Morphological and Functional Rescue in RCS Rats after RPE Cell Line Transplantation at a Later Stage of Degeneration

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PURPOSE. It is well documented that grafting of cells in the subretinal space of Royal College of Surgeons (RCS) rats limits deterioration of vision and loss of photoreceptors if performed early in postnatal life. What is unclear is whether cells introduced later, when photoreceptor degeneration is already advanced, can still be effective. This possibility was examined in the present study, using the human retinal pigment epithelial cell line, ARPE-19.

METHODS. Dystrophic RCS rats (postnatal day [P] 60) received subretinal injection of ARPE-19 cells (2 × 10^5/3 μL/eye). Spatial frequency was measured by recording optomotor responses at P100 and P150, and luminance threshold responses were recorded from the superior colliculus at P150. Retinas were stained with cresyl violet, retinal cell–specific markers, and a human nuclear marker. Control animals were injected with medium alone. Animals comparably treated with grafts at P21 were available for comparison. All animals were treated with immunosuppression.

RESULTS. Later grafts preserved both spatial frequency and threshold responses over the control and delayed photoreceptor degeneration. There were two to three layers of rescued photoreceptors even at P150, compared with a scattered single layer in sham and untreated control retinas. Retinal cell marker staining showed an orderly array of the inner retinal lamina tion. The morphology of the second-order neurons was better preserved around the grafted area than in regions distant from graft. Sham injection had little effect in rescuing the photoreceptors.

CONCLUSIONS. RPE cell line transplants delivered later in the course of degeneration can preserve not only the photoreceptors and inner retinal lamination but also visual function in RCS rats. However, early intervention can achieve better rescue.

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D egeneration of photoreceptors as a result of genetic defec ts affecting either the photoreceptors themselves or associated cells such as the retinal pigment epithelium (RPE) represents the leading cause of blindness in humans for which no suitable treatment exists. While there has been very little work correlating the degree of photoreceptor survival with preserved vision in these patient groups, it is clear from what has been reported that surprisingly good vision can be achieved by a very poorly organized retina characterized by advanced photoreceptor loss. This finding raises the possibility that it is not necessary to have a perfectly structured outer retina for competent vision and/or that there may be adaptive changes within the inner retina or in central visual pathways to compensate for the poor input signal: this raises the hope that attempts at rescue even at a later stage in the course of retinal degeneration may still be effective up to a point.

The presence of animal models, particularly rodents with diseases homologous or analogous to human disorders, provides an opportunity to explore therapeutic approaches that might eventually be applied in the clinic. Among these is examination of the potential of cell-based therapies in the Royal College of Surgeons (RCS) rat to rescue photoreceptors from death. In this animal, photoreceptor loss is secondary to a defect in the RPE. Approaches include transplanting healthy RPE cells or cell lines into the subretinal space, with the object of replacing the defective host RPE cells, grafting iris pigment epithelium, injecting cells that release trophic factors to improve the chemical environment in diseased eyes, and introducing stem or progenitor cells. These studies have for the most part achieved successful morphologic and functional rescue when intervention was performed early in the course of degeneration, but whether they may be effective if introduced at a later stage in the progress of degeneration is not at all clear. The one comprehensive study that was undertaken to investigate this question showed that grafts, introduced later in the course of degeneration, up to age 38 days in RCS rats, were ineffective in achieving rescue of photoreceptors. We have explored further by implanting cells of the human ARPE-19 line into the subretinal space of RCS rats at a time when there was already significant loss of photoreceptors and the electroretinogram (ERG) a-wave had disappeared. Would such cells be effective in maintaining the remaining retinal morphology and visual functions? This is relevant clinically, where cell-based therapies to slow progress of retinal degeneration are most likely to be applied at least at a time when most patients have seriously compromised low-luminance vision.

MATERIAL AND METHODS

Animals

Pigmented dystrophic RCS rats (n = 12) at postnatal day (P) 60 received subretinal injections of ARPE-19 cells (2 × 10^5/3 μL/eye); control rats (n = 6) received medium alone. Nonsurgical and early-stage (P21) grafted animals were available for comparison. All animals were maintained on cyclosporine A (CyA; Novartis, Basel Switzerland), administered in the drinking water (210 mg/L; resultant blood concentr-
tation of ~300 µg/L) from 1 day before transplantation until they were killed. These studies were conducted with the approval and under the supervision of the Institutional Animal Care Committee at the University of Utah. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Transplantation

ARPE-19 donor cells were obtained from ATCC (American Type Culture Collection, Manassas, VA). Cells were stored in liquid nitrogen and on thawing were grown to confluence in flasks at which point they were trypsinized and washed: a cell suspension containing approximately $2 \times 10^5$ cells was delivered into the subretinal space through a small scleral incision in 3 µL of DMEM/F12 carrying medium (Invitrogen, Carlsbad, CA) with a fine glass pipette (internal diameter, 75–150 µm) attached by tubing to a 25-µL syringe (Hamilton, Reno, NV). The cornea was punctured to reduce intraocular pressure and to limit the efflux of cells. A sham-surgery group was treated the same, except carrying fundus alone was injected. Immediately after injection, the fundus was examined to check for retinal damage or signs of vascular distress. Any animals showing such problems were removed from the study and are not included in the animal counts herein.

Spatial Frequency Records

Animals were tested at P100 and P150 (40–90 days after surgery). With an optomotor testing apparatus (Optomotry; Cerebral Mechanics, Inc., Lethbridge, Alberta, Canada). The system comprises a rotating cylinder displaying a vertical sine wave grating presented in virtual three-dimensional (3-D) space on four computer monitors arranged in a square. Rats standing unrestrained on a platform in the center of the square track the grating with reflexive head movements. The spatial frequency of the grating is clamped at the viewing position by repeatedly recentering the cylinder on the head of the test subject. Acuity is quantified by increasing the spatial frequency of the grating until an optomotor response is longer elicited.

Luminance Threshold Responses

To assess luminance thresholds, we recorded single and multiunit activity in the superficial layers of the superior colliculus (SC) at P150, by using a modification of a procedure we had developed in previous work.30 Recordings were made from the superficial layers of the SC to a depth of 100 to 300 µm, using glass-coated tungsten electrodes (resistance: 0.5 MΩ; band-pass 500 Hz–5 kHz). A uniform background of 0.2 to 0.4 log units was sustained on a hemisphere with an opaque surface. The brightness of the spot projected onto the hemisphere was varied with neutral-density filters (minimal steps of 0.1 log unit) until a response was obtained that was double the background activity. This method gave the threshold level for that point on the visual field calculated as 5.2 log units minus the value of the filter. A total of 16 to 20 positions were recorded from each SC.

Histology

Rats were given an overdose of pentobarbital sodium (Sigma-Aldrich, St. Louis, MO) and perfused with phosphate-buffered saline (PBS) at either P100 or P150. The superior pole of each eye was marked with a suture to maintain orientation. The eyes were removed and immersed either P100 or P150. The superior pole of each eye was marked with a suture to maintain orientation. The eyes were removed and immersed in 4% paraformaldehyde for 1 hour, after which they were infiltrated with sucrose and embedded in OCT. Horizontal sections (10 µm) were collected in 5 series. One series was stained with cresyl violet (CV) for assessing the injection site and retinal lamination, and the others were used for antibody staining: rhodopsin (Robert Molday, University of British Columbia, Vancouver) for rod photoreceptors; α-PKC (Sigma-Aldrich) for rod bipolar cells; recoverin (James McGinnis, University of Oklahoma, Tulsa, OK) for photoreceptors and ON cone bipolar cells; RT97 (Sigma-Aldrich) for neurofilaments in ganglion cell axons and horizontal cells; von Willebrand factor (vWF; Chemicon, Temecula, CA) for blood vessels; and anti-human nuclear marker (MAB1281 Chemicon) for ARPE-19 donor cells (see Ref. 10 for further details). Retinal sections were examined by regular and confocal microscopy.

**Results**

**Functional Assessment**

Spatial Frequency. In untreated RCS rats at P60, the visual acuity was $0.33 \pm 0.01$ c/deg. Eyes grafted at that age and then tested at P100 showed no diminution in spatial frequency over this period, showing even a slight improvement ($0.39 \pm 0.02$ c/deg) with the best eyes performing at 0.41 c/deg, similar to that recorded in dystrophic eyes of the same age receiving grafts at P21 ($0.45 \pm 0.026$ c/deg; Holmes TM, et al. *IOVS* 2004;45;ARVO E-Abstract 489), although it was significantly below the level recorded in nondystrophic eyes ($0.52 \pm 0.03$ c/deg). Sham surgeries resulted in $0.14 \pm 0.06$ c/deg and untreated in $0.15 \pm 0.06$ c/deg ($P = NS$). Between 100 and 150 days of age there was, however, a rapid deterioration of performance to 0.16 $\pm 0.06$ c/deg in later- and 0.34 $\pm 0.02$ in early-grafted eyes, although both were still significantly better than age-matched untreated eyes ($0.028 \pm 0.04$c/deg; see details in Fig. 1A, Table 1).

Luminance Threshold Responses. By recording thresholds from 16 to 20 positions across the area of the SC, it was possible to provide indication of the relative sensitivity of the retina over the visual field and therefore of local efficacy of the

**Figure 1.** The spatial resolution (A) as measured by the optomotor test at P100 shows that the ARPE-19-treated eyes performed significantly better than the vehicle-treated and untreated eyes ($P < 0.01$, t-test), although the early graft gave an even better response. (B, C) Luminance threshold responses recorded across the SC in early- and later-grafted, untreated control animals: each curve (average ± SEM) shows the percent of retinal area (y-axis) where the visual threshold was less than the corresponding value on the x-axis (log units, relative to background illumination of 0.02 cd/m²). *Points at which the curves for grafted and sham-grafted eyes are significantly different (t-test, $P < 0.05$).
cell treatment in rescuing low-threshold luminance responses. When points were recorded across the visual map of transplant-recipient eyes at P150, response sensitivity was not uniform, being best in the retinal region around the area into which the cells were injected. With this method, 17.4% of the colliculus area gave thresholds better than 2.2 log units above background and 24.7% was better than 2.7 log units against untreated eyes, which gave 0% better than 2.2 log units, 4.2% better than 2.2 log units (Fig. 1B). For comparison, early ARPE-19 grafts (Fig. 1C, Table 2) showed that 26.9% of the area of the SC gave thresholds better than 2.2 log units and 52.3% better than 2.7 log units, whereas the shams for later graft group gave the same result as the untreated group. In nondystrophic eyes, previous work showed responses recorded over the whole area of the colliculus to have thresholds better than 0.5 log units above background. Figures 1B and 1C represent a compression of the whole data set in which the area under the curve to the left indicates rescue. Point-to-point statistical comparison of the curves obtained for grafted and nongrafted eyes showed significant rescue effects of ARPE-19 grafts (P < 0.05). In untreated RCS rats at P60, 1.6 ± 0.52 log units was recorded across the colliculus area; whereas in later-grafted eyes a threshold of 2.2 log units over 17.4% of colliculus area (vs. 26.9% in early-grafted eyes) was recorded at P150, indicating that the later graft sustained the same visual response as P60 for at least 3 months after transplantation.

**General Retinal Morphology**

At P60, photoreceptor cell bodies in untreated dystrophic RCS rats were reduced to three to four layers (this was the time when ARPE-19 cells were injected) (Fig. 2A); by P150 only a few scattered photoreceptors remained (Fig. 2B). ARPE-19 transplantation performed at P60 promoted some delay in photoreceptor degeneration: at P110 (50 days after cell injection), the outer nuclear layer (ONL) was two to three cells deep (Fig. 2C) compared with one cell deep in untreated retina (data not shown) and five to six cells deep in early-grafted retina (Fig. 2D) at similar survival times. At P150 (90 days after cell injection), there was further photoreceptor loss (the ONL was reduced to a single layer), but in best grafted eyes, there were still two to three layers of photoreceptors remaining (Fig. 2E). A discontinuous single layer of photoreceptors was observed in sham grafts, with a group of cells surviving immediately adjacent to the injection site (Fig. 2F, double arrows).

**Antibody Staining**

In later-grafted eyes, donor cells were detected next to the host RPE layer in the subretinal space by human nuclear antibody only at P110. By P150, they were no longer detected. Antibody staining for specific retinal cell types showed a normal distribution of cells within the inner retina and no disruption of lamination within the inner nuclear layer. Figures 3A–C show retinal sections (at P110) double stained with antibodies against rhodopsin (red) and PKCγ (green) in the graft-protected area (Fig. 3A), distant from the graft (Fig. 3B), and in an age-matched retina that had received a graft at P21 (Fig. 3C) for comparison. There were rhodopsin-positive cells in the graft-protected area, and rod bipolar cells were maintained in an orderly array (Figs. 3A, 3C). It is noted that the rhodopsin also stained cell bodies in the later-grafted (Fig. 3A), but not in the early-grafted (Fig. 3C) retinas. Distinct from the graft (Fig. 3B), there was little rhodopsin-positive material, and the dendrites of rod bipolar cells had begun sprouting (Fig. 3B, arrows). Figures 3D–F are retinal sections (at P150) double stained with recoverin (green) and PKC (red). Even at P150, there were still two to three layers of recoverin-stained photoreceptors associated with later grafts (Fig. 3D) compared with four to five layers after early (Fig. 3F) grafts. The second-order neurons (rod bipolar cells) showed a normal disposition in the graft-protected area, whereas distant from the graft (Fig. 3E) there were only scattered recoverin-stained photoreceptors (arrows); smaller rod bipolar terminal end bulbs were clearly evident (left-pointing arrows, Fig. 3E versus Figs. 3D and 3F). Figures 3G–I are retinal sections double-stained with calbindin (green) and neurofilament protein-RT97 (red). At P150, the horizontal axons (revealed by RT97) were greatly reduced in density in the later-graft–protected area (Fig. 3G, arrows) compared with that in the early-graft–protected area (Fig. 3I, arrows), whereas distant from the graft (Fig. 3H); extensive horizontal axonal sprouting into both the subretinal space (arrows) and the inner retina (left-pointing arrow) was evident.

There were no abnormal vascular changes around the graft-protected area, whereas distant from the graft and in untreated eyes, secondary vascular disease was observed.

**DISCUSSION**

ARPE-19 transplants delivered at a later stage in the sequence of photoreceptor degeneration (P60) in RCS rats sustained not only the photoreceptors and inner retinal lamination, but also visual function, although early intervention achieved better morphologic rescue and sustained functional rescue over a longer period.

In the only previous study of later RPE grafts performed in RCS rats, freshly harvested cells taken from normal Long-Evans rats were grafted at P38, P43, and P48, and rescue was examined at P60. In marked contrast to the present study, little or no rescue was found. The investigators concluded that effective rescue of photoreceptor cells in the RCS rat requires...
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Late-Stage RPE Transplantation

FIGURE 2. Dystrophic RCS retinal sections stained with cresyl violet: photoreceptors were three to four cells thick with organized retinal lamination at P60 (A), whereas there were only scattered photoreceptors remaining with clear secondary disease at P150 (B). Left-pointing arrow: an abnormal blood vessel; right-pointing arrow: RPE cells migrating along a vessel. (C, D) RCS retinas at P110 with ARPE-19 grafts at P60 (C) and P21 (D). Photoreceptor layers were five to six cells thick in early-grafted retinas (D), compared with two to three cells thick in later-grafted retina (C). (E, F) Retinas at P150 with later graft (E) and sham injection (F). (E, arrows) Scattered photoreceptors. (F, arrows) Scattered photoreceptors around the injection site (double arrows) with a disorganized inner retina. Scale bars, 50 µm.

early-stage transplantation, a conclusion that would severely limit the application of transplantation in a clinical setting. Whether the difference between the prior and the present studies is due to cell-related effects (their fresh allografted RPE cells versus our xenografted RPE cell line) or to consequences of immune responses to allografted cells (no immunosuppression was used) that may be more extreme at later transplantation is not clear. Previous work has shown that even allografts to young recipients are susceptible to rejection and that the subretinal space is not an absolutely immunoprivileged site, and this may become more acute in the diseased RCS retina with age. Furthermore, earlier studies in a different eye transplant paradigm from that in rats showed that immunogenetically mismatched grafts were more susceptible to rejection in older hosts. In accord with the present observations, several studies have shown that growth factor delivery at a later stage in degeneration can still be effective in sustaining photoreceptors. In P23H transgenic rats, the delivery of ribozyme-mediated factor was highly effective when injection was performed at later stages of photoreceptor degeneration. Gliarial neurotrophic factor (CNTF) injection into P23H and S334 rats at a time when 20% of the photoreceptors were lost still had a preservative effect on the remaining photoreceptors. In other work, sheets of embryonic retina including RPE cells implanted as late as P69 sustained visual responses for as long as 10 months in the RCS rat, although it is not clear whether, in this instance, the graft exercised its effect by rescuing host photoreceptors or by making new connections with the host retina. Certainly the longevity of effect may in part reflect the low immunogenicity of embryonic neural tissue.

Comparison between the Effect of Early and Later Cell Transplantation on Photoreceptor Rescue

Morphologically, in best grafted animals, later ARPE-19 grafts can maintain photoreceptors for at least 3 months at almost the same level as that on the day of injection. The ONL was still two to three cells thick at P150 in the rescued area compared with sparsely distributed photoreceptors in untreated retina. However, early ARPE-19 graft slowed the degeneration process more effectively: the ONL was five to six cells thick by P110 (3 months after grafting), and four to five cells thick at P150. Both early and later grafts also maintained the orderly lamination of the inner retina. There were no vascular changes around the graft-protected area, even at P150, whereas distant from graft or untreated area there was clear evidence of vascular disease. Such secondary vascular disease after photoreceptor loss in the RCS rat has been well documented.

Functionally, both early and later grafts can maintain visual function, and while early intervention was more effective, the difference between the two groups was not as great as might be expected based on the morphologic studies. The optomotor test at P100, after cell grafting at P21 gave an average 0.43 ± 0.02 cyc/deg with best-performing eyes at 0.45 cyc/deg, whereas eyes grafted at P60 and tested at P100 gave an average of 0.39 ± 0.02 cyc/deg, with best-performing eyes giving 0.41 cyc/deg. Given that the acuity threshold of this test in normal animals (Holmes TM, et al. IOVS 2004;45:ARVO E-Abstract 489) was 0.52 ± 0.01 and in untreated RCS rats was 0.33 ± 0.01 at P60 (at the time when cells were injected), this result is very encouraging, as it suggests that there was no deterioration over this time period. Luminance threshold recording from the SC also revealed that later grafts sustained for 3 months the same level of luminance threshold (over 17.4% of area in the SC) as the level when the cells were injected.

There was both morphologic and functional deterioration between P110 and 150. This may be correlated with an inability to identify donor cells at this age, possibly because of inadequacy of oral CyA in providing suitable immune protection for these xenografts. The CyA is poorly absorbed. Some RPE cells in culture may be activated, and these cells may accelerate the rejection cascade after transplantation. An additional issue is that it has been found that recruitment of microglia to the outer retina as well as upregulation of MHC antigens is limited by giving dexamethasone. This drug is used routinely in grafts in young animals, but it has been found to cause significant weight loss and compromise viability in later-grafted rats, and so it was not administered at the time of transplantation in the present study. Furthermore, a recent study showed that the number of donor cells declined even under triple immunosuppression. Another confounding factor is the RCS rats, in which like some forms of age-related macular degeneration, leaky vessels appear. These vessels may play a role in abrogating the immune-privileged status of the eye, thus compromising CyA efficacy.

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It should be noted that some individual animals showed severe decline, whereas others sustained performance levels for as much as 3 months after grafting, raising the possibility that the absorption of CyA may vary among animals.

**Limitations of Later Intervention**

In general, there are some limitations of later intervention: (1) There was already significant photoreceptor loss at the time when ARPE-19 was introduced (from 10 cells thick at P21 to 3 to 4 cells thick at P60). There was obvious accumulation of undigested outer segment material, which would affect the efficacy of donor cells (including interactions between donor cells and host retina). (2) Our previous study showed that secondary modifications occur as photoreceptor loss progresses: these changes include bipolar dendrite atrophy and horizontal cell sprouting. (3) There are already vascular changes, especially vascular leakage, which could compromise the beneficial effect of grafted cells. (4) Donor cells were not detectable at P150, whereas our previous study revealed that donor cells grafted at early stage of degeneration survived at P150, although the number decreased substantially. The reason for failure of donor cells to survive was discussed earlier.

**CONCLUSIONS**

ARPE-19 cells grafted into the subretinal space of RCS rat at the later stage of degeneration can maintain both retinal morphology and visual function, and although the degree of rescue is generally less than that achieved by introducing grafts early in the course of the degenerative process, the degree of sustained performance especially at the P150 survival time suggests that later grafts are not ineffective, as has been reported in previous work.
References