Feet on the Ground: Physical Support of the Inner Retina Is a Strong Determinant for Cell Survival and Structural Preservation In Vitro

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Purpose. The purpose of this study was to explore the importance of local physical tissue support for homeostasis in the isolated retina.

Methods. Full-thickness retinal sheets were isolated from adult porcine eyes. Retinas were cultured for 5 or 10 days using the previously established explant protocol with photoreceptors positioned against the culture membrane (porous polycarbonate) or the Müller cell endfeet and inner limiting membrane (ILM) apposed against the membrane. The explants were analyzed morphologically using hematoxylin and eosin staining, immunohistochemistry, TUNEL labeling, and transmission electron microscopy (TEM).

Results. Standard cultures displayed a progressive loss of retinal lamination and extensive cell death, with activated, hypertrophic Müller cells. In contrast, explants cultured with the ILM facing the membrane displayed a maintenance of the retinal laminar architecture, and a statistically significant attenuation of photoreceptor and ganglion cell death. Transmission electron microscopy revealed intact synapses as well as preservation of normal cellular membrane structures. Immunohistochemistry showed no signs of Müller cell activation (glial fibrillary acidic protein [GFAP]), with maintained expression of important metabolic markers (glutamine synthetase [GS], bFGF).

Conclusions. Providing physical support to the inner but not the outer retina appears to prevent the tissue collapse resulting from perturbation of the normal biomechanical milieu in the isolated retinal sheet. Using this novel paradigm, gliotic reactions are attenuated and metabolic processes vital for tissue health are preserved, which significantly increases neuronal cell survival. This finding opens up new avenues of adult retinal tissue culture research and increases our understanding of pathological reactions in biomechanically related conditions in vivo.

Keywords: retinal biomechanics, neuron-glia interactions, Müller cell, photoreceptors, tissue culture

In the living eye, the neuroretina is stretched and fixated against the interior wall by means of hydrostatic pressure and adhesion with the posterior vitreous membrane and the retinal pigment epithelium.\(^1\)\(^,\)\(^2\) Interestingly, conditions in which the mature neuroretinal sheet is removed from these normal forces, such as retinal detachment in vivo and explant cultures in vitro, elicit similar tissue reactions, that is, gliosis and neuronal degeneration.\(^3\)\(^,\)\(^4\) We have previously explored the relationship between biomechanical force and retinal homeostasis and have shown that when in vivo tension is emulated in vitro, by laterally stretching the tissue, gliosis and neuronal cell death in adult porcine explants are significantly attenuated.\(^5\)\(^,\)\(^6\)\(^,\)\(^7\) However, the exact mechanism behind this phenomenon remains to be explained. Intriguingly, Müller cells have recently been shown to possess mechanosensory and mechanoregulatory properties.\(^2\)\(^,\)\(^6\)\(^,\)\(^7\) The Müller cells, previously viewed in this context as a passive scaffold for the retinal neurons, are aptly placed to act as mechanosensors, as they vertically span the retinal layers from the outer to the inner limiting membrane (ILM). The laminar architecture as well as cell density contributes to the differential viscoelastic properties of the retina, which vary not only from center to periphery, but also from inner to outer border. The outer retina consists of pliable inner and outer segments, which provide little in the form of structural stability, whereas at the inner retinal perimeter, the Müller cells form stiff endfeet rich in mechanosensitive ion channels, indicating that the biomechanical responsive element of the retina is located in this region.

The retinal organ culture paradigm has been in use since the early 1930s,\(^9\)\(^,\)\(^10\) and has since been employed as a model to study the central nervous system in a variety of species. The most common method entails dissecting the retinal sheet free from the surrounding tissues and placing it with the outer layers apposed to a culture membrane in medium for incubation.\(^9\)\(^,\)\(^10\)\(^,\)\(^11\) This polarity of the retinal tissue in culture was most likely chosen due to the high metabolic rate of the photoreceptors, with the rationale that survival of these cells would be enhanced by the close proximity to the culture medium. In addition, it was believed that the support provided by the culture membrane would mimic physical RPE-outer segment interaction in vivo. This approach has been used successfully for the study and modulation of immature tissue, which has
been found to survive well with several signs of normal development.\(^9-13\) In contrast, as mentioned above, isolated adult retinal sheets cultured under standard conditions display gliosis and neuronal degeneration very early.\(^14-17\) The discrepancy of cell survival in vitro depending on stage of maturity is well established but not yet fully understood.

For the present paper, based on our previously published results on the importance of a biomimetic physical environment for retinal homeostasis, we hypothesize that apposing the comparatively stiff adult inner retina against the nonelastic culture membrane may provide better stability compared with traditional explants in which the culture membrane is apposed to the outer retina. Thus, an improved physical interaction between the tissue and culture membrane may help to restore the collapsed network structure in the isolated retinal sheet removed from the normal stabilizing forces present in the eye. We have thus cultured adult retinal explants “upside down,” that is, with the ILM facing the culture membrane, and compared their neuronal survival and Müller cell reactions with conventionally cultured counterparts.

**Materials and Methods**

**Tissue Culturing**

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 on the Use of Animals in Ophthalmic and Vision Research. Eyes were harvested from adult pigs aged between 4 and 6 months that were euthanized by an overdose of sodium pentobarbital (Apoteket, Umeå, Sweden). The neuroretinas were removed using the method described previously by Engelsberg and Ghosh\(^ {12} \) in 2007. To summarize, the eyes were enucleated immediately after euthanization and immersed in CO\(_2\)-independent medium (Invitrogen, Paisley, UK). The anterior segment was excised by a sharp incision in the pars plana and cut 360\(^\circ\). The vitreous was removed in one piece by carefully pulling it from the eyecup using sterilized tissue paper. The neuroretinas were gently dissected free from the pigment epithelium with microforceps, and the optic nerve head was carefully cut using microscissors. Each neuroretina was sectioned into six pieces measuring approximately 6 mm\(^2\). In total, 18 eyes from 9 animals were used, yielding 91 specimens for culture and 2 eyes serving as normal adult in vivo controls. The 91 neuroretinal pieces were explanted onto Millicell-PCF 0.4-\(\mu\)m culture plate inserts (Millipore, Billerica, MA, USA; Fig. 1), with either the photoreceptors (standard protocol; CT) or the ILM positioned against the culture membrane, providing the explants with inner retinal support (IRS; Fig. 2). Specimens were cultured in 1.5 mL Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Invitrogen) supplemented with 10% fetal calf serum (Sigma-Aldrich, St. Louis, MO, USA) as well as a cocktail containing 2 mM L-glutamine, 100 U/mL penicillin, and 100 ng/mL streptomycin (Sigma-Aldrich) for 5 and 10 days. The explants were maintained in an incubator at 37\(^\circ\)C at 95% humidity and 5% CO\(_2\). The medium was exchanged every second day.

**Histology**

Histological examinations were performed as previously described\(^ {12} \) and are only briefly recapped here. After culturing, the explants were fixed in 4\% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 2 hours in 4\(^\circ\)C. The normal adult in vivo controls were fixed immediately after harvest using the same paraformaldehyde concentration for 4 hours in 4\(^\circ\)C. The explants were then infiltrated with 0.1 M Sörensen’s medium with increasing concentrations of sucrose up to 25\%. They were then embedded in egg albumin/gelatin medium for cryosectioning at –20\(^\circ\)C with a section thickness of 12 \(\mu\)m. For light microscopy, every 10th slide was stained with hematoxylin and eosin. For immunohistochemical labeling, adjoining slides with sections originating from the center of the explants (the area centralis in the normal control) were chosen. The specimens were rinsed three times with PBS containing 0.1\% Triton X-100, and then incubated with PBS containing 0.1\% Triton X-100 and 1\% bovine serum albumin (BSA) for 20 minutes at room temperature. After this, the specimens were incubated overnight at 4\(^\circ\)C with the respective primary antibody (Table). In the double labeling for glutamine synthetase (GS)/bFGF, both primary antibodies were added at this stage. The specimens were then rinsed in PBS–Triton X-100 (0.1\%) and incubated for 45 minutes with a secondary fluorescein isothiocyanate (FITC) or Texas Red-conjugated

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**Figure 1.** Scanning electron microscopy image of the culture well membrane. Porous polycarbonate culture membrane. Pore size 0.4 \(\mu\)m. Scale bar: 2 \(\mu\)m.

**Figure 2.** Illustration of the positioning of the retina on the culture membrane in the different culture groups. Illustration of the inner retinal support (IRS) explant, cultured with the inner limiting membrane facing the culture membrane (A), compared with the standard explant (CT) in which the photoreceptor outer segments are apposed to the membrane (B).
antibody (Table). In the double labeling for GS/bFGF, both secondary antibodies were added at this stage. The specimens were then mounted in Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA, USA). Negative control experiments were performed as above, replacing the primary antibody with PBS containing 0.1% Triton X-100 and 1% BSA. Normal porcine adult retina was used as a positive control.

For transmission electron microscopy (TEM), cultured explants were fixed in 4% glutaraldehyde in phosphate buffer overnight at 4°C. The fixation was followed by repeated rinsing in cacodylate buffer, after which the explants were postfixed in 1% osmium tetroxide. The explants were dehydrated using increasing concentrations of ethanol and embedded in Epon resin. Ultrathin sections (50 nm) were then taken from the central part of the specimens for TEM.

Microscopy and Image Analysis. The histological sections and immunohistochemically labeled specimens were examined using an epifluorescence microscope (Eclipse E800; Nikon, Tokyo, Japan) equipped with an Olympus digital camera system (Olympus, Tokyo, Japan) and a digital acquisition system (DP 70; Olympus). Photographs were taken at each end of the section and in the center. Transmission electron microscopy specimens were examined using a 120-kV JEOL 1230 (JEOL, Welwyn Garden City, UK), equipped with a cooled high-resolution digital camera (Gatan, Abingdon Oxon, UK). Images were viewed and processed using Photoshop (Adobe Systems, Mountain View, CA, USA).

Statistical Analysis. Immunohistochemically labeled sections were used to statistically quantify survival of individual cell types. One central section per cultured specimen was analyzed for TUNEL, rhodopsin, and neuronal nuclei (NeuN) labeling, along with one section per in vivo reference eye. In vivo reference tissue and cultured specimens were processed in the same batch for each immunohistochemical labeling. Three photographs were obtained from the sections, and labeled cells (TUNEL and NeuN) and labeled cell rows in the outer nuclear layer (ONL); rhodopsin were counted at \( \times 40 \) magnification. Normal control tissue and cultured specimens were processed in the same batch for each immunohistochemical labeling. Data were analyzed using ANOVA with a Tukey post hoc test (GraphPad InStat; GraphPad Software, San Diego, CA, USA). Raw data from cell counts were used to generate mean values for each of the groups. Values of \( P < 0.05 \) were considered significant.

RESULTS

In Vivo Controls

The overall morphology and immunohistochemical characteristics of the normal adult porcine retina have been well...
described previously but will be summarized here. 4,5,12–18 Hematoxylin and eosin staining of in vivo controls revealed clearly defined nuclear and plexiform layers as previously described (Fig. 3A). Rhodopsin immunohistochemistry revealed strong labeling of outer segments at the outer border of the specimen, as well as weaker labeling of inner segments and photoreceptor cell bodies (Fig. 3B). Transducin labeling showed cone photoreceptor cell bodies in the outer part of the ONL, as well as labeled processes in the outer plexiform layer (OPL; Fig. 3C). Strong labeling was also present in cone bipolar cell perikarya located in the inner nuclear layer (INL), and in their processes vertically spanning the INL and inner plexiform layer (IPL). NeuN labeling showed a multitude of large cell bodies in the ganglion cell layer (GCL) corresponding to ganglion cells (Fig. 3D). Strong labeling of synaptic vesicles in both the OPL and IPL was seen using the synaptophysin antibody (Fig. 3E). Specimens labeled with glial fibrillary acidic protein (GFAP) displayed strong labeling of astrocytes and Müller cell endfeet at the innermost part of the specimens (Fig. 3F). Weak labeling of vertical Müller cell fibers was present throughout the retina, which is normal for this species. 18 Double labeling with GS and bFGF showed diffuse GS expression throughout the retinal layers, with bFGF labeling of Müller cell bodies in the INL and amacrine cells in the GCL, which is normal for this species (Fig. 3G). 18

Cultured Explants

Overview Morphology and Apoptosis. Hematoxylin and eosin staining of 5 and 10 days in vitro (DIV) IRS specimens revealed an overall retained laminar architecture with well-populated nuclear layers. The ILM appeared to be adherent to the culture membrane, although no growth into the membrane was observed. Inner and outer segments appeared present, albeit slightly disorganized (Figs. 4A, 4B). The standard cultured counterparts (CT), in contrast, contained multiple vacuoles at 5 DIV with a multitude of pyknotic cells present in all nuclear layers (Fig. 4C). No inner or outer segments could be identified, with cellular debris lining the outer border toward the culture membrane. At 10 DIV, the ONL appeared degenerated, with some remaining cells in the INL and GCL (Fig. 4D). Subretinal Müller cell growth was observed lining the outer border of the specimen.

The TUNEL labeling revealed apoptotic cells in the ONL and GCL in 5 DIV IRS explants (Fig. 4E). Similarly, at 10 DIV, labeled cells were observed in the ONL (Fig. 4F). The CT specimens displayed a multitude of labeled cells in all nuclear layers after 5 DIV (Fig. 4G). After 10 DIV, scattered labeled cells were present, mostly in the remaining outer retina (Fig. 4H). Statistical analysis revealed significantly fewer labeled cells in 5 DIV IRS specimens compared with their CT counterparts ($P < 0.001$; Fig. 5A). No significant difference was found between IRS specimens and CT specimens after 10 DIV.
Immunohistochemistry. Inner retinal support specimens labeled with rhodopsin revealed labeling of rod photoreceptors in the entire ONL with somewhat higher labeling intensity corresponding to the inner and outer segment area at both 5 and 10 DIV (Figs. 6A, 6B). The CT specimens revealed strong labeling of disorganized structures in the outer part of the specimen at 5 DIV (Fig. 6C). At 10 DIV, strong labeling of scattered structures and isolated cell bodies at the outer border was observed (Fig. 6D). Statistical analysis of rhodopsin-labeled cell rows revealed a significant preservation of the ONL of IRS specimens at both 5 and 10 DIV compared with their CT counterparts ($P < 0.001$). Transducin labeling of 5 DIV IRS specimens revealed cone photoreceptor cell bodies, inner and outer segments in the outer ONL, and cone pedicles in the OPL (Fig. 6E). Labeling was also found in a population of bipolar cells in the INL. A similar labeling pattern was observed in the 10 DIV specimens, but cones as well as bipolar cells were labeled.

### Table

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<th>Dilution</th>
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**FIGURE 5.** Statistical analysis of immunohistochemically labeled cells. (A) TUNEL-labeled cells per image. Significantly fewer labeled cells were found in 5 DIV IRS specimens compared with their CT counterparts ($P < 0.001$). No significant difference was found between the groups at 10 DIV. *Error bars: SEM.* (B) NeuN-labeled cells per image. Significantly more NeuN-labeled ganglion cells were found in IRS specimens at both 5 DIV ($P < 0.001$) and 10 DIV ($P < 0.01$) compared with their CT counterparts. *Error bars: SEM.* (C) Rhodopsin-labeled cell rows per image. A significant preservation of rhodopsin-labeled photoreceptor cell rows was found in IRS specimens at both time points compared with the CT cultured specimens ($P < 0.001$). *Error bars: SEM.*
were not as strictly organized (Fig. 6F). In contrast, 5 DIV CT explants almost completely lacked the normal organized morphology, displaying scattered labeled cells in the ONL (Fig. 6G). After 10 DIV only isolated labeled cone cells remained, whereas labeling of cone bipolar cells was still present (Fig. 6H).

NeuN labeling of IRS specimens revealed numerous large cell bodies in the GCL at both 5 and 10 DIV, with significantly more labeled cells than in their CT counterparts (P < 0.001 at 5 DIV and P < 0.01 at 10 DIV, respectively; Figs. 5C, 7A–D). Synaptophysin labeling of 5 and 10 DIV IRS specimens revealed strong labeling of synaptic vesicles in the OPL, with slightly weaker labeling of the IPL (Figs. 7E, 7F). In contrast to observations in normal in vivo controls, autofluorescence was present in the inner and outer segment area. The corresponding 5 DIV CT specimens displayed strong labeling of the IPL and OPL, with both layers appearing more disorganized compared with their IRS cultured counterparts (Fig. 7G). After 10 DIV, only isolated labeling was found in the highly disorganized OPL, whereas stronger labeling was present in the thin, vacuolized IPL (Fig. 7H).

Glia fibrillary acidic protein labeling of IRS specimens cultured for 5 and 10 DIV revealed strong labeling at the inner border, with weaker labeling of vertical fibers, comparable to that found in the in vivo controls (Figs. 8A, 8B). No gliotic remodeling was evident. Corresponding CT specimens revealed an upregulation of GFAP expression with high-intensity labeling present throughout the sections (Figs. 8C, 8D). Progressive gliotic remodeling was observed from 5 to 10 DIV, where the 10 DIV specimens displayed folding and subretinal fibrotic growth.

Glutamine synthetase/bFGF double labeling of IRS specimens revealed strong labeling of GS at the inner border of the specimen at 5 DIV, with slightly weaker labeling present throughout the section, comparable to the in vivo controls (Fig. 8E). At 10 DIV, strong GS labeling was present throughout the Müller cells (Fig. 8F). Basic FGF labeling of both 5 and 10 DIV specimens revealed a multitude of Müller cell nuclei in the INL, as well as scattered amacrine cells in the GCL. Labeling of CT counterparts revealed a downregulation of GS at both 5 and 10 DIV (Figs. 8G, 8H). An upregulation of bFGF in cells located mostly in the INL, but also at the innermost and outermost borders of the specimen, was found at 5 DIV. At 10 DIV, only scattered cells were labeled with bFGF.

**Ultrastructure.** Transmission electron microscopy revealed intact inner and outer segments in 5 DIV IRS specimens, with a well-delimitied OLM (Figs. 9A, 9B). Ribbon synapses with intact triads were present, as well as conventional synapses.
In 5 DIV IRS specimens, intact ribbon synapses as well as electrical synapses were present in the OPL (Figs. 9D, 9E). Both ribbon synapses and conventional synapses were found in the IPL (Figs. 9F, 9G). At the inner border, Müller cell endfeet were found with the intact ILM facing the culture membrane (Fig. 9H). No Müller cell sprouting into the membrane pores was found.

Five DIV CT specimens showed no intact inner or outer segments, although isolated phagocytosed outer segment debris was found in the IPL (Figs. 9E, 9G). At the inner border, Müller cell endfeet were found with the intact ILM facing the culture membrane (Fig. 9H). No Müller cell sprouting into the membrane pores was found.

In 5 DIV IRS specimens, intact ribbon synapses as well as electrical synapses were present in the OPL (Figs. 9D, 9E). Both ribbon synapses and conventional synapses were found in the IPL (Figs. 9E, 9G). At the inner border, Müller cell endfeet were found with the intact ILM facing the culture membrane (Fig. 9H). No Müller cell sprouting into the membrane pores was found.

Five DIV CT specimens showed no intact inner or outer segments, although isolated phagocytosed outer segment debris was found (Fig. 10A). Müller cell growth was present subretinally, with processes sprouting into the culture membrane pores. The ONL appeared disorganized with pyknotic cell bodies present (Fig. 10B). In standard cultured specimens, short, dislocated ribbon synapse structures could be found in the OPL (Fig. 10C), although the OPL appeared to consist mainly of cellular debris (Fig. 10D). Isolated short ribbon synapses and conventional synapses were present in the IPL (Figs. 10E, 10F). The IPL, similar to the OPL, contained cellular debris and electron-dense material (Fig. 10G). In the inner part of the explants, large vacuoles were found (Fig. 10H). The ILM appeared intact, although Müller cell growth was present.

Inner retinal support specimens cultured for 10 DIV displayed photoreceptor inner segments with occasional outer segment ciliae, as well as an intact OLM (Fig. 11A). No normal outer segments could be found. A multitude of photoreceptor nuclei were present, and their processes could be followed inward toward the OPL (Fig. 11B). Numerous intact ribbon synapses were present at the inner border of the ONL (Fig. 11C). Large cell bodies were found at the inner border apposed against the culture membrane (Fig. 11D). No Müller cell growth into the membrane was observed. In comparison, CT specimens displayed subretinal gliotic remodeling with Müller cell growth, as well as only a few shrunken unidentifiable nuclei present (Fig. 11E). Isolated, small photoreceptor nuclei were found in the ONL (Fig. 11F). Pyknotic nuclei were present in the INL, as well as scattered Müller cell nuclei (Fig. 11G). The inner retina appeared highly disorganized with dark, gliotic Müller cell processes lining the inner border (Fig. 11H).

**DISCUSSION**

**Summary**

In this study, we used the explant culture paradigm to explore the importance of IRS for tissue homeostasis in adult porcine retina. Based on our previous findings that biomimetically stretching retinal explants significantly extends adult tissue survival time in culture, coupled with the discovery of mechanoreceptors concentrated to the inner retina, we reinvented the traditional culture paradigm through the inversion of the explant, instead using the culture membrane to provide support for the inner retina. Culturing the retina with inner layer support has been reported previously by Wang et al. In this video article, Wang and colleagues described cultures of adult porcine retina explanted onto sterile filter paper to facilitate attachment, and found that retinal morphol-
ogy, investigated using GFAP, propidium iodide, and synaptic vesicle protein 2, was preserved for up to 7 DIV. In our study, in which we provide support to the inner retina by placing it directly onto the culture membrane and expand the morphological analysis, we can confirm that IRS is of vital importance for structural preservation and synaptic maintenance, as well as cell survival and metabolic health in adult retinal cultures.

In contrast to immature tissue, which has successfully been used in culture for many decades, the use of adult retinal tissue in vitro has been limited due to the fact that it cannot be kept for extended time periods in culture using standard methods. Due to significant neuronal degeneration and gliosis, the time restriction has been 3 to 4 DIV.5–11,15,17 Improved survival of adult rodent retinal tissue has previously been demonstrated on biochemically and structurally modified substrates, indicating that the local biomechanical environment is important for cell health.20 Using our novel approach, we have shown that these pathological changes can be significantly attenuated by providing mechanical support to the inner retina.

Survival of Inner and Outer Retinal Cells
The culture procedure entails axotomy of retinal ganglion cells, which both in vitro and in vivo rapidly induces apoptosis.5,14,15,17,21–23 Several approaches have been explored to minimize the axotomy effect on ganglion cells. One common strategy in vitro is to supplement the medium with neurotrophic factors.17,22,24–27 Despite this treatment, only limited numbers of ganglion cells have been shown to survive, which has at least in part been attributed to a reduced responsiveness to neurotrophins.17,28–31 However, even when the expression of neurotrophic receptors is enhanced through cAMP elevation and a cocktail of suitable support factors is delivered, only approximately 50% of cells can be saved 3 days after axotomy in vivo.30,31 We observed a preservation of approximately 40% of ganglion cells in our IRS cultured explants after 5 DIV without using neurotrophic treatment, indicating that inner retinal biomechanics may be important for tissue homeostasis.

Another striking pathological alteration in previous experiments involving adult retinal explants takes place in the outer retina.5,14–17 The deconstructive process leading to photoreceptor cell death is highly similar in the adult explant culture system and retinal detachment in vivo.52,53 After detachment, photoreceptors die mainly by apoptosis, which occurs quite rapidly.5,12,14–17,54,55 In vitro under standard conditions, the degenerative process also progresses quickly, with the majority of the photoreceptors appearing pyknotic after 3 to 4
FIGURE 9. Transmission electron microscopy of 5 DIV adult porcine retinal explants cultured with inner retinal support. Layer morphology and synapses. (A) IRS specimens display intact photoreceptor inner and outer segments (IS and OS). The photoreceptor cilia appear intact, and the IS mitochondria appear healthy. (B) IRS specimens show a well-delimited outer limiting membrane (OLM) with a multitude of photoreceptor nuclei (PR) present in the ONL. (C) Intact ribbon synapses were found in the outer plexiform layer (OPL; arrows) in IRS specimens. (D) An intact ribbon synapse in the OPL. (E) An electrical synapse in the OPL. (F) A ribbon synapse present in the inner plexiform layer (IPL). (G) An intact conventional synapse in the IPL. (H) IRS specimens display an intact ILM apposed against the culture membrane (MEM). Scale bars: 2 μm (A–C); 0.5 μm (H); 0.5 μm (D–G).
FIGURE 10. Transmission electron microscopy of 5 DIV standard cultured adult porcine retinal explants. Layer morphology and synapses. (A) CT specimens show subretinal Müller cell growth (arrow) along and into the pores of the culture membrane (MEM), with no identifiable photoreceptor IS evident. The occasional phagocytosed outer segment (OS) is present. (B) Isolated, shrunken PR nuclei were found in the outer part of the CT specimens. (C) A damaged, displaced ribbon synapse (arrow) in the OPL. (D) The OPL consists largely of degenerating and swollen processes and electron-dense debris. (E) A damaged ribbon synapse (arrow) present in the IPL. (F) An intact conventional synapse (arrow) in the IPL. (G) CT specimen in which the IPL appears disorganized with large electron-dense structures, presumed Müller cell processes, filled with cellular debris. (H) CT specimens also display an intact ILM; however, the innermost part of the specimen displays a Müller cell process but is otherwise void of cellular material. Scale bars: 2 μm (A, B, G); 0.5 μm (C-F); 1 μm (H).
FIGURE 11. Transmission electron microscopy of 10 DIV adult porcine retinal explants. Layer morphology. (A) IRS specimens display intact photoreceptor inner segments (IS) with outer segment ciliae (CL) and a continuous outer limiting membrane (OLM). The photoreceptor cilia appear intact, and the IS mitochondria appear healthy. (B) Photoreceptor nuclei (PR) and their processes appear healthy in the IRS specimens. (C) A multitude of intact ribbon synapses (arrows) were found at the inner border of the ONL in the IRS specimens. (D) Large cell bodies line the culture membrane in the IRS specimens. No Müller cell growth into the membrane could be found. (E) CT specimens display subretinal Müller cell growth and severe gliotic remodeling with only a few shrunken unidentifiable nuclei present. (F) Isolated, small photoreceptor nuclei were found in the ONL of CT specimens. (G) Müller cell nuclei, and unidentifiable apoptotic nuclei, were present in the INL of CT specimens. (H) The inner retina appeared disorganized with dark Müller cell processes lining the inner border. Scale bars: 2 μm (A, B); 1 μm (C, D); 5 μm (E-H).
involve a loss of tissue tensility and a collapse of the network. However, full-thickness retinal explants cultured using the conventional protocol but with an intact RPE still display photoreceptor degeneration and gliosis, even when placed in perfusion culture. Other indications that these factors may not be an acute concern are found in patients with central serous chorioretinopathy, where the detachment is often left to spontaneously resolve and in most cases results in only minor vision loss. The high oxygen consumption of the photoreceptors is well known, and the cell survival may therefore be increased by the increased air exposure of the outer layers by the inversion of the explants. However, the preservation of ganglion cells in the IRS specimens, as well as our previously published results on significantly preserved photoreceptor health using the standard culture procedure with the addition of biomimetic stretch, indicates that this is not the primary determinant for the increased photoreceptor survival. The significant preservation of the photoreceptors and ganglion cells in our IRS specimens therefore suggests that neither nutritive support from the choroid, oxygen supply, or physical interactions between outer segments and RPE are the primary determinants of cell survival in the isolated retinal sheet, but that the biomechanical milieu may in fact be pivotal.

**Mechanisms and Biomechanics**

In vivo, the retina resides in a highly mechanical environment where adhesive, tensile, and hydrostatic pressure forces come into play.

During development, the retinal sheet is passively stretched during expansion of the globe. In the mature eye, it is sandwiched between the posterior vitreous membrane and the RPE and maintained in a stretched state by the intraocular pressure (IOP). During the culture procedure, the IOP is lost when the eye is opened and the retina is dissected free from the vitreous and the RPE, leading to a loss of tissue tensility and a collapse of the network structure of the tissue (Figs. 12A–C).

The elastic retinal network consists of soft Müller glia interposed with stiffer neuronal cell bodies. The viscoelastic properties of the retina vary not only from center to periphery but also from inner to outer border. Our group has previously demonstrated the importance of preserving the normal retinal lateral tension for explant health in vitro. We found that free-floating cultured explants, which lacked all physical support, displayed rapid disintegration with the majority of cells appearing pyknotic after 2 DIV. Standard cultured explants, placed on the membrane over the pliable inner and outer segments that undergo rapid degeneration, left the explant network structure distorted, and as in the current experiment, quickly became gliotic with extensive neuronal cell death. In comparison, specimens cultured under standard conditions with the addition of biomimetic stretch displayed increased survival of both ganglion cells and photoreceptors, as well as an attenuation of the gliotic process for up to 10 days. In the present work we found strikingly similar results by providing the inner retina with mechanical support. Thus, IRS and lateral stretch appears to reproduce a common permissive biomechanical environment with stability of the retinal glioneuronal network as a key factor.

In the retina, the biomechanical scaffolding as well as the biochemical homeostatic upkeep is maintained by the Müller cells. These cells can alter their elasticity and stiffness through up- and downregulation of cytoskeletal intermediary filaments, the most common of which are GFAP and vimentin, thereby altering both the tissue-wide and cellular biomechanical milieu. Interestingly, mechanosensory Ca$^{2+}$ ion channels such as TRPV4 are known to be present on Müller cells, particularly on the comparatively stiff and intermediate filament-rich Müller cell endfeet at the inner retinal border. These channels have been shown to respond to changes in cell membrane tensility by Ca$^{2+}$ influx, which in turn can cause upregulation of intermediate filaments in Müller cells. Ca$^{2+}$ influx is a common response to mechanical stimuli seen in several types of mechanosensitive cells. The presence of TRPV4 channels in the Müller cell endfeet may thus provide the retina with a sensor of biomechanical changes. During injury and disease, the Müller cells become activated, losing their metabolic functions and structural integrity. These gliotic changes lead to a highly detrimental environment for the neuronal cells, thereby accelerating cell death. Because of this, gliosis has been considered the limiting factor behind long-term cultures of adult retinal explants using the standard method. In our standard cultured explants, Müller cell hypertrophy and subretinal growth were widespread, with a multitude of glial processes sprouting into the culture membrane. The IRS specimens, in contrast, showed no signs of Müller cell activation, with a preservation of GS as well as bFGF
expression. The lack of gliotic response thus may allow the Müller cells to preserve their normal regulatory and metabolic functions, which in turn promotes neuronal health.46,47

Conclusions

In this paper we have explored the effects of biomimetic structural stability on adult retinal explants. Specimens cultured with IRS displayed a significant preservation of photoreceptors and ganglion cells at 5 and 10 DIV, with a profound attenuation of the gliotic response. Control explants cultured using the standard culture method displayed extensive degenerative changes in a deconstructive process similar to that observed in retinal detachment. The relationship presented herein between biomechanical environment and retinal cell health enhances possibilities of using adult retinal tissue for culture-related research, and may increase our understanding of pathological events in biomechanically related conditions in vivo.

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