Adult Rabbit Retinal Transplants

Johan Wassellius and Fredrik Ghosh

PURPOSE. To study the survival of adult retinal grafts prepared in a physiologically optimized way.

METHODS. Twenty-three rabbits received an adult full-thickness rabbit retinal transplant positioned under the host retina, using a vitrectomy technique. The transplants were prepared using a procedure based on a previously described in vitro model used for physiological experiments on the adult retina. Five rabbits received a fragmented graft. All grafts were prelabeled with 4′,6-diamidin-2-phenylindol (DAPI; Boehringer Mannheim, Indianapolis, IN) in aqueous solution to allow identification. The eyes were examined by light and fluorescence microscopy 6 to 174 days after surgery. To assess the amount of cell death in the graft before actual transplantation, in vitro experiments were performed. The extent of cell death in retinas prepared by the optimized protocol was examined and compared with a simpler preparation previously used successfully for embryonic grafts. The amount of cell death in the in vitro experiments was examined using a fluorescent green nucleic acid stain that penetrates dying cells.

RESULTS. In 21 of the 23 animals that received full-thickness grafts prepared in an optimized way, the transplant survived. Sixteen grafts, including all four with a 174-day survival time, displayed normal morphology, with all retinal layers preserved. The fragmented grafts survived poorly. The in vitro experiments showed minimal cell death in retinas prepared according to the optimized protocol, whereas control retinas displayed extensive cell death after 5 hours.

CONCLUSIONS. The results showed that it is possible to transplant adult retina in the rabbit and that the grafts survive well if they are prepared under physiologically optimized conditions and the integrity of the grafted tissue is kept intact. (Invest Ophthalmol Vis Sci. 2001;42:2632–2638)

Transplantation within the central nervous system offers the possibility of replacing neurons lost in degenerative diseases. Experimental transplantations have been performed for a number of years to various parts of the brain (for a review, see Ref. 1) and retina. Neuroretinal transplantation to the retina is intended to alleviate symptoms caused by degenerative diseases, such as retinitis pigmentosa and age-related macular degeneration. Various forms of grafts from full-thickness sheets to suspensions of neural progenitor cells derived from the brain have been extensively studied in animal models as well as in limited clinical trials. In most transplantation protocols, both in the eye and brain, fetal or neonatal donor tissue has been preferred because of a substantially better survival than adult counterparts. In the clinical setting, however, access to immature donor tissue is limited, and investigating other transplant sources such as adult neuronal grafts may therefore be valuable. The retina is a highly organized tissue, and another reason for exploring adult retinal grafts is that in many of the models using immature donor tissue, the delicate retinal architecture and neurocircuity are not reproduced.

The use of adult donor tissue is not uncomplicated. The adult mammalian retina is vulnerable, and it has been shown that degenerative changes occur as early as 15 minutes after death. However, found that the adult retina can be kept alive and functional in vitro for many hours if dissection and handling of the retina are undertaken in a physiologically optimized way. Crucial factors in this process included access to oxygen and nutrients, stability of pH at a physiological level, and minimizing of stress caused by bright light or physical manipulation.

For this study we wanted to explore whether careful handling of the donor tissue would promote better survival of adult retinal grafts than has previously been reported. Therefore transplanted adult full-thickness rabbit retina prepared in a physiologically optimized way to adult hosts and studied their survival in the light microscope.

MATERIALS AND METHODS

Animals

Twenty-eight pigmented mixed-strain rabbits, 2 to 4 months old, from a local breeder were used as hosts. Nine adult rabbits of the same age as the recipients (littermates) were used as donors. Surgery was performed at nine different sessions. In each session, tissue from one donor animal was used. Twenty-three of the hosts received a full-thickness graft and the remaining five a fragmented one. Each host received one graft. Four additional rabbits were used for in vitro experiments in which the extent of cell death in retinal explants was measured.

To establish the immunocompetence of the host animals from our local breeder, three separate rabbits (littermates) received skin transplants from each other. All skin grafts were rejected, suggesting a low degree of inbreeding among the animals and that they were immunocompetent.

All proceedings and animal treatment were in accordance with the guidelines and requirements of the Government Committee on Animal Experimentation at Lund University and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Graft Preparation

To label the graft retina, the donor rabbit received an intravitreal injection of 200 ng 4′,6-diamidino-2-phenylindol (DAPI; Boehringer Mannheim, Indianapolis, IN) in aqueous solution to a volume of 50 µl 24 hours before surgery. DAPI injected by this method is known to result in a bright blue nuclear staining of retinal neurons. Cholinergic neurons are most prominently labeled but most other neurons are also labeled and can be identified based on the size and shape of their nuclei and their localization.

Further preparation of the donor tissue was performed according to the in vitro model developed by and Ames and

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The surgical procedure for full-thickness transplantation has been described in detail in previous articles. In summary, a three-port surgical procedure was performed using a threeport vitrectomy system (DEI-750; Optronics, Goleta, CA). The anterior segment was placed in Ames medium. The posterior segment was cut, the lens and vitreous body removed, and the posterior segment placed in Ames medium. The posterior segment was cut, the lens and vitreous body removed, and the posterior segment placed in Ames medium. The anterior segment was placed in Ames medium and kept in darkness on a gently rocking platform. The final preparation was made minutes before actual transplantation when the neuroretina was transferred briefly from the epithelium with a glass rod, until it was attached only at the optic nerve head. The optic nerve was then cut, and the eye was removed. Within a few seconds, the anterior segment was cut, the lens and vitreous body removed, and the posterior segment placed in Ames medium. The posterior segment was cut, the lens and vitreous body removed, and the posterior segment placed in Ames medium. The anterior segment was placed in Ames medium and kept in darkness on a gently rocking platform. The final preparation was made minutes before actual transplantation when the neuroretina was transferred briefly from the epithelium with a glass rod, until it was attached only at the optic nerve head. The optic nerve was then cut, together with a small piece of choroid-sclera that was used as a handle to lift the retina. The retina was transferred to a sealed chamber containing Ames medium and kept in darkness on a gently rocking platform. The final preparation was made minutes before actual transplantation when the neuroretina was transferred briefly from the epithelium with a glass rod, until it was attached only at the optic nerve head. The optic nerve was then cut, together with a small piece of choroid-sclera that was used as a handle to lift the retina. The retina was transferred to a sealed chamber containing Ames medium and kept in darkness on a gently rocking platform. The final preparation was made minutes before actual transplantation when the neuroretina was transferred briefly from the epithelium with a glass rod, until it was attached only at the optic nerve head.

For the fragment transplantation experiments, the preparation of the retina was the same up to this point, but the donor retina was then treated differently (described later).

The time between donor eye removal and actual transplantation ranged from 50 to 430 minutes.

### Transplantation Procedure

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The surgical procedure for full-thickness transplantation has been described in detail in previous articles. In summary, a three-port vitrectomy was performed, after which a local retinal bleb was created 2 to 3 mm inferior to the optic nerve by infusing Ames medium subretinally. A second retinotomy was then made to provide a valuable. The full-thickness piece of donor retina was drawn into a glass cannula, and the instrument was introduced into the eye and advanced until its tip was adjacent to the first retinotomy. The graft, together with its accompanying fluid, was then pushed out and into the subretinal space.

For fragment transplantation, the method developed by Bergström et al. was used. Briefly described, the grafts were obtained by drawing a full-thickness piece of neuroretina and the surrounding Ames medium into a syringe through a thin polyethylene capillary, which fragmented the tissue. The capillary was supported by a blunt metal cannula with an attached metal handle. A contact lens was placed on the cornea of the host eye, and a small incision was made in the sclera 1 to 2 mm behind the limbus. Under visual guidance through an operating microscope, the polyethylene capillary with its supporting cannula was introduced into the vitreous cavity and then advanced until it penetrated the host retina 2 to 3 mm inferior to the optic nerve. The transplant solution was then placed in the subretinal space.

### Postoperative Management and Follow-Up

No postoperative treatment was given. Ophthalmoscopic examinations were made on postoperative days 1 and 7 and monthly thereafter. The animals that had received full-thickness transplants were killed at 6 to 174 days after the transplantation (Table 1). The animals that had received fragmented transplants were killed at 11 or 14 days after the transplantation.

### Tissue Preparation

For light microscopy, the transplanted eyes were enucleated and fixed for 30 minutes in 4% formaldehyde (generated from parafomaldehyde) at pH 7.4 in 0.1 M phosphate buffer. The anterior segment was then removed, and the posterior eyecup was fixed in the same solution for 4 hours. Tissue specimens were obtained as approximately 4-mm wide sections, including the area of the transplant together with parts of the myelinated fibers and optic nerve. After fixation, the specimens were washed in phosphate buffer (0.1 M, pH 7.4) and then were washed with the same phosphate buffer with added sucrose of increasing concentrations (5%, 10%, and 20%). Cryostat sectioning (12 μm) and hematoxylin-eosin staining were performed with standard procedures. Cryostat sections were used to enable future immunohistochemical analysis.

### Microscopy and Image Analysis

The specimens were examined using an epifluorescence microscope (Eclipse E800; Nikon, Tokyo, Japan) equipped with a digital acquisition system (DEI-750; Optronics, Goleta, CA). Images were viewed and processed by computer (Photoshop; Adobe Systems, Mountain View, CA).

### In Vitro Experiments

For measuring the extent of cell death in the donor tissue, four DAPI-labeled retinas from two rabbits were prepared according to the graft preparation procedure described earlier, with the addition of 0.005% green nucleic acid stain (Sytox; catalog number S-11368; Molecular Probes, Eugene, OR) in the medium. This stain is a nuclear dye that specifically labels dying or dead cells. A normal cell membrane is not permeable by the stain and hence cannot be labeled, but a dying or dead cell looses its membrane integrity and is then labeled by the stain.

As a control, four DAPI-labeled retinas from two other rabbits were prepared according to the protocol described earlier, except that all dissection was performed in bright light, and the tissue was kept in Ames medium that was not continuously bubbled with O₂ and CO₂.

The retinas were allowed to incubate in their respective media in room temperature for either 2 or 5 hours. They were then washed in Ames medium without the green fluorescent stain and fixed, using 4% formaldehyde at pH 7.4 in a 0.1-M phosphate buffer for 30 minutes, washed in phosphate buffer (0.1 M, pH 7.4) and then in the same

### Table 1. Overview of the Morphology of Full-Thickness Grafts

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<td>Total</td>
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Data are number of grafts for each survival period.

Masland. On the day of surgery, the donor animal was dark adapted for at least 30 minutes. All work with the donor tissue was performed in dim red light. The buffer used at all times was Ames medium kept at room temperature and prepared the same day. The medium was continuously bubbled with 95% O₂ and 5% CO₂ to secure an ample supply of oxygen for the donor tissue and to keep the pH stable. The latter was monitored with indicator strips (Merck KGaA, Darmstadt, Germany) at various times up to 7 hours and was found to be stable at pH 7.4.

The donor rabbit was deeply anesthetized using intramuscular ketamine (40 mg/kg) and xylazine (5 mg/kg). In addition, 0.5% tetracaine HCl was applied to the ocular surface. The conjunctiva and external eye muscles were cut, the optic nerve and retinal vessels were cut, and the eye was removed. Within a few seconds, the anterior segment was cut, the lens and vitreous body removed, and the posterior segment placed in Ames medium. The posterior segment was cut, the lens and vitreous body removed, and the posterior segment placed in Ames medium. The anterior segment was placed in Ames medium and kept in darkness on a gently rocking platform. The final preparation was made minutes before actual transplantation when the neuroretina was transferred briefly from the chamber to a beaker. A full-thickness piece of neuroretina (approximately 3 × 3 mm) was cut with microscissors, and the remaining tissue was returned to the sealed chamber to await the operations.

For the fragment transplantation experiments, the preparation of the retina was the same up to this point, but the donor retina was then treated differently (described later).

The time between donor eye removal and actual transplantation ranged from 50 to 430 minutes.
buffer with added sucrose of increasing concentrations (5%, 10%, and 20%), and examined as wholemount preparations with fluorescence microscopy, using filters optimized for Texas red and DAPI. To identify the different retinal nuclear layers, a filter optimized for DAPI was used and the microscope focused until a layer where most cells were labeled was reached. This layer was presumed to be the inner nuclear layer (INL), and the outer nuclear layer (ONL) and ganglion cell layer (GCL) were identified by changing the plane of focus appropriately. Fluorescent green labeling was visualized in each layer by switching to a filter optimized for Texas red. Dying cells were presumed to be located in the respective layer if they were intensely labeled by the stain and were of the same size as DAPI-positive cells in the same focus plane. No exact cell count of fluorescently labeled cells was made. Instead, images were obtained from the different nuclear layers in both preparations, and the corresponding images compared.

RESULTS

Full-Thickness Transplants

Surviving grafts were found in 21 of the 23 eyes that received transplants of a full-thickness adult neuroretina. The identification was made by observing serial sections, and also from the DAPI labeling of the transplant. Table 1 gives an overview of the light microscopic appearance of the 23 specimens.

Specimens with Grafts

Nineteen grafts were correctly oriented with photoreceptors adjacent to the RPE and ganglion cell layer toward the host retina. Sixteen of these grafts showed a normal laminated morphology with all retinal layers present (Figs. 1, 2, 3). Photoreceptor outer segments of normal length apposed to the host RPE were present in all grafts except in three short-term grafts (6–14 days after surgery), in which normal inner but only short outer segments were found (Fig. 1). In all 16 grafts, the nuclear and plexiform layers appeared normal. The appearance of the GCL changed with increasing surviving times. In the youngest grafts (6 days after surgery), large cells could be seen in the GCL, but in older grafts, remaining cells in this layer were small with round nuclei. In two of the four long-term specimens (173 and 174 days after surgery), the GCL of the graft had degenerated to a large extent. In these specimens, a varying degree of fusion could be seen between the graft’s innermost layers and the host retina (Fig. 3). The 16-well laminated grafts measured from 0.5 to 3.6 mm in length. The three longest grafts (2.2, 3.0, and 3.6 mm) were long-term implants (173–174 days after surgery). The time from donor eye removal to actual transplantation in these specimens ranged between 50 and 360 minutes (median, 165). In five of the specimens, the grafts were folded, at least at the edges. The
folded part of the graft facing the host retina displayed a degenerated morphology. Three of the correctly oriented grafts had degenerated to a varying degree, and displayed thin layers, no photoreceptor outer segments, and occasional large macrophage-like cells within the graft. In these three specimens the grafts had been placed 260, 300, and 430 minutes after donor eye removal. Two grafts (7 and 36 days after surgery) were found upside down, with the GCL oriented toward the host retinal pigment epithelium (RPE). The younger graft measured 1.0 mm, was laminated, and displayed all retinal layers, including short photoreceptor outer segments oriented toward the overlying host retina. The older graft measured 0.5 mm and displayed thin layers. The time from donor eye removal to transplantation in these specimens was 110 and 140 minutes, respectively.

Specimens without Graft

In two animals (35 and 50 days after surgery), no positive identification of a transplant could be made, either from morphology or from DAPI-labeled cells, even though the transplants had been positively identified clinically at 7 days after surgery. The grafts in these two specimens were placed 260 and 300 minutes after donor eye removal.

Host

In all specimens, including the two without a graft, the host retina had degenerated in the transplant area. In all other areas, it appeared normal. In the youngest specimen with a graft (6 days after surgery), the host outer retina in the area of the transplant was completely missing, whereas some parts of the inner retina remained (Fig. 1). In specimens 14 days and older after surgery, the INL and inner plexiform layer (IPL) appeared more degenerated (Figs. 2, 3C). DAPI-labeled cells were not detected in the host retina.

The host RPE was continuous in the majority of specimens. A few RPE defects were noted, but these were never correlated with any alterations in the graft or choroid morphology.

In approximately half of the younger specimens with grafts (6–50 days after surgery), vessels in the host choroid appeared more dilated than normal in the transplant area, but no signs of inflammatory cells were seen. In the remaining young specimens, as well as in all long-term specimens (173–174 days after surgery), the choroid appeared normal.

Fragmented Transplants

In two of the five specimens with a fragmented graft (11 days after surgery), transplanted cells could be positively identified by DAPI (Fig. 4). These grafts did not display any of the normal retinal architecture or even rosettes, but were composed of small clusters of cells. In both specimens, defects in the host RPE were found, but no signs of inflammation were present in the choroid.

No transplant could be found in either DAPI- or hematoxylin and cosin-stained sections in the remaining three specimens. RPE defects were found in all three of these specimens, and one of them displayed dilated blood vessels in the choroid along with a massive invasion of presumed inflammatory cells in the choroid and subretinal space.
The time from donor eye removal to transplantation in the five eyes receiving fragmented grafts ranged between 90 to 210 minutes (median, 150).

In Vitro Experiments
A few scattered fluorescence-labeled cells were found in the GCL and INL in the wholemount retinal preparations fixed after 2 hours (Figs. 5A, 5C). There was no clear difference in the number of labeled cells at this time between the retinas prepared according to the two different preparation procedures. In specimens fixed after 5 hours, the retinas that had been prepared according to the optimized graft preparation procedure displayed a moderate increase in fluorescence-labeled cells when compared with retinas fixed after 2 hours (Fig. 5B). The retinas prepared according to the simple preparation procedure after 5 hours displayed fluorescent labeling of virtually all cells in the INL and GCL (Fig. 5D).

Patches of fluorescence-labeled cells in the ONL were found in all retinas examined. Fluorescence-labeled cells could also be found in all cell layers around the cut edges of all examined retinas. We concluded that these findings were caused by dissection trauma.

DISCUSSION
Graft Survival
Previous studies on adult retinal transplants are few and have generally shown poor graft survival.22–24 Immature donor tissue, on the other hand, has been successfully used by a number of groups. We reported earlier that embryonic rabbit grafts develop and survive well for at least 10 months when transplanted as a full-thickness sheet.18 These embryonic grafts were prepared in the same manner as the adult control retinas in our present in vitro experiment—that is, dissected in bright light and kept in Ames medium that was not continuously bubbled with O2 and CO2. With adult full-thickness grafts, however, we previously found that the same protocol results in very poor survival, even in short-term specimens.24 and we thus drew the same conclusion as earlier investigators—that is, adult donor tissue could not be used.

For the present study, we hypothesized that adult donor tissue can survive transplantation if care is taken to avoid cell death in the graft before actual transplantation. In our in vitro experiment we found a massive cell death in specimens treated according to the same protocol as our previously reported adult and embryonic grafts. These results suggest that such a protocol is not suitable for adult donor tissue and may help to explain our earlier reported poor survival of adult grafts.

Further indications of the vulnerability of adult donor tissue can be gained from examining the survival rate of the current adult grafts in correlation to time from donor eye removal to actual transplantation. If specimens in which the surviving graft appeared degenerated and specimens in which no graft was found are grouped (altogether five specimens) and compared with specimens with well-laminated surviving grafts (n = 16), a significant difference in time before transplantation is obtained (P < 0.05, median test). No such correlation between time before transplantation and survival rate has been noted in our earlier reported experiments involving embryonic grafts,18 and it indicates that a prolonged time between donor eye removal and actual transplantation is not favorable for adult graft survival.

To limit cell death in the graft tissue before transplantation and thereby possibly enhance the chance of graft survival, we have concentrated on environmental factors such as light conditions, changes in pH, and oxygen concentration. Light exposure not only elicits a visual response in the retina, but can also damage the tissue by inducing apoptosis.25 Dissecting the donor eye in dim red light and keeping the tissue in darkness minimizes the potential of bright light damaging the retina in vitro. Extensive work on the connection between ischemia and cell death in in vitro preparations of the retina has been performed by Ames and Nesbett.15–17 In the protocol used for our experiments, slowly rocking the chamber and bubbling the medium with gas containing 95% O2 assures continuous access of oxygen to the retina. Keeping the pH at a stable physiological level is an important factor for cell survival in vitro,26 and it is accomplished by adding 5% CO2 to the gas mixture bubbling the medium.

It is not enough, however, to optimize the environment in which the adult retinal tissue is kept. From our results we can
also conclude that donor tissue integrity is important for graft survival. Fragmented grafts, prepared according to the optimized protocol apparently do not survive well at all. The technique of fragmenting immature neuroretinal grafts was first reported by Turner and Blair in 1986.\(^{27}\) The great advantage of using fragmented donor tissue is that it can be transplanted to the subretinal space with relative ease, and this kind of tissue has therefore been widely used experimentally. Recently, Humayun et al.\(^{3}\) reported the first clinical trial involving fragment transplants even in human patients. Earlier work on embryonic fragmented grafts have shown that they can survive and retain their rosetted morphology for up to 3 months. After this time, the major part of the graft starts to degenerate, and only small pieces where the photoreceptors are apposed to the host RPE can survive longer.\(^{3,28}\) We have noted that embryonic fragmented grafts elicit a low-key immune response from the host that is not present in the full-thickness grafts.\(^{27}\) The exact mechanism by which fragmented grafts are rejected, however, remains unclear. The RPE defects found in our earlier reported embryonic grafts and in the adult fragmented grafts in the present study indicate that RPE wound healing in these specimens is delayed, thereby exposing the normally immune-privileged subretinal space to the host immune system present in the choroid.\(^{30–32}\)

To summarize, our results still support the principle that adult donor tissue is more vulnerable than its embryonic counterpart. However, they also show that if cell death is minimized in the adult graft before transplantation and the tissue integrity is kept intact, a graft survival rate close to the one found using embryonic grafts can be achieved.\(^{18}\)

**Graft Morphology**

The normal mammalian retina is a highly complex structure, composed of a multitude of specialized neuronal types organized for the purpose of processing and transmitting useful visual information to the brain.\(^{25}\) We therefore believe that the first step toward replacing diseased photoreceptors with a retinal transplant is to achieve good long-term survival of grafted photoreceptors with an architecture resembling that found in the normal retina.

The current trend in neuronal transplantation experiments is to move toward more immature donor tissue, with stem-cell grafting at its pinnacle. The rationale for this is that the multipotency and plasticity of noncommitted cells will assure its desired differentiation and also facilitate neuronal contacts with host neural elements. One objection that can be raised against this paradigm lies in the complexity of neural development. Both migration of determined neurons and dendritic and axonal pathfinding are dependent on neighboring cells, especially glia.\(^{2,3}\) The intricate interaction between developing glia and neuron, governed by a multitude of adhesion molecules, intercellular signals, and growth factors, is a process that is at this stage not possible to reproduce in vitro. In addition, experiments involving transplantation of suspensions of immature neuronal cells have shown that even if some cells show development of the characteristics of specialized neurons, the grafts show very little of the normal organization of their surroundings in vivo.\(^{8,10}\)

The use of adult donor tissue may offer an advantage, in that the neuronal architecture is already established. The laminated morphology and normal appearance of photoreceptor outer segments in the majority of our full-thickness grafts, even 6 months after surgery, suggests that these adult grafts thrive in spite of their foreign environment. These findings are encouraging and inevitably raise the question of whether useful visual information be transferred from the graft to the remaining host inner retinal cells.

**CONCLUSION**

In this study we have shown that adult neuroretinal grafts can survive and keep their laminated morphology including photoreceptor outer segments for at least 6 months. Important factors for survival include optimizing the in vitro conditions before transplantation and keeping the integrity of the donor tissue intact.

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