A reliable method for organ culture of neonatal mouse retina with long-term survival

Judith Mosinger Ogilvie a,b,*, Judith D. Speck a,b, Jaclynn M. Lett a, Timothy T. Fleming b

a Central Institute for the Deaf, 818 South Euclid Avenue, St. Louis, MO 63110, USA
b Department of Ophthalmology and Visual Sciences, Washington University School of Medicine, St. Louis, MO, USA

Received 24 July 1998; received in revised form 30 September 1998; accepted 1 October 1998

Abstract

Organ culture systems of the central nervous system have proven to be useful tools for the study of development, differentiation, and degeneration. Some studies have been limited by the inability to maintain the cultures over an extended period. Here we describe an organ culture technique for the mouse retina. This method uses commercially available supplies and reproducible procedures to maintain healthy retinas with normal architecture for 4 weeks in vitro. The system is amenable to quantitative analysis. It can be used with both normal and retinal degeneration (rd) retinas to study the role of various factors in photoreceptor degeneration in retinal cell fate determination and development. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Organ culture; Retinal explant, mouse; Development; Photoreceptor degeneration

1. Introduction

Mammalian retinal organ cultures can provide a useful model for the study of retinal development (Tansley, 1933; Hild and Callas, 1967; LaVail and Hild, 1971; Tamai et al., 1978; Sparrow et al., 1990; Söderpalm et al., 1994; Sheedlo and Turner, 1996), degeneration (Lucas, 1958; Sidman, 1961, 1963; Tamai et al., 1978; Caffè et al., 1993) and rescue of sensory cells (Caffè et al., 1993; Mosinger Ogilvie et al., 1998). The ability to control the in vivo environment offers an opportunity to study the effects on the retina of long-term application of substances and to test hypotheses which can not be tested in vivo. As early as the 1920s, Strangeways and Fell (1926) cultivated embryonic chick retina in organ culture using plasma clots. Within a short time the technique had been extended to the mammalian retina (Tansley, 1933). While brain slice cultures have found wide spread application over the last decade, the use of retinal organ culture has been more limited. This may be due, in part, to the perceived difficulty in setting up and maintaining the preparation. Here we describe a retinal organ culture technique using commercially available supplies and reproducible procedures that consistently maintain healthy mouse retinas for 4 weeks in vitro.

2. Methods

2.1. Animals

Mice used for these experiments came from a C57BL/6 background with either the wild type (+/+ ) or retinal dystrophic (rd/rd) allele. The rd mouse has a defect in the β-subunit of the cGMP phosphodiesterase gene which results in the degeneration of rod photoreceptors within approximately the first 3 weeks after birth (Farber et al., 1994). One hundred and seven wild type and 104 dystrophic retinas were used with at least four retinas in each condition. Twenty-one retinas were...
used in preliminary experiments described below. Animals were handled in accordance with institutional guidelines and the National Institutes of Health Guidelines on Laboratory Animal Welfare.

2.2. Organ cultures

Mouse pups at postnatal day 2 (P2) were anesthetized on crushed ice and decapitated. The head was dipped in 70% ethanol and moved to a laminar flow hood where sterile procedures were maintained for the remainder of the preparation. Eyes were enucleated and placed in cold Dulbecco’s modified Eagle’s media (DMEM, Gibco #11965) plus 2.5 μg/ml Fungizone (Sigma, St. Louis, MO) in a 35-mm petri dish. Using fine forceps, extraneous connective tissue was removed from the globe which was then placed in fresh DMEM containing 0.5% proteinase K (Boehringer Mannheim, Germany) for approximately 7 min at 37°C. The tissue was rinsed at room temperature in DMEM plus 10% fetal calf serum (FCS) and 1.25 μg/ml Fungizone and then replaced with the same media without serum. Starting at the limbus border, the sclera and choroid were gently peeled away using two pair of #5 forceps leaving the retinal pigment epithelia (RPE) attached to the retina. Using the same forceps, each retina was isolated from its anterior segment (cornea, lens and vitreous) with as little disruption and manipulation as possible. The tissue was then gently lifted with a pair of forceps and transferred to a fresh petri dish containing DMEM plus 10% FCS and 1.25 μg/ml Fungizone and incubated for approximately 30 min at 37°C. RPE sheets, which detach from the retinas during this process and begin to roll up, were gently teased away from the retina using fine forceps, leaving a few RPE cells behind. Using a disposable transfer pipette which had the tip cut to enlarge the opening, each retina was gently transferred with a few drops of media onto the Millicell membranes.

Early experiments with Anocell (#25) and Falcon (#3090) inserts were less successful than organ cultures grown on the Millicell-CM insert; we have not tested other culture inserts. In some preliminary experiments, after the eyes were enucleated and placed in cold DMEM plus 10% FCS, the sclera, choroid and RPE were peeled away mechanically without enzyme treatment. In other experiments, only the sclera and choroid were removed and the retina and RPE were co-cultured on the Millicell membranes.

2.3. Histology

Organ cultures were terminated after a period ranging from 4 h to 41 days in vitro (DIV 0–41) by placing them in fixative with 2.5% glutaraldehyde and 2% paraformaldehyde overnight. Eyecups from age-matched control animals, were also isolated and fixed to provide an in vitro comparison. Surviving littermates were used when available. Tissue was postfixed for 1 h in 1% osmium tetroxide, stained en bloc with 1% uranyl acetate for 1 h, rinsed, dehydrated through an acetone series and embedded in Epon-Araldite. For electron microscopy, silver or light gold sections were cut on a diamond knife and post-stained with uranyl acetate and lead citrate.

2.4. Data analysis

A trained observer, blind to the experimental conditions, viewed the retinal sections through a light microscope at 40 × magnification. Using a grid reticule to determine length, the center of the section was identified and two regions, 100 μm on either side of the center point, were randomly selected for three quantitative measurements. First, the thickness of the outer nuclear layer (ONL) was determined by counting the number of ONL cells in a vertical column touching a single grid line on the reticle. Five counts were taken across an 80 μm region (20 μm intervals) on both sides of the center point for a total of ten measures, which were averaged for each retina. The total number of ONL nuclei and the number of pyknotic nuclei were counted in both regions to determine the percent of pyknotic cells in the ONL. Finally, inner and outer segments within each 80 μm region were ranked on a scale of 0–3 where: 3, full-length inner segments (IS) and many identifiable outer segments (OS); 2, full-to-moderate-length IS and few OS; 1, moderate-to-shortened IS and no OS; 0, few small, rounded IS or none remaining. In some cases, the region 100 μm to the side of the center point contained a rosette or distortion due to folding. These retinas were counted both in the designated region and in the first adjacent flat area. Since the distortions generally resulted in a thickening of the ONL at the center of the rosette with an adjacent
thinning, no significant difference was found between randomly selected areas compared to flat selected areas in the average thickness of the ONL or the percent of pyknotic cells, although the standard deviation was larger for the former. In order to minimize any observer bias that might impinge on the selection of the region to be counted, the randomly selected area (100 μm from the center point) was used for all reported measures. However, adjacent flat areas were observed for determination of the IS and OS integrity. These observations varied little across the length of the retinas. A one way analysis of variance (ANOVA) on ranks was performed on the data from different experimental conditions.

3. Results

3.1. Development of normal retina in vitro

At P2, when tissue is harvested for organ culture, the retina is comprised primarily of a neuroblast layer (NBL). The ganglion cell layer (GCL) and inner plexiform layer (IPL) have differentiated; some cells on the inner margin of the NBL have the appearance of amacrine cells (Young, 1984). Cells undergoing mitosis can be identified at the outer margin of the NBL. After 24 h in vitro, organ culture retinas show inner retinal swelling and cell loss in the GCL (Fig. 1A). This observation is consistent with previous findings that neonatal axotomy is followed by ganglion cell death (Miller and Oberdorfer, 1981) but has only a minimal effect on the survival of other retinal neurons (Beazley et al., 1987). Mitotic profiles at the outer margin provide evidence of continued differentiation of the NBL. Horizontal cell differentiation first appears at this stage both in vitro and in vivo and is characterized by differentiated cell nuclei in the center of the NBL. Although retinas grown in vitro are thinner than their age-matched controls (Fig. 1B), they otherwise look very similar to retinas from their P3 littermates.

By 8 DIV the retina has differentiated into three nuclear and two plexiform layers similar in appearance to the in vivo control retinas (Fig. 1C, D). The GCL is comprised of a monolayer of cell bodies. Cells in the ONL have differentiated with the characteristic chromatin pattern of photoreceptors. Dense, round apoptotic cells can be seen in both the in vitro and in vivo retinas, consistent with developmental programmed cell death which occurs in the retina through P18 (Young, 1984). IS development in the organ culture is comparable in length to in vivo controls at this age, but does not have the tightly-packed appearance. By 12 DIV, thin processes extending from the IS with overlying wispy material can be seen. These are identified as connecting cilia and OS disk membranes in the electron microscope (EM, see below). Although RPE sheets are detached...
during the isolation process, a few RPE cells always remain. At early times points, rounded cells with long thin processes extending laterally are frequently seen at the outer margin of the tissue, sitting in the position of RPE above the developing IS. The cells often have pigment granules at early time points identifying them as RPE cells. As the organ culture develops, these cells become more elongated, loose their pigmentation, but can be identified as RPE cells by their characteristic apical processes and EM appearance (see below). The edges of the organ culture are very thin and may be disorganized reflecting any folding or disruption to the edge of the tissue that occurred at the time of isolation and plating.

The overall retinal architecture in organ cultures is maintained through 28 DIV. Other than an overall thinning of the retina, few differences are seen between 20 and 28 DIV (Fig. 2A, B). The IS extend further from the external limiting membrane and many thin wispy processes can be seen. In vivo littermates have mature OS at this age (Fig. 2C). Cell bodies in the GCL are still present after 4 weeks in culture but comprise less
Fig. 3. Light micrographs from rd mouse retinas grown to maturity in organ culture (A, B, D) or 22 day in vivo control (C). (A) At 20 DIV, the outer nuclear layer is reduced to two to four rows of cells and inner segments are stunted (arrowheads). (B) By 27 DIV, the outer nuclear layer is reduced to one to two rows of cells and only residual inner segments remain similar to the in vivo rd retina at P22 (C). Overall retinal architecture is maintained through 27 DIV (D) without folds or rosettes. RPE, retinal pigment epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. A–C, scale bar = 20 μm; D, scale bar = 200 μm.

3.2. Alternate isolation techniques

In preliminary experiments, we tried to transfer the RPE intact with the retina into organ culture. On the Millicell membranes, the RPE lost its epithelial integrity resulting either in large clumps of RPE cells, which would cause displacement of retinal layers, or in migration and invasion of RPE cells into the retina with disruption of retinal architecture. Removal of the RPE cells through mechanical isolation caused more extensive folding and rosette formation in the ONL than isolation with proteinase K treatment. This is likely to be a result of subjecting the sensitive retina to additional handling during mechanical isolation. Some RPE cells remained attached to the retina with either treatment. Over a period of weeks, these RPE cells developed into a layer underlying the organ culture, that varied from an intact monolayer in some areas to sparse, thin cells in other areas while a few regions appear devoid of RPE cells. Few pigment granules were seen consistent with the progressive depigmentation of isolated RPE cells grown in culture. These residual RPE cells were not observed to invade the retina or disrupt the retinal architecture.

3.3. Development of rd retina in vitro

During the first week in organ culture, retinas from rd mice cannot be distinguished from wild type retinas. By 20 DIV, the ONL is reduced to an average of 2.8 rows of cells in the rd retina and many pyknotic cells can be identified (Figs. 3A and 4). However, the ONL of the rd organ culture is thicker than in vivo
littermates where only a monolayer of cells remain in the ONL (Fig. 3C). IS are still apparent in vitro, but have a stubby appearance and the fine wispy OS processes seen in the normal retinas cannot be identified. RPE cells, when present, often sit directly above or surrounding IS. Inner retinal layers appear similar in both the rd and wild type organ cultures. By 27 DIV, the ONL in the rd retina has narrowed to approximately 1.5 cells thick (Figs. 3B and 4), approaching the monolayer appearance of the in vivo littermates. ONL folding and rosette formation are rarely seen in the rd organ cultures (Fig. 3D). Organ cultures can be maintained for as long as 6 weeks in vitro, however, they become so thin that retinal architecture is compromised.

3.4. Evaluation of retinal cultures using EM

IS with connecting cilia can clearly be identified extending beyond the outer limiting membrane in 3–4 week organ cultures of wild type retina (Fig. 5A). Stacked and free-floating OS membranes, which clearly correspond to the thin wispy processes identified in the light microscope, extend from the cilia into a subretinal space separated from the RPE. These OS disks are sparsely scattered beyond the IS, but are more densely packed adjacent to RPE. RPE cells may appear rounded or elongated with nuclei that are easily distinguished from displaced photoreceptors by the diffuse heterochromatin pattern. Microvillar processes extend toward the OS from overlying RPE cells. Thin Müller cell processes extend beyond the junctions which form the external limiting membrane. A row of photoreceptor synaptic terminals in the outer plexiform layer (OPL) form invaginating ribbon synapses (Fig. 5B) with some vacuolization in the terminals. The IPL has an overall normal appearance with both ribbon and conventional synapses (Fig. 5C).

In the 3 week rd organ culture, IS, some with connecting cilia, can be clearly identified, but OS membranes are very sparse (Fig. 5D). Some free-floating membranes, which may be residual OS, are present. The thin Müller cell processes appear more numerous than in wild type organ cultures, consistent with gliosis seen in the in vivo rd retina. After 4 weeks in vitro, IS become smaller and more stunted. Synaptic terminals can still be seen although they are fewer in number and ribbons often appear in the cytoplasm of the cell body (Fig. 5E), as is seen in the in vivo degenerating photoreceptors (Carter-Dawson et al., 1978). The IPL shows no obvious differences from the wild type.

4. Discussion

The first organ culture of eye tissue was performed by placing whole embryonic chick eyes in plasma clots (Strangeways and Fell, 1926). Tansley (1933) advanced the technique by extending the methods to mammalian
tissue and by isolating the rat retina from the whole eyecup. Retinal explant cultures in the first half of the century were primarily grown in plasma clots using a roller tube method. The roller tube culture, also known as the flying coverslip method, maintains a stable attachment of the tissue by embedding it in a plasma clot or collagen matrix affixed to a coverslip which undergoes a continuous, slow rotation. Variations on this method have continued to be used (Hild and Callas, 1967; LaVail and Hild, 1971; Feigenspan et al., 1993).

In the 1950s, Trowell (1954) developed the membrane culture in which the tissue is placed on a porous membrane such as lens or filter paper on top of a wire grid and maintained at the air-media interface. This technique was used by him and others for retinal explants (Lucas, 1958; Lucas and Trowell, 1958; Sidman, 1961; Tamai et al., 1978). Caffé et al. (Caffé et al., 1989; Caffé and Sanyal, 1991) have developed a method which combines some features of both membrane and roller tube techniques by placing rafts made of nitrocel-
lulose filters and polyamide gauze grids on a rocking device.

The techniques described here rely on commercially available membranes and reproducible procedures to consistently produce healthy mouse retinas for 4 weeks in vitro. These retinas follow the same developmental time course as in vivo littersmates. However, they show incomplete development of OS and formation of rosettes in the ONL. Rosette formation is a common feature of explants of developing retina and has been attributed to the absence of intraocular pressure, a condition which also produces rosettes and folding in vivo (Tansley, 1933; Coulombre, 1956; Hild and Callas, 1967). The incomplete development of OS is also a common feature of photoreceptors in culture. It may be partially due to the sparseness of the overlying RPE since the pigment epithelium has been shown to be important for photoreceptor development (Sheetlo and Turner, 1995) and specifically for OS development in culture (Hollyfield and Witkovsky, 1974; Spoerri et al., 1988). No difference in OS development is seen between regions with continuous overlying RPE and regions with no overlying RPE, consistent with a diffusible factor. This retinal organ culture model differs from others previously described in the growth of a new layer of RPE from a few residual cells. The nature of the underlying membrane may play a role in RPE growth. Interestingly, a subretinal space is maintained between the normal retina and the RPE even though this space has been shown to be amenable to quantitative analysis which will support continued studies of underlying mechanisms.

The techniques described here rely on commercially available membranes and reproducible procedures to consistently produce healthy mouse retinas for 4 weeks in vitro. These retinas follow the same developmental time course as in vivo littersmates. However, they show incomplete development of OS and formation of rosettes in the ONL. Rosette formation is a common feature of explants of developing retina and has been attributed to the absence of intraocular pressure, a condition which also produces rosettes and folding in vivo (Tansley, 1933; Coulombre, 1956; Hild and Callas, 1967). The incomplete development of OS is also a common feature of photoreceptors in culture. It may be partially due to the sparseness of the overlying RPE since the pigment epithelium has been shown to be important for photoreceptor development (Sheetlo and Turner, 1995) and specifically for OS development in culture (Hollyfield and Witkovsky, 1974; Spoerri et al., 1988). No difference in OS development is seen between regions with continuous overlying RPE and regions with no overlying RPE, consistent with a diffusible factor. This retinal organ culture model differs from others previously described in the growth of a new layer of RPE from a few residual cells. The nature of the underlying membrane may play a role in RPE growth. Interestingly, a subretinal space is maintained between the normal retina and the RPE even though this space is only sparsely filled with OS membranes. In the rd organ cultures, little separation is observed between the degenerating photoreceptors and the RPE.

The rd mouse carries a genetic mutation leading to the degeneration of the rod photoreceptors during the first 3 weeks. This mouse has proved an excellent model for the study of Retinitis Pigmentosa, a human retinal degenerative disease. The development of an organ culture model of the rd retina allows for investigations that have been limited by the small size of the mouse eye. Photoreceptor degeneration can be described qualitatively in this organ culture model of the rd mouse retina. Loss of photoreceptors is slightly delayed in the explants of rd mouse retinas compared to in vivo littersmates. Lucas (1958) made a similar observation and suggested that photoreceptor degeneration in vitro occurs at a slower rate because of delayed differentiation. This hypothesis is based on his observation that photoreceptors in organ culture failed to develop IS and OS, although they do undergo nuclear differentiation at the same time as in vivo photoreceptors (Lucas and Trowell, 1958). In the organ culture system described here, however, IS and OS development is not delayed although development appears incomplete. Alternatively, the slower rate of photoreceptor degeneration may be due to survival factors in the fetal calf serum in the media. In addition to quantitative studies of photoreceptor degeneration, the organ culture model provides a system for studies of ganglion cell death following axotomy, making the model a valuable tool for the study of degeneration of two different cell types in the retina. The organ culture also provides a controlled environment for the study of the role of various factors in retinal cell fate determination and development.

We have described here a retinal organ culture model which can play an important role in understanding the mechanism of photoreceptor degeneration and rescue. This model provides several advantages over other methods that have been used for screening possible therapeutic agents. Isolated photoreceptor cultures may be simpler than organ cultures, but lack the cell–cell interactions of normal retinal architecture that may play a role in cell survival. Injection of growth factors directly into the vitreous has the advantages of an in vivo model, but the small eye of the young mouse makes it extremely difficult, if not impossible, to ensure that a consistent dose is injected without leakage. Furthermore, with intravitreal injections, the dose decreases with the metabolic turnover of the vitreous, requiring multiple injections for 4 week survival times. The organ culture model provides a middle course which allows for a constant and controlled dose for the duration of the 4 week exposure while maintaining the integrity of the retinal architecture and cell–cell interactions. It is amenable to quantitative analysis which will support further studies of underlying mechanisms.

Acknowledgements

The authors would like to thank Dr Martin S. Silverman for his many useful contributions and Ann Benz for technical advice. This work was supported by NIH Grants NS01756 (JMO), EY11360 (TTF), EY02687, the Foundation Fighting Blindness (JMO) and an unrestricted grant to the Department of Ophthalmology and Visual Sciences from Research to Prevent Blindness.

References


