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Functional and Anatomic Consequences of Subretinal Dosing in the Cynomolgus Macaque

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Objective: To characterize functional and anatomic sequelae of a bleb induced by subretinal injection.

Methods: Subretinal injections (100 µL) of balanced salt solution were placed in the superotemporal macula of 1 eye in 3 cynomolgus macaques. Fellow eyes received intravitreal injections (100 µL) of balanced salt solution. Fundus photography, ocular coherence tomography, and multifocal electroretinography were performed before and immediately after injection and again at intervals up to 3 months postinjection. Histopathologic analyses included transmission electron microscopy and immunohistochemistry for glial fibrillary acidic protein, rhodopsin, M/L-cone opsin, and S-cone opsin.

Results: Retinas were reattached by 2 days postinjection (seen by ocular coherence tomography). Multifocal electroretinography waveforms were suppressed post–subretinal injection within the subretinal injection bleb and, surprisingly, also in regions far peripheral to this area. Multifocal electroretinography amplitudes were nearly completely recovered by 90 days. The spectral-domain ocular coherence tomography inner segment–outer segment line had decreased reflectivity at 92 days. Glial fibrillary acidic protein and S-cone opsin staining were unaffected. Rhodopsin and M/L-cone opsins were partially displaced into the inner segments. Transmission electron microscopy revealed disorganization of the outer segment rod (but not cone) discs. At all postinjection intervals, eyes with intravitreal injection were similar to baseline.

Conclusions: Subretinal injection is a promising route for drug delivery to the eye. Three months post–subretinal injection, retinal function was nearly recovered, although reorganization of the outer segment rod disc remained disrupted. Understanding the functional and anatomic effects of subretinal injection is important for interpretation of the effects of compounds delivered to the subretinal space.

Clinical Relevance: Subretinal injection is a new potential route for drug delivery to the eye. Separating drug effects from the procedural effects is critical.


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The eye poses numerous challenges for drug delivery. This is especially the case for biologics, ie, large molecules. The transformative success of the intravitreally injected anti–vascular endothelial growth factor agents including ranibizumab, bevacizumab, and pegaptanib for the treatment of neovascular age-related macular degeneration as well as certain aspects of diabetic retinopathy have increased interest in novel means of drug delivery. In addition, aflibercept (VEGF Trap-Eye) is now completing phase 3 clinical trials, and other intravitreal biologics, such as inhibitors of tumor necrosis factor α, show promise.

Another possible site for delivery of pharmaceuticals is the so-called subretinal space (embryologically, a collapsed brain ventricle) located between the neurosensory retina and the retinal pigment epithelium (RPE). Although drugs introduced into the vitreous do not need to cross a blood-brain barrier to reach this space, the neurosensory retina acts to limit transport of certain large molecules. If the target is the photoreceptors or RPE, as in the case of viral gene transfection, delivery of the therapeutic agent to this space can focus its action to the intended cells.

Increased interest in subretinal injections has been generated by recent studies showing successful viral transfection of the RPE65 gene into the RPE cells as a possible treatment for a rare form of Leber congenital amaurosis. Initial studies in a dog model were followed by human oculocutaneous trials involving therapeutic gene transfection via viral vector. Improvement in visual function was demonstrated in RPE65-deficient dogs and in humans with Leber congenital amaurosis.
Work with other types of specific gene transfer via subretinal injections is also in progress. For example, Kong et al\textsuperscript{33} inserted the human ABCA4 gene into the rods and cones of a murine model of Stargardt disease, a somewhat more common inherited retinal degeneration than Leber congenital amaurosis.

Recent work with subretinal delivery for one of the leading causes of blindness, neovascular age-related macular degeneration, has produced promising results in animal models. Although intravitreal anti-vascular endothelial growth factor agents are highly effective, they need to be injected on a monthly basis, perhaps for years. This has spurred research into sustained-release formulations and transfection of the RPE cells via long-acting viral vectors containing therapeutic transgenes. Ikeda et al\textsuperscript{21} have shown long-term (4-year) expression of enhanced green fluorescent protein as well as human pigment epithelium–derived factor using a lentiviral vector based on simian immunodeficiency virus from African green monkeys (SIVagm) injected subretinally in nonhuman primates. Such an approach has been shown to be effective in inhibiting choroidal neovascularization in a murine laser model using equine infectious anemia viral vector, with delivery of endostatin and angiotatin driven by the RPE-specific VMD2 promoter.\textsuperscript{22-25}

Work with subretinally injected human neuroprogenitor stem cells and RPE cells generated from human embryonic stem cells has shown promise in rescuing photoreceptors in the Elov14 mouse (a model of Stargardt disease) and the RCS rat (a model of retinal degeneration).\textsuperscript{26-30} Although inherited retinal degenerations, such as retinitis pigmentosa and Stargardt disease, are fairly uncommon in humans, treatment with stem cells (where the cells presumably release neuroprotective cytokines and/or replace degenerating RPE cells) might have applications in treating one of the most common causes of vision loss, age-related macular degeneration. A phase 1 human trial using RPE generated from human embryonic stem cells is in progress to treat Stargardt disease. A second phase 1 trial using human embryonic stem cells to treat the dry (nonneovascular) form of age-related macular degeneration has recently been approved by the US Food and Drug Administration.\textsuperscript{31}

Basic research is also progressing. Novel agents, such as nonviral gene delivery using nanoparticles placed into the subretinal space, have promise.\textsuperscript{32} Viral genes have been targeted to specific cell types. For example, Komaromy et al\textsuperscript{33} targeted green fluorescent protein expression to the L/M cones in the canine retina using human cone opsin promoters in recombinant adeno-associated virus and subsequently used a similar vector to rescue cone function in 2 canine models of congenital achromatopsia.\textsuperscript{34} This was carried one step further by Mancuso et al,\textsuperscript{35} who reported transfecting adult squirrel monkeys that were missing the L-opsin gene with a recombinant adeno-associated virus containing a human L-opsin gene, thus producing animals that were behaviorally trichromatic.

Despite these exciting advances in drug and vector delivery to the subretinal space, a review of the literature shows little analysis of potential toxic effects of subretinal injections using rigorous controls and modern methods of functional and morphologic analysis. In this study, we compared the effects of subretinal injection of balanced salt solution in nonhuman primates with fellow-eye sham intravitreal injections with the use of functional testing, in vivo imaging, and histopathologic analysis.

### METHODS

#### SUBJECT ANIMALS

Three cynomolgus (Macaca fascicularis) monkeys (denoted in this article as Cy1, Cy2, and Cy3) were used for collection of data. All experimental methods and techniques adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by our institution’s animal care and use committee.

#### ANESTHESIA

Multifocal electroretinography (mERG), ocular coherence tomography (OCT), and fundus photography were performed under ketamine, dexmedetomidine hydrochloride, and oxymorphone hydrochloride anesthesia. Animals were pretreated with midazolam and anesthesia reversed with a combination of atipamezole and naloxone hydrochloride. Subretinal dose administration used a similar protocol but excluded oxymorphone.

#### SURGICAL PROCEDURES

Surgical procedures were identical for all animals. Right eyes were dosed subretinally and left eyes were dosed intravitreally with balanced salt solution (Alcon Laboratories, Fort Worth, Texas). Both eyes were prepped with a 2.5% povidone-iodine solution and rinsed with sterile saline. A wire pediatric-sized eyelid speculum was used for eyelid retraction. A disposable 4-mm-diameter plano-convex plastic vitrectomy lens with the flange filed off (Dutch Ophthalmic USA, Exeter, New Hampshire) was used to visualize the posterior pole with an operating microscope. Hydroxypropyl methyl cellulose served as an optical couple.

The subretinal bleb was formed in the superior macular region using a RetinaJect apparatus (SurModics, Inc, Eden Prairie, Minnesota) and a 1-mL syringe (Luer-Lok; Becton Dickinson, Durham, North Carolina). The injection was performed as follows. A 25-gauge cannula was inserted with a trocar through the conjunctiva and sclera approximately 2.5 mm posterior to the corneal limbus in the 2-o’clock (right eyes) or 10-o’clock (left eyes) meridian. A 25-gauge fiber-optic light pipe was inserted through the preplaced cannula and used to illuminate the retina. The sharpened 23-gauge RetinaJect needle was passed through the conjunctiva and sclera at the 10-o’clock (right eyes) meridian approximately 2.5 mm posterior to the corneal limbus and moved through the vitreous under visual control using a surgical microscope viewing through a dilated pupil with a plano-convex lens on the cornea. The 39-gauge cannula tip was advanced from the needle and gently touched the retinal surface in the superior macular region adjacent to a retinal vessel. With the tip adjacent to the retina, the force of the balanced salt solution stream emerging from the fine-diameter cannula perforated the neural retina to gain initial access and subsequently expand the subretinal space. The instruments were then withdrawn.

For the intravitreous dose to the left eye, a 25-gauge needle connected to a 1-mL syringe was advanced to the vitreous at the 2-o’clock meridian approximately 2.5 mm posterior to the corneal limbus. To provide for similar treatment of the right and left eyes, 100 µL were delivered into the posterior vitreous and a 25-gauge fiber-optic light pipe was also introduced.
The 25-gauge needle sclerotomies were self-sealing, and thus, suturing was not required. A topical antibiotic-steroid (Tobradex; Alcon Laboratories) was instilled in each eye after the procedures were completed. In addition, animals were treated with topical atropine, 1%, (anti-inflammatory and functional analgesic) and a nonsteroidal anti-inflammatory (flunixin meglumine, intramuscularly). Treatment continued for 2 days postdose.

MULTIFOCAL ELECTRORETINOGRAPHY

The mfERG recording procedure was performed as previously reported. Briefly, all animals were premedicated with atropine (0.05 mg/kg intramuscularly) and midazolam (0.2 mg/kg intramuscularly) at 20 minutes prior to medicating with ketamine (5.0 mg/kg intramuscularly)/dexmedetomidine (0.025 mg/kg intramuscularly). Oxy-morphine was administered intravenously (0.15 mg/kg) after the animal was sedated and stable. Animals were maintained on oxygen while anesthetized. The combination of medications was sufficient to suppress eye movements. Redoses of ketamine/dexmedetomidine (generally 50% of initial dose) were given as needed to continue suppression of eye movement. Heart rate and blood oxygen saturation were monitored continuously with a pulse oximeter. Position was maintained with a bite-bar head holder. Wire specula were used for eyelid retraction. Mydriasis was produced by topical tropicamide, 1%, and phenylephrine hydrochloride, 2.5%. Electroretinograph-jet contact lens electrodes were applied to the corneas with hydroxypropyl methylcellulose, 2.5%. Reference electrodes for each eye consisted of subdermal needles that were placed at the ipsilateral outer canthus and the ground electrode was inserted sub-dermally in the upper lid. VERIS Science version 4.9 (Electro-Diagnostic Imaging, Inc, San Mateo, California) was used for stimulus generation, data collection, and preliminary analyses. The visual stimulus consisted of 241 unstretched hexagonal elements that were displayed on a 21-inch monochromatic monitor (Model MGD403; Koninklijke Philips Electronics NV, Eindhoven, the Netherlands). The VERIS autocalibration software/hardware system was used for calibration of monitor stimulus luminance. Calibration procedures were carried out at the initiation of the study and approximately monthly throughout the study. The VERIS fast m-sequence (binary maximum-length sequence cycle of 215-1) was used with a frame rate of 75 Hz (13.3 milliseconds per frame). Maximum and minimum luminances of the display were 200 cd/m² and about 1 cd/m². Mean luminance was about 100 cd/m². Sampling rate of the signal was 1200 Hz (0.83 milliseconds per frame).

IMAGING

A fundus camera (50EX; Topcon, Oakland, New Jersey) was used to acquire color fundus images using Kodak Elite-Chrome film (ASA 100) (Kodak, Rochester, New York). Color photographs of each eye included stereoscopic photographs of the posterior pole and the borders of the subretinal bleb.

A 10-mm plano contact lens was applied to ensure adequate corneal hydration. A Stratus model 3000 OCT (software version 4.0.3; Carl Zeiss Meditec, Dublin, California) was used to obtain horizontal line scan images. Ninety-two days after bleb formation, a spectral-domain OCT instrument (Cirrus HD-OCT; Carl Zeiss Meditec) was used to obtain horizontal line scans just prior to euthanasia.

Fundus photography and OCT were obtained before and after injection and again 2 (OCT only), 9, 22, 36, 64, and 92 days later.

LIGHT MICROSCOPY AND IMMUNOHISTOCHEMICAL ANALYSIS

All 3 animals underwent upper body perfusion with fixative at the time of euthanasia. Animals Cy1 and Cy2 were perfused with paraformaldehyde, 4%, in 0.1M sodium phosphate buffer, pH 7.6. Both eyes of each animal were then promptly enucleated and placed in the same fixative as was used for the perfusate. The eyes were kept at 4°C for 1 day, then transferred to 0.1M sodium phosphate buffer and stored at 4°C.

Retinal sections were embedded in epoxy (Durcupan; Polysciences, Warrington, Pennsylvania). One-micrometer-thick sections were placed on glass slides (ProbeOn Plus; Fisher Scientific, Pittsburgh, Pennsylvania). One tissue section on each slide was stained with Richardson stain.

The immunohistochemistry (IHC) procedures were performed on both eyes of Cy1 and Cy2. Four areas were sampled: the subretinal bleb area of the right eye, the nonbleb area of the right eye (located in the inferior macula), and 2 similar regions of the fellow eye (left eye). Prior to immunoreaction, the Durcupan epoxy resin was removed according to the method of Mayor et al. Once rehydrated, the sections were incubated in a trypsin at 37°C for 8 minutes. After cold double-deionized water rinses, the sections were incubated with primary antibodies including anti-glial fibrillary acidic protein (GFAP) (1:2000 dilution, rabbit polyclonal; Dako Corp, Carpinteria, California) and mouse monoclonal antibody for rhodopsin staining or a rabbit polyclonal antibody for M/L-cone opsin staining (both at 1:2000, catalog numbers MAB5316 and AB5405, respectively; Chemicon/Millipore, Billerica, Massachusetts) overnight at 4°C. After washing, the sections were incubated with biotinylated antimouse IgG and biotinylated antirabbit IgG, respectively, for 30 minutes and then incubated for 1 hour at room temperature with avidin-biotin complexes (Vectastain ABC Kit Elite PK-6102 with the rhodopsin primary and Vectastain ABC Kit Elite PK-6103 with the GFAP and cone opsin primaries; Vector Laboratories, Burlingame, California). 3, 3-Diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St Louis, Missouri) served as the chromogen. Digital photomicrographs were obtained (Olympus CH-2; Olympus Corp, Lake Success, New York).

ELECTRON MICROSCOPY

Animal Cy3 was perfused with a mixture of glutaraldehyde, 2.5%, and paraformaldehyde, 2%, in 0.1M sodium phosphate buffer, pH 7.6. Tissues were then immersed in the same fixative overnight at 4°C and stored in 0.1M sodium phosphate buffer. Prior to embedding, the tissues were postfixed in osmium tetroxide, 1%, in the same buffer for 2 hours at room temperature. Following osmium tetroxide postfixation, the samples were dehydrated in a graded ethanol series, then further dehydrated in propylene oxide and embedded in epoxy resin (Epon; Miller-Stephenson, Sylmar, California).
Semithin (1-µm) sections were cut with an ultramicrotome (EM UC6; Leica Microsystems GmbH, Wetzlar, Germany) and observed under a light microscope to establish proper orientation. At the appropriate level, ultrathin sections were cut with the same microtome and collected on 200 mesh copper grids. Sections were then contrasted with Reynolds lead citrate and uranyl acetate, 8%, in ethanol, 50%. Ultrathin sections were observed with an electron microscope (CM120; Koninklijke Philips Electronics NV) and images were captured with a side-mounted digital camera (MegaView III; Olympus Soft Imaging Solutions GmbH, Münster, Germany).

RESULTS

The subretinal blebs in all 3 animals were similar in location and size (Figure 1). A small hemorrhage was sometimes observed at the site of the injection, which resolved within a few days. Once the subretinal fluid had resorbed, RPE pigmentation mottling was evident (Figure 1C). Specifically, there was a generalized depigmentation in the bed of the former bleb with a band of hyperpigmentation inferiorly. The area of the RPE pigmentation alteration was larger than the size of the original bleb, giving the appearance of a ring around the region of the resorbed bleb.

The OCT scans obtained within an hour of the bleb formation documented the presence of subretinal fluid. When the animals were rescanned 2 days later, the subretinal fluid was found to have completely reabsorbed (Figure 2).

Ophthalmoscopy performed 2 days postinjection revealed either no or mild intraocular inflammation, characterized by small numbers of cells in the aqueous and/or vitreous. Mild focal chemosis was noted in the region of the injection and cannula sites. Intraocular pressure was normal in all eyes. No definite signs of inflammation were present 92 days following injection.

MULTIFOCAL ELECTRORETINOGRAPHY

The mfERG root-mean-square (RMS) surface plots of the K1 5- to 60-millisecond interval showed the characteristic central peak in RMS amplitude corresponding to the foveal retina (Figure 3A). Within 1 hour of the subretinal injection, there was marked depression in the waveforms, not only in the region of the bleb but, unexpectedly, throughout the central retina (Figure 3B). At 90 days postinjection, the mfERG responses had recovered to nearly baseline levels (Figure 3C).

Quantitative analysis of the mfERG responses was done by analyzing a group of array traces within and peripheral to the location of the subretinal bleb and comparing them with similar regions in the fellow (left) eyes that had been given only intravitreal injections. Figure 4 illustrates a typical 241-element mfERG K1 baseline trace array with the approximate location of the optic nerve and the region corresponding to the retinal detachment in the 3 monkeys. The mfERG clusters of 7 elements used for analysis were shown as groups of colored hexagons. The red cluster was located within the retinal detachment area and directly superior to the fovea in all 3 right eyes. The blue cluster was directly inferior to the fovea and outside of the detached retina in all 3 right eyes. The blue elements are more peripheral than the red elements and thus have smaller response amplitudes.

Figure 5 shows plots of the mean values for the 3 animals at each of the 4 groups of 7 elements (shown in Figure 4) at each test day, including the 2 baseline tests. A marked decrease in mean K1 RMS (5- to 60-millisecond interval) response amplitudes occurred shortly following the subretinal injection (day 0) in the cluster of 7 elements located within the region of the subretinal bleb (dashed red line). A similar decrement in signal...
strength was also observed inferior to the bleb in the attached retinas of the eyes that had undergone subretinal injections (the right eyes). Such strong signal attenuation was not observed in the fellow eyes. By postinjection day 9, the amplitudes in both former bleb and nonbleb regions had returned to nearly normal levels. As with the amplitude responses, the P1 latencies were markedly increased in both the bleb and nonbleb areas of the right eyes. (P1 latency is the time to the peak of the first positive wavelet.) These also returned to nearly baseline levels by 9 days after the injection and remained stable thereafter.

**LIGHT MICROSCOPY AND IHC ANALYSIS**

At the light microscopic level, Richardson-stained sections of the bleb-associated retinas were seen to have all cellular layers intact with no apparent abnormalities except for mild irregularity to the pigment granules in the RPE cells (*Figure 6*).

The IHC analysis was performed on the 2 monkeys (Cy1 and Cy2) that had been fixed with paraformaldehyde only. The findings were similar in both animals. Anti-GFAP staining was identical in bleb and nonbleb areas (*Figure 7*) and was limited to the inner retina, especially the nerve fiber layer. No appreciable GFAP stain-
ing was seen in the outer retina in either the bleb or non-
bleb section.

A difference was found in antirhodopsin IHC in the
bleb and nonbleb areas. In the nonbleb regions of the right
eyes and in the corresponding locations of the fellow left
eyes, the antirhodopsin staining was limited to the outer
segments. However, in the bleb area, many of the rods
showed staining activity in their inner segments as well
(Figure 8). A similar pattern was found for the anti–
M/L-cone opsin antibody staining (Figure 9).

Anti–S-cone opsin staining was indistinguishable in
all 4 regions tested. That is, the outer segments but not
the inner segments were positively labeled. The distri-
bution of S-cones did not appear to be altered in the bleb
region (not shown).

ELECTRON MICROSCOPY

Only 1 of the 3 animals (Cy3) had the eyes perfused with
glutaraldehyde. Segments from the other 2 animals were
postfixed in glutaraldehyde and used to confirm the elec-
tron microscopy results even though the morphology was
not ideal. Consistent with light microscopic findings, the
inner retinas in the area of the former subretinal blebs
had normal-appearing ultrastructure. However, there was
a marked difference in the outer segment photoreceptor
disc spacing, predominantly in the rods. The bleb-
associated rod discs were not as tightly packed, having
markedly increased intradiscal spaces (Figure 10 and
Figure 11). Spectral-domain OCT scanning done just
prior to death showed a marked decrement in the inner
segment–outer segment (IS/OS) line in the region of the
former subretinal blebs (Figure 12).

The ultrastructure of the RPE-photoreceptor inter-
face also appeared to be altered in the region of the for-
mer subretinal blebs. The number and size of the intra-
cellular spaces were markedly increased in the former bleb
areas (Figure 13).
The subretinal injections were well tolerated, although some residual effects remained after 90 days. These changes were absent from fellow eyes injected intravitreally.

To our knowledge, the most recent detailed studies of the effects of transient retinal detachment in the nonhuman primate were reported by Guérin et al38-40 more than 17 years ago, prior to the development of OCT and mERG. These studies lacked rigorous controls for the subretinal injection. In our study, we performed intravitreal injections in the fellow (left) eyes to control for other aspects of the injection procedure, such as trauma from passing the needles through the conjunctiva and eye wall, transient elevation of intraocular pressure following injection of a volume of liquid, possible effects of light on the retina, and any chemical/osmotic effects of the balanced salt solution on the retina. Thus, the subretinal injection should be the only variable.

**BLEB PLACEMENT**

From our previous experience with creating subretinal blebs in monkeys (T.M.N, C.J.M., P.E.M., and B.J.C., unpublished data, 2006-2008), we learned that blebs are most reliably formed when the injection site is...
near a large retinal vessel. It may also help to perform the injections along the vascular arcades where the retina is thicker because of the increased depth of the nerve fiber layer. Presumably, this is because the retina in this region is stiffer and thus provides more resistance to the stream of injected fluid, thus allowing the fluid stream to create a hole in the retina. We also found that attempts at making a more centrally located bleb often result in macular hole formation. Injections adjacent to the optic nerve end up encircling the nerve. Another reason to position the blebs eccentrically is that anatomically similar areas of retina in the same eye can be compared (bleb: superior macula and nonbleb: inferior macula).

**RETINAL FUNCTION—mfERG**

The preinjection baseline mfERG recordings showed some mild test-retest variability, which was within the previously described range for this species. \(^{30,41,42}\) Not surprisingly, the mfERG responses from the region of retina cor-
responding to the subretinal bleb were markedly reduced immediately following the subretinal injection. Such a focal diminution of mfERG responses has been observed in acute spontaneous rhegmatogenous retinal detachment in humans and it might be expected considering that, although the mfERG waveforms represent functioning of the various component cells throughout the retina, the signals are driven by the photoreceptors and it is the photoreceptors that are farthest removed from their primary blood supply (the choriocapillaris) during retinal detachment. What was unexpected was the marked generalized reduction of the mfERG waveforms and increase in P1 latency even in areas far removed from the region of the subretinal injection (Figure 3 and Figure 5). A generalized reduction in relative RMS amplitude (but not latency) was seen in the fellow eyes as well. However, this was a relatively mild effect. Reduced response in the fellow eye could be due to a number of minor factors including longer anesthesia period on the day of injection (compared with the duration of anesthesia on the other testing days), the exposure to the operating light, the transient elevation of intraocular pressure following both intravitreal and subretinal injections, and the penetration of the eye. Nevertheless, the generalized RMS reduction was greater in the subretinal bleb eyes. A similar generalized reduction in the mfERG has been observed in human eyes with partial spontaneous rhegmatogenous retinal detachments. Chisholm et al observed depressed macular function in patients who had only peripheral retinal detachments. A morphologic study of rabbit eyes with partial detachments created by subretinal injection of hyaluronic acid found some signs of photoreceptor degeneration even in areas distal to the detachment. In our study, the generalized mfERG depression had resolved at the first test interval 9 days after the subretinal injection. By 3 months, the RMS amplitudes and P1 latencies had recovered to normal or nearly normal levels. Consistent with our study, Kyhn et al in a study of subretinal injections in porcine eyes showed return of retinal mfERG function after 6 weeks of reattachment.

RETINAL MORPHOLOGY—CLINICAL

The subretinal fluid in all 3 blebs was found to be completely resorbed as observed with OCT testing 2 days after the subretinal injection (Figure 2). However, considerable RPE pigmented mottling was evident on both the color fundus photographs (Figure 1) and at the light microscopic level (Figure 6). The pattern of the distribution had a ringlike configuration. The central area corresponded to the original bleb and was more lightly pigmented than the retina distal to the bleb. There was also a surrounding ring showing a different pattern of pigmentation, especially inferior to the original bleb where the pigment was much darker. The surrounding ring could be a reactive change or, more likely, once the animal was awake and moving, there was a mild spreading of the detached retina before it reattached. Such pigmented changes are uncommon in humans except for chronic detachments (greater than 3 months), which can result in a demarcation line. We also have personal experience with subretinal injections in pigmented rabbits and have not observed a similar RPE pigmented mottling. The difference may be related to greater neurosensory retina/RPE adherence in the monkey compared with the rabbit. Human eyes have a retina/RPE adherence that is intermediate between monkey and rabbit. Szurman et al showed that cellular damage is worse in experimental detachments as a function of retinal adhesion. So, the monkey may be somewhat atypical in this regard with its higher retina/RPE adhesion compared with other species.

Peripheral retinal tears or detachments were not observed in the 3 animals in this study. However, rhegmatogenous retinal detachment is a known (although usually small) risk associated with any vitreous manipulation in human eyes.

LIGHT MICROSCOPY AND IHC

The IHC analysis was performed 3 months following subretinal injections. No difference was found in GFAP labeling between the retina in the former subretinal bleb area and in the corresponding area of the fellow eye (Figure 7). Glial fibrillary acidic protein is known to be rapidly upregulated in the Muller cells with retinal stress, such as glaucoma, light toxic effects, ischemia, and retinal detachment. However, Lewis et al showed that GFAP returns to nearly normal levels in cat eyes with a 1-day retinal detachment followed by 2 days of reattachment. In our model, 3 months of reattachment was an adequate interval to permit the GFAP IHC labeling to normalize, consistent with a good healing response.

The IHC analysis for the 3 opsins (rhodopsin, M/L-cone opsin, and S-cone opsin) was nearly, but not entirely, normal in the reattached retinas. Gross photoreceptor loss was not evident. However, some redistribution of both rhodopsin and M/L-cone opsin was evident even after 3 months of reattachment (Figure 8 and Figure 9). Dislocation of opsin staining into the photoreceptor inner segments has been observed in experimental cat retina. At least partial recovery of the normal opsin IHC pattern was observed following short-term (2 days) reattachment. That the irregular distribution of opsins should persist after 3 months in the monkey suggests either a permanent alteration of the photoreceptors or that the healing process was not complete.

PHOTORECEPTOR ULTRASTRUCTURE AND THE IS/OS LINE

The spectral-domain OCT showed a marked decrease in the reflectivity of the IS/OS line at 3 months in the formerly detached compared with the attached retina of the same eyes and corresponding regions of the fellow control eyes (Figure 12). A similar decrement in the IS/OS line is seen in reattached human retinas, which may persist for months after surgical repair. To our knowledge, retinas from reattached human eyes that also underwent spectral-domain OCT testing have not yet become available for ultrastructural analysis. So, our animal model might help to explain the origin of this decrement in IS/OS reflectivity.

On transmission electron microscopy, the photoreceptors had a subjectively normal morphology except that...
the normal close stacking of the outer segment discs (much more so in the rods than the cones) was lacking. Instead, large intradiscal spaces were seen throughout the outer segment stacks in the areas of the former subretinal blebs. Consistent with this finding, Guérin et al. noted abnormal disc stacking in their rhesus monkey model of retinal detachment (for 7 days) and reattachment (after 14 days). However, in our experiment, the duration of the detachments was much shorter (less than 2 days) and the period of reattachment, much longer. Even so, there was a marked difference in the density of rod disc packing in our animals. Taken together, the decreased IS/OS reflectivity and reduced disc packing are probably related. That is, an outer segment–RPE junction (Figure 13). Persistent subretinal fluid that has not clinically apparent, as we saw on transmission electron microscopy.

**RETINAL RECOVERY**

Overall, our results show nearly complete recovery of the retina 3 months after subretinal injection of balanced salt solution. This is consistent with observations from human rhegmatogenous retinal detachments. Vision recovery following macula-off detachments often continues for 6 months to 1 year after reattachment. The reason for this may be persistence of subretinal fluid that is not clinically apparent, as we saw on transmission electron microscopy at the outer segment–RPE junction (Figure 13). Persistent subretinal fluid has also been observed by OCT in human retinal detachments even many months following surgical repair. In addition, our finding that outer segment disc stacking has not fully recovered at 3 months may also explain some of the slow visual recovery in humans.

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