Photoreceptor Cell Rescue at Early and Late RPE-Cell Transplantation Periods During Retinal Disease in RCS Dystrophic Rats

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ABSTRACT

Maximal PRC rescue was affected by RPE-cell transplantation in retinas of RCS dystrophic rats at early stages of the retinal disease, while little or no rescue was detected when transplantation was performed at late time periods.

Key words: Retinal pigment epithelial cells, RCS dystrophic rat, photoreceptor cell, transplantation.

SUMMARY

Normal retinal pigment epithelial (RPE) cells were transplanted into retinas of Royal College of Surgeons (RCS) dystrophic rats at different stages of the retinal disease process. RPE-cell transplantation at 10, 17 and 26 days resulted in rescue of photoreceptor cells, such that at 4 months the outer nuclear layer (ONL) was 8-10 cells in thickness as shown in retinas of age-matched control rats. Of these transplantation times, day 17 appeared to affect the best rescue of photoreceptor cells. Nongrafted retinas of 4 month-old RCS dystrophic rats exhibited scattered PRC's, most prevalent in the peripheral retina. In addition, a small, but significant increase in the ONL thickness was detected in vehicle-injected retinas (sham control) of 17 day-old RCS dystrophic rats at 2 months; however, at 3 months, the ONL thickness was reduced to control levels. A normal distribution of $(Na^+ + K^+)$-ATPase immunostain was demonstrated beneath grafted RPE cells in retinas of 4 month-old RCS dystrophic rats. Dense immunostaining was shown along rescued photoreceptor cell inner segments (IS), within the inner (IPL) and outer (OPL) plexiform layers and on plasmalemma of cell bodies in the inner nuclear layer (INL). In nongrafted retinas of age-matched RCS dystrophic rats, immunostaining for $(Na^+ + K^+)$-ATPase was observed only in the INL and IPL. Under RPE-cell transplants in retinas of 4 month-old RCS dystrophic rats, opsin immunostaining was detected along both rescued photoreceptor cell inner and outer (OS) segments and on plasmalemma of ONL cell bodies. However, immunostaining for opsin was restricted to a debris zone in nongrafted retinas of age-matched RCS dystrophic rats. In contrast to PRC rescue resulting from early stage transplantation at 10 and 17 days, little or no rescue was observed in retinas of 4 month-old RCS dystrophic rats with RPE cell transplantation performed at 43 and 68 days. Interestingly, even lateral to RPE cell grafts, beneath dystrophic RPE cells and membrane debris, rescued inner segment-bearing photoreceptor cells were detected in RPE cell-grafted retinas of 4 month-old RCS dystrophic rats when transplanted at 26 days. Also, in these retinas, melanosome-containing RPE cells were attached to Bruch's membrane and rescued segment-bearing photoreceptor cells beneath the RPE-cell graft appeared to have a normal ultrastructure.

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INTRODUCTION

Photoreceptor cell (PRC) degeneration in Royal College of Surgeons (RCS) rats begins in the second postnatal week /3,5/. PRC loss proceeds in a central-to-peripheral gradient and by 6 months, the few remaining PRC's are located only in the peripheral retina /8,10,16/. Defective phagocytosis of shed rod outer segments (ROS) by retinal pigment epithelial (RPE) cells results in an accumulation of membrane debris in the subretinal space of RCS dystrophic rats /1/. The resultant opsin-immunostained debris zone is thickest at 2 months, while little debris remains at 6 months /8,16/. Also, in the third postnatal week, junctional complexes between dystrophic RPE cells begin to break down. Moreover, by postnatal week 10, many of these cells become detached from Bruch's membrane and degenerate /2/. These reports clearly show that RPE cells of RCS dystrophic rats are unhealthy and suggest that other metabolic functions typical of these cells may also be defective. Earlier experiments have shown that defective RPE cells are the cause of this animal model of inherited retinal dystrophy /1,3,5/. This may also be the case in some forms of retinitis pigmentosa and macular degenerations in humans. Based on the above observations, transplantation of normal, healthy RPE cells into retinas of RCS dystrophic rats may be a possible approach to arrest PRC degeneration. In turn, if successful in rats, this approach may be applicable to similar forms of human eye diseases.

Two laboratories have reported successful PRC rescue in RCS dystrophic rats by RPE-cell transplants, using different transplantation methods /7,9,15/. The results of those studies revealed PRC rescue with transplantation in retinas of 26 and 30 day-old RCS dystrophic rats for at least 3 months after transplantation. In one study /7,15/, transplanted RPE cells were observed attached to Bruch's membrane and their microvilli were detected between rod outer segments of rescued PRC's. Furthermore, rescued PRC's were observed at the lateral edge of the RPE-cell transplant, beneath host dystrophic RPE and membrane debris. These observations suggest the existence of an RPE-cell diffusible trophic factor which may affect PRC survival and development /6,8,15/. Also, as shown by immunocytochemistry, the ubiquitous membrane-bound enzyme ($Na^+ + K^+$)-ATPase and the photopigment opsin were normally distributed beneath RPE-cell transplants /14,15/.

The purpose of this study was to determine by microscopic and immunocytochemical methods the effects of RPE-cell transplantation in retinas of RCS dystrophic rats at early (prior to PRC degeneration) and late (during the period of advanced PRC degeneration) stages of the retinal disease process. Specifically, we wanted to know how far the disease process would progress before normal RPE-cell transplantation had little or no therapeutic value in regard to photoreceptor cell rescue.

MATERIALS AND METHODS

Animals

Eyes of Long Evans rat pups between 8-10 days of age were the source of normal RPE cells. Isolated pigmented RPE cells were injected into the retinas of pink-eyed RCS dystrophic rats at 10, 17, 26, 39, 43, 61 and 68 days and retinas were examined when these rats were 2 to 6 months of age. Control animals were age-matched nongrafted RCS dystrophic, pigmented Long Evans, nonpigmented Sprague-Dawley rats and vehicle-injected (sham control) RCS dystrophic rats.

RPE Cell Isolation and Transplantation

RPE cells were isolated from Long Evans rat pups using a previously described procedure /6,11/. The cells were concentrated to 60,000 cells/μl for transplantation. The isolated RPE cells were injected into the subretinal space of RCS dystrophic rats using a dorsal lesion method /6,15/. Briefly, the dorsal surface of the eye was exposed, then a small lesion was made through the sclera, choroid and Bruch's membrane between the two vorticose veins. The cells were injected into the retina with a 32 gauge blunt-edged needle at the lateral edge of the lesion. As sham controls, retinas of age-matched dystrophic rats were injected with 1 μl of vehicle (calcium ion-magnesium ion free Hank's balanced salt solution, HBSS).

Tissue Preparation, Microscopy and Morphometric Analysis

Eyes were removed from rats which were administered an overdose of sodium pentobarbital and these eyes were fixed in Bouin's fixative for 5 hours. Following dehydration, the eyes were embedded in paraffin using standard procedures. Paraffin-embedded eyes were sectioned at 5-10 μm and deparaffinized sections were then either stained with Hematoxylin-eosin or treated immunocytochemically. For electron microscopy (EM), eyes were fixed in 2.5% glutaraldehyde in 0.1M cacodylate, pH 7.4, trimmed to the limits of the RPE-cell graft, then embedded in Epon. Epon-embedded eyes were sectioned and these sections were poststained with uranyl acetate and lead citrate, then examined with a Zeiss EM-10CA electron microscope.

ONL thicknesses and total rescued ONL area were measured using JAVA software and an IBM computer. Any statistical differences of corresponding ONL measurements were determined by the students t-test.

Immunocytochemistry

The immunocytochemical method followed in this study has been described previously /13,14/. Briefly, deparaffinized sections were treated with 20% normal goat or rabbit serum prior to incubation with primary rabbit antibovine brain (Na^+ + K^+)-ATPase /4/ or sheep antibovine opsin /12/ antisera, respectively. Sections were then incubated in the appropriate secondary antibody conjugated to horseradish peroxidase ($F(ab')_2$-HRP), then treated with the chromogen diaminobenzidine in Tris buffer. Finally, sections were rehydrated, cleared in xylene, mounted in Permount, then examined with a Zeiss Universal light microscope using Nomarski optics.

RESULTS

The outer nuclear layer (ONL) in retinas of 4 month-old nonpigmented Sprague-Dawley rats was about 10 cells in thickness (Fig. 1A). Retinas of 4 month-old RCS dystrophic rats transplanted with RPE cells at 17 days exhibited an ONL of 8-10 cells in thickness. Also, outer (OS) and inner (IS) segments were observed beneath grafted pigmented RPE cells (Fig. 1B). However, in nongrafted retinas of age-matched dystrophic rats, few photoreceptors were detected at 4 months (Fig. 1C). Immunostaining for (Na^+ + K^+)-ATPase under RPE-cell transplants was dense along IS, in the inner (IPL) and outer (OPL) plexiform layers and on plasmalemma of rescued photoreceptor cells and inner nuclear layer (INL) cell bodies (Fig. 1D). Immunostaining in respective regions of nongrafted retinas of 4 month-old dystrophic rats was still detected within the IPL and INL (Fig. 1E). Also, beneath RPE-cell grafts, opsin immunostaining was observed along OS, IS and on the plasmalemma of rescued photoreceptor cells (Fig. 1F), while only the debris zone immunostained for opsin in corresponding regions of nongrafted retinas (Fig. 1G).

Examination of retinas of 4 month-old RCS dystrophic rats transplanted with RPE cells at 10 days revealed somewhat similar PRC rescue as shown for the 17 day transplantations. The distribution of (Na^+ + K^+)-ATPase beneath the RPE-cell graft was shown along IS, in the plexiform layers and surrounding INL cell bodies. Also, the immunostained ONL was almost as thick as observed at the 17 day grafting period and (Na^+ + K^+)-ATPase immunostained IS were clearly demonstrated (Fig. 2A). Opsin immunostaining under RPE-cell transplants were detected along OS, IS and on the plasmalemma of rescued photoreceptor cells (Fig. 2B).

RPE-cell transplantation into retinas of 43 (Fig. 3A) and 68 (Fig. 3B) day-old RCS dystrophic rats resulted in very little, if any, PRC rescue, as shown 17 and 52 days post transplantation, respectively. Opsin-immunostained photoreceptor cells were scarce, indicating no rescue effect at both transplantation periods.

Photoreceptor cell rescue was clearly shown lateral to RPE-cell transplants and beneath membrane debris in 4 month-old RCS dystrophic rats grafted at 26 days (Fig. 4A). EM examination of these retinas revealed grafted pigmented RPE cells attached to Bruch's membrane (Fig. 4B). Also, rescued segment-bearing photoreceptor cells under the RPE-cell graft showed a normal-appearing ultrastructure (Fig. 4C).

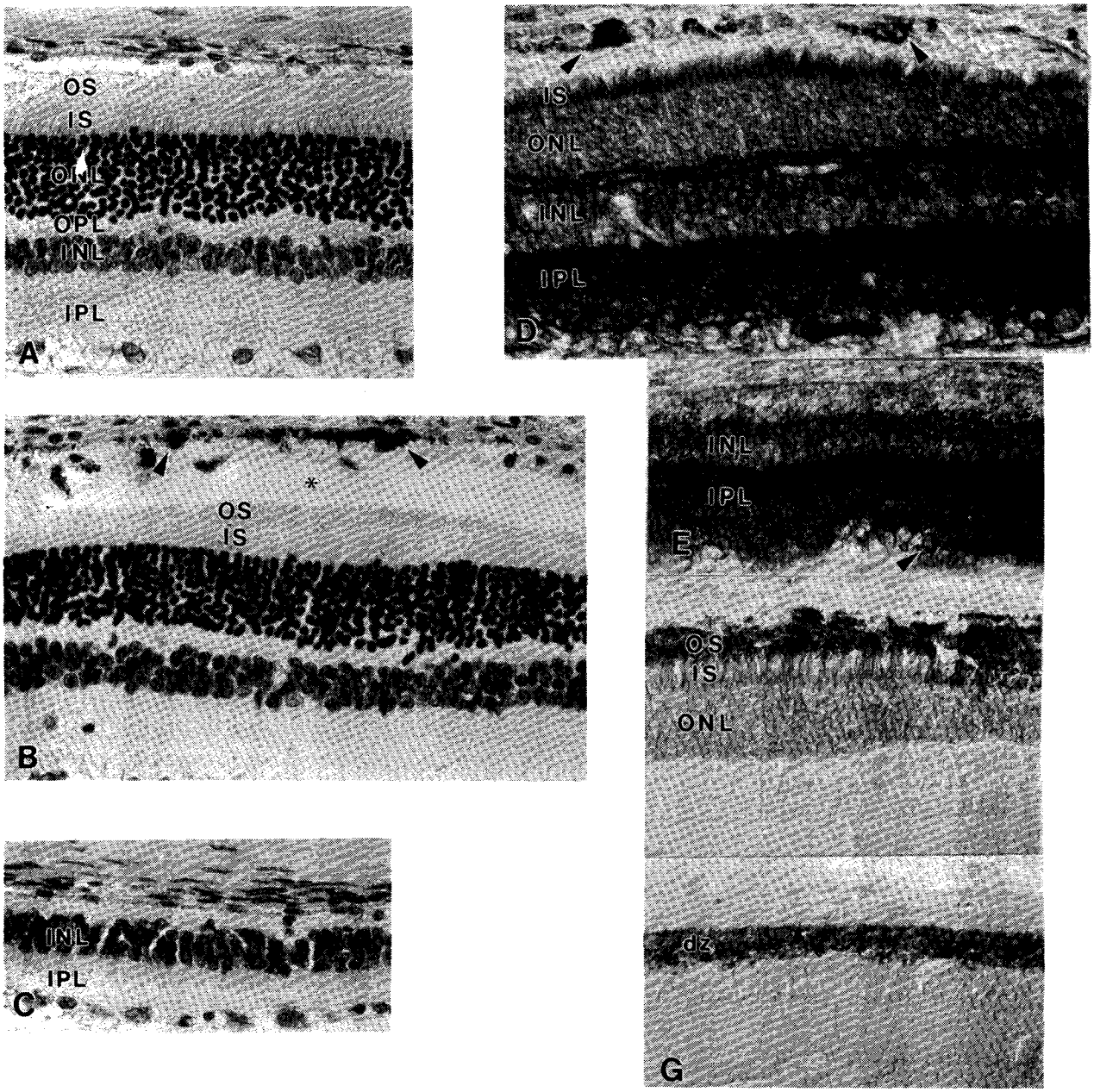

Fig. 1: Effects of RPE-cell transplantation in retinas of 17 day-old RCS dystrophic rats, examined at 4 months. A) Retinas of 4 month-old control Sprague-Dawley rats showed an ONL about 10 cells in thickness. B) Beneath RPE-cell transplants (arrowheads) in retinas of 4 month-old RCS dystrophic rats, PRC's bearing IS and OS are shown. Asterisk indicates separation of OS from RPE due to tissue processing. C) In nongrafted retinas of 4 month-old RCS dystrophic rats, few PRC's are detectable. D) $(Na^+ + K^+)$-ATPase immunostain under the graft (arrowheads) is distributed along rescued photoreceptor cell IS, in both plexiform layers and surrounds cell bodies in the INL. E) in nongrafted retinas, $(Na^+ + K^+)$-ATPase immunostain is demonstrated in the IPL and INL and at the periphery of ganglion cell bodies (arrowhead). F) Beneath RPE-cell grafts, opsin immunostain is localized along OS and IS and on plasmalemma of rescued photoreceptor cells. G) Opsin immunostaining in nongrafted retinas of 4 month-old RCS dystrophic rats is distributed in a debris zone (dz), which is only detectable in the peripheral retina. Original magnification: 375x. A-C are Hematoxylin-Eosin (H&E) stained sections.

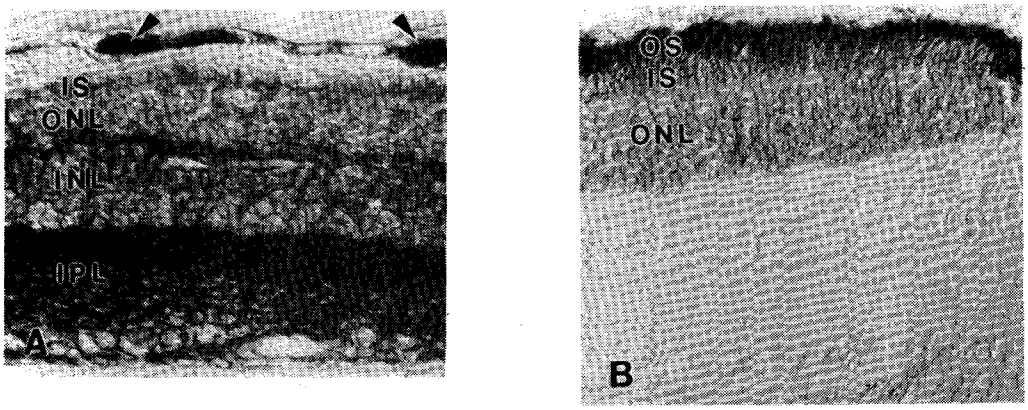

Fig. 2: Effects of RPE-cell transplantation in retinas of 10 day-old RCS dystrophic rats and examined at 4 months. A) A normal distribution of $(Na^+ + K^+)$-ATPase immunostaining is detected beneath RPE-cell transplants (arrowheads), especially dense along IS of rescued photoreceptor cells and within the plexiform layers. PRC rescue, as shown by the $(Na^+ + K^+)$-ATPase immunostained ONL, is clearly evident beneath RPE-cell transplants. B) Immunostaining for opsin under RPE-cell transplants is demonstrated along OS, IS and on plasmalemma of rescued photoreceptor cells. Original magnification: 375x.

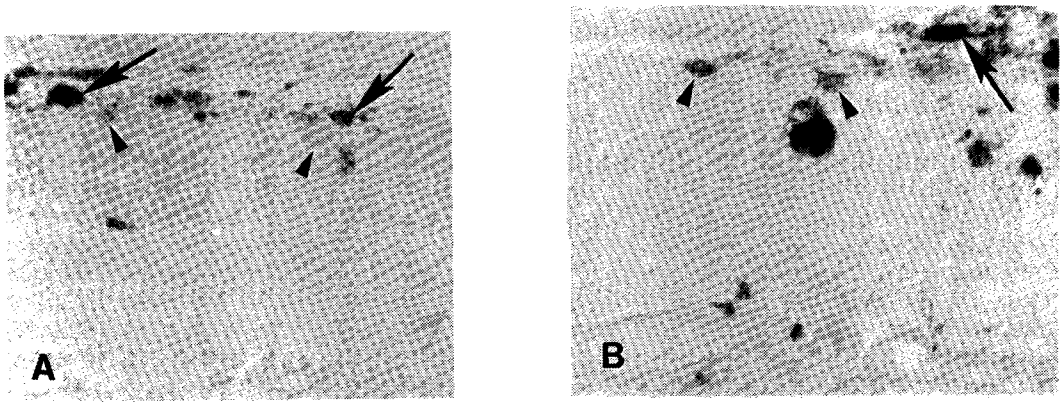

Fig. 3: Effects of RPE-cell transplantation in retinas of 43 and 68 day-old RCS dystrophic rats, when examined at 2 and 4 months, respectively. No PRC rescue was observed under grafted RPE cells (arrowheads), when RPE cells were transplanted at 43 (A) or 68 (B) days. A few opsin-immunostained PRC's (arrows) are present near transplanted pigmented RPE cells (arrowheads). Original magnification: 375x.

As shown in Figure 5, RPE-cell transplantation in retinas of RCS dystrophic rats at 17 days caused rescue of photoreceptor cells, such that at 4 months the ONL thicknesses approximated that of control retinas of age-matched Sprague-Dawley rats. ONL thicknesses in RPE-cell transplanted dystrophic retinas at postnatal days 10 and 26 when examined at 4 months were slightly, but significantly, decreased from retinas of both age-matched control retinas and 17 day transplanted dystrophic retinas. RPE-cell transplantation at 39 days resulted in no significant photoreceptor cell rescue when retinas were examined about 3 months after grafting (at 4 months). When vehicle (1 μl of Ca,Mg-free HBSS) was injected into retinas of 17 day-old RCS dystrophic rats, ONL thicknesses were slightly, but significantly increased above thicknesses of nontreated retinas at 2 months. However, this vehicle effect on the ONL was

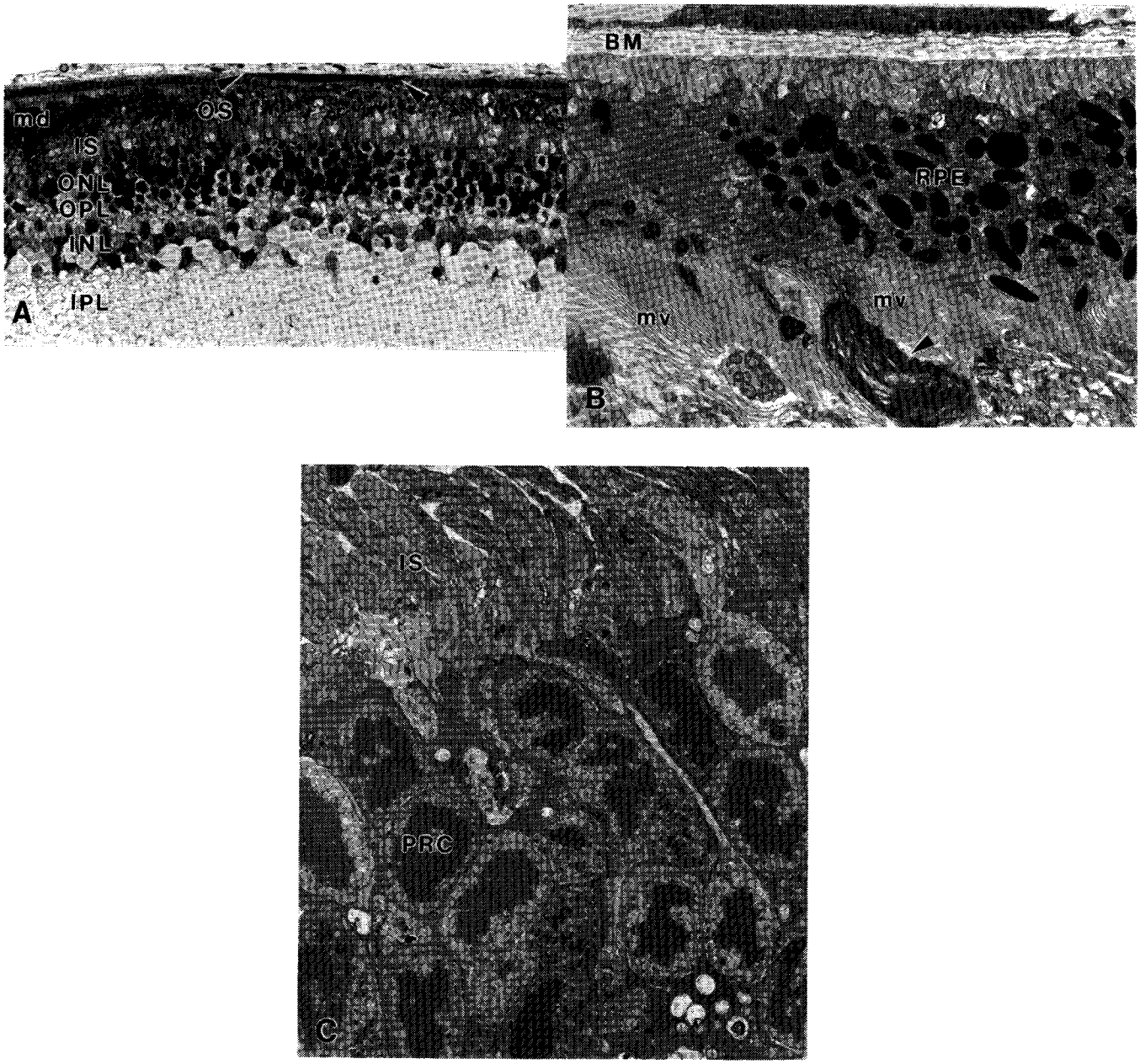

Fig. 4: Examination of RPE-cell grafted retinas of 4 month-old RCS dystrophic rats transplanted at 26 days. A) As shown in this toluidine-blue stained Epon section, rescued PRC's were observed not only beneath but lateral to RPE-cell transplants (outer limits of graft are indicated by arrowheads) and even under membrane debris (md). B) At the EM level, a melanosome-containing grafted RPE cells is shown attached to Bruch's membrane (BM). Typically, these grafted cells phagocytose shed ROS. In this case, a shed ROS (arrowhead) is surrounded by microvilli (mv) of a grafted pigmented RPE cell. C) Surviving host PRC's beneath RPE cell transplants have a normal structural appearance and, here, are shown bearing inner segments (IS). Original magnification: A, 375x; B, 6500x; C, 3300x.

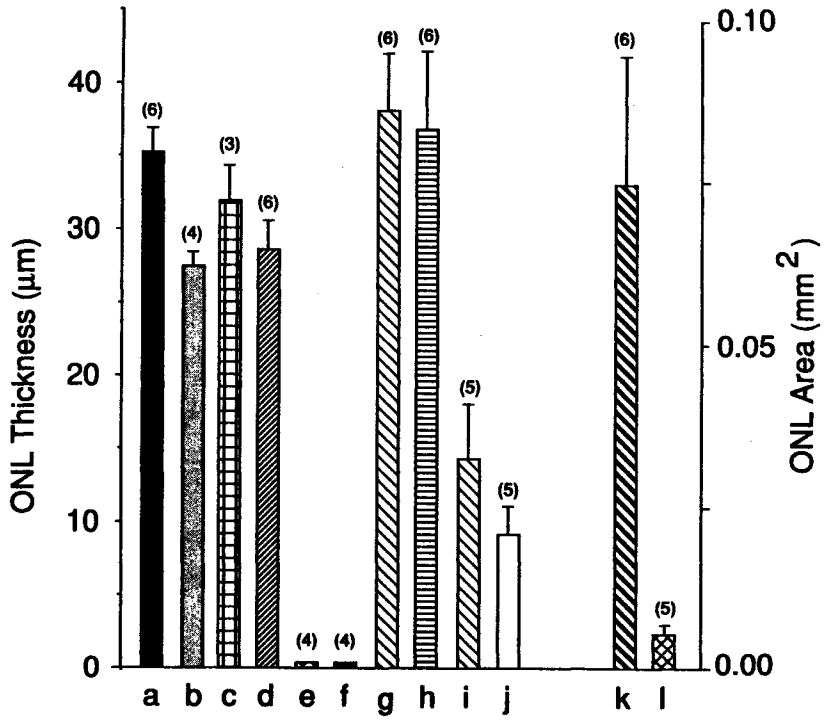

Fig. 5: Graph showing effects of RPE-cell transplantation on the outer nuclear layer (ONL) at early and late stages of degeneration in retinas of RCS dystrophic rats. The ONL thicknesses in retinas of 4 month-old RCS dystrophic rats when transplanted at 17 days (c) are not significantly different from ONL thicknesses of age-matched control Sprague-Dawley rats (a). However, in retinas of 4 month-old RCS dystrophic rats when transplanted at 10 (b) and 26 (d) days, the ONL thicknesses are significantly different ($p<0.001$) from ONL thicknesses of control retinas (a). Also, retinas of 4 month-old RCS dystrophic rats when grafted at 39 days (e) exhibit an ONL not appreciably thicker than nongrafted dystrophic retinas (f) at the same age. Furthermore, ONL thicknesses in retinas of 2 month-old RCS dystrophic rats, when transplanted at 17 days (h) are not significantly different from ONL measurements of 2 month-old Sprague-Dawley rats (g). When retinas of 17 day-old dystrophic rats are injected with vehicle, a slight increase in the ONL is detected at 2 months (i). This resulting ONL thickness is statistically greater ($p<0.025$) than the ONL of nontreated retinas of age-matched dystrophic rats (j), but significantly less than ($p<0.001$) the ONL thickness of 2 month-old control Sprague-Dawley (g) and RCS dystrophic rats, when transplanted at 17 days (h). In addition, the rescued ONL area beneath transplanted RPE cells in 17 day-old RCS dystrophic rats, when examined at 2 months (k), is vastly increased when compared to respective areas of sham control dystrophic retinas treated and examined at the same age (l). The number of eyes examined for each study is in parentheses. The bars represent the standard deviations for each measurement.

dwarfed when contrasted to the RPE-cell transplantation effect on the ONL of age-matched dystrophic retinas. Furthermore, the effect of the vehicle injection was short-lived, as ONL thicknesses at 3 months (5.2 ± 1.3 μm) were reduced to control age-matched dystrophic retina levels (4.3 ± 0.45 μm). Also, the area of the ONL affected by RPE-cell transplants in 17 day-old RCS dystrophic rats, when examined at 2 months, was over 10 times greater than respective ONL areas in vehicle-injected dystrophic retinas examined at the same time period.

DISCUSSION

The results of this study suggested that after a specific time period of disease progression in retinas of RCS dystrophic rats (about 30 days), RPE cell transplantation will not cause a photoreceptor cell rescue, at least on a long term

basis (4 months). For example, RPE cell transplantation in retinas of RCS dystrophic rats prior to (10 days) and during the initial stages of (17-26 days) PRC degeneration caused PRC rescue to an extent similar to that observed in earlier transplantation studies /7,8,15/. However, little or no PRC survival was detected when transplantation was performed 2 weeks later at stages of advanced PRC degeneration (43 days).

As shown in earlier studies /7,15/, photoreceptor cell rescue was observed lateral to grafted RPE cells and beneath host dystrophic RPE cells and membrane debris. In those studies, transplanted RPE cells were distributed in a patchy mosaic pattern. Therefore, even though rescued photoreceptor cells may appear to be distant from areas of normal RPE cells, these transplanted cells are not far removed from the affected site. These results support the hypothesis that RPE cells release a diffusible trophic factor(s) which is essential for PRC development and survival. In fact, a preliminary study in our laboratory has shown that normal RPE-cell conditioned medium (CM) injected into retinas of 26-27 day-old RCS dystrophic rats arrested PRC degeneration for at least 2 weeks after treatment /17/.

Injection of vehicle (sham control) into retinas of 17 day-old RCS dystrophic rats appeared to have a slight beneficial effect on photoreceptor cells about one month after treatment, but 2 months after the injection, the ONL thicknesses were reduced to control dystrophic retina levels and was confined only to the area of the needle track. The reason for this transitory effect on PRC's by vehicle-injection in retinas of RCS dystrophic rats remains unknown. This phenomenon is currently under investigation in this laboratory.

In conclusion, RPE cell transplantation into retinas of RCS dystrophic rats at early, but not late, stages of retinal degeneration caused extensive photoreceptor cell rescue. From these studies it appears that the optimum period for RPE-cell transplantation in the retinas of RCS dystrophic rats to achieve maximal photoreceptor cell rescue is at approximately postnatal day 17. RPE-cell transplantation in retinas of 10 day-old RCS dystrophic rats may have caused some trauma to the small eye, thus resulting in a lessened effect on photoreceptor cell survival than was shown at 17 days. Also, photoreceptor cells in retinas of 26 day-old RCS dystrophic rats have already begun to degenerate, which may explain the reduced effect of RPE-cell transplantation when compared to the 17 day transplants. These and earlier studies /7,9,15/ are the first demonstrations of the rescue of neurons by a transplantation procedure that would normally die due to an inherited disease process.

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