chilled 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for a minimum of 2–6 h, rinsed with the phosphate buffer, trimmed, and dehydrated through graded ethanol con-

centrations before processing for embedding in JB-4 (Sigma). The embedded retinal strips were sectioned at 3 μ m with tooled steel knives in an AO Spencer 820 Microtome; after flattening in a 68–80°F water bath, the sections were transferred to a slide, heated (120°F for 5–10 min), and stained with methylene blue/Azure II. The sections were mounted with Permount (Fisher Scientific, Fair Lawn, NJ), coverslipped, and evaluated and photographed with a Zeiss Axioplan light microscope (Zeiss, Inc., White Plains, NY).

RESULTS

Clinical Assessment

The injection procedure was well tolerated in the 12 eyes that received subretinal cell injections. Postsurgical observation periods ranged from 0 days to 24.5 weeks with a median survival time of 5 weeks (Table 1). Mild conjunctivitis was noted in five eyes (three dogs), and a small subconjunctival hemorrhage was present in one eye of a different animal; these generally resolved within 1–2 weeks following surgery. Transient, low-grade anterior uveitis and vitreal flare was found in four eyes (two dogs), and vitreal condensations or strands were noted unilaterally in four other dogs. Cataracts did not develop as a result of the surgical manipulation or injection.

Because most of the injections were made through the tapetum lucidum in the superior quadrant, the hallmark finding of the injection was the instantaneous focal color change in the tapetal layer as the result of penetration of the injection cannula. This lesion appeared black, presumably the result of a focal change in the cellular organization of this layer, and this color change was evident ophthalmoscopically regardless of whether the injection volume was media or cells (data not shown). The appearance of the fundus immediately following the injection was dependent on the volume of fluid injected. Apart from the focal tapetal puncture wound, injection volumes of 10 μ l were not visible clinically. Small blebs resulted from volumes of 50 μ l, and these usually resorbed in 2–3 days (Fig. 2). Medium-sized blebs resulted from injections of 100 μ l, which resorbed within 1 week. Retinal folds were associated with the peripheral border of small and medium-sized blebs, and these flattened out with fluid resorption from the subretinal space. Larger injection volumes (e.g., 150 and 200 μ l) resulted in very large blebs that occupied one quarter to one half of the subretinal expanse and, in some cases, resulted in an increased intraocular pressure immediately after injection.

The appearance of the injection site was dependent on the number of RPE cells injected, their level of pigmentation, which is very variable in the dog, and whether the cells were dispersed throughout the subreti-

Table 1. Details of the Animals and Methods Used in the Subretinal Injection Studies

Animal No./Eye	Volume/Injection	No. of Injections and Site	Total No. RPE Cells/Eye	Postinjection Period
India ink				
#1/OD	10 μ l	4 (2 sup./2 inf.)	—	0 days
#1/OS	10 μ l	4 (2 sup./2 inf.)	—	0 days
#2/OD	10 μ l	2 (sup.)	—	0 days
#2/OS	10 μ l	2 (sup.)	—	0 days
#3/OD	40 μ l	2 (1 sup./1 inf.)	—	0 days
#3/OS	40 μ l	2 (1 sup./1 inf.)	—	0 days
RPE autografts				
#4/OS	10 μ l	2 (1 sup./1 inf.)	4.5×10^5	5.1 weeks
#5/OD	55 μ l	2 (sup.)	1×10^5	16 weeks
RPE (or CF) allografts				
#6/OD	100 μ l	1 (sup.)	1.4×10^5	0 days
#6/OS	200 μ l	1 (sup.)	1.4×10^5	0 days
#7/OD	125 μ l	2 (sup.)	1×10^6	3 weeks
#7/OS	125 μ l	2 (sup.)	1×10^6 (CF)	3 weeks
#8/OD	10 μ l	2 (sup.)	3.8×10^5	3.2 weeks
#8/OS	10 μ l	2 (sup.)	5.2×10^5	3.2 weeks
#9/OD	10 μ l	2 (1 sup./1 inf.)	1.5×10^6	4 weeks
#9/OS	20 μ l	2 (1 sup./1 inf.)	1.5×10^6	4 weeks
#10/OD	40 μ l	2 (sup.)	4.6×10^6	5 weeks
#10/OS	50 μ l	2 (sup.)	4.6×10^6	5 weeks
#11/OD	150 μ l	1 (sup.)	1×10^6	5 weeks
#12/OD	10 μ l	2 (1 sup./1 inf.)	6.5×10^5	6 weeks
#13/OD	15 μ l	2 (1 sup./1 inf.)	9.5×10^5	7.1 weeks
#13/OS	15 μ l	2 (1 sup./1 inf.)	9.5×10^5	7.1 weeks
#14/OD	40 μ l	2 (1 sup./1 inf.)	5.6×10^5	21 weeks
#14/OS	50 μ l	2 (1 sup./1 inf.)	5.4×10^5	21 weeks
#15/OD	25 μ l	2 (1 sup./1 inf.)	5×10^5	24.5 weeks
#15/OS	25 μ l	2 (1 sup./1 inf.)	5×10^5	24.5 weeks

OD: right eye; OS: left eye. sup.: superior quadrant; inf.: inferior quadrant. CF: choroidal fibroblasts.

nal bleb or were clustered at the site of injection. When using low injection volumes and a high concentration of RPE cells, the cells formed a darkly pigmented cluster in the subretinal space; larger volumes with similar concentration of cells dispersed the cells over a larger area of the subretinal space and effectively eliminated the cellular aggregates found with the lower volumes (Fig. 2, compare A, B with C, D). Moreover, the presence of hemorrhage, either subretinal (six eyes of five dogs) or vitreal (one eye), influenced the appearance of the injection site. In all cases, the blood was resorbed within 1–2 weeks and was never larger than the example illustrated in Figure 2A. Serial examination of the eyes during the

postinjection period did not demonstrate changes in the size or appearance of the transplant in animals that received either autografts or allografts, and there was no retinal or choroidal edema or inflammation, which could have resulted from an immune-mediated response to the transplanted cells.

In 5 of the 12 eyes, pigmented cells were found on the retinal surface or posterior vitreous. In three of the eyes, the cells formed very small aggregates in the posterior vitreal cortex or along strands of vitreous. In one case, a focal cluster of RPE cells accumulated on the retinal surface (Fig. 3). The presence of these cells on the retinal surface or vitreous indicated partial or full

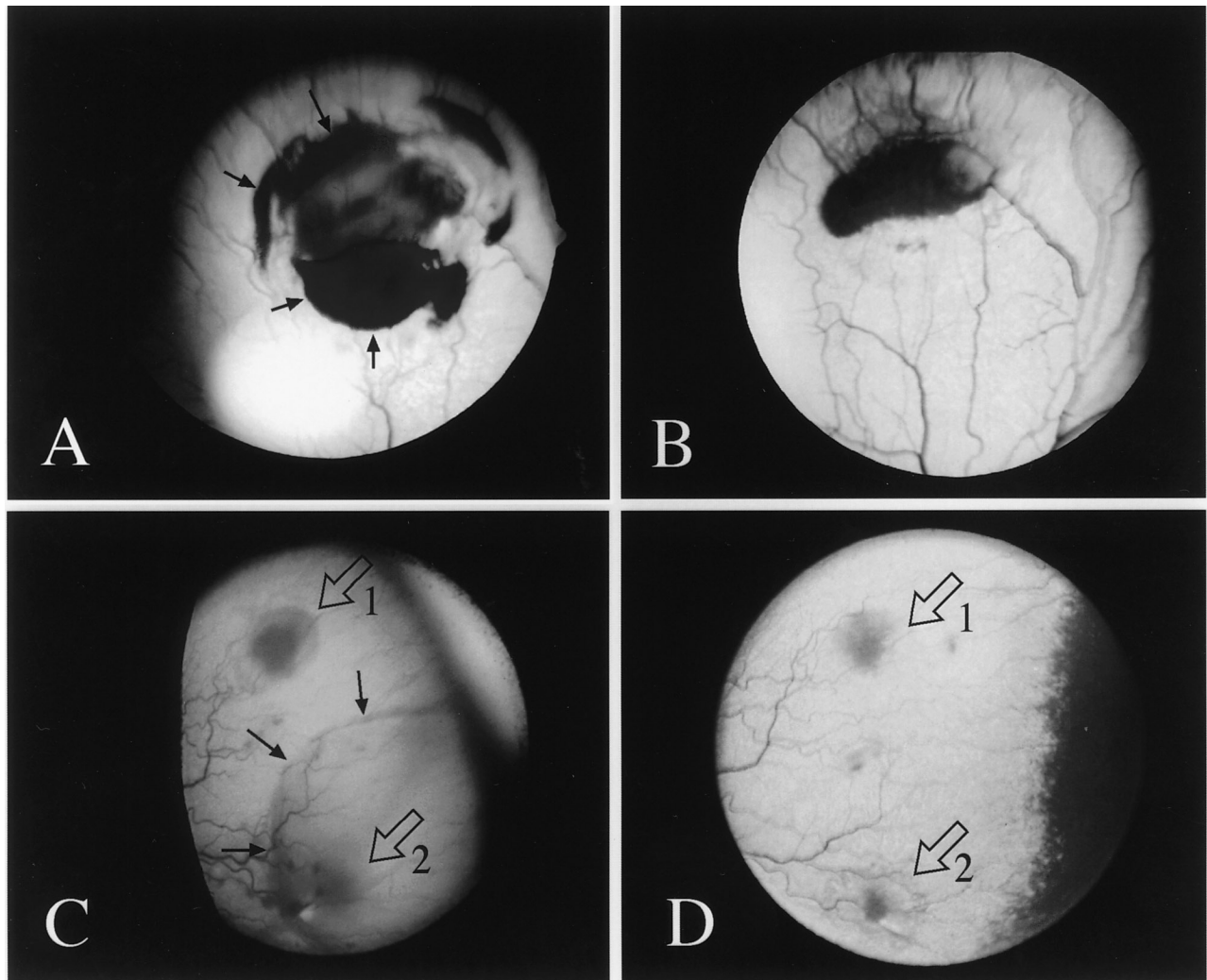


Figure 2. Fundus photographs of experimental animals at different postinjection intervals. (A, B) Dog #13 at 2 days (A) and 7.1 weeks (B) following injection of RPE cells (allograft) in a 15 μ l volume. At the early time point there is a darkly pigmented subretinal lesion resulting from the injection. Surrounding the transplanted cells is a zone of subretinal edema and hemorrhage (arrows), which disappears by the 7.1 week time point without any alteration in the surrounding retina. (C, D) Dog #5 at 2 days (C) and 2 weeks (D) following injection of autologous RPE cells in a 55 μ l volume. Two injection sites are visible on the second postoperative day, and these are located beneath a fluid bleb (C, small arrows). By 2 weeks the fluid has been resorbed and the retina is flat.

retinal penetration by the injection cannula. Serial examination and photography of these eyes indicated that the small vitreal pigment cell clusters did not change in size or extend into the retinal surface and/or vitreous in the animals with postinjection survival periods of 3, 5, 21, and 24.5 weeks. In contrast, the one eye that received allografts of choroidal fibroblasts (subretinal injection with secondary vitreal extension) showed by 3 weeks postinjection retinal traction that resulted from fibroblast proliferation along the vitreal cortex and retinal surface (data not shown).

Histologic Results

In developing the surgical technique, we initially injected India ink as a marker. Injections resulted in the separation of the retina from the pigment epithelium and the accumulation of injected fluid volume in the subretinal space. There was minimal damage to the retina adjacent to the injection site (Fig. 4A, B). In the absence of retinal penetration or reflux around the scleral puncture, the size of the subretinal bleb was dependent on the volume of fluid injected. Similar results were obtained in short-term injections of RPE cells suspended in DMEM

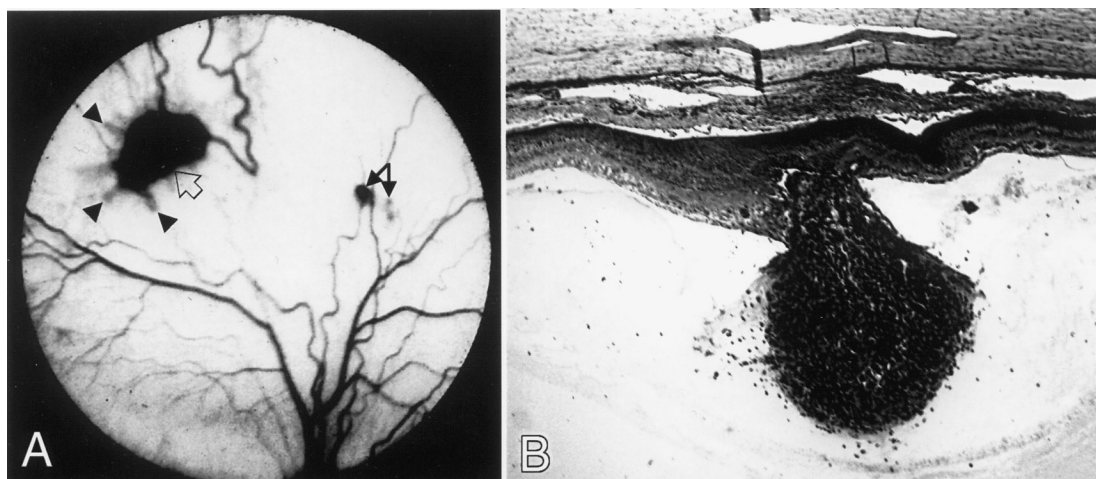


Figure 3. Fundus photograph and photomicrograph of the right eye of dog #7, 3 weeks after the injection of RPE cells. (A) Accidental penetration of the retina resulted in a preretinal cluster of pigmented cells (large open arrow) that obscures the underlying retina and vessels; retinal folds (arrowheads) are at the margins of the pigment cell cluster. A smaller group of pigmented cells (small arrows) is located over the posterior vitreous over two retinal vessels. (B) Histological section showing the cluster of RPE cells attached to the inner retinal surface. Note that the pigment cells remain well circumscribed without extension into the retina or vitreous (130 \times).

using larger fluid volumes. The separation of the retina from the RPE occurred at the tip of the photoreceptor outer segments, and caused minimal to no retinal damage either at the injection site or in adjacent areas (Fig. 5A, B). Because of the larger fluid volumes used, and loss of cells during tissue processing, we could not identify the transplanted cells in the subretinal space.

With postinjection intervals of 3 weeks or longer, cells were readily identified in the subretinal space, which had the cytologic characteristics of RPE and likely were of donor origin. Very rarely the cells formed a partial monolayer located between the host RPE and retina (Fig. 6A). More frequently, single or isolated clusters of RPE cells were found subretinally in the previously detached regions; again, these cells were interposed between the host RPE cells and the photoreceptors (Fig. 6B, C). Because cell marking studies were not carried out, it was not possible to determine if the transplanted cells replaced the host RPE cells on Bruch's membrane. In parallel with the clinical observations, we did not find vasculitis of the preretinal vessels nor cellular infiltration of mononuclear cells in the choroid, either at the transplant site or elsewhere, that could represent a rejection response to the transplant. The two eyes receiving RPE autografts showed the same response as those that received allografts.

The most common surgical complication was penetration of the retina by the injection cannula. At these sites the transplanted RPE cells formed cellular aggregates attached to the retinal surface (Fig. 3B). These cell clusters remained aggregated and did not extend into the adjacent retina. In cases where the retina was penetrated,

but the injection cannula was withdrawn without injection, a focal retinal scar formed, often with limited fibrous connective tissue that extended from the overlying choroid (Fig. 7). The retina adjacent to these focal scars was normal.

DISCUSSION

This study demonstrates the development of a posterior approach for subretinal injection or cell transplantation in the canine model. The procedure is atraumatic and has minimal morbidity; long-term observation of those animals that survived for nearly 6 months has indicated no major complications, either in the immediate postoperative period or later. This procedure is potentially adaptable for use in other large animal models (e.g., primates and cats), which are commonly used in ophthalmic research studies.

Studies requiring access to the subretinal space have used either anterior or posterior approaches. The anterior approach is favored by surgeons working with nonrodent eyes, because direct visualization of the placement of the injection cannula in the subretinal space allows for greater success in placing the transplanted cells (3,6,21). In addition to the greater need for surgical instrumentation and dexterity, the major limitations of the anterior approach include damage to the photoreceptors and nerve fiber layer and the presence of a retinal hole that can leak RPE cells into the vitreous where they might eventually support proliferative vitreoretinopathy. The posterior or transcleral approach has been successfully used in RPE transplantation studies to prevent photoreceptor degeneration in RCS rats (18,19). Wong-

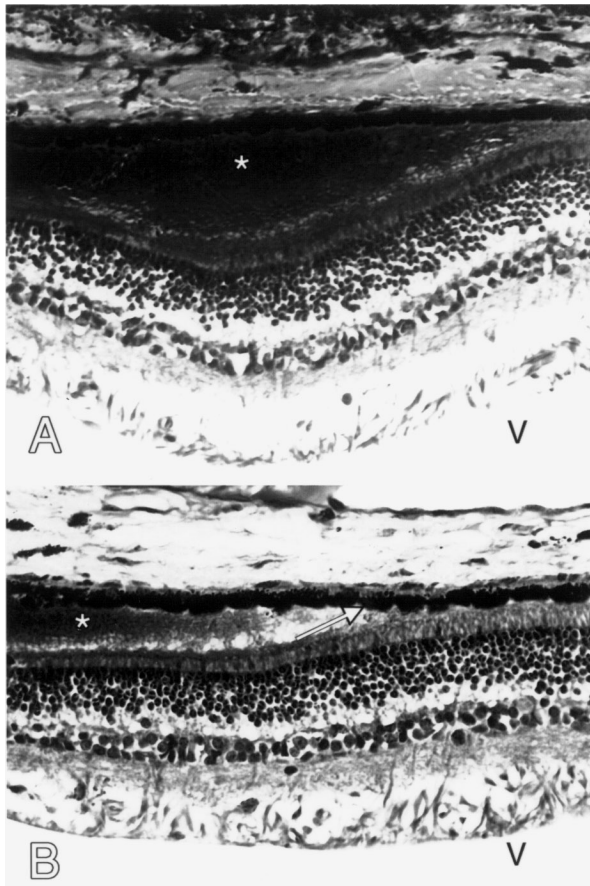


Figure 4. Photomicrographs of an eye immediately following the subretinal injection of 10 μ l of India ink. (A) The fluid (*) is confined to the subretinal space with a gradual elevation of the retina. (B) The edge of the subretinal bleb shows the minimal damage that has occurred away from the injection site. The India ink is confined to the subretinal space, and the vitreous (V) is free of injection product. (A = 250 \times).

pichedchai et al. (26) compared results of RPE transplantation in rabbits using the internal and external approaches and found that the external approach resulted in better grafts, although the lack of direct visualization was a limitation.

Lazar and del Cerro (17) have developed a precise means of accessing the subretinal space of rats for transplantation or injection. Their method is based on the principle of using a preset stop to limit penetration of the needle, and direct visual control through the dilated pupil of the host eye. The eye is maintained in the primary gaze position, and, because of the optical properties of the rat eye, one can directly monitor the penetration of the needle into the subretinal space and the formation of the bleb through the operating microscope. Such a method has the added advantage of allowing real-time photography or video to document the transplantation procedure.

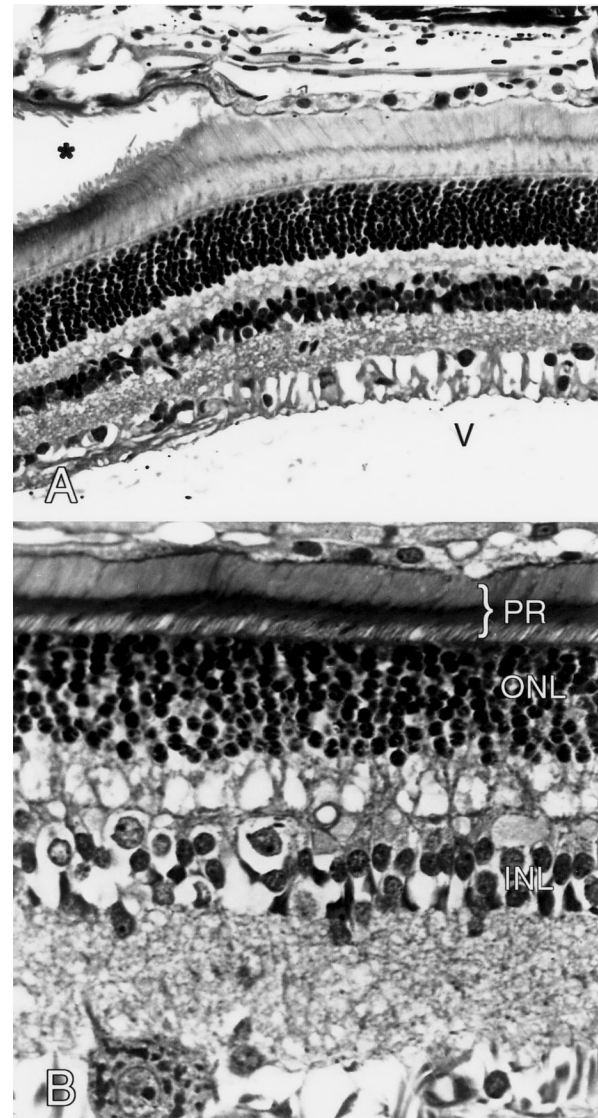


Figure 5. Photomicrographs of an eye immediately following the injection of 100 μ l of RPE cells in DMEM. (A) The fluid (*) has separated the retina from the RPE, but no transplanted RPE cells are visible at the edge of the subretinal bleb. (B) Away from the detachment site, the retina is intact. V, vitreous cavity; PR, photoreceptor layer; ONL, outer nuclear layer; INL, inner nuclear layer. (A = 250 \times ; B = 500 \times).

We have modified this technique for use in a larger species such as the dog, and have found it to provide consistent results once the technique is mastered. However, the lack of direct visualization during the injection procedure is a major limitation. Unlike the rodent, whose readily mobile and anteriorly placed eye allows access to the posterior sclera, the dog eye is large and deeply set in the orbit. In order to access the posterior surface of the globe for injections, the eye must be rotated away from the site of injection. Thus, the injections

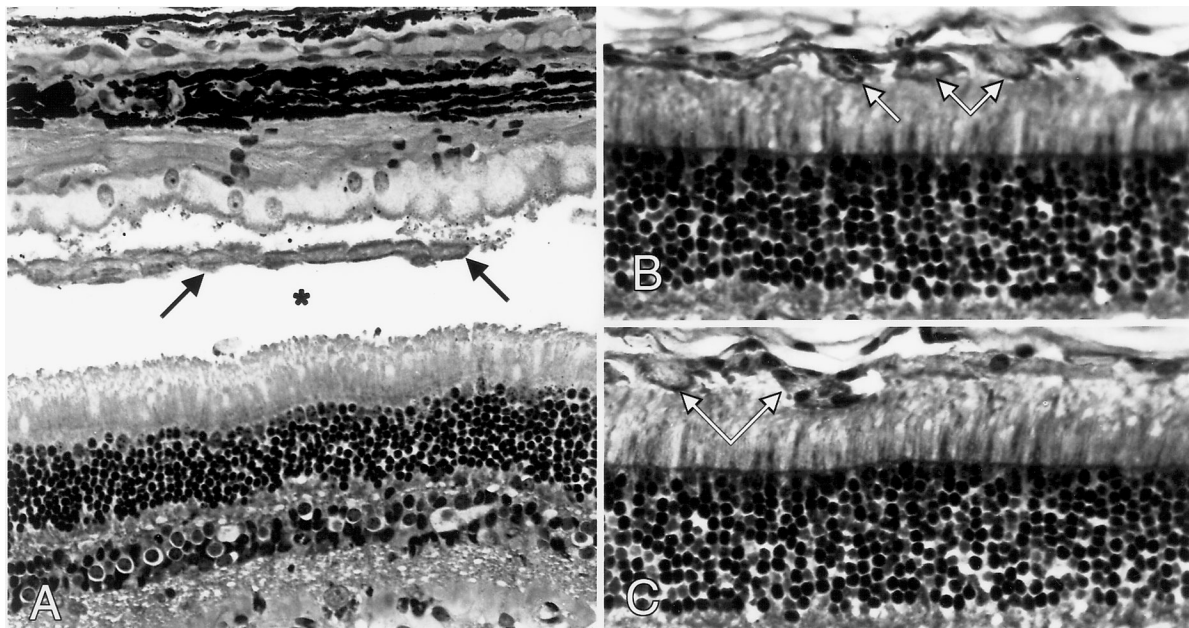


Figure 6. Photomicrographs of dogs following subretinal injection of RPE cells, which survived for periods of 3 weeks or longer. (A) Monolayer of transplanted cells (arrows) is present in the subretinal space (*) between the host RPE and the retina. The normal photoreceptor integrity indicates that the separation of the retina is a processing artifact. (B, C) Transplanted RPE cells (white arrows) located in the subretinal space adjacent to the host RPE. (A = 250 \times ; B, C = 400 \times).

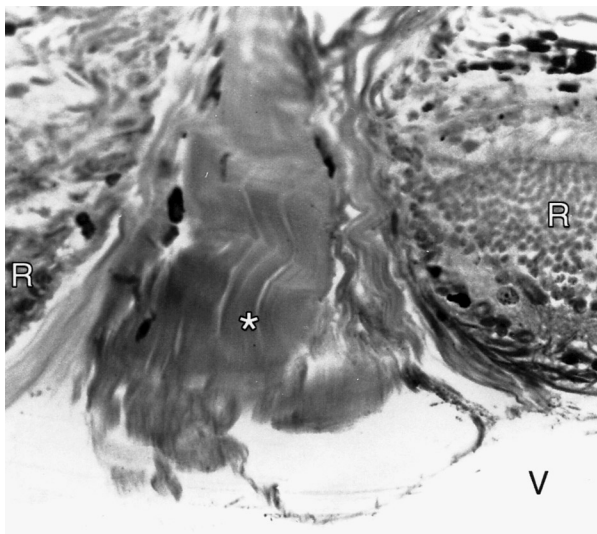


Figure 7. Photomicrograph illustrating a focal retinal scar following penetration by the injection cannula. Connective tissue extends from the choroid and fills the retinal defect (250 \times).

are made by “feel” (i.e., sensing the resistance to the needle penetration as it goes first through the base of the scleral flap and subsequently through Bruch’s membrane). The lack of direct observation resulted in the puncture of the retina in the initial surgeries even when the plastic sheath around the injection cannula was set

to limit the depth of needle penetration. Subsequent surgeries used the blunt end of the injection cannula for tissue penetration, and, together with the plastic sheath near the tip of the injection cannula, this prevented the puncture of the retina as the needle entered the subretinal space.

Even when the retina is penetrated and RPE cells gained access to the vitreous cavity, we found no cellular proliferation or secondary complications in the animals. Although labeling to identify cell division was not used, the RPE cell cluster that formed immediately after the injection remained stable in size, did not extend into the adjacent retina or vitreous cortex, and did not show any evidence of proliferative activity and retinal traction, such as was produced when autologous choroidal fibroblasts were used. This situation appears quite different from the human, where vitreal RPE proliferation results in retinal membranes and proliferative vitreoretinopathy (11,13). One possible difference is that we used normal eyes from healthy young adult animals for these studies, and there was no evidence of concurrent retinal or vitreal disease prior to the injection or transplantation procedure. In contrast, proliferative vitreoretinopathy develops in older human eyes that have abnormalities of the vitreous, retinal detachment, or other retinal surgeries and, as a result, may be predisposed for this devastating complication (9,13).

We have used a large range of injection volumes with

or without RPE cells. A volume of 10 μ l resulted in a nondetectable retinal bleb that covered a minimal retinal expanse. Volumes of 150–200 μ l were much more effective in spreading through the subretinal space but, especially for injections of 200 μ l, resulted in transient increases in intraocular pressure. If such large volumes are required, then decompression of the globe by anterior chamber paracentesis could be readily performed following the subretinal injection. From these results, we estimate that a volume of 50–75 μ l injected into the subretinal space in each of four quadrants (supero nasal and temporal; infero nasal and temporal) would be required to distribute the fluid over most of the subretinal expanse. Such a volume would likely result in a complete serous retinal detachment. However, based on prior observations in dogs with total serous detachments secondary to acute hypertension, reattachment of the retina without secondary complications would be expected to occur within 1–2 weeks following the injection (2).

The distribution of transplanted RPE cells within the subretinal space was more dependent on the injection volume than the cell concentration. Low volumes resulted in deeply pigmented subretinal RPE cell foci, while larger volumes showed more uniform distribution of small numbers of RPE cells. In this case, cells were found throughout the interphotoreceptor space adjacent to the RPE layer. Very rarely was a monolayer of donor RPE cells present in the subretinal space, and, additionally, we did not identify the presence of transplanted pigmented RPE cells that had replaced donor cells on Bruch's membrane. Although donor cell labeling was not done before transplantation, a prerequisite for identifying donor vs. host cells, similar studies using normal or β -glucuronidase (GUSB)-corrected RPE cells transplanted into GUSB-deficient recipients have shown the same results (Verdugo et al., unpublished results). That is, the donor cells do not fully repopulate Bruch's membrane, but are located as single or multiple cell clusters in the interphotoreceptor space. In regards to the transplantation of RPE cells, the surgical approach we have developed would be more applicable to returning to the subretinal space cells that could secrete trophic factors or act as enzyme pumps to release to the extracellular environment enzymes that are deficient in the host RPE cell layer. Additionally, the method is also applicable for the subretinal delivery of fluids or viral vectors. It will require further development of the technique to have the transplanted RPE cells replace, to a large extent, the host population of cells.

Zhang and Bok (28) have shown that transplanted RPE cells in rats that have incompatible MHC haplotypes with the host are slowly rejected even though there is no histologically evident acute immune rejection. In this study, however, whether the transplanted cells were

located in the subretinal space or accidentally injected into the vitreous cavity, there was no evidence of immune rejection of RPE cells. Neither was there evidence of retinal perivasculitis, edema, or cellular infiltration, either at the transplantation site or further away. This lack of response to RPE allografts was similar to what was observed in the two RPE autographs. Because no effort was made to tissue type the donor and host to maximize tissue compatibility, it is possible that the lack of response could indicate that in the dog model the transplant sites were immunologically privileged.

ACKNOWLEDGMENTS: This work was supported by NEI/NIH grants EY11142, 13132, and 06855, and the Foundation Fighting Blindness. The authors are grateful to Sue Pearce-Kelling for supervision of the histologic studies, and to Dr. Sinoj John for expert assistance with graphic programs.

REFERENCES

1. Aguirre, G. D.; Baldwin, B.; Pearce-Kelling, S.; Narfström, K.; Ray, K.; Acland, G. M. Congenital stationary night blindness in the dog: Common mutation in the RPE65 gene indicates founder effect. *Mol. Vision* 4:23–29; 1998.
2. Aguirre, G. D.; Gross, S. L. Ocular manifestations of selected systemic diseases. *The Compendium on Continuing Education* 2:144; 1980.
3. Alverez P.; Berglin L.; Gouras P.; Sheng Y.; Kopp, E. Transplantation of RPE in age-related macular degeneration: Observations in disciform lesions and dry RPE atrophy. *Graefes Arch. Clin. Exp. Ophthalmol.* 235:149–158; 1997.
4. Buyukmihci, N.; Aguirre, G. D. Rod disc turnover in the dog. *Invest. Ophthalmol.* 15:579–584; 1976.
5. Castillo, B. V., Jr.; Little, C. W.; del Cerro, C.; del Cerro, M. An improved method of isolating fetal human retinal pigment epithelium. *Curr. Eye Res.* 14:677–683; 1995.
6. Das, T.; del Cerro, M.; Jalali, S.; Rao, V. S.; Gullapalli, V. K.; Little, C.; Loreto, D. A.; Sharma, S.; Sreedharan A.; del Cerro, C.; Rao, G. N. The transplantation of human fetal neuroretinal cells in advanced retinitis pigmentosa patients: Results of a long-term safety study. *Exp. Neurol.* 157:58–68; 1999.
7. Dudus, L.; Anand, V.; Acland, G. M.; Chen, S. J.; Wilson, J. M.; Fisher, K. J.; Maguire, A. M.; Bennett, J. Persistent transgene product in retina, optic nerve and brain after intraocular injection of rAAV. *Vision Res.* 39:2545–2553; 1999.
8. Faktorovich, E. G.; Steinberg, R. H.; Yasumura, D.; Matthes, M. T.; LaVail, M. M. Photoreceptor degeneration in inherited retinal dystrophy delayed by basic fibroblast growth factor. *Nature* 347:83–86; 1990.
9. Glaser, B. M.; Vidaurri-Leal, J.; Michels, R. G.; Campochiaro, P. A. Cryotherapy during surgery for giant retinal tears and intravitreal dispersion of viable retinal pigment epithelial cells. *Ophthalmology* 100:466–470; 1993.
10. Gouras, P.; Flood, M. T.; Kjeldbye, H.; Bilek, M. K.; Eggers, H. Transplantation of cultured human retinal epithelium to Bruch's membrane of the owl monkey's eye. *Curr. Eye Res.* 4:253–265; 1985.
11. Grisanti, S.; Guidry, C. Transdifferentiation of retinal pigment epithelial cells from epithelial to mesenchymal phenotype. *Invest. Ophthalmol. Vis. Sci.* 36:391–405; 1995.

12. He, S.; Wang, H.-M.; Ogden, T. E.; Ryan, S. J. Transplantation of cultured human retinal pigment epithelium into rabbit subretina. *Graefes Arch. Clin. Exp. Ophthalmol.* 231:737–742; 1993.
13. Hiscott, P.; Sheridan, C.; Magee, R. M.; Grierson, I. Matrix and the retinal pigment epithelium in proliferative retinal disease. *Prog. Retin. Eye Res.* 18:167–190; 1999.
14. Lane, C.; Boulton, M.; Marshall, J. Transplantation of retinal pigment epithelium using a pars plana approach. *Eye* 3:27–32; 1989.
15. LaVail, M. M.; Li, L.; Turner, J. E.; Yasumura, D. Retinal pigment epithelial cell transplantation in RCS rats: Normal metabolism in rescued photoreceptors. *Exp. Eye Res.* 55:555–562; 1992.
16. LaVail, M. M.; Unoki, K.; Yasumura, D.; Matthes, M. T.; Yaneopoulos, G.; Steinberg, R. H. Multiple growth factors, cytokines, and neurotrophins rescue photoreceptors from the damaging effects of constant light. *Proc. Natl. Acad. Sci. USA.* 89:11249–11253; 1992.
17. Lazar, E.; del Cerro, M. A new procedure for multiple intraretinal transplantation into mammalian eyes. *J. Neurosci. Methods.* 43:157–169; 1992.
18. Li, L.; Turner, J. E. Inherited retinal dystrophy in the RCS rat: Prevention of photoreceptor degeneration by pigment epithelial cell transplantation. *Exp. Eye Res.* 47:911–917; 1988.
19. Little, C. W.; Castillo, B.; DiLoreto, D. A.; Cox, C.; Wyatt, J.; del Cerro, C.; del Cerro, M. Transplantation of human fetal retinal pigment epithelium rescues photoreceptor cells from degeneration in the Royal College of Surgeons rat retina. *Invest. Ophthalmol. Vis. Sci.* 37:204–211; 1996.
20. Little, C. W.; Cox, C.; Wyatt, J.; del Cerro, C.; del Cerro, M. Correlates of photoreceptor rescue by transplantation of human fetal RPE in the RCS rat. *Exp. Neurol.* 149:151–160; 1998.
21. Lopez, R.; Gouras, P.; Brittis, M.; Kjeldbye, H. Transplantation of cultured rabbit retinal epithelium to rabbit retina using a closed-eyed method. *Invest. Ophthalmol. Vis. Sci.* 28:1131; 1987.
22. Lopez, R.; Gouras, P.; Kjeldbye, H. Transplanted retinal pigment epithelium modifies the retinal degeneration in the RCS rat. *Invest. Ophthalmol. Vis. Sci.* 30:586–588; 1989.
23. Marlhens, F.; Bareil, C.; Griffoin, J.-M.; Zrenner, E.; Amahic, P.; Eliaou, C.; Liu, S.-Y.; Harris, E.; Rechonond, T. M.; Arnaud, B.; Claustres, M.; Harnel, C. P. Mutations in RPE65 cause Leber's congenital amaurosis. *Nat. Genet.* 17:139–140; 1997.
24. Maw, M. A.; Kennedy, B.; Knight, A.; Bridges, R.; Roth, K. E.; Mani, E. J.; Mukkadan, J. K.; Nancarrow, D.; Crabb, J. W.; Denton, M. J. Mutation of the gene encoding cellular retinaldehyde-binding protein in autosomal recessive retinitis pigmentosa. *Nat. Genet.* 17:198–200; 1997.
25. Ray, J.; Wu, Y.; Aguirre, G. D. Characterization of β -glucuronidase in the retinal pigment epithelium. *Curr. Eye Res.* 16:131–143; 1997.
26. Wongpichedchai, S.; Weiter, J. J.; Weber, P.; Dorey, C. K. Comparison of external and internal approaches for transplantation of autologous retinal pigment epithelium. *Invest. Ophthalmol. Vis. Sci.* 33:3341–3352; 1992.
27. Yamamoto, H.; Simon, A.; Eriksson, U.; Harris, E.; Berenson, E. L.; Dryja, T. P. Mutations in the gene encoding 11-cis retinol dehydrogenase cause delayed dark adaptation and fundus albipunctatus. *Nat. Genet.* 22:188–191; 1999.
28. Zhang, X.; Bok, D. Transplantation of retinal pigment epithelial cells and immune response in the subretinal space. *Invest. Ophthalmol. Vis. Sci.* 39:1021–1027; 1998.