Selective Transplantation of Rods Delays Cone Loss in a Retinitis Pigmentosa Model

Saddek Mohand-Said, MD; David Hicks, PhD; Henri Dreyfus, PhD; José Alain Sahel, MD

Background: Rod-cone retinal degenerations (retinitis pigmentosa) are typified by initial rod loss followed by secondary cone death. Rod death, predominantly caused by gene mutations expressed specifically in these cells, induces scotopic vision loss. Cone death, the overriding cause of blindness, has no current explanation. Disease progression and preliminary data suggest that cone survival depends on rods.

Objective: To establish whether rod transplantation into mutant rodless retinas could halt cone loss.

Methods: We transplanted pure sheets of rods isolated from normal-sighted mice into the subretinal space of recipient retinal degeneration mice lacking rods but possessing approximately 30% residual cones. Control animals were unoperated on or grafted with inner retinal cells from young normal donors, entire retinas from aged retinal degeneration mice, or gelatin. Two weeks after surgery, we quantified by an unbiased method the numbers of host retinal cones after immunolabeling with specific markers.

Results: Only mice receiving rod-rich transplants demonstrated statistically significant greater cone numbers, with rescue of 40% of host cones normally destined to die during this period.

Conclusion: Cone survival depends specifically on rods.

Clinical Relevance: Such findings indicate that transplantation of rods could limit loss of cones, thus preserving useful vision in human retinitis pigmentosa.


Retinal dystrophies such as retinitis pigmentosa (RP) and age-related macular degeneration (ARMD) affect a large and growing number of people (1 birth in 3000 to 1 birth in 7000 for RP and >25% of persons aged ≥75 years for ARMD). These diseases are incurable, but several avenues of research are being actively pursued, ie, replacement strategies (transplantation and corrective gene therapy) and pharmacological treatment (vitamin A supplementation, channel activity modulators,1 or neurotrophic factors2-4). Replacement of defective genes and neurotrophic factor application have met with some success in animal studies3,5 but have yet to undergo clinical trials.

During the past 5 years, several unsuccessful attempts to treat RP and ARMD through retinal cell transplantation have been performed in the clinical setting.6-8 Some studies have contributed to establishment of the surgical procedure as relatively safe, albeit ineffective.9 Critical analysis of these attempts reveals possible reasons underlying the lack of success, including the lack of experimental models of ARMD precluding any preclinical validation and, especially, that the prospects of visual recovery in legally blind patients were envisioned despite the fact that in pertinent animal models of RP no definite demonstration of connectivity or functionality of transplanted cells has been reported.10,11

However, the pattern and time frame of photoreceptor degeneration has provided clues for a therapeutic approach using photoreceptor transplantation, as well as possibly consolidating transplantation with pharmacological treatments. The fact that most RP mutations are expressed exclusively in rods suggests that cone death, observed in all human and animal conditions of rod degeneration, represented the consequence of this event and the key pathway leading to blindness.12-17 In several cases of RP with rhodopsin mutations, loss of cone function occurred after functional evidence of disappearance of these attempts reveals possible reasons underlying the lack of success, including the lack of experimental models of ARMD precluding any preclinical validation and, especially, that the prospects of visual recovery in legally blind patients were envisioned despite the fact that in pertinent animal models of RP no definite demonstration of connectivity or functionality of transplanted cells has been reported.10,11

However, the pattern and time frame of photoreceptor degeneration has provided clues for a therapeutic approach using photoreceptor transplantation, as well as possibly consolidating transplantation with pharmacological treatments. The fact that most RP mutations are expressed exclusively in rods suggests that cone death, observed in all human and animal conditions of rod degeneration, represented the consequence of this event and the key pathway leading to blindness.12-17 In several cases of RP with rhodopsin mutations, loss of cone function occurred after functional evidence of disappearance
MATERIALS AND METHODS

Experimental procedures adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmology and Vision Research.

ANIMALS

Normal C57BL/6 and mutant C3H/He/N strains were obtained from Charles River France, Saint-Aubain-Lès-Elbeuf, France. All animals were reared in cyclic light (12 hours on and 12 hours off, with a room illumination of approximately 15 lux) at 25°C from birth.

PREPARATION OF THE TRANSPLANTS

Photoreceptor-pure sheets were prepared from normal 8-day-old C57 mice by planar vibratome sectioning exactly as previously described.18,20 To obtain preparations of inner layers from normal retina, 8-day-old C57 retinas were cemented to the gelatin block with the photoreceptors facing up, and the outer nuclear layer was progressively shaved off until the photoreceptors had been completely removed. Whole retinas were dissected from 8-week-old rd mice, an age at which rods are completely missing. Slabs of gelatin were also prepared for transplanting. In each case, equal sizes of samples (a lozenge of approximately 3 mm on its longest side) were prepared for grafting. Histological observations showed that a pure preparation of photoreceptor was obtained. Although this implies elimination of the synaptic region of most cells, the live dead assay assessment of photoreceptor viability and in vitro studies using vibratome-isolated photoreceptors showed that these regions were viable more than 7 days after isolation.21

SURGERY

Recipient mice (C3H/He, rd/rd) aged 35 days—an age at which more than 99.7% of rods have degenerated, leaving a monolayer of cones—were operated on as previously described.18,19; to ensure, despite the difficulties encountered in such small eyes, reproducible data, the shape of the transplant was standardized by trephination (trapezoidal shape of 3.0 × 1.5 mm). We also used a custom-made remote advanced injector (0.9 mm in diameter). The experimental series comprised (1) normal 8-day-old C57BL/6 mouse photoreceptors transplanted into 3-week-old rd mice (n=12), (2) normal 8-day-old C57BL/6 mouse inner retinal layers transplanted into 3-week-old rd mice (n=12), (3) 8-week-old rd mouse whole retinas transplanted into 5-week-old rd mice (n=12), and (4) gelatin transplanted into 5-week-old rd mice (n=7) as a control for surgical effects. In all animals, only the right eye was operated on, and the left eye served as the nonoperated control; the mice were killed 2 weeks after the grafting procedure to monitor cone survival. Five-week-old rd nontransplanted mice (n=5) were used as a reference group, from which were calculated the rate of normal cone degeneration during the same 2-week period in this animal model.

MORPHOLOGIC STUDIES

These studies were conducted according to previously published methods.18 The different probes used were screened initially on intact retinas isolated from 3-week-old normal and rd mice to ensure their specificity. Immunolabeling of retinas was performed as described previously,19 using peanut agglutinin (PNA) lectin from arachis hypogae (Sigma-Aldrich, Saint Quentin Fallavier, France) to label cones and anti-opsin Rho-4D2 antibody22 to label rods. Prelabeling of grafts before transplantation was performed by briefly incubating the tissue in PKH26-GL lipophilic dye (Sigma-Aldrich).

CELL COUNTS

The total numbers of labeled cones were estimated in flat-mounted retinas using a stereological approach to obtain unbiased samples, as previously described.13,23 Use of such an approach eliminated regional variations in cone numbers, which are known to exist in this strain,17 and reduced sampling error to 5% to 10% of the mean. The cells were counted on 200 sampled, nonoverlapping, 1825-µm² zones determined in a systematic random fashion to sample equally the whole retinal surface extending from the center of the optic nerve head over a radius of 2 mm.

STATISTICAL ANALYSIS

For comparisons of grafted vs control retinas, difference analysis was performed with computer software (StatWorks Data Students t Statistic; Cricket Software, Malvern, Pa) using the parametric method of the t test for unpaired series13,23 for one variable, ie, the total cone number estimates per retina. We performed the Scheffé t test after variance analysis and the Bartlett test.

of at least 75% of rod outer segments.13 To test the hypothesis that reinsertion of rods into degenerating retinas could improve residual cone survival, we performed an initial experimental study in the retinal degeneration (rd) mouse, the murine counterpart of one form of human RP exhibiting mutations in the β subunit of cyclic guanosine monophosphate rod-specific phosphodiesterase. This provided preliminary data suggesting that rod-rich transplants delayed cone cell death.18 Yet, the data sampling methods in existence at the time led to measures performed over only a limited central retinal surface, and the high variability of cone densities obscured the potential clinical significance. Nevertheless, the data prompted us to test whether paracrine interactions underlay cone survival through the use of an in vitro coculture model. The design of an unbiased, reproducible stereological method to reliably estimate total cone numbers was critical for this study. This method unambiguously demonstrated that retinas containing rods release a diffusible factor that promotes cone survival in the rd mouse.20

While awaiting identification of this factor, rod transplantation hence offers a potentially valuable ophthalmicologic remedy to cone loss in human patients with RP. Definitive proof of this beneficial effect in intact animal models is paramount before photoreceptor transplanta-
tion in patients with RP could be attempted with reasonable hope for delay in central visual loss. We provide herein the first unequivocal data showing that the total cone population can be significantly rescued in an animal model of RP by transplanting small amounts of rod photoreceptors and that no other grafted retinal cells induce such paracrine effects. These findings validate the concept of transplantation as a means to promote survival of degenerating neurons, shifting the emphasis to trophic interactions rather than reconstruction of circuitry, and eventually lay the basis for clinical trials in patients with RP affected with mutations in rod-specific proteins.

RESULTS

The different experimental groups of recipient 5-week-old rd mice received subretinal transplants of either outer retina (containing only photoreceptors) or inner retina (containing retinal interneurons, ganglion cells, and glia) prepared from normal 8-day-old C57 mice. Another group received fragments of the entire retina of 8-week-old rd mice (lacking photoreceptors but containing the other retinal cell types). A final group received sheets of gelatin alone. All animals were killed after 2 weeks. Grafts were visualized through prelabeling with lipophilic fluorescent dyes before transplantation. Label was always confined to the grafts, indicating that cells did not spread to colonize host tissue (Figure 1). Because the normal murine retinas from which the photoreceptor transplants were isolated are heavily rod dominant (97% rods), photoreceptor grafts within fixed, flattened whole mounts could also be visualized against the rodless rd host retina through immunohistochemical labeling with a specific antirod opsin antibody (Figure 1). Photoreceptor grafts were detected in 100% of the group receiving such transplants and were of variable size and shape, routinely appearing as a sheet or scattered islands covering less than 10% of the total retinal surface. The grafts were located in the middle to far periphery of the host retina.

Cone photoreceptors were visualized using PNA lectin, which selectively binds to their outer segments and cone matrix sheaths (Figure 1). The stereological nonbiased counting frame enabled reproducible counting of PNA-labeled cones over the entire retinal surface. Quantification of PNA-labeled cones in flattened retinal whole mounts from unoperated 5-week-old C3H/He/N rd mice gave mean±SEM total numbers of 119 415±5160 (n=10). Because no statistically significant differences were noted among unoperated left eyes of the 4 experimental groups, we averaged cone numbers from these eyes to obtain mean±SEM total cone numbers at 7 weeks, which were 88 515±6778 (n=42) (Table). This represents a loss of more than 30000 cones during the investigation, ie, approximately one quarter of the cone population between the fifth and seventh weeks of life. This difference was statistically significant (t=11.01 for unpaired groups; P<.001).

Four paired groups were obtained, and to verify whether the variance test analysis was applicable, we performed the Bartlett test. The calculated χ² = 5.97 was below 14.06 for α = .05. There was no statistically significant difference between the variance of the 8 groups, so we performed variance analysis and the results showed that the comparisons between the 8 groups were allowed (F=2.60>2.15 [I₁=7, I₂=76]). Among the 8 groups, only the rodless rd mice grafted with C57 mouse photoreceptor sheets showed higher mean±SEM residual cone numbers (98 913±5713), and these numbers were statistically significantly different from those of the other 7 groups (Scheffé test; t₁₀=3.79, P<.001).

The degree of cone survival was comparable to that in the previous in vitro data, although the quantity of transplanted tissue was small relative to the host retina (<10%) and the survival time was longer (2 weeks in vivo as opposed to 1 week in vitro). These data unambiguously show that transplantation of limited amounts of normal rods results in significant neuroprotection against secondary cone death. The differences in mean±SEM cone numbers observed in the other 7 experimental groups (operated and unoperated eyes) were never statistically significant: transplanted normal inner retina, 90 846±8688; transplanted entire aged rd retina, 91 780±3738; or transplanted gelatin, 86 026±4595 (Table and Figure 2). These paradigms indicate that only transplantation of rods,
not that of other retinal neuronal and glial populations or surgery by itself, elicits preservation of host cones.

We provide the first unequivocal data showing that the total cone population can be significantly rescued in an animal model of RP by transplanting small amounts of photoreceptor cells and that no other retinal cell can induce such a paracrine effect.

The recent development of retinal degeneration knowledge suggests that interactions between photoreceptors play a major role in the cell death mechanisms. Therefore, their elucidation and regulation is paramount for the development of therapeutic strategies for retinal degenerations. The link between the rod loss and the cone death is demonstrated by studying the sequence from mutant rhodopsin allele to rod and cone degeneration in humans. From flies to humans, in most cases of rod-cone degenerations the retina affected shows the same degeneration sequence of photoreceptors. Constructed chimeric mice expressing a mutant rhodopsin gene in some retinal areas adjacent to other areas expressing a normal rhodopsin gene exhibited uniform photoreceptor degeneration similar to that observed in transgenic rd mice but at a slower rate. It is also reported that in rd fruit flies, only the 6 photoreceptor cells (R1-R6) in every ommatidium expressing a mutant form of rhodopsin die first and therefore the other 2 photoreceptor cells (R7 and R8) expressing a different form of rhodopsin, so not affected directly, die shortly after. This suggests strongly that the survival of the R7 and R8 photoreceptor requires a signal from R1-R6 cells.

Previous work suggested that normal retina releases a diffusible signal that stimulates cone survival in the rd mouse. The present data demonstrate that transplantation of healthy photoreceptors into degenerating retinas reduces host cone loss and that this effect is induced by photoreceptors but not other retinal cells. This trophic effect is observed in the total cone population. Although this is probably mediated by a diffusible signal, the regional variations of cone population and transplant placement preclude the quantitative assessment of the amount of rescue as a function of distance from the transplant. This might be feasible in larger animal models (eg, transgenic rats or pigs). We cannot ascertain at the present time whether such effect is induced directly by rods themselves or as a consequence of their interaction with other retinal populations and whether this effect is mediated by a diffusible factor or by other mechanisms (eg, structural and protection from toxins released by dying cells). Clues might come from the identification of the underlying molecular mechanisms, as currently undertaken in our group.

Despite the small size of the mouse eye, the surgical technique is reproducible. Its use in other larger animal models should be more straightforward, facilitating detailed examination of possible correlation between graft size and the magnitude of cone survival, long-term transplant survival, and graft-host retina interactions (integration). Furthermore, the present data are limited to histological findings, and it will be necessary to demonstrate functional visual improvement in grafted retina as well. The availability of transgenic pigs expressing mutations in the rhodopsin gene as observed in some forms of autosomal dominant RP should be of considerable value in such studies.

Rod transplantation was performed after almost complete rd host rod disappearance and during the progression of host cone degeneration, suggesting a relatively broad window of applicability of such treatments during human retinal degeneration. In human patients, cone loss occurs gradually over many years. Such results support the concept of transplanting neural cells as a means
to promote survival of degenerating neurons and lay the basis of a clinical trial in a group of 10 patients with RP affected with mutations in rod-specific proteins (eg, rhodopsin and phosphodiesterase). This trial, herein outlined, was recently approved by the relevant local and national ethical, scientific, and safety regulatory authorities. It will compare the decay in cone function (ETDRS visual acuity, standardized color vision, visual field, and cone and multifocal electroretinogram) between the operated and nonoperated eye in patients with RP affecting both eyes symmetrically. Transplantation of small (<10 mm²) sheets of photoreceptors isolated from adult donors in the pericentral retina in patients with end-stage RP (≤3° of visual fields, measurable acuity inferior to 20/40) should minimize surgical trauma to the functional central part of the retina, as shown in a previous study. We envision that after a minimum of 3 years, a significant difference in cone function in the treated eye should be observable. This clinical application of neural retinal transplantation might, in the long term, prove to be the most effective in preventing blindness from hereditary conditions.

Finally, the results of these transplantation studies are in line with the existence of rod-derived cone trophic factors and with recent demonstration that protection of rods might indirectly affect cone survival and function. The identification and pharmacological application of such rod-derived molecules will be of paramount importance for future treatment of RP and ARMD.

Accepted for publication January 5, 2000.

This work was supported by Fédération Nationale des Aveugles et Handicapés Visuels de France, La Fondation de l’Avenir, INSERM, Le Ministère de l’Education Nationale, de la Recherche et de la Technologie, Association Française contre les Myopathies, L’Institut d’Etudes des Greffes, Paris, France; Retina France, Coulomier; and CIBA Vision, Buelach, Switzerland.

We thank N. Sadeg, MD (Laboratoire Claude Bernard, Hôpital de Pontoise, Pontoise, France), for assistance with the statistical analysis; M. Simonutti for technical assistance; and T. Léveillard, PhD, for useful discussion.

Reprints: José Alain Sahel, MD, Laboratoire de Physiopathologie Cellulaire et Moléculaire de la Répine, EMI 9918 INSERM, Clinique Médicale A, Hôpitaux Universitaires de Strasbourg, 1 Place de l’Hôpital, 67091 Strasbourg Cédex, France (e-mail: sahel@neurochem.u-strasbg.fr).

REFERENCES

3. LaVail MM, Unoхи K, Yasumura D, Mattes MT, Yancopoulos GD, Steinberg RH. Multiple growth factors, cytokines and neurotrophins rescue photoreceptors from the damaging effects of constant light. Proc Natl Acad Sci U S A. 1992;89:11249-11253.