Retinal Detachment Prevents Normal Assembly of Disk Membranes in Vitro

Michael W. Kaplan, Roderick T. Iwata, and Craig B. Sterrett

The effects of retinal detachment upon disk membrane assembly in rod outer segments were assessed in *Xenopus laevis* retinas that had been maintained in eyecup cultures for up to 4 days. In these cultures, assembly of disk membranes occurred at a normal rate in regions of the retina that remained attached to the retinal pigment epithelium. In regions of the retina that were detached from the pigment epithelium, the assembly of new disk membranes either was abnormal or was inhibited. This result cannot be attributed to reduced access of cells in the detached retina to oxygen and metabolites. The experiments described here suggest that the apposition of the retina with the pigment epithelium is a necessary condition for normal disk membrane assembly in *Xenopus* retinas. This effect may be mediated by contact between the rod outer segments and the pigment epithelium, or by trophic factors in the subretinal space.
Eyecup Cultures

Eyes were enucleated from light-adapted, adult *Xenopus laevis* frogs. Eyecups were prepared in one of two ways. For most experiments, the globe was hemisected at the equator while the anterior portion of the eye was carefully retracted. This procedure removed the lens and most of the vitreous. A partial detachment was produced by pulling gently on the peripheral retina with fine forceps. The *Xenopus* retina spontaneously curled away from the RPE when detached, so a permanent detachment was achieved by this procedure. In order to make comparisons between photoreceptors from both attached and detached regions in the same retina, a significant portion of the retina was usually left attached to the RPE. In the second method, designed for experiments in which it was preferable to leave the entire retina attached to the RPE, incisions were made in the cornea and iris, and pressure was applied at the lateral margins of the eye until the lens and most of the vitreous were extruded. This procedure preserved the mechanical integrity of the ora serrata, and usually left the retina fully attached to the RPE.

The effects of retinal detachment were studied in eyecup cultures in order to avoid the potential effects of anoxia and metabolite depletion inherent in studies of retinal detachment in vivo. In these cultures, the cells in detached portions of the retina can be expected to have at least as much access to oxygen and metabolites in the tissue culture medium as the cells in attached portions. Eyecups were incubated for up to 4 days in sterile 25-ml side-arm Erlenmeyer flasks containing 7.5 ml of Wolf-Quimby amphibian tissue culture medium (ATCM) (GIBCO, Grand Island, NY) that had been supplemented with 4.3 mM KCl, 0.14 mM MgSO4, 4.6 mM NaCl, 1.4 mM NaHCO3, 0.56 mM glucose, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. This medium supports disk-shedding in short-term cultures of *Xenopus* eyecups. Culture medium was replaced twice daily. Eyecups to be used in electron microscopy were rinsed in bicarbonate-buffered saline solution and then fixed for 30 min at 4°C in 0.1 M cacodylate buffer containing 4% paraformaldehyde and 0.1% glutaraldehyde; after this step, the eyecups were fixed overnight at 4°C in cacodylate buffer containing 4% paraformaldehyde. Prior to adding fixatives, the osmolarity of buffer solutions was adjusted with sucrose to 235 mOsm. Tissues were then embedded and processed in Spurr’s resin, sectioned at 1.5 μm, and mounted for fluorescence microscopy.

Dye Labeling and Morphometric Analysis

Patent disks at the proximal and distal ends of ROSs were labeled by incubating retinas initially for 6 hr in ATCM that contained 1.3 mg/ml of the fluorescent dye Lucifer yellow CH. Assembly of ROS disk membranes in vitro was assessed by monitoring the axial displacement of dye-labeled disks as unlabeled disks formed subsequently in dye-free ATCM. In most experiments, Lucifer yellow dye was also included in the final aliquot of culture medium at the end of the incubation period in order to mark the proximal ends of outer segments (double-labeled retinas). The mean rate of assembly of disk membranes in vitro could then be quantified by measuring the distance between the dye bands. In some experiments, the second incubation in dye-containing medium was omitted (single-labeled retinas). At the end of the incubation period, eyecups to be used in histologic studies were rinsed in bicarbonate-buffered saline solution and then fixed for 30 min at 4°C in 0.1 M cacodylate buffer containing 4% paraformaldehyde and 0.1% glutaraldehyde; after this step, the eyecups were fixed overnight at 4°C in cacodylate buffer containing 4% paraformaldehyde. Prior to adding fixatives, the osmolarity of buffer solutions was adjusted with sucrose to 235 mOsm. Tissues were then embedded and processed in Spurr’s resin, sectioned at 1.5 μm, and mounted for fluorescence microscopy.

Eyecups to be used in electron microscopy were rinsed in buffer containing 0.1 M Na-cacodylate, 3.4 mM CaCl2, and sucrose to adjust the osmolarity to 235 mOsm. Tissues were fixed for 1.5 hr at 4°C in cacodylate buffer containing 1.0% OsO4 and 2.0% glutaraldehyde. After rinsing with buffer, tissues were dehydrated by immersion for 5 min each in 50%, 70%, 80% and 90% ethanol; then for 15 min each in two changes of 100% ethanol; and finally for 15 min each in 50% ethanol–50% propylene oxide and 100% propylene oxide. Eyecups were infiltrated with 50% propylene oxide–50% Spurr’s resin for 1 hr, and then with 100% Spurr’s resin for 4 hr. Blocks were cured overnight at 60°C, and silver sections were cut and mounted on formvar-coated grids for observation in a Zeiss (Oberkochen, West Germany) TEM10CR microscope.

Because of shrinkage artifacts that occur in fixed tissues, measurements of dye-band displacement, morphology and birefringence were made using unfixed ROSs that had been isolated from either attached or detached portions of retina and then mounted in gelled 1.3% agar, as described previously. Data were recorded with fluorescence or POL photomicrography, or with a silicon-intensifier-tube video camera coupled to analog and digital video processors and an image analyzer.

Results

Histological Sections

Sections of double-labeled eyecups that had been in culture for 3 days were examined for evidence of
disk assembly. As shown in the representative fluorescence micrograph of Figure 1A, ROSs in regions of retinas that remained attached to the RPE exhibited two distinct bands of disks that were labeled with Lucifer yellow dye. The distal dye band was produced at the onset of the 3-day incubation period. The proximal band was formed at the end of the incubation period. Therefore, the section of the outer segment between the dye bands was composed of disks that had been assembled in vitro. When viewed with a light microscope, the morphology of both the inner segments and the outer segments of rod cells in attached regions of the retina appeared normal. Figure 1B shows the transition zone between ROSs with two dye bands in the attached region and ROSs with one dye band in the detached region of the same retina shown in Figure 1A.

The photoreceptors shown in Figure 1C also are 

![Fig. 1. Fluorescence micrographs of retinas from Xenopus eyecup cultures after 3 days in vitro. (A) Attached region of a retina double-labeled with Lucifer yellow dye (ie, labeled at the beginning and the end of the incubation) and showing two bands of dye-labeled disk membranes (inset). Calibration bar = 20 μm. (B) Transition zone (arrow) between ROSs with two dye bands in the attached region and with one dye band in the detached region of the same retina shown in (A). (C) Detached region of the same retina as in (A) showing only a single dye band and vacuolated stained material in the junction region between the inner and outer segments (inset). (D) Detached region of a retina that was single-labeled with dye at the end of the incubation period. (E) Detached region of a retina that was single-labeled with dye at the beginning of the incubation period. (F) Attached region of the same retina shown in (E).]
from the same doubly-labeled retina shown in Figure 1A, but are from a region that was detached from the RPE during the 72-hr culture period. The ROSs in detached regions contained only one band of dye-labeled disks at the proximal end. A single band of dye-labeled disk membranes also was seen at the proximal end of ROSs in detached regions in experiments in which dye-labeling at the end of the incubation period was omitted. This means that the disk membranes at the proximal end of the ROSs in detached regions of the retina at the end of the 3 days in vitro were the same disks that had been at the proximal end at the beginning of the incubation. It also shows that patent disks at the proximal end of the ROSs in detached regions of *Xenopus* retinas had incorporated dye during the initial incubation with Lucifer yellow, but that the subsequent assembly of disks in vitro was either absent or abnormal.

**Fig. 2.** Electron micrographs of the disoriented and vacuolated lamellar membranous material found in the region between the inner and outer segments of rod cells in detached portions of the retina. (A) After 24 hr in vitro (x11,500). (B) After 3 days in vitro (x6000).

Rod cells located in detached portions of the retina usually appeared distended and vacuolated in the region proximal to the dye band, in the space between the outer segment and the inner segment. This enlarged intersegment region contained material that was weakly stained by Lucifer yellow in double-label experiments (Fig. 1C), and in experiments in which dye was included only in the final aliquot of ATCM (Fig. 1D). The intersegment region was not stained in single-label experiments in which dye was included only during the initial 6 hr in vitro (Figs. 1E, F). Electron micrographs show that the vacuolated spaces between the inner and outer segments of rods in detached portions of the retina contained whorls of lamellar membranous material (Figs. 2A, B). Experiments are in progress to determine unambiguously whether these membranes were formed de novo in culture, or whether they were formed from degenerated disk membranes that were at the base of the outer segment at the start of the incubation period.

The amount of disoriented membranous material in the intersegment space of ROSs in the detached retina usually appeared greater after 3 days in culture (Fig. 2B) than after 24 hr in culture (Fig. 2A). This observation and the results of the dye-labeling experiments support the conclusion that the disoriented membranes in the intersegment region were assembled in vitro. The proximal end of ROSs from regions that remained attached to the RPE in culture had disk lamellae and intersegment spaces (Fig. 3) that appeared very similar to equivalent regions of cells in freshly isolated eyecups.

When *Xenopus* retinas were incubated in ATCM containing Lucifer yellow, dye was in many cases in-
corporated into the distal tips of a subpopulation of the ROSs. When retinas were incubated in dye only during the initial 6 hr of the incubation period, many of the ROSs in the detached portions of retinas had distal tips that remained stained after 4 days in culture (Fig. 1E). This finding supports the conclusion of Williams and Fisher that detachment of Xenopus retinas from the RPE inhibits normal shedding of disk membranes from the distal end of ROSs. No staining of distal tips was found in single-labeled ROSs in regions of retina that remained attached to the RPE (Fig. 1F). In addition, after 3 days in culture, many of the ROSs in attached regions were shorter than ROSs in nearby detached regions. This observation suggests that shedding of the distal tips occurred in vitro in attached regions of the retina, and that frequently the amount of material shed exceeded the amount of material added to the proximal end of the ROSs by the assembly of new disks.

The pronounced degenerative changes in ROS morphology produced in vivo in experimentally detached retinas of monkeys and cats were not seen in detached Xenopus retinas after as many as 4 days in vitro. In Xenopus, the ROSs in detached regions of the retina were well preserved. Cone outer segments, however, were more susceptible to damage and frequently appeared disrupted. In attached regions, the morphology of cultured rod and cone inner segments was comparable to that of freshly isolated tissue. In detached regions, distortion of the distal surface of the inner segment often was apparent (Fig. 2B). The frequency of pyknotic nuclei in the outer nuclear layer was not noticeably different in detached regions as compared to attached regions of the same retina (data not shown).

Morphometric Analysis

Disk assembly in vitro was assessed quantitatively in unfixed ROSs that were isolated from either attached or detached regions of the retina. Figure 4A shows superimposed bright-field and fluorescence images of a ROS isolated from a section of retina that had been attached to the RPE; this retina sample was obtained from an eyecup that had been cultured for 4 days at 24°C on a 12:12-hr light-dark cycle. Figure 4B shows the same cell viewed with polarized light optics. The position of the dye band indicates that the proximal portion of the outer segment was composed of disks formed in vitro. In the proximal region of the ROSs, light-dark-dependent birefringence bands showed the daily growth rate due to addition of newly assembled disk membranes. There were 4 birefringence bands formed between the dye band and the proximal end of the ROSs, corresponding to the 4 light-dark cycles that were completed during the incubation. A comparison of the period of the birefringence bands in the proximal and distal regions of these ROSs showed that the mean rate of addition of new disks, assuming a constant packing density along the ROS axis, was the same for disks assembled in vivo and in vitro. At a temperature of 24°C, approximately 2.2 μm was added to the length of the ROSs each day in both conditions. The light-dark-dependent bands had a normal appearance and birefringence contrast in the proximal region that had formed in vitro. The proximal ends of isolated ROSs also demonstrated a pronounced gradient of birefringence similar to that seen in freshly isolated frog ROSs. These results from cultured eyecups show that the light-dark-dependent differences between disk membranes assembled in the light and in the dark, and the age-dependent changes in disk structure that cause the birefringence gradient, are controlled by factors that are endogenous to the eye rather than by systemic factors.

The displacement of dye-labeled disks from the proximal end was measured in isolated ROSs from both attached and detached retinas that had been...
stained only during the initial 6 hr of the incubation period. The measure used for assessing growth was the displacement distance produced by the addition of disk membranes during one 24-hr light–dark cycle, as determined by the period of light–dark–dependent birefringence bands. The ROSs measured in the histograms of Figure 5A were from four retinas that were fully detached from the RPE during the entire 4-day culture period. In the overwhelming majority of ROSs from detached retinas, the dye-labeled disk membranes remained at the proximal end. The ROSs in the histograms of Figure 5B were isolated from two retinas that were attached during the initial 2 days of the incubation period and then detached for the final 2 days. The majority of these ROSs had dye-labeled disks that had been displaced the equivalent of 2 days' growth. The ROSs measured in the histograms of Figure 5C were from six retinas that had remained attached to the RPE for the entire 4-day culture period. In most of these cells the dye band was displaced from the proximal end of the ROSs a distance equivalent to 4 days' growth. In some of the ROSs putatively from attached retinas, the dye-labeled disks remained at the proximal end (indicating no growth). In some ROSs the dye-labeled disks were displaced away from the proximal end, but less than the equivalent of 4 days' growth. These results are consistent with the conclusion that the assembly and distal displacement of new disk membranes in cultured Xenopus eyecups occurred only while the retina was attached to the RPE.

Discussion

Results reported here suggest that normal assembly of Xenopus rod disk membranes in eyecup cultures requires close apposition of the distal ends of the ROSs with the RPE. One possible explanation is that a factor or mechanism that mediates normal disk assembly is missing in detached portions of the retina. Normal disk assembly could require specific contact between the cell types. Alternatively, disk assembly may involve a diffusible trophic factor in the subretinal space; this factor becomes greatly diluted when the volume of the subretinal space is increased by detachment of the retina. The subretinal space is a highly regulated environment for photoreceptors and RPE cells. Metabolites, organic and inorganic ions, amino acids, growth factors, and retinoids are all transported into or out of the subretinal space by photoreceptors, RPE cells, and Müller cells. The concentration of any or all of these factors may be important in the regulation of disk membrane assembly. A third possibility is that detachment causes the abnormal release of factors into the subretinal space which interfere with the normal assembly of disks. 

Fig. 5. Histograms of the displacement of dye-labeled disks from the proximal ends of ROSs that were isolated from retinas cultured for 4 days. Cells were classified as either no growth (dye band at the proximal end of the isolated ROSs), 4d growth (dye band displaced the equivalent of 4 days' growth as measured by light–dark–dependent birefringence bands), or <4d growth (dye band displaced from proximal end, but less than 4 days' equivalent growth). Differently shaded histogram bars in each classification distinguish data from separate retinas. Disks were labeled during the initial 6 hr in vitro. (A) Retinas detached from the RPE for 4 days in vitro. With few exceptions, no displacement of dye bands was seen. Data recorded for 4 retinas; total number of ROSs measured for each retina, n = 40, 110, 84, and 85, respectively. (B) Retinas left attached for 2 days, and then detached for the final 2 days in vitro. The majority of ROSs exhibited displacement of dye-labeled disks equivalent to 2 days of disk assembly. Therefore, most ROSs were classified as <4d growth. Data recorded for 2 retinas; n = 166 and 95, respectively. (C) Retinas attached to the RPE for 4 days in vitro. A plurality of ROSs had dye bands displaced the equivalent of 4 days of disk assembly. Data recorded for 6 retinas; n = 96, 103, 184, 165, 141, and 311, respectively.
This explanation, however, requires that putative disruptive or inhibitory factors affect photoreceptors only in the detached portion of the retina. The abnormal disk assembly seen in detached regions of cultured *Xenopus* retinas was probably not attributable simply to deficiencies in oxygen or diffusible metabolites. It is likely that the detached regions had better access to the culture medium than did attached regions of the same retina, where disk assembly apparently was normal.

In a previous study of the synthesis and assembly of disk membranes in isolated *Xenopus* retinas that had been cultured for up to 6 hr, Hollyfield et al. found abnormal, undulating basal disk membranes and swelling of the junction region between the inner and outer segments of rod photoreceptors. These changes in morphology were attributed to artifacts of culturing, but the similarity of these changes with results reported here suggests that the abnormality of the basal disk membranes was related to the detachment of the isolated retinas from the pigment epithelium.

Other studies of the biosynthesis of disk membrane components in vitro have been performed with isolated retinas from *Rana pipiens*. Basinger et al. and Bok et al. cultured frog retinas in the dark for up to 32 hr, and found with electron microscopy that basal disk membranes had relatively normal morphology. The discrepancy of this finding from results reported here may be due to differences of species, culture medium, or lighting conditions. However, the rate of disk assembly reported for isolated *Rana pipiens* retinas was at most only one disk during the 32 hr of incubation, which is less than 2% of the rate of assembly in vivo. Therefore, it seems likely that disk assembly was inhibited by the culturing conditions used in prior studies, even though some components that normally would be incorporated into the disk membranes were being synthesized. It is clear that in vitro, processes of membrane assembly near the junction of the inner and outer segments must be sustained at a nearly normal rate in order to produce the abnormal lamellar membranes that were observed in detached portions of *Xenopus* retinas.

It has been shown that contact of the retina with the RPE is essential for normal growth and development of photoreceptor cells in embryonic vertebrate retinas. For example, Hollyfield and Witkovsky found that rod cells in retinal rudiments from embryonic *Rana pipiens* tadpoles did not produce normal disk membranes in culture unless the tissue was in contact with RPE cells. Rudiments grown in the absence of RPE cells produced photoreceptors with cilia that either lacked lamellar disk membrane material or that produced disklike material that became highly disorganized and vacuolated. By contrast, rudiments grown in the presence of RPE cells developed well-ordered disks. In more recent experiments, factors which promote the growth and differentiation of embryonic chicken photoreceptors in culture have been isolated from the interphotoreceptor matrix and from the RPE cells of chicken and bovine eyes. These results may be relevant to those described in this paper for fully developed, adult *Xenopus* photoreceptors. It is tempting to speculate that factors or mechanisms which involve contact of the retina with the RPE and which mediate the normal development of disk membranes in embryonic photoreceptors may be analogous to or similar to factors or mechanisms required to maintain normal disk assembly in mature photoreceptors.

The specific causes of abnormal disk assembly in detached retinas in vitro remain to be determined. The normal assembly of disk membranes at the proximal end of ROSs is known to be affected by drugs that disrupt carbohydrate processing during glycoprotein synthesis. Tunicamycin blocks the synthesis of N-acetyl-glucosaminylpyrophosphoryl polylisoprenol, an intermediate in the pathway of the formation of asparagine-linked oligosaccharides. The region between the inner and outer segments of rod cells from tunicamycin-treated *Xenopus* retinas contains whorls of vacuolated membranous material that resembles the lamellar membranes found in the detached regions of cultured retinas described here. Similarly, treating *Xenopus* eyecups with cytochalasin D depolymerizes actin found normally in the inner segment, in the calycal processes, and in the proximal region of outer segment of rod cells. Depolymerization of actin is accompanied by aberrant disk assembly, in which newly forming disk lamellae grow beyond the normal boundaries of previously formed disk membranes. Future work investigating the mechanisms responsible for aberrant assembly of ROS disk membranes in detached retinas in eyecup cultures will include studies of membrane glycoproteins and cytoskeletal proteins to determine whether or not these elements become abnormal in the absence of the RPE.

**Key words:** retinal detachment, rod outer segment, disk membranes, retinal pigment epithelium

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**References**


