Cell Attachment to, and Contraction of, the Retina
In Vitro

David Allamby, David Foreman, Louise Carrington, David McLeod, and Mike Boulton

Purpose. To examine the behavior of fibroblasts and retinal pigment epithelial cells after attachment to the retinal surface in vitro to elucidate the pathobiology of the early stages of epiretinal membrane formation.

Methods. Human retinal pigment epithelial (HRPE) cells and bovine Tenon’s capsule fibroblasts (BTFs) were seeded onto the surface of bovine retinal explants maintained in organ culture. The changes induced in the underlying retina, including contraction, were assessed during a period of up to 10 days. Immunohistochemistry was used to assess proliferation of the seeded cells and to determine deposition of extracellular matrix.

Results. Explants of bovine neuroretina were maintained in organ culture, with good morphologic preservation of the inner limiting lamina and inner retinal layers, for 7 to 10 days. The HRPE cells and the BTFs attached to the retinal surface and exerted partial- and full-thickness retinal folding. Contraction commenced within 24 hours of attachment of the cells and continued for several days, with most of the contraction occurring within the next 48 to 72 hours. The HRPE cells and BTFs were found to be equally contractile. Deposition of cellular fibronectin (but not collagen type I) was demonstrated.

Conclusions. The contractile cellular membranes generated in this organ culture system exhibit many of the morphologic and functional features of epiretinal membranes found in the early stages of proliferative vitreoretinopathy. Invest Ophthalmol Vis Sci. 1997; 38:2064-2072.

Proliferative vitreoretinopathy (PVR) is characterized by the formation of contractile cellular membranes on the inner and outer surfaces of the retina and within the vitreous gel after rhegmatogenous retinal detachment. Contraction of these membranes, producing retinal distortion and secondary tractional retinal detachment, is causally or etiologically associated with failure of retinal reattachment surgery.1,2

There is general agreement that the cellular components of PVR membranes are derived from the retinal pigment epithelium (RPE),3-6 retinal glia (Müller cells, astrocytes),7-9 circulating macrophages,10 and fibroblast-like cells.6,11 The RPE cells are dispersed into the vitreous cavity in eyes with rhegmatogenous retinal detachment1 and during its surgical repair.12,13 They settle on the retinal surface, especially inferiorly, where they are thought to attach and proliferate. The presence of RPE cells in the vitreous and on the retina has been proposed as the initiating factor for the migration and accumulation of glial cells and macrophages and the subsequent development of complex fibrocellular membranes.3,4,14,15 The fibroblast-like cells may represent RPE cells that have undergone a change in phenotype owing to their new environment, reflecting their exposure to the vitreous and to growth factors accumulating through breakdown of the blood–retinal barrier.16-18 Once initiated, a complex fibrocellular cascade ensues with proliferation, migration, contraction, and extracellular matrix synthesis, leading to established PVR with a median interval of 2 months for primary and recurrent disease.19

Much of our knowledge of the cellular and extracellular components of epiretinal membranes (ERMs) in PVR has been derived from electron microscopic and immunohistologic analysis of membranes excised during vitrectomy and therefore representing established or end-stage disease. However, we possess very limited information regarding the early pathogenesis of these membranes, and especially the behavior of

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Contraction of the Retina In Vitro

RPE cells once dispersed within the vitreous gel and into the retrohyaloid space. Also, the role of the fibroblast-like cell remains unclear. We have attempted to overcome this deficit in knowledge by studying the behavior of RPE cells and fibroblasts that have been seeded onto the surface of the retina in organ culture. Our results demonstrate that RPE cells and fibroblasts attach to the retinal surface, producing contractile cellular ERMs that demonstrate many of the characteristics of ERMs observed in PVR.

MATERIALS AND METHODS

Retinal Organ Culture

Bovine eyes were obtained from a local abattoir, kept on ice, and used within 4 hours after slaughter. After the extraocular tissue had been removed, all further procedures were performed under aseptic conditions. The eyes were briefly immersed in 70% alcohol and washed in sterile phosphate-buffered saline (PBS). The globes were bisected, the anterior portion discarded, and, by engaging the cortical gel (which readily separates from the retinal surface), the remaining vitreous was removed intact. A small amount of PBS solution supplemented with 20 mM HEPES buffer was added to the eyecup to facilitate removal of the neuroretina, which was performed with a loop spatula. The retina was cut at the optic nerve, placed in PBS (supplemented as above), and mounted on Whatman chromatography filter paper or a cellulose ester membrane (Supor, Gelman Sciences, Northampton, UK). Explants approximately $5 \times 5$ mm$^2$ were cut from the equatorial or the posterior regions of mounted retina. This produced a preparation with an identical area of retina and support and allowed nontraumatic manipulation of the tissue. The preparations were transferred to specially designed organ culture dishes constructed from 35-mm and 50-mm Petri dishes; the preparations rested on a stainless steel mesh (Fig. 1) with up to 16 samples per dish. The level of the medium was maintained at the top of the mesh and in contact with the support membrane beneath the explant. The dishes were then incubated at 37°C in a humidified atmosphere of air and 5% CO$_2$.

Extensive experimentation was performed to determine the best medium for the optimal morphologic preservation of bovine retina in organ culture. It was demonstrated that this could be achieved with Trowel's T8 as a basal medium, with 10% fetal calf serum (Gibco, Paisley, UK), 20 mM HEPES buffer, 200 mg/l streptomycin, 200 mg/l kanamycin, 120 mg/l benzyl penicillin, and 70 mg/l glutamine (Sigma, UK). The medium (designated 10% T8) was changed on days 3, 6, and 9 and was adjusted daily to maintain the fluid at the correct level.

Explants were observed daily before removal at intervals up to 10 days in culture and were fixed in 10% neutral-buffered formalin for 24 hours, dehydrated, embedded in paraffin wax, serial sectioned (7 μm), and stained with hematoxylin–eosin, or were immersed in OCT (R Lamb, London, UK), frozen in isopentane–liquid nitrogen, sectioned (7 μm), fixed in acetone, and stained with toluidine blue or stored at −80°C for immunohistochemical analysis.

Evaluation of the Retinal Surface

Scanning and transmission electron microscopy were used to ascertain whether there were remnants of cortical gel attached to the inner limiting lamina after mechanical separation of the vitreous. Explants from 10 bovine eyes (10 animals) taken from the equator and posterior pole (from an area immediately temporal to the optic disc) were fixed in 2.5% glutaraldehyde, buffered to pH 7.4 with 0.1 M sodium cacodylate-HCl, with 0.75 mg/ml CaCl$_2$, snap frozen, freeze dried, gold-sputter coated and examined on a Cambridge 360 scanning electron microscope (Cambridge, Cambridge, UK). Further samples were fixed as before, postfixed in 2% osmium tetroxide, dehydrated, and embedded in SPURR resin (TABB, Aldermaston, UK) before transmission electron microscopy.

Cell Culture

Human retinal pigment epithelial (HRPE) cells were routinely isolated and cultured in Ham's F10 medium supplemented with 10% fetal calf serum, as previously described. Donor eyes were obtained with informed consent from responsible relatives, and the tenets of the Declaration of Helsinki were followed. This research is in accordance with institutional guidelines.
on human research. Donor age ranged from 44 to 76 years. Bovine Tenon's capsule fibroblasts (BTFs) were routinely isolated and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were used between passages four and nine (P4 to P9).

**Seeding, Cell Attachment, and Contraction**

Retinal explants and their underlying membrane support were cultured for 2 days before transfer to a 96-well dish. To determine the effect of seeding density on attachment and contraction, HRPE cells or BTFs were seeded into each attachment well at one of the following densities: 5, 10, 20, 40, and 80 × 10^3. All quoted cell numbers refer to the total cells added to the attachment well suspended in 150 μl 10% T8. The cells were allowed to attach to the whole explant surface for 2 hours. The explants were then transferred back to the organ culture dish. All experiments were performed on at least five explants and repeated with more than one cell line. The cultures were continued for up to 10 days.

Explants were assessed macroscopically (before fixation), and gross changes in retinal explants were recorded photographically. Contraction was assessed from Seescan image analysis (Seescan Imaging, Cambridge, UK) of photographs taken daily for the first 4 days by measuring the final area of the explant viewed from above, expressed as a percentage of the area of the support membrane.

In some instances melanin granules were fed to HRPE cells to observe their behavior macroscopically after seeding. Melanin granules were prepared from HRPE cells, as previously described; 20 μl of the melanin extract was added to each 75 cm² flask of HRPE cells and incubated overnight before the medium was replaced. Explants were assessed histologically, using wax and frozen sections prepared as described previously.

**Immunohistochemistry**

Immunostaining was performed using primary antibodies against the following: proliferating cell nuclear antigen (1:40), a marker of cell division; cellular fibronectin (1:400), a glycoprotein synthesized by many cells—the antibody used does not cross-react with plasma fibronectin; collagen type I (1:100); vimentin (1:40), a mesenchymal cell marker to demonstrate fibroblasts but also expressed by Müller cells; pancytokeratin (1:100), a cocktail of monoclonal antibodies to cytokeratins 1, 4, 5, 6, 8, 10, 13, 18, and 19 and a marker for RPE cells.

With the exception of collagen I, which was polyclonal and obtained from Southern Biotechnology (Euro-path, Bude, UK), all antibodies were monoclonal and were obtained from Sigma.

Sections were used from explants frozen at intervals from 6 hours to 6 days after cell seeding. Frozen sections were used for the majority of immunostaining procedures, except when enhanced morphology necessitated the use of wax-embedded material. The immunostaining procedure for wax sections has been described previously. Frozen sections, fixed in acetone for 5 minutes, were processed as for dewaxed and rehydrated wax sections. Goat antimonoclonal rabbit antigoat immunoglobulin G (IgG), conjugated to biotin (Sigma) were used as secondary antibodies at a dilution of 1:150. Antibody binding was demonstrated with a naphthol phosphate—fast red substrate mixture, which resulted in the formation of a red product. In some instances immunostained sections were counterstained with hematoxylin to enhance visualization.

Frozen sections of bovine retina and RPE cells were used as positive controls for anti-glial fibrillary acidic protein (GFAP) and antipancytokeratin, respectively. Frozen sections of bovine cornea, lens epithelium, and healing porcine dermal wounds (4 days after injury) were used as positive controls for anticollagen I, antivimentin, and cellular fibronectin, respectively. Negative controls included omission of the primary antibody and substitution of the primary antibody with an affinity-purified IgG at the same concentration as the primary antibody.

**RESULTS**

**Morphology of Retina in Organ Culture**

The organ culture technique allowed the successful maintenance of bovine retina with good morphologic preservation. Explants remained transparent during 7 to 10 days in culture, with thinning occurring in the later stages, evidenced by the increasing prominence of the blood vessels protruding through the contour of the retinal surface. In a small number of samples significant edema developed, which could be assessed macroscopically by the loss of transparency and by the creamy, yellow color of the explant, which correlated well with later histologic analysis showing intraretinal vacuolization. Explants with such marked edema after 2 days in organ culture were discarded.

Immediately after mechanical removal of the vitreous, examination with scanning electron microscopy confirmed a consistent, clean separation of the bovine gel from the retinal surface and an inner limiting lamina that remained intact at the retinal equator and the posterior pole (Fig. 2A). This result was confirmed by transmission electron microscopy, in which no residual cortical vitreous collagen was also demonstrated (Fig. 2B).

Thereafter, in light microscopy, it was observed that the internal limiting membrane was consistently attached to the retina and retained its structural integrity up to 7 days in culture. All samples showed some
Contraction of the Retina In Vitro

degree of retinal edema 24 hours after culture. Vacuoles were present primarily in the inner plexiform and ganglion cell layers. The histologic appearance of the retina was generally improved when explants were examined 48 hours after culture, with edema essentially confined to the outer plexiform layer at this stage. The retinas then retained good morphology until days 7 to 10 (Fig. 3), but thereafter became markedly thinned.

Behavior of Explants after Cell Seeding

Retinal explants seeded with cells exhibited varying degrees of contraction, ranging from mild inner-retina distortion to full-thickness folding. At the lowest seeding densities (10 x 10³), there was no or minimal contraction of the retina. At all other seeding densities (20 to 80 x 10³), contraction commenced within the first 24 hours after seeding and continued for up to 7 to 10 days, with most of the contraction occurring within the first 4 days. The explant margins were pulled centripetally and became separated from the underlying support membrane, which remained undistorted. As contraction proceeded, the retinal explants lost their transparency and became creamy yellow (Figs. 4A, 4B).

The use of melanin-fed HRPE cells allowed observation of their behavior on the retina. After initial attachment, HRPE were evenly distributed across the retinal surface; but during the next 12 hours dehiscences were seen within the seeded layer (Fig. 5A). By 24 hours, some cells were concentrated at irregular intervals into cellular clumps, with pigment-free areas between them (Fig. 5B). With lower cell numbers (10 to 20 x 10³), these clumps remained essentially unchanged for several days. With higher numbers (40 to 80 x 10³), gross explant contraction ensued.

Measurement of Contraction

With HRPE cells and BTFs, contraction commenced within 24 hours after attachment of the cells to the retinal surface and continued for several days, with the most significant effect occurring during the first 48 to 72 hours. The degree of contraction was dependent on the number of seeded cells. There was no statistically significant difference (P > 0.08) in contraction between HRPE cells and BTFs (Figs. 6A, 6B). Melanin-fed HRPE cells appeared to behave in a manner similar to that of routinely cultured cells. No difference was observed in the degree of contractility between melanin-fed cells and unpigmented cells (data not shown).

Histology of Cell-Seeded Explants

Histologic assessment of explants 4 hours after seeding using a range of cell densities (10 to 80 x 10³) demonstrated that HRPE and BTFs had attached to the inner retinal surface; the lower seeding densities produced isolated cells or an incomplete layer,
FIGURE 4. Macroscopic photographs of bovine retina mounted on Whatman filter paper. Explants have been seeded with 40,000 passage-4 human retinal pigment epithelial cells. (A) Immediately after cell seeding, showing attachment of cell to the retinal surface, and (B) 4 days after cell seeding, showing gross contraction of the retinal explant. Bar = 1 mm.

whereas higher cell numbers produced a monolayer over the entire explant (Fig. 7A). During the following 7 to 10 days in culture, the histologic appearances induced by the BTFs and HRPE cells were similar; there was no statistical difference in the rate of contraction ($P > 0.08$) for 4 days between HRPE and BTFs, at all cell densities.

Using low cell densities, the initial monolayer often contracted in a multifocal manner, with splits occurring in the sheet of cells after 24 to 48 hours, correlating with the macroscopic appearance seen in Figure 5A. The edges of the divided monolayer retracted, and cells became aggregated at the margins of the split; gross contraction of the explant normally did not occur. After 4 days, the cells remained as an attached monolayer or they formed cellular clumps, but they did not appear to be exerting significant traction in either case (Fig. 7B).

By contrast, the histologic examination after seeding with high cell numbers (for example, 40 to 80 X $10^3$) demonstrated significant traction on the retina after 24 hours (Fig. 7C). Most commonly, the edges of the explant were pulled inward and over the retinal surface around a mass of seeded cells that had become aggregated at the periphery of the explant (Fig. 7D). It was difficult to observe the inner limiting membrane at this stage. Variation from the vertical parallel arrangement of Müller's cells (as seen in the normal
Contraction of the Retina In Vitro

FIGURE 7. Photomicrographs showing histologic features of bovine retinal explants after seeding with 40,000 passage 4 human retinal pigment epithelial cells: (A) 4 hours after seeding to produce uniform cell attachment and (B) 24 hours after seeding, forming a cellular clump, as seen in Figure 5B. (C) Inner retinal traction is evident 24 hours after seeding, and (D) full-thickness folding is seen after 48 hours. (E) Gross retinal traction is induced by a mass of human retinal pigment epithelial cells. (F) After 4 days, cells are buried within a grossly contracted retinal explant. Bar = 50 μm (A); 200 μm (B,C); 100 μm (D,E,F).

The contractile process continued during the next 48 to 72 hours, producing retinal distortion by a mass of cells (Fig. 7E), with the explant edges pulled further over the retinal surface, sometimes causing the HRPE cells or the BTFs to become buried within the folded explant (Fig. 7F).

Immunohistochemistry

The antipancytokeratin antibody produced staining of the seeded HRPE cells at all time points (Figs. 8A, 8B). In many of these cells, anti-vimentin demonstrated the presence of an additional network of intermediate filaments (Fig. 8C). The BTFs stained positively for vimentin alone.

Cellular fibronectin was detected, associated with foci of HRPE cells and BTFs 3 to 6 days after seeding, but not at earlier time points (Fig. 8D and Fig. 8F, control). Cellular fibronectin was also found within the walls of intraretinal vessels. No staining for collagen type I was demonstrated at any time point.

Found only in the nerve fiber layer of freshly isolated retina, GFAP was detectable throughout the retina from the inner limiting lamina to the outer plexiform layer after 3 days in culture, when associated with foci of contractile cells (Fig. 8E). During organ culture of unseeded explants, GFAP became detectable to a similar degree after approximately 7 days in culture. No GFAP was detected within the foci of seeded cells at any time point.

DISCUSSION

Several investigators have attributed a pivotal role for the RPE cell in the initiation of the cellular cascade, leading to contractile epiretinal membrane formation in PVR, a primary event of which is cell attachment to the retinal surface.\(^{3,4,15}\) We have very limited knowledge of the cellular processes during these early stages, because much of our knowledge is derived from analysis of ERMs excised at PVR surgery (which
usually represent established or end-stage disease). By seeding cell types found in PVR membranes onto isolated retina maintained in organ culture, we have successfully induced the formation of contractile cellular epiretinal membranes representing the early pathobiology of ERM s. In particular, we have shown attachment of RPE cells and fibroblasts to the inner limiting membrane of the retina, deposition of fibronectin, and contraction of the underlying retina. Within our experimental conditions, HRPE cells and BTFs attached to the retina as a monolayer that, at higher cell densities, proceeded to form clumps comparable to the appearance in grade-A PVR. In grade-A PVR, proliferation within pigmented clumps of cells is considered to represent the first stage of the proliferative process and the noncontractile phase of the disease. However, although we observed cell attachment to the retina, clumping, and retinal contrac-

FIGURE 8. Photomicrographs showing the immunohistologic staining of bovine retina and seeded cells for cytokeratin, vimentin, and fibronectin. (A) Human retinal pigment epithelial cells on the surface of the retina are positive for pan-cytokeratin. (B) An embedded mass of human retinal pigment epithelial cells is positive for pan-cytokeratin. (C) A mass of human retinal pigment epithelial cells within a contracted retina. The seeded cells and the inner nuclear layer of the retina are positive for vimentin. (D) Neighboring section to that seen in (B) is positive for cellular fibronectin. (E) Area within the retina around a focus of human retinal pigment epithelial cells is positive for glial fibrillary acidic protein. (F) Control section. Bar = 50 μm.
tion, we did not observe significant proliferation by immunostaining for proliferating cell nuclear antigen during the course of the experiment.

Extracellular matrix proteins (fibronectin and collagen type I) are believed to play a prominent role in the onset, course, and severity of PVR.\(^{2,24}\) Fibronectin, a major matrix component of wound healing, which through cell adhesion receptors results in cell attachment to fibrillar collagens, has been confirmed in excised epiretinal membranes by immunohistochemistry\(^{25,26}\) and by hybridization in situ.\(^{27}\) We have demonstrated cellular fibronectin colocalizing with cellular foci of RPE cells on and within the retina after 3 days in culture, suggesting that it may play a role in the ensuing retinal contraction. Collagen type I appeared to be absent during the experiment. This was not surprising, because in PVR, collagen type I is a prominent component of excised membranes, with this tissue usually representing disease of several months' duration.\(^{28}\)

The question of the mechanism of cellular contraction in PVR has not yet been resolved, with two theories dominating current thinking: Isolated cells produce contractile forces as a result of locomotion,\(^{29}\) and a syncytium of cells produces contractile forces by contracting in a coordinated manner.\(^{30}\) Our results support the latter theory, with cell contraction of organ-cultured retina dependent on seeding density. Contraction was minimal at low seeding density in the presence of isolated cells and was greatest at high seeding density in which clumps of cells were evident. Investigation into the mechanism of cellular contraction in PVR has essentially been limited to investigating contraction of fibrillar substrates (collagen gels)\(^{31-35}\) and vitreous explants.\(^{34}\) Glaser et al have proposed a hand-over-hand theory for RPE cells involving the extension and retraction of lamellipodia attached to individual collagen fibrils.\(^{30}\) However, the mechanism of contraction of the retina by cells within ERMs may be different from that within the vitreous and other fibrillar substrates, in that we repeatedly observed clumps of cells exerting traction to produce full-thickness retinal folding. Furthermore, cellular contraction of the retina occurred, although no fibrillar collagen was present on the explant surface. Whether cell-induced contraction of the organ-cultured retina was exerted via the inner limiting membrane or some other substrate is difficult to ascertain. Certainly traction involved the Müller cells, which is logical because they are structural cells extending almost full thickness within the retina.

No statistically significant difference was found between the contractility of HRPE and BTFs on the retina in vitro, suggesting that HRPE cells may play a role in the contractile process in PVR. This finding is in contrast to results in previous studies that showed the contraction of collagen gels by bovine RPE cells was significantly less than that by bovine fibroblasts.\(^{31,36}\) However, these results are difficult to compare, because of the differences in the substrate used and the experimental technique employed. Although collagen gels have been deemed to mimic the contractile mechanisms associated with PVR, the gel routinely uses collagen type I, which is not present in the vitreous; and the gel does not reflect the retinal substrate.

The situation is further complicated by the observations that RPE-induced contraction in vitro is dependent on passage number: high-passage cells (higher than P8) show greater contraction than is observed in low-passage cells (lower than P5);\(^{16}\) and contraction by RPE cells may be dependent on the presence of intracellular pigment granules;\(^{36}\) no difference was observed between the degree of contractility of pigmented and nonpigmented RPE cells in this study.

In conclusion, we have demonstrated cell attachment to, and contraction of, the retina in vitro, with many similarities to effects seen in PVR. The reproducibility of histologic features and contractile response make this an ideal system with which to elucidate further the early cellular events of PVR—the effect of growth factors, the role of integrins, the effect of mixed populations of cells, and screening for inhibitors of contraction.

**Key Words**
contraction, extracellular matrix, organ culture, proliferative vitreoretinopathy, retinal pigment epithelium

**References**

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