Transplantation of Cultured Rabbit Retinal Epithelium to Rabbit Retina Using a Closed-Eye Method

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We have developed a closed-eye technique for transplanting cultured rabbit retinal epithelial cells to Bruch's membrane of the rabbit. A glass micropipette containing a suspension of 3H-thymidine-labeled, cultured retinal pigment epithelial (RPE) cells is inserted through a pars plana incision and positioned adjacent to the neural retina. A jet stream from the pipette is used to make a small retinal hole and bleb detachment. Patches of host retinal epithelium lift off with the neural retina, creating areas of bare Bruch's membrane. The cell suspension is injected into the subretinal space, and labeled cells can be seen attached to Bruch's membrane as early as 1 hr later. The neural retina spontaneously reattaches within 24 to 48 hr, bringing photoreceptor outer segments in direct contact with the transplanted cells. Phagocytosis of outer segment material by transplanted cells can be seen as early as 24 hr after surgery. This closed-eye technique offers an advantage over the open-sky method used previously in that it allows for reattachment of the neural retina and at least a partial return of function in the transplanted retinal epithelium. Invest Ophthalmol Vis Sci 28:1131-1137, 1987

Our laboratory has previously reported the transplantation of cultured human retinal pigment epithelial (RPE) cells to Bruch's membrane of the owl monkey.1,2 The open-sky technique was employed, whereby the anterior segment was opened, a small retinal flap created, and the underlying RPE cells removed using a combination of trypsinization and gentle rubbing. Cultured, 3H-thymidine-labeled human RPE cells were placed onto the denuded Bruch's membrane and could be seen attached to it as early as 1 to 2 hr later.

The major drawback with the open-sky technique is the difficulty with which the retinal flap can be reaposed to the underlying transplanted cells. However, the encouraging results obtained using the open-sky technique allowed us to speculate that reattachment of the neural retina to the transplant site could be better accomplished using a pars plana incision for surgical entry. A closed-system approach would also be less traumatic to the retina, vitreous, and anterior segment.

We now present a closed-eye method for the transplantation of cultured rabbit RPE cells to Bruch's membrane of the rabbit. The method itself has led to novel insights into the cell biology and physiology of this problem.

Materials and Methods

Cultured Rabbit RPE Cells

These investigations adhered to the ARVO Resolution on the Use of Animals in Research. The technique used for the isolation and culture of rabbit RPE cells is similar to that previously reported for human RPE.3 Eyes harvested from adult albino and pigmented rabbits were allowed to stand at 4°C for approximately 18 hr to facilitate separation of the retina from the RPE. The anterior segment, vitreous, and retina were removed and 0.25% trypsin was placed into the eye cups. The eyes were incubated at 37°C for 1 to 2 hr, after which time the RPE cells were removed with gentle pipetting and placed at a concentration of 3 to 10 x 10^4 cells per mm^2 in Minimal Essential Medium (MEM) with 20% fetal calf serum (fcs). After 24 hr, the medium was changed to 20% fcs/MEM with 0.2 µCi/ml 3H-thymidine. The medium was changed every 48 hr until the cells were used.

The cells were prepared for transplantation by replacing the medium with 0.05% trypsin with EDTA and incubating at 37°C for 5 to 10 min until dissociated. The cells were taken up in 20% fcs/MEM and an aliquot containing approximately 2 x 10^5 cells was centrifuged at 800 RPM for 5 min. The pellet was re-suspended in MEM to a total volume of approximately 50–100 µL.
Surgery

Adult pigmented rabbits were anesthetized with an intravenous injection of 20 to 30 mg/kg sodium pentobarbital, with additional IV pentobarbital given as needed. The pupil was dilated with 1% tropicamide and 2.5% phenylephrine HCl and a 3-cc retrobulbar injection of 2% lidocaine was administered. The eye was proptosed and held in position with a lid speculum. A conjunctival flap was formed and a pars plana incision was made with a stiletto blade. The previously prepared, cultured RPE cells were taken up into a glass micro-cannula filled with balanced salt solution (BSS). The internal diameter of the cannula measured approximately 100 μm. An additional volume of approximately 50 μL clear BSS was then withdrawn into the tip of the cannula. The cannula was inserted through the pars plana incision and was visualized in the vitreous cavity using an operating microscope and contact lens. The cannula was positioned adjacent to the neural retina approximately 2 to 3 disc diameters from the optic nerve. A jet-stream of the clear BSS was used to produce a small retinal hole, and once the BSS entered the subretinal space, a small bleb detachment formed. The detachment formed, patches of RPE cells could be seen still adherent to the neural retina. With continued slow injection, the cell suspension would enter the subretinal space. Figure 1 illustrates this approach schematically. Care was taken to keep the retinal hole as small as possible and to avoid seeding the vitreous with cells when the cannula was withdrawn. The total detachment volume was approximately 100 to 200 μL. The sclerotomy site and conjunctival flap were closed using 6-0 vicryl sutures.

Histological Preparation

The animals were sacrificed at time intervals of 1, 2, and 24 hr. The eyes, punctured at the pars plana to facilitate entry of the fixative, were placed into 3% glutaraldehyde in Earle’s buffer for 24 hr at 4°C and then transferred into Earle’s buffer. After dissection, the tissue was post-fixed in 1% osmic acid in Earle’s buffer for 1 hr, dehydrated with ethanol, and embedded in Epon. Sections were cut 1 to 2 μm thick and were stained with toluidine blue for examination by light microscopy. Selected blocks were trimmed and thin sections cut for examination by electron microscopy. Autoradiography was performed by mounting thick sections onto gelatin-coated slides, dipping them into Kodak NTB2 emulsion (Rochester, NY), and leaving them in the dark at 18°C for 2 weeks.

Results

Tissue Culture of Retinal Epithelium

Dissociated rabbit retinal epithelial cells form epitheloid-appearing monolayers by cell division after several weeks in culture. Rabbit retinal epithelium can be distinguished by the relatively large cytoplasmic oil droplets, about 5-10 estimated per cell. These oil droplets are gradually lost in culture, either by exocytosis or by dilution through repeated cell division. Some droplets can still be seen in those cells, however, in primary cultures after confluency occurs. With tritiated thymidine in the culture media from the start, a large fraction of the cell nuclei in these primary cultures become radiolabelled.

Removal of Host Epithelium

Although in initial experiments we were able to remove host retinal epithelium by gentle rubbing with a diamond-dusted needle under the detached neural retina, we found after examining the histology that this frequently caused breaks in Bruch’s membrane, which resulted in cellular proliferation from the choroid into the subretinal space. We discovered that a less traumatic way to remove the host retinal epithelium was to use the force of the fluid being injected from the glass cannula. Patches of host retinal epithelium could be lifted off Bruch’s membrane, still adherent to the undersurface of the detached neural retina. A similar phenomenon has also been observed by Marmor (personal communication). This method of removing the host retinal epithelium is much less prone to breakage of Bruch’s
membrane and has the advantage of allowing the surgeon to determine how successful the removal has been, since the patches of removed RPE can be clearly visualized during surgery. It is our impression that this phenomenon, which we have called “lift off,” is more easily produced in the more light-adapted retina, but this has not been tested experimentally. Most of the cells which lift off may not survive; those that do will settle back down with the neural retina, in some cases landing on top of transplanted cells (Fig. 6A).

Figures 2A and B illustrate examples of how well the host retinal epithelium can be removed and how the previously detached neural retina can reattach to these denuded areas of Bruch’s membrane. This illustration demonstrates that it is possible to perform this procedure without having any significant inflammatory response in either the subretinal space or the choroid, and that the neural retina will spontaneously reattach to the denuded area of Bruch’s membrane and retain intact photoreceptors in contact with bare Bruch’s membrane.

Transplantation of Cultured Retinal Epithelium

After dissociated cultured retinal epithelial cells have been injected under the neural retina, clusters of such cells can be found in the subretinal space. These cells can be identified by their decreased content of pigment granules as compared to the host epithelium. Some of these cells can be seen sitting on top of unremoved host retinal epithelium but more are found near or on areas of denuded Bruch’s membrane (Fig. 3A, B, C). Transplant cells do not appear to float out through the small retinal hole into the vitreous cavity but seem to be drawn toward Bruch’s membrane. Some of these cells make close attachment to Bruch’s membrane within 1 hr (Fig. 3A, B) or 2 hr (Fig. 3C) after being injected into the subretinal space.

Figures 4A and B demonstrate by autoradiography that the former cells, previously identified by light microscopy, are indeed the cultured retinal epithelium. More than 50% of these cells have tritiated thymidine-produced grains in their nuclei. In both Figures 4A and B, these transplanted epithelial cells can be seen to be closely attached to Bruch’s membrane as well as to neighboring host retinal epithelium that was not dislodged from Bruch’s membrane.

Figure 5 shows an autoradiograph from a rabbit sacrificed 24 hr after the cultured epithelial cells had been introduced into the subretinal space. Four radio-labelled cells (arrows) can be seen attached to Bruch’s membrane and have formed a pseudomonolayer juxtaposed to a reattached neural retina. Outer segments of photoreceptors can be seen in close proximity to the transplanted cells. At the light microscopic level there is relatively little evidence of
Fig. 3. (A) A cluster of previously cultured RPE cells in the subretinal space 1 hr after injection. An area of bare Bruch's membrane (arrow) can be seen adjacent to intact host RPE (X200). (B) A higher power view of Fig. 3a shows a transplanted cell beginning to attach to Bruch's membrane (X540). (C) Two previously cultured RPE cells can be seen attached to Bruch's membrane 2 hr after injection (X540).

Discussion

The results show that a closed-eye approach with a small pars plana port can be used to transplant cultured retinal epithelium to Bruch's membrane of rabbit retina. This approach is less traumatic than the open-sky method used previously for such experiments.1,2,8 The hydrodynamics of the ocular chambers are only minimally disturbed in this method, so that the neural retinal spontaneously reattaches within hours to the transplant site. An important aspect of retinal epithelial function, phagocytosis of outer segment material, can be recovered by these transplanted cells within 24 hr after surgery. This result indicates that at least partial reestablishment of function is possible even in the central nervous system using transplantation techniques.

The rabbit retina is especially suited to such a procedure because it is relatively avascular, and therefore the small hole, which must be produced in the neural retina to induce its detachment, does not cause any hemorrhage. We have, however, produced such holes in monkey retina, where a small hemorrhage does occur, but this usually resorbs within a week, so the procedure can also be performed in primates.

The discovery that a fluid stream from a micropipette can be used to dislodge host retinal epithelium was extremely important because it is difficult to use either rubbing or aspirating under the detached neural retina, since the detached retina obscures the view of the underlying retinal epithelium. The fact...
that patches of retinal epithelium stick to the undersurface of the neural retina provides the experimenter with a degree of feedback on how much epithelium has been removed, since this can be visualized while the surgery is occurring. We have employed the same cannula used to detach the neural retina and dislodge the host epithelium to inject the transplant inoculum. This method obviates the need for using a second cannula, since the retinal hole becomes difficult to reenter when the neural retina becomes detached. Conceivably a double-barreled cannula might prove to be more effective in this procedure.

A valuable finding in these experiments was that the dissociated cells injected into the subretinal space

![Fig. 4. Autoradiographs of cells shown in Fig. 3 (serial sections). Intracellular H-thymidine granules identify these as previously cultured RPE cells attached to Bruch’s membrane one hour (A) and two hours (B) after injection (×500).](image)

![Fig. 5. Autoradiograph showing a near-monolayer of previously cultured RPE cells (arrows) lying on Bruch’s membrane 24 hr after injection. The neural retina has reattached and photoreceptor outer segments can be seen in close proximity to the transplanted cells. The open arrow indicates the area that was subsequently sectioned for electron microscopy shown in Fig. 6 (×300).](image)
Fig. 6. Electron microscopy of transplanted RPE cells attached to Bruch's membrane 24 hr after injection. (A) Arrow points to a row of electron-dense intracytoplasmic material, indicating that this transplanted cell is undergoing mitosis. Host RPE cells which have lifted off with the neural retina have settled on top of the transplanted cell, preventing its contact with the photoreceptors (X2400). (B) Photoreceptor outer segments in direct contact with transplanted cell. Inset at upper right shows phagocytosis of outer segment material by transplanted cell (X3500; inset X6000).

will rapidly reach and attach to Bruch's membrane. There may be a bulk flow of fluid from the subretinal space into the choriocapillaris, driven by the oncotic pressure of proteins, which could be the factor that draws the transplant cells toward Bruch's membrane. This idea is in agreement with what is known about the forces that bind the neural retina to the epithelial layer. Not only do the transplant cells reach Bruch's membrane but they may be preferentially drawn to the patches devoid of host epithelium. This makes sense physiologically, if one assumes that the retinal epithelium forms a significant resistance to the bulk flow of fluid toward the choriocapillaris. This idea is in agreement with what is known about the forces that bind the neural retina to the epithelial layer. 

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The previously cultured, transplanted cells closely attach within an hour to Bruch's membrane, as seen in both light and electron microscopy. Apical villi can be seen facing the subretinal space and not on the basal surface. However, the basal surface does not show the characteristic infoldings of well-differentiated retinal epithelium; this may require longer periods of time than we have studied so far. Some transplanted cells are capable of phagocytosing outer segment material through their apical processes. It is interesting to consider how these epithelial cells, which establish a distinct apical-basal polarity in vitro, adjust to their contact with Bruch's membrane. We do not know whether they tend to attach with their original basal surface or whether they transform their plasma membrane to suit their new environment. Recent experiments have indicated that there is considerable plasticity in epithelial polarity, so that the microenvironment can influence what proteins and, concomitantly, what membrane features are expressed at either side of such cells.

There are a number of potential pitfalls associated with this technique which may become even more important in longer-term experiments. For example, in order to transplant cultured cells to bare Bruch's membrane, the host epithelium must first be detached. Retinal epithelial detachments in man are known to be associated with subretinal neovascularization, although this may be related to damage to Bruch's membrane. We are aware of the importance of keeping Bruch's membrane intact and have taken great care to preserve its integrity during this procedure. Another potential problem is proliferative vitreoretinopathy (PVR), which is felt to be due in part to the proliferation of RPE cells along the pre- and subretinal surfaces and the posterior hyaloid. Retinal epithelium usually starts to divide only when the neural retina is detached, in response to injury, or when placed in tissue culture. When the retina reattaches or when
cultured cells reach confluency, cell division stops. Some of the transplanted cells appeared to be in a mitotic state. It is possible that the transplanted cells in the subretinal space may respond to the same forces that regulate retinal epithelial division during detachment and reattachment of the neural retina or in tissue culture. However, cultured RPE cells in the vitreous may continue to proliferate, producing a condition similar to PVR. Although we try to make the retinal hole as small as possible and are careful not to seed the vitreous with cultured RPE cells, it may be difficult to be certain that no cells enter the vitreous cavity. A third problem is reattachment of the retina and closure of the retinal hole. The small bleb detachments spontaneously reattach in the rabbit, but this may not be the case in other animals. Perhaps a vitrectomy in combination with an intraocular gas may be required to effect reattachment and closure of the retinal hole. Not addressed in this study is the possibility of immune rejection. Longer-term transplant experiments are now underway which will help clarify the extent of the immune reaction, if any, produced against these allografted cells.

Key words: retinal epithelium, transplantation

Acknowledgments

We thank Anne M. Leitch and Mary K. Bilek for their assistance.

References