Graft–Host Connections in Long-term Full-Thickness Embryonic Rabbit Retinal Transplants

Fredrik Ghosh, Anitha Bruun, and Berndt Ebinger

PURPOSE. To establish neuronal connections in the rod and cone pathway between laminated rabbit retinal transplants and the host retina.

METHODS. Fourteen adult rabbits received a complete full-thickness embryonic transplant. After survival times of 3 to 10 months, the retinas were studied under light microscopy and with immunohistochemistry. Antibodies against protein kinase C (PKC), parvalbumin, and calbindin were used to label rod bipolar cells, All amacrine cells, and cone bipolar cells, respectively. The AB5 antibody was used to label ganglion cells.

RESULTS. The transplants displayed laminated morphology with layers parallel to the host retinal pigment epithelium. In the oldest specimens (10 months after surgery), laminated layers of graft and host approached each other and almost reconstructed the normal retinal appearance. The ganglion and cone bipolar cells of the host survived well, as was seen with AB5 and calbindin double-labeling. Connections between cone bipolar cells in the graft and ganglion cells in the host were not common. PKC-labeled rod bipolar cells and parvalbumin-labeled All amacrine cells of host and graft showed sprouting activity directed toward an intermediate plexiform layer located between the graft and host. In specimens double-labeled with PKC and parvalbumin, this intermediate plexiform layer was seen to contain numerous PKC- and parvalbumin-labeled processes. Direct connections between rod bipolar and All amacrine cells in host and graft were seen in the 10-month specimens.

CONCLUSIONS. Full-thickness embryonic transplants survive for at least 10 months, and normal laminated morphology develops. Host and graft fuse and together constitute nerve cell processes to an intermediate plexiform layer. Direct graft-host contacts are also present between neuronal types that in the normal retina participate in the rod pathway. (Invest Ophthalmol Vis Sci. 1999; 40:126-132)

Retinal transplantation offers the possibility of restoring visual function in eyes affected by degenerative disease. Different techniques have been used, and reports on the survival of transplants are numerous.1-4 To serve its purpose, however, the retinal transplant must form functional contacts with the host retina. Reports on such graft-host connections in retina-to-retina transplantation are few. Contacts between lacZ-labeled photoreceptor and host retinal cells have been investigated,5 but the results were inconclusive, because the origin of their postsynaptic targets have not been established. Sprouting activity has been reported in Dil-labeled grafts,6 but to our knowledge, no study has shown connections between selectively labeled neurons of the graft and host.

We have developed a method for producing well-laminated transplants in the rabbit7 and have also reported on the long-term survival of these transplants.8 To summarize the results of these studies, a laminated morphology developed in the short-term transplants with all retinal layers present. With longer survival times, fusion of graft and host took place at the level of the inner plexiform layer (IPL) of the transplant. Under the electron microscope, many normal synapses were seen within the transplants, and an extensive, organized sprouting in the region of graft-host fusion was evident. In the present study, we used immunohistochemistry to characterize further the sprouting neurons in the graft and host. We also wanted to investigate whether these neurons could form parts of a normal rod or cone pathway.

The rod pathway of the rabbit has been well characterized9 and can be summarized as follows: The rod cells contact rod bipolar cells, which in turn synapse on All amacrine cells in the most vitreal part of the IPL. The All amacrine cells contact cone bipolar cells that transmit information to ganglion cells. Most of the cells in this chain can be labeled with immunohistochemical markers. Rod cells and rod bipolar cells can be selectively labeled by antibodies against rhodopsin and protein kinase C (PKC), respectively.10,11 All amacrine cells are labeled by antibodies against parvalbumin12 and ganglion cells by the AB5 antibody.13 The whole population of cone bipolar cells cannot be identified by any single antibody. Anti-calbindin is the most commonly used commercial antibody, but it labels...
TABLE 1. Number and Survival Times of All Transplants

<table>
<thead>
<tr>
<th>Postoperative Days</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>86</td>
<td>6</td>
</tr>
<tr>
<td>126–185</td>
<td>4</td>
</tr>
<tr>
<td>302–309</td>
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only approximately one twelfth of the cone bipolar cells,14 and also labels horizontal cells. The cone pathway is built from only three types of neurons. The color-sensitive cone cells contact cone bipolar cells, which in turn synapse on ganglion cells. Horizontal and amacrine cells modify the signal, but these cells do not participate directly in the chain.

For the investigation of graft–host connections we chose two sets of double-labeling experiments, PKC-parvalbumin and calbindin-AB5. With this method we labeled the retinal neurons proximal to the photoreceptor cells in the rod and cone pathway.

METHODS

All animals included in the present study were part of an earlier study in which the light and electron microscopic morphology of 18 long-term transplants was reported.28 In this earlier study, two transplants were prepared and examined by electron microscopy, and two transplants displayed rosettes. The remaining 14 animals were included in the present study in which we concentrated on connections between graft and host, using immunohistochemical markers.

Surgery

The surgical procedure has been described in detail elsewhere.27 To summarize, complete full-thickness embryonic rabbit neuroretinas 19 days after conception were dissected from the donor eyes using jeweler’s-type forceps. Specimens were kept in +4°C Ames’ solution and then transplanted to the subretinal space of 14 adult pigmented mixed-strain rabbits by means of vitrectomy and retinotomy. Each animal received one transplant. The animals were killed 3 to 10 months (86-309 days) after the transplantation. For the number of transplants and their survival times, see Table 1. All proceedings and animal treatment were in accordance with the guidelines and requirements of the Government Committee on Animal Experimentation at Lund University and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Tissue Preparation

The eyes were enucleated and fixed for 30 minutes in 4% formaldehyde, generated from paraformaldehyde (Merck, Darmstadt, Germany) at pH 7.4 in 0.1 M Sørensen’s phosphate buffer (primary and secondary NaHPO4; Merck). The anterior segment was then removed and the posterior eyecup was postfixed in the same solution for 4 hours. Tissue specimens were approximately 3.5-mm wide and included the area of the transplant, parts of the myelinated fibers, and optic nerve. After fixation, the specimens were washed with 0.1 M Sørensen’s phosphate buffer (pH 7.4) and then washed again using the same solution with added sucrose of increasing concentrations (5%, 10%, 15%, and 20%) before serial sectioning in the vertical plane at 12 μm on a cryostat. Slides from all transplants were stained with hematoxylin (according to Mayer, Apothekebolaget, Stockholm, Sweden, 1985) and 0.1% eosin (BDH, Dorset, England) for routine microscopy.

Immunohistochemistry

The sections were thawed and washed in 0.1 M sodium phosphate-buffered saline (PBS) pH 7.2 (Merck) with 0.25% Triton X-100 (PBS-Triton; ICN Biomedicals, Aurora, OH). For parvalbumin and PKC double-labeling, the tissue was incubated with the parvalbumin antibodies for 18 to 20 hours, rinsed in PBS-Triton and then incubated with the PKC antibodies for 18 to 20 hours. After rinsing in PBS-Triton, the tissue was incubated for 45 minutes in darkness with a mixture of the secondary antibody conjugated with two fluorophores: anti-rabbit fluorescein isothiocyanate (FITC; Southern Biotechnology, Birmingham, AL) and anti-mouse Texas red (Jackson Immunoresearch, West Grove, PA). The dilution of each secondary antibody was 1:100. For calbindin and AB5 double-labeling, a different approach was chosen because these antibodies were made in the same species (mouse). The sections were first incubated with the calbindin antibody for 18 to 20 hours. They were then rinsed in PBS-Triton and incubated with anti-mouse Texas red. After another thorough rinsing in PBS-Triton, the AB5 immunolabeling was performed in a similar manner but with anti-mouse FITC (Sigma, St. Louis, MO) as a secondary antibody. This secondary antibody now recognized all mouse antigen in the tissue, and consequently, the FITC fluorescence was present at anti-calbindin- and AB5-positive sites. To differentiate the AB5 and calbindin labeling, separate images of the

TABLE 2. Antigen and Antibody Specifications

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Polyclonal or Monoclonal</th>
<th>Working Dilution</th>
<th>Fluorophore</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB5 (bovine brain extract)</td>
<td>AB5</td>
<td>Mono</td>
<td>1:10,000</td>
<td>Texas red</td>
<td>Keith R. Fry, The Woodlands, TX</td>
</tr>
<tr>
<td>PKC (human 80 kDa PKC)</td>
<td>Human protein kinase C</td>
<td>Poly</td>
<td>1:3,000</td>
<td>FITC</td>
<td>Chemicon, Temecula, CA</td>
</tr>
<tr>
<td>Calbindin (chicken gut calbindin 28kDa)</td>
<td>Calbindin D</td>
<td>Mono</td>
<td>1:200</td>
<td>FITC</td>
<td>Sigma, St. Louis, MO</td>
</tr>
<tr>
<td>Parvalbumin (carp muscle)</td>
<td>Parvalbumin</td>
<td>Mono</td>
<td>1:1,000</td>
<td>Texas red</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

PKC, protein kinase C; FITC, fluorescein isothiocyanate.
tissue activity of the fluorophores were superimposed. In this composite image, calbindin-positive sites were yellow (green + red), whereas Aβ5 was seen as green. For antigen and antibody specifications, see Table 2. Control experiments on normal adult rabbit retina were made with both sets of antibodies. The complete labeling procedures without primary antibodies were also performed. Photographs of all specimens were obtained with a digital camera system using image management software (Adobe Photoshop, San Jose, CA) was used.

RESULTS

Light Microscopy

The light microscopic findings have been reported in detail earlier.* The host retina covering the transplant showed degeneration of its outer layers, whereas the inner layers remained in varying proportions. Between laminated layers of host and graft, an array of disorganized cells was often seen in the youngest specimens (3 months after surgery). These cells were fewer in older specimens (4–10 months after surgery). The transplants displayed laminated morphology with well-developed photoreceptor outer segments facing the host retinal pigment epithelium. In the 4- to 10-month grafts, the lamination of the innermost retinal layers was often dissolved and fused with the host inner retina in an intermediate plexiform layer located between host and graft (Fig. 1). Differentiating laminated layers of host from graft was fairly easy by viewing consecutive sections, but nonlaminated cells between the two could not be defined with certainty as graft- or host-derived.

Immunohistochemistry

PKC. In all transplants, PKC-labeled rod bipolar cell bodies were identified in their normal position in the scleral part of the inner nuclear layer (INL). In the 3-month transplants, they seemed to be as common as in the normal rabbit retina,

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FIGURE 1. Hematoxylin and eosin staining of a specimen 309 days after surgery. The host (H) outer retina has degenerated completely, and the inner layers are affected. The transplant (T) displays good lamination, with photoreceptor outer segments (OS) facing the host retinal pigment epithelium (RPE). The transplant also displays a laminated outer nuclear layer (ONL) and outer plexiform layer (OPL). The lamination of the inner nuclear layer (INL) is dissolved, and graft and host have fused. An intermediate plexiform layer (i-Plex) is apparent. Scale bar, 50 μm.

FIGURE 2. Protein kinase C (PKC) immunohistochemistry. Transplant 86 days after surgery. PKC-labeled structures interpreted as growth cones in the inner plexiform layer (IPL) of the transplant (arrows). One of the growth cones is an extension of a labeled rod bipolar axon (arrowhead). INL, inner nuclear layer of the transplant. Scale bar, 20 μm.

FIGURE 3. Protein kinase C (PKC) immunohistochemistry. Transplant 86 days after surgery. PKC-labeled rod bipolar cell axons from the transplant (T) contribute to the inner plexiform layer (IPL). Rod bipolar cells from the host (H) are fewer than normal, and some sprout dendrites toward the transplant (arrows). They also extend axons with terminals in the host inner plexiform layer (h-IPL). INL, inner nuclear layer of the transplant; ONL, outer nuclear layer of the transplant. Scale bar, 20 μm.
although no precise cell count was made. In 4- to 10-month grafts, their number had decreased moderately. The cells extended vertical axons with terminal bulbs in the vitreal part of the transplant IPL. The axons in some cases appeared longer than in the normal retina. Large and well-labeled structures consisting of minute fibers were seen in a zone more to the vitreal side of the transplant IPL (Fig. 2). These structures, which were most common in the 3-month transplants, were often in contact with bipolar cell axons and were interpreted as collections of growth cones. The PKC-labeled rod bipolar cells extended dendritic branches to the most scleral part of the normal retina. Large and well-labeled structures consisting of minute fibers were seen in a zone more to the vitreal side of the transplant IPL (Fig. 2). These structures, which were most common in the 3-month transplants, were often in contact with bipolar cell axons and were interpreted as collections of growth cones. The PKC-labeled rod bipolar cells extended dendritic branches to the most vitreal part of the host IPL. Dendrites extending toward the transplant (Fig. 3) were seen in specimens of all ages. These dendrites often displayed growth cones with minute filopodialike processes at their tips (see Fig. 5D).

Parvalbumin. Parvalbumin-labeled All amacrine cells in the 3-month specimens were often organized in three layers: in the transplant, with cell bodies in the inner nuclear and ganglion cell layer and with processes in the IPL; in an intermediate plexiform layer on the vitreal side of the transplant IPL; and in the host INL with processes in the host IPL (Fig. 4A). In the 4- to 10-month specimens, the All amacrine cells around the transplant IPL were fewer, and more labeled cell bodies and processes were seen in the intermediate plexiform layer (Fig. 4B). The All amacrine cells in the host retina of these specimens displayed branches in the host IPL. There was no apparent decrease in the total number of labeled cells in the oldest specimens (10 months after surgery), when compared with the number in the younger ones.

PKC plus Parvalbumin. In the eight oldest transplants (4-10 months after surgery), double-labeling with PKC and parvalbumin showed numerous small PKC- and parvalbumin-labeled processes close together in a thick intermediate plexiform layer located between host and graft (Figs. 5C, 5D). These processes seemed to be derived from host and graft rod bipolar and All amacrine cells. In all these specimens, rod bipolar cells in the host extended, sprouting dendrites toward the intermediate plexiform layer (Fig. 5D). Sprouting fibers from host All amacrine cells were also common. Direct contacts between rod bipolar cells in the transplant and All amacrine cells in the host were seen in three of these specimens (Fig. 5B). All amacrine cells in the transplant were sometimes seen to extend fibers terminating on rod bipolar dendrites in the host (Fig. 5C).

AB5. AB5-labeled ganglion cells were found in the ganglion cell layer of the host in all 14 specimens (Figs. 6B, 6C, 6D). Their number seemed to be relatively constant in all specimens, and they displayed branches in the host IPL (Fig. 6B). A few AB5-labeled large cells were seen in the ganglion cell layer of two transplants (86 and 126 days after surgery).

Calbindin. Calbindin-labeled cone bipolar and horizontal cells were found in all transplants. The cone bipolar cells were identified by their small size and their thinner, more vertical processes. These processes often ended in a network of fibers on the vitreal side of the transplant IPL (Fig. 6D). The cells were not as common as in the normal rabbit retina. In the host, labeled cone bipolar cells with processes terminating in the host IPL were seen (Fig. 6B), although these cells were not as numerous as in the normal retina. Some remaining horizontal cell processes were also noted in the host retina.

Calbindin plus AB5. Contacts between calbindin-labeled bipolar cells in the graft and AB5-labeled ganglion cells were not apparent. In one specimen, 126 days after surgery, a few
FIGURE 5. Protein kinase C (PKC) (green) and parvalbumin (red) double-labeling. (A) Normal adult rabbit retina. The PKC-labeled rod bipolar cells in the inner nuclear layer (INL) extend axons that branch and terminate in the most vitreal part of the inner plexiform layer (IPL). The parvalbumin immunoreactive AII amacrine cells are bistratified with branches in the vitreal and scleral part of the IPL. Transplant 178 (B) and 309 (C and D) days after surgery. (B) PKC-labeled rod bipolar cell from the transplant inner nuclear layer (t-INL) extends an axon toward the host (H) and displays terminal bulbs (arrow) on a parvalbumin-labeled AII amacrine cell in the inner nuclear layer (h-INL). (C) Parvalbumin-labeled AII amacrine cell in the t-INL extends a process (arrow) toward a PKC-labeled rod bipolar cell of the host (H). (D) PKC-labeled rod bipolar cells from the host sprout fibers with growth cones (arrows) toward the intermediate plexiform layer (i-PL). In (C) and (D) the i-PL displays numerous small PKC- and parvalbumin-labeled processes (arrowheads). T, transplant; ONL, outer nuclear layer of the transplant; h-IPL, inner plexiform layer of the host. Scale bar, 20 μm.

AB5-labeled fibers from the host came markedly close to calbindin-labeled cone bipolar axons from the graft, but no definite contact was seen (Fig. 6C). In the 10-month specimens, the distance between ganglion cell processes from the host and cone bipolar cell processes from the transplant was short; yet even then, no direct contacts were seen (Fig. 6D).

DISCUSSION

The Intermediate Plexiform Layer
The outer retina of the host degenerated completely in all specimens, which made fusion of host and graft inner retina possible. The retina of the rabbit is merangiotic (i.e., vessels are
Connections in Long-Term Retinal Transplants

FIGURE 6. AB5 (green) and calbindin (yellow) double-labeling. (A) Normal rabbit retina. The calbindin-labeled cone bipolar cells in the inner nuclear layer (INL) extend vertical axons that terminate in the vitreal part of the inner plexiform layer (IPL). The AB5-labeled ganglion cells extend processes in the vitreal and the sceral part of the IPL. (B) Transplant 126 days after surgery. In the host (H), AB5-labeled ganglion cell processes and calbindin-labeled cone bipolar cell axons meet in the host inner calbindin (h-INL, arrows). (C) Transplant (T) 126 days after surgery. An AB5-labeled ganglion cell in the host (H) extends a fiber (arrow) toward a calbindin-labeled fiber (arrowhead) from a cone bipolar cell in the transplant inner nuclear layer (t-INL). (D) Transplant 309 days after surgery. Numerous small calbindin-labeled processes are present (arrowheads) between the host and transplant. The distance between the transplant inner plexiform layer (t-IPL) and the AB5-labeled ganglion cell is small, but no established contact can be seen. ONL, outer nuclear layer of the transplant; h-INL, inner nuclear layer of the host. Scale bar, 20 μm.

The results of this study show that retinal neurons, predominantly rod bipolar and all amacrine cells, of host and graft are capable of sprouting. This sprouting activity seems directed toward the formation of an intermediate plexiform layer that most likely is a vitreally displaced transplant IPL fused with inner layers of the host. In this plexiform layer, parvalbumin- and PKC-labeled small processes are numerous, but they do not form organized sublaminae. It is probable that calbindin-labeled cone bipolar cell processes also contribute to this layer, but further double-labeling experiments are needed to establish this fact.

Graft-Host Connections

Few of the cone bipolar cells found in the transplant made direct contact with ganglion cell processes. The cone pathway is difficult to analyze because only a fraction of the cone

confined to a horizontal band at the myelinated nerve fibers), making it vulnerable when separated from the retinal pigment epithelium. Degeneration of the outer nuclear layer of the host has also been reported in fragment transplants to holangiotic (completely vascularized) retina, indicating that mechanisms other than the separation from the retinal pigment epithelium may be involved. In the youngest specimens of this study (3 months after surgery), graft-host fusion was not prominent because of the presence of disorganized cells between graft and host. The well-developed inner retina of the transplant, often with all layers present, also suggests that there is not much fusion in these specimens. We have reported on a shortened distance between laminated layers of graft and host and fusion of the two with longer postoperative times. With immunohistochemical markers we have now been able to characterize the zone of fusion further.
bipolar cells can be labeled with commercially available antibodies. In the normal rabbit retina, however, contacts between calbindin-labeled cone bipolar and AB5-labeled ganglion cell processes were readily seen, suggesting that graft-host connections in the cone pathway of our specimens were not common.

Graft-host connections in the rod pathway were more frequent. Cell counts extending over many sections will be needed to find out more exactly how common these connections are. Such determinations were beyond the scope of this study. It was clear, however, that they were less numerous than rod bipolar and amacrine cell contacts in the normal rabbit retina. All amacrine and rod bipolar cells interacted intimately in several ways in our specimens. In the intermediate plexiform layer, all amacrine cell processes were in contact with bipolar cell axons from the graft and bipolar cell dendrites from the host. The intermediate plexiform layer thus may function as a switchboard between neurons of graft and host. Further, all amacrine cells in the transplant were seen to sprout fibers directly onto host bipolar dendrites. The normal output of the all amacrine cells are cone bipolar cells, which in turn contact ganglion cells. Whether transplanted all amacrine cells can contact cone bipolar cells in the host remains to be investigated. That which is probably most compatible with normal retinal function occurs when all amacrine cells in the host are postsynaptic to rod bipolar cells from the transplant, which was seen in three of the eight oldest specimens (4-10 months).

One prominent feature of most specimens was the dendritic sprouting from rod bipolar cells in the host. These processes have also been found in detached retina and may represent an effort to maintain contacts between rod cells and bipolar cells. Their presence in our specimens even 10 months after surgery indicates a prolonged ability of these cells to form connections in the cone pathway of our specimens were not common.

To conclude, we have shown that transplantation of full-thickness embryonic retina can lead to a graft-host adaptation in which neurons from both entities coalesce after several months to form an intermediate plexiform layer. Graft-host connections exist, and the participating neuronal types are the ones seen in the normal rod pathway of the retina.

Acknowledgments

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References