INDUCTION OF PROLIFERATIVE VITREORETINOPATHY BY HUMAN RETINAL PIGMENT EPITHELIAL CELLS

by

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ABSTRACT

Proliferative vitreoretinopathy (PVR) is characterized by the formation of fibrocellular membranes in the vitreous and on the surfaces of the retina. These membranes exert tractional forces on the retina, causing retinal tears and/or detachments. The purpose of this study is to develop a reproducible animal model that closely mimics PVR in the clinical setting.

The retinal pigment epithelial (RPE) cell is the major cell type found in PVR membranes. We cultured RPE cells from a human epiretinal membrane (ERM) obtained at surgery from a patient with PVR, and injected them into the right eye of 24 albino rabbits. The eyes were examined by indirect ophthalmoscopy over 4 weeks. By day 7, all but one of the 24 eyes developed proliferative vitreoretinopathy, with 8 progressing to localized tractional retinal detachment (TRD). By day 21, 17 out of the 24 eyes had developed localized TRD. One eye went on to develop an extensive TRD by day 28. Immunostaining showed that mostly RPE cells, but also myofibroblasts, glial cells, and collagen were present in the newly formed rabbit PVR membranes. Thus, human RPE cells cultured from a human PVR membrane appear to be capable of inducing PVR in rabbits. The resultant epiretinal membranes are similar to those formed in human PVR, and consist mainly of RPE cells.
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Chapter I

GENERAL INTRODUCTION

Proliferative vitreoretinopathy (PVR) is the most common cause for failure of retinal detachment surgery and is characterized by the formation of epiretinal membranes (ERMs). These membranes contract, causing retinal tears and detachments. Over the last thirty years, a considerable amount of research time has been spent looking for an experimental model that closely resembles PVR (Weller et al., 1990). An accurate model of PVR could ultimately lead to prevention of this complication, for which there is no adequate treatment or prophylaxis. The incidence of PVR remains at approximately 10% of retinal detachments that come to surgery (Retina Society Terminology Committee, 1983). The incidence rises with repeated surgical trauma (19%) (Bonnet, 1984; Rachal & Burton, 1979), long-standing retinal detachment (32%) (Bonnet, 1984), and with vitreal hemorrhage and/or large retinal holes (24%) (Bonnet, 1984).

The most widely used models of PVR rely on injection of cells into the vitreous of experimental animals. Cells found in PVR membranes, such as retinal pigment epithelial (RPE) cells, fibroblasts, macrophages, and glial cells, have been injected (Mendelcorn et al., 1975; Fastenberg et al., 1982; Peters et al., 1986; Hui et al., 1987). The cells are usually cultured from healthy, homologous eyes or tissue.

Recent studies have shown that RPE cells isolated from human PVR membranes have different gene expression, and morphological characteristics, compared with healthy human RPE cells in vitro (Abe et al., 1996; Hiscott PS et al., 1983). It is believed that
growth factors and cytokines in the vitreous are responsible for this transdifferentiation (Vidaurri-Leal J et al., 1984; Casaroli-Marano RP et al., 1999; Kirchhof B & Sorgente N, 1989). For example, interleukin-6, a cytokine believed to play an important role in the pathogenesis of PVR is produced in higher levels by RPE cells from human PVR membranes. Therefore, using RPE cells from human PVR membranes may produce a more accurate model of human PVR.

This study was performed to determine whether human RPE cells derived from a single ERM, from a patient with a retinal detachment and PVR, are capable of inducing the same disease in the rabbit eye. I sought to examine whether the induced ERMs had cellular components similar to those of human PVR membranes.

ANATOMY OF THE EYE

Basic Anatomy of the Globe

The eye consists of three concentric layers, surrounding three compartments. (Fig. 1) The outermost layer (tunica fibrosa) consists of the opaque sclera and the transparent cornea; the corneoscleral junction is the limbus. The middle layer (tunica vasculosa) -- also called the vascular layer or the uveal tract -- consists of the choroid (posterior), ciliary body, and iris (anterior). The inner layer (tunica interna) consists of nervous tissue called the retina. The retina receives its oxygen and nutrients from the choroid (Junqueira et al., 1998; Burkitt et al., 1993).

The three compartments of the eye are the (1) anterior chamber, (2) the posterior chamber, and (3) the vitreous cavity. The cornea, iris and lens bind the anterior chamber,
whereas the iris, ciliary processes, zonular attachments, and lens bind the posterior chamber. Flowing throughout the anterior and posterior chambers is a protein-poor fluid called aqueous humor. The vitreous cavity is largest of the three cavities. It is bound anteriorly by the lens, zonular attachments, and ciliary bodies and posteriorly by the retina and optic nerve head. The vitreous cavity contains vitreous humor, a transparent gel composed of a random network of collagen (Type II) fibrils suspended in a solution composed of water (98.5-99.7%), salts, protein, and hyaluronic acid (Junqueira et al., 1998; Burkitt et al., 1993).

**Basic Anatomy of the Retina**

The retina consists of two layers: (1) an outer layer, the retinal pigment epithelium, and (2) an inner layer, the sensory retina. Both layers develop as a result of invagination of the primitive optic cup, with the inner (sensory retina) layer facing the vitreous, and the outer RPE layer facing the choroid layer (Junqueira et al., 1998; Burkitt et al., 1993) (Figure 2).

**Retinal Pigment Epithelium (RPE)**

The retinal pigment epithelium is a single layer of cells involved in activities essential to retinal homeostasis and visual function. RPE cells metabolize vitamin A for the visual (or retinol) cycle, phagocytose spent photoreceptor outer segment discs, absorb light energy to improve image resolution, synthesize glycoaminoglycans, transport metabolites, and constitute an important part of the blood-retinal barrier (Bok, 1993).
Figure 1 Anatomy of the Human Eye.
(From Burkitt HG, In: Wheater's Functional Histology, 1993)
The RPE monolayer extends from the optic nerve margin of the posterior retina, to the ora serrata of the anterior retina. Anterior to the ora serrata, the RPE continues as the pigmented ciliary epithelium. Beneath the RPE is Bruch’s membrane, of which the RPE basement membrane is a component. Posterior to Bruch’s membrane is the capillaries of the choroid (choriocapillaries) where the RPE receives its nutrients (Bok, 1993; Grierson et al., 1994).

The RPE develops from neuroectoderm. RPE precursors do not differentiate into neural tissue because its embryonic tissue develops along the outside wall of the developing optic cup. By the end of the second year of life in humans, RPE cell replication is complete and the numbers of RPE cells remain static at around 5 million cells, even though the cells retain the capacity to divide. The mechanism of replicative suppression remains obscure, however, tight junctions (zonula occludens) and intermediate junctions (zonula adherens) that form between neighboring cells are thought to play key roles. As the developing eye enlarges, the 5 million RPE cells enlarge in size and cover an expanding area from about 8 cm$^2$ at age 2 to over 11 cm$^2$ at age 45 (Bok, 1993; Grierson et al., 1994).

If the eye is subject to trauma, such as a retinal detachment or tear, RPE cells can re-enter the mitotic cycle. Characteristically, the RPE cells undergo migration and massive proliferation on both sides of the retina and within the vitreous, and do not become self-limiting until advanced changes take place. Several pathological conditions can then develop, such as PVR (Hiscott & Sheridan, 1998).
The apical surface of RPE contains numerous microvilli that extend into the subretinal “space” (i.e. between the sensory retina and RPE) and form sheaths around the photoreceptor (rod and cone) outersegments. The RPE and photoreceptor outersegments are not firmly attached but are kept in close contact (while minimizing the subretinal space) through the action of the RPE “pump.” The “pump” mechanism effectively removes fluid from subretinal space thereby exerting a negative pressure between the two tissue layers. The adhesion can be easily overcome resulting in retina tears or detachments (Bok, 1993; Grierson et al., 1994; Hiscott & Sheridan, 1998).

The lateral surfaces of RPE cells are bound by terminal bar complexes composed of an apical zonula occludens and a basilar zonula adherens. These junctions encircle the apical portion of the cell, and serve to join neighboring RPE cells. The tight seal that arises between neighboring cells forms the inner blood retinal barrier. Together with the non-fenestrated retinal blood vessels (choriocapillaries) of the outer blood retinal barrier, they effectively prevent the exchange of macromolecules between the photoreceptors and the blood flowing through the capillaries (Bok, 1993; Grierson et al., 1994).

**Sensory Retina**

The sensory retina is responsible for receiving light stimuli, converting it into a neural impulse, and transmitting the signal to the optic nerve for relay to the brain. It consists of several layers: three layers of nuclei and three layers of nerve fibers. (Fig. 2) The three nuclear layers are (1) the outer nuclear layer, which contains the nuclei of photoreceptors (rods and cones); (2) the inner nuclear layer, which contains the nuclei of
bipolar, horizontal, amacrine, and Müller glial cells; and (3) the ganglion cell layer, which contains the nuclei of ganglion cells. The three nerve layers are (1) the outer plexiform layer, where the rods and cones synapse with the dendrites of bipolar and horizontal cells; (2) the inner plexiform layer, where there is synapsis between bipolar cells, amacrine cells, and ganglion cells; and (3) the nerve fiber layer, composed of axons of ganglion cells (Junqueira et al., 1998; Burkitt et al., 1993).
Figure 2. Histology of the Retina. The retina is divided into 11 distinct layers. The outermost layer (1) consists of the pigmented epithelial cells forming a single layer resting on Bruch’s membrane, which separates them from the choroid. Rod and cone outer (2) and inner (3) segment processes of the photoreceptor cells comprise the next layer with a thin structure known as the outer limiting membrane (4) that separates them from the receptor nuclear layer (cell bodies of the rods and cones (5)). Deep to the cell bodies is the outer plexiform layer (6), which contains synaptic connections between the photoreceptor cells and the bipolar neurons, whose cell bodies lie in the inner nuclear layer (bipolar cell layer) (7). In the inner plexiform layer (8), the bipolar neurons make synaptic connections with the ganglion cells of the ganglion cell layer (9). Deep to this layer is the optic nerve fiber layer, which contains afferent fibers that form the optic nerve head and eventually become the optic nerve. The deepest layer is the inner limiting membrane (11), which separates the innermost layer of the retina from the vitreous. (Adapted from Burkitt HG, In: Wheater’s Functional Histology, 1993; H & E x 640)
11 Inner limiting membrane
10 Nerve fibre layer
9 Ganglion cell layer
8 Inner plexiform layer
7 Inner nuclear layer
6 Outer plexiform layer
5 Receptor nuclear layer
4 Outer limiting membrane
3 Inner segment
2 Outer segment
1 Pigment epithelium
Choroid layer

Figure 2
PROLIFERATIVE VITREORETINOPATHY: PATHOGENESIS

Proliferative vitreoretinopathy (PVR) is a serious disorder that commonly develops after a rhegmatogenous retinal detachment or break, especially in cases where giant retinal tears and/or perforating trauma to the posterior segment is present. When PVR takes place before retinal reattachment surgery, the disease process is termed “primary PVR.” This disease is termed “post-operative PVR” when the process occurs after retinal reattachment surgery. Postoperative PVR is the primary reason why retinal reattachment surgery fails.

At the time of a retinal break, retinal pigment epithelial (RPE) cells, from the pigment epithelial layer, gain access to the vitreous cavity either by active migration or by passive movement driven by intraocular fluid currents (Pastor, 1998; Weller et al., 1990). Once in the vitreous, the cells settle at the inferior circumference, and appear clinically as “tobacco dust” (Hamilton & Taylor, 1972) reflecting the presence of pigment in RPE cells. Components within the vitreous, and serum components released into the vitreous during the time of retinal detachment (i.e. breakdown of the blood-retinal barrier), stimulate the cells to proliferate, dedifferentiate, and secrete additional growth factors. Chemotactic factors are also released, which stimulate other cell types to migrate from the retina into the vitreous. Clinical signs of active cell proliferation in the early stages of PVR are gray cell clusters (cell clumping), increased vitreous haze, and the formation of proteinaceous rays composed of leaked serum proteins. Eventually, fibrocellular membranes develop along the anterior surface of the retina (epiretinal membranes or ERMs) and/or along collagen strands within the vitreous (intravitreous membranes).
Subretinal membranes can also develop, but only contribute to approximately 13% of all PVR cases (Lewis et al). The RPE cell is the predominant cell type present in PVR membranes (Machemer R et al., 1978b; Shirakawa H et al., 1987; Van Horn DL et al., 1977; Newsome et al., 1981).

As the disease process continues, the cells within the membranes “contract,” causing tractional forces on the retina. These forces result in irregular retinal folding (cellophane) and/or stretching of retinal blood vessels. Once the normal adhesion of the retina to the RPE layer is overcome, the retina detaches.

**Incidence and Time Course**

In spite of continuing refinements in microsurgical instruments and vitreoretinal surgical techniques, approximately 10% of patients with a rhegmatogenous retinal detachment or break develop PVR (Retina Society Terminology Committee, 1983). Moreover, the incidence rises with repeated surgical trauma (19%) (Bonnet, 1984; Rachal & Burton, 1979), long-standing retinal detachment (32%) (Bonnet, 1984), and with vitreal hemorrhage and/or large retinal holes (24%) (Bonnet, 1984). Eyes with giant equatorial tears demonstrate an even higher incidence of developing PVR (Scott, 1976).

The time course for the development of a tractional PVR membrane is approximately 2 months after the initial retinal break (Mietz & Heimann, 1995). PVR cannot develop immediately since the cells involved need time for migration, transdifferentiation, proliferation, and contraction. Since this process commonly
develops after retinal reattachment surgery, post-operative PVR is the principle cause for failure of surgery.

Once detached, however, it is important that patients with retinal detachment seek immediate attention. The visual outcomes after retinal reattachment surgery demonstrate an inverse relationship with prolonged retinal detachment.

CLASSIFICATION OF PVR

Clinical Classification of PVR

Machemer and colleagues (1989) developed a revised classification to the scheme introduced by the Retina Society Terminology Committee (1983). The revised classification was proposed with a more detailed description of PVR pathogenesis to reflect the major advances in understanding the disease.

The new classification consists of three grades of severity: A, B and C. Grade C is further classified into anterior PVR and posterior PVR (i.e. whether the proliferative membrane develops anterior or posterior to the equator of the eye), the extent of the proliferation in each area expressed by the number of clock hours of retina involved (1-12), and by five different contraction types (Type 1-5). Tables 1A and 1B outline the revised classification in detail.

Experimental Classification of PVR

To evaluate the severity of PVR in animal models, the scheme described by Fastenberg is commonly used (1982). This method is simpler and more straightforward.
### TABLE IA**

Proliferative Vitreoretinopathy Described by Grade

<table>
<thead>
<tr>
<th>GRADE</th>
<th>FEATURES</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Vitreous haze; vitreous pigment clumps; pigment clusters on inferior retina</td>
</tr>
<tr>
<td>B</td>
<td>Wrinkling of inner retinal surface; retinal stiffness; vessel tortuosity; rolled and irregular edge of retinal break; decreased mobility of vitreous</td>
</tr>
<tr>
<td>C P 1-12</td>
<td>Posterior to equator: focal, diffuse, or circumferential full-thickness folds*; subretinal strands</td>
</tr>
<tr>
<td>C A 1-12</td>
<td>Posterior to equator: focal, diffuse, or circumferential full-thickness folds*; subretinal strands; anterior displacement*; condensed vitreous with strands</td>
</tr>
</tbody>
</table>

*Expressed in the number of clock hours involved  
**Adapted from Machemer et al., 1991

### TABLE IB**

Grade C PVR Described by Contraction Type

<table>
<thead>
<tr>
<th>Type</th>
<th>Location (in relation to equator)</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Focal</td>
<td>Posterior</td>
<td>Starfold posterior to vitreous base</td>
</tr>
<tr>
<td>Diffuse</td>
<td>Posterior</td>
<td>Confluent starfolds posterior to vitreous base. Optic disc may not be visible</td>
</tr>
<tr>
<td>Subretinal</td>
<td>Posterior/Anterior</td>
<td>Proliferations under the retina: Annular strand near disc; linear strands; motheaten-appearing sheets</td>
</tr>
<tr>
<td>Circumferential</td>
<td>Anterior</td>
<td>Contraction along posterior edge of vitreous base with central displacement of the retina; peripheral retina stretched; posterior retina in radial folds</td>
</tr>
<tr>
<td>Anterior displacement</td>
<td>Anterior</td>
<td>Vitreous base pulled anteriorly by proliferative tissue; peripheral retinal trough; ciliary processes may be stretched, may be covered by membrane; iris may be retracted</td>
</tr>
</tbody>
</table>

**Adapted from Machemer et al., 1991
(for animal model purposes) than Machemer's clinical classification. Table 2 outlines the five stages according to Fastenberg.

### TABLE 2*

<table>
<thead>
<tr>
<th>Stage</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Intravitreal membrane</td>
</tr>
<tr>
<td>2</td>
<td>Focal Traction; localized vascular changes; hyperemia; engorgement; dilation; blood vessel elevation</td>
</tr>
<tr>
<td>3</td>
<td>Localized detachment of medullary ray</td>
</tr>
<tr>
<td>4</td>
<td>Extensive retinal detachment; total medullary detachment; peripapillary retinal detachment</td>
</tr>
<tr>
<td>5</td>
<td>Total retinal detachment; retinal folds and holes</td>
</tr>
</tbody>
</table>

* Adapted from Fastenberg et al., 1982

### CLINICAL RISK FACTORS

This section will describe the clinical risk factors for “primary” PVR, defined as PVR that develops preoperatively, and “postoperative” PVR, defined as PVR that develops as a complication of retinal reattachment surgery.

#### Primary PVR

Risk factors for primary PVR are associated with intravitreal dispersion of RPE cells and breakdown of the blood ocular barrier. The risk factors include giant retinal tears, long duration of retinal detachment, and vitreous hemorrhages (Yoshino et al., 1989; Nagasaki et al., 1998).

In cases with giant retinal tears and long standing retinal detachment, large areas of RPE cells are exposed to vitreous, allowing individual cells ample opportunity to migrate
or "fall" into the vitreous gel. Larger numbers of RPE cells directly correlate with severity of PVR. Vitreous hemorrhages release serum components, such as fibronectin and PDGF into the vitreous cavity. These growth factors enhance RPE cell proliferation, migration, and contraction. Almost all risk factors can be minimized by early retinal detachment surgery.

Postoperative PVR

The risk factors for postoperative PVR are related to the procedures employed during surgery. Almost all are associated with intravitreal dispersion of RPE cells and breakdown of the blood-ocular barrier, as seen for primary PVR. These include the preoperative presence of PVR grades A and B, uveitis, vitreous hemorrhage intraoperatively or postoperatively, excessive cryotherapy, diathermy or photocoagulation, repeated surgical procedures, and the use of air tamponade (Bonnet et al., 1989; Cowley et al., 1989; Girard et al., 1994; Nagasaki H et al., 1998).

PATHOBIOLOGY

Types of Cells

Contractile fibrocellular (PVR) membranes are composed of RPE cells, glial cells, macrophages, fibroblasts, and fibroblast-like cells (Charteris, 1995; Clarkson et al., 1977; Constable et al., 1975; Green et al., 1979; Hiscott et al., 1984, 1985; Kampik et al., 1981; Laqua & Machemer, 1975a,b); Machemer & Laqua, 1975; Machemer et al., 1978). Among them, RPE cells are the predominant cell type, and the principle cell involved in
the initiation of PVR. (Machemer R et al., 1978b; Shirakawa H et al., 1987; Van Horn DL et al., 1977; Newsome et al., 1981)

Retinal pigment epithelium

The RPE is a single layer of ectoderm-derived pigmented cells forming the outermost layer of the retina. In the normal eye, RPE cells are virtually stationary and exhibit a low proliferation rate. RPE cells serve several functions (as mentioned above): they form the inner blood-retinal barrier (via its tight junctions), absorb light, phagocytose rod and cone outer segment fragments, and sub serve vision by being involved in the uptake, processing, transport, and release of vitamin A (retinol) (Bok, 1993).

Several studies have shown that RPE cells play a major role in the pathogenesis of PVR and are usually the predominant cell type within epiretinal membranes (ERMs) removed from eyes with PVR (Machemer, 1975; Shirakawa et al., 1987; Newsome, 1981). Studies have also established that RPE cells possess the characteristics necessary to produce PVR: they are able to migrate (Compochiaro et al, 1984; Johnson & Foulds, 1977), proliferate (Machemer & Laqua, 1975), transdifferentiate into epitheloid-shaped and fibroblast/fusiform-shaped cells (Mandelcorn et al, 1975; Muller-Jensen et al, 1975; Casaroli-Marano et al. 1999), “contract” collagen (Burnside, 1976; Forrester et al, 1986; Raymond & Thompson, 1990; Wong et al, 1999), and synthesize a collagenous extracellular matrix (Kigasawa et al., 1998).
Glia

Glia make their way from the retina to the vitreous through breaks in the internal limiting membrane (Laqua & Machemer, 1975). Although glial cells play important roles in the recruitment of proliferation of other cell types in PVR (Burke & Foster, 1985), *in vivo* and *in vitro* studies have shown that they proliferate less rapidly than RPE and fibroblasts, and contribute less to the extracellular matrix of intravitreal membranes (Peters et al., 1986). Also, glial cells have been shown to contract, but do so in a lesser degree than RPE and fibroblasts (Forrester et al., 1986; Hui et al., 1987). It has therefore been suggested that glial cells mainly act as scaffolds and/or anchor points for other cells (Cleary & Ryan 1981), and that epiretinal membranes composed chiefly of glial cells would not exert severe tractional forces on the retina (Hiscott et al., 1984). The characteristic intermediate filament found in glial cells is glial fibillary acidic protein (GFAP).

Macrophages

The inflammatory response at the time of a retinal break is modulated mainly by the influx of macrophages from the ciliary body, optic nerve head, and the posterior choroid (Burke & Foster, 1982; Johnson & Foulds, 1977; Cleary and Ryan, 1979). The majority of macrophages are circulating monocytes rather than tissue macrophages. Although macrophages and have been localized to almost all PVR membranes (Kampik et al., 1981), their role was once thought to be limited to phagocytosis (Newsome et al., 1981), since macrophages do not proliferate, secrete extracellular matrix, transform into...
fibroblastic-like cells, or have the ability to contract (Ross et al., 1970; Forrester et al., 1986). However, macrophages are now believed to be important contributors to PVR pathogenesis.

Studies have shown that macrophages stimulate RPE migration and proliferation in vitro and in vivo (Kirchhof et al., 1989). The exact mechanisms remain unclear, but secreted growth factors and cytokines, such as platelet-derived growth factor (PDGF), fibroblast growth factor, TFG-β, IL-1, and IL-6 are believed to play important roles. Furthermore, recent studies have demonstrated that the contributions of macrophages principally occur during the initial stages of the disease process (Nathan et al., 1980; Wahl & Wahl, 1985; Esser et al., 1994).

**Fibroblasts and fibroblast-like cells**

Fibroblasts represent only a small population of cells in PVR membranes (Machemer et al, 1978). These cells most likely arise from the adventitia of the retinal vessels or from the optic nerve head through breaks in the internal limiting membrane (Newsome et al., 1981).

Several histological and electron-microscopic studies have identified fibroblast-like cells in ERMs. Due to their altered phenotypes, these cells were believed to be either fibroblasts or transformed (de-differentiated) RPE cells (Machemer et al., 1978; Newsome et al., 1981; Hiscott et al., 1983; Kampik, 1981). Recent evidence suggests that these cells are indeed RPE cells that have transdifferentiated into epitheloid-shaped and
fibroblast/fusiform-shaped cells (Mandelcorn et al., 1975; Muller-Jensen et al., 1975; Casaroli-Marano RP et al., 1999). These studies provide further support implicating the RPE cell as the primary cell type involved in PVR.

CHARACTERISTICS OF RPE

RPE Cytoskeleton

The RPE cytoskeleton consists of different cytoplasmic filaments (intermediate and actin filaments), microtubules, and other structural proteins that provide for the shaping of cells, assist in the movements of organelles and intracytoplasmic vesicles, and participate in cellular movement. Expression of the cytoskeletal proteins varies between species: rabbit RPE cells, for example, are devoid of vimentin, an intermediate filament found in humans and other species. In culture conditions and in pathologic eyes, RPE expression of intermediate filaments show the greatest variability compared to that seen in normal eyes.

Cytokeratin (CK) is the name given to intermediate filaments found predominantly in epithelial cells. Although cytokeratin expression varies between species, approximately 20 forms of cytokeratins have been identified in human epithelia. The type I keratins (#9-19) are acidic proteins and weigh between 40-57 kd, whereas the type II keratins (1-8) are basic proteins that weigh between 52-67 kd. Each intermediate filament is composed of keratin dimers, consisting of one acidic and one basic keratin. The cytokeratin dimer found in RPE cells of the normal human (fetal and adult) eye is CK
8 and 18. No other cytokeratins are present (Hiscott et al., 1984; Kasper et al., 1988; McKechnie et al., 1988; Hunt & Davis, 1990; Uusitalo & Kivela, 1995).

Vimentin is a second intermediate filament found in certain types of epithelial cells. These filaments are usually found in cells of mesenchymal origin, such as fibroblasts, macrophages, sertoli cells and endothelial cells. Human RPE cells of the normal adult eye do not express vimentin (Hunt & Davis, 1990), whereas RPE cells of normal fetal eyes do (Uusitalo & Kivela, 1995).

When in diseased eyes (i.e. when present in ERMs associated with PVR) or in culture, RPE cells undergo changes in shape, ranging from cuboidal-like to fibroblast/fusiform (Casaroli-Marano et al, 1999; Matsumoto et al, 1990). These changes in morphology are most likely correlated with changes in cytokeratin and vimentin expression (Hunt & Davis, 1989; McKechnie et al., 1988; Kivela & Uusitalo, 1998). CK 8 and 18 expression is reduced while CK 19 (strong) as well as vimentin (weak) and CK 7 (weak) is increased. Recent studies have shown that CK19 is associated with invasive ability and cell migration (Robey et al., 1992). This would seem plausible since the initial stages of PVR involve the migration of de-differentiated RPE cells from their monolayer to the vitreous.

**Contraction**

Previous studies have suggested that PVR membranes are composed of a continuum of cells that contract like muscle cells, causing tractional retinal detachments (Ryan SJ, 1985). Recent studies of RPE-mediated gel contraction *in vitro* have shown that
the cells do not act as a continuum of muscle-like cells, but rather act as individual cells that pull collagen in a hand-over-hand manner (Glaser et al., 1987). In 24 hours, 5 mm of collagen can be pulled causing rapid collapse of the gel (Glaser et al., 1987). This mechanism is analogous to sailors pulling in sheets of a sail in a hand-over-hand manner.

Collagen Production

Collagen types I through V are present in epiretinal membranes associated with PVR (Scheiffarth et al., 1988, 1989; Jerdan et al., 1989; Morino et al., 1990). Since normal vitreous contains Type II collagen, the presence of the other types implies that the cells in the membranes are synthesizing the collagen. Cell culture studies demonstrate that RPE cells secrete collagen types I-V, although the production of type II is minor when compared to types I, III and IV (Campochiaro et al., 1986; Newsome et al., 1988; Mueller-Jensen et al., 1975; Morino et al., 1990).

IMMUNE PRIVILEGE AND THE EYE

Immune privilege refers to a deviated immune response in sites that protects grafted tissue from rejection. Privileged sites include the brain, pregnant uterus, ovary, testis, adrenal cortex, tumors, and several sites in the eye, such as the cornea, lens, subretinal space, anterior chamber, and vitreous cavity (Streilein, 1999).

Medawar (1946) performed the initial studies on immune privilege. He believed that immune privilege was "immune ignorance," such that antigenic material trapped in privileged sites (i.e. isolated behind blood-tissue barriers and lacking lymphatic drainage)
would remain “hidden” from the immune system. Evidence over the last 30 years suggests that this is far from the truth.

Ocular immune privilege was first described in studies that placed antigenic tumor cells into the anterior chamber (Kaplan and Streilein, 1974, 1977, 1978; Kaplan et al., 1975; Niederkorn et al., 1980; Streilein et al., 1980). This led to the discovery of Anterior Chamber Associated Immune Deviation, or ACAID. The term ACAID refers to a distinctive and dynamic immune response in which T cells that mediate delayed hypersensitivity and B cells that secrete complement fixing antibodies are inhibited, while cytotoxic T cells and B cells that secrete non-complement fixing antibodies are enhanced. In addition, regulatory T cells are generated which inhibit induction and expression of delayed hypersensitivity. The net effect of ACAID induction is a systemic immune response to the intraocular antigens without causing ocular inflammation, since the mediators of immunogenic inflammation, delayed hypersensitivity and complement fixing antibodies, are quenched (Niederkorn, 1990; Streilein, 1987, 1995, 1999).

Studies have shown that virtually all types of antigens placed in the anterior chamber induce ACAID, and that this stereotypic immune response appears to be a general feature of mammalian eyes, having been induced in the eyes of mice, rats, guinea pigs, rabbits, and cynomologus monkeys. The mechanisms of ACAID are also responsible for immune privilege in other eye sites, such as the vitreous cavity (Jiang & Streilein, 1991).
EXPERIMENTAL MODELS OF PVR

The rationale for creating models for PVR is three-fold: 1) to reproduce the findings observed in human disease; 2) to obtain a high PVR-rate; and 3) to evaluate surgical techniques and therapeutic drugs. Several models have been presented, and all involve some form of trauma applied to the vitreoretinal surface, such as an incision, (Cleary & Ryan, 1979a; 1979b) a perforating trauma (Topping et al., 1979), or the inoculation of agents into the vitreous (e.g. freshly harvested or cultured cells) (Algvere et al., 1988; Fastenberg et al., 1982; Frenzel et al., 1998; Hitchins et al., 1985; Hsu et al., 1984; Hui et al., 1988; Hui et al., 1987; Lean et al., 1984; Mandelcorn et al., 1975; Miller et al., 1986; Peters et al., 1986; Shabo and Maxwell, 1977; Topping et al., 1979).

Early cell-injection models were aimed at inducing PVR at high rates while attempting to mimic the natural course of the disease with regard to earlier stages of pathogenesis. The most common model involved the injection of autologous or homologous dermal fibroblasts into the rabbit eye (Sugita et al., 1980; Fastenberg et al., 1982; Gonvers & Thresher, 1983; Hsu et al., 1984; Hitchins et al., 1985). Fibroblasts are easy to obtain, are highly contractile, and are able to cause tractional retinal detachments in a short time course.

Recently, however, it became clear that the real value of experimental models of PVR lies in the evaluation of surgical techniques and therapeutic drugs. The major drawback of the fibroblast model is that the induced (contractile) PVR membranes are unlike those found in human PVR (i.e. fibroblasts represent only a small population of cells compared to RPE cells, myofibroblast-like cells, and glial cells in human ERMs).
Since the RPE cell is the cell responsible for inducing PVR and the most common cell type present in ERMs, researchers sought to create models that allowed them to examine RPE behavior.

The studies of Mandelcorn (1976) were the first involving the intravitreal injection of RPE cells to induce PVR. After injecting autologous RPE cells into the vitreous cavity of the owl monkey, the authors reported that the cells developed into membranes but were not able to produce tractional retinal detachments. When Grierson (1986) injected 400,000 human adult RPE cells into the rabbit eye, they were also not able to induce tractional retinal detachments. Cui (1998) showed that an injection of exogenous platelet-derived growth factor (PDGF) was necessary along with adult rabbit RPE cells to induce tractional retinal detachments, and produce membranes composed of RPE cells, myofibroblasts, and glial cells.

In the following thesis, I will describe how my study, involving the intravitreal injection of human RPE cells alone, induced tractional retinal detachments and produced membranes composed of the cell types found in human PVR. I will then follow with a discussion of why I feel my model more closely mimics clinical PVR and can be a powerful tool for evaluating novel surgical techniques and therapeutic drugs.
PVR Membranes: Explant Culture

A single ERM from a patient with a retinal detachment and PVR was surgically removed and placed in Ham’s F12 medium (Gibco BRL, NY). The membrane was washed with Hank’s balanced salt solution (BSS), dissected into twenty 0.3 x 0.3 cm pieces, and placed into separate 35 mm culture dishes. Ham’s F12 medium, 30% fetal bovine serum (FBS; Gibco, NY), 100 units/ml of penicillin G sodium, and 100 mg/ml of streptomycin was added to the dishes, which were then placed in a humidified 37°C incubator containing 5% CO₂. After cell migration from the membrane explants had occurred (Fig. 1), the cells were dissociated with 0.25% trypsin (Gibco, NY), and placed into culture dishes containing growth medium. The growth medium consists of Ham’s F12 medium supplemented with 10% FBS and antibiotics as described above (Figure 3).

Cell Culture Immunohistochemistry

Sub-confluent cultures were fixed with cold methanol/acetone (1:1 v/v) at -20°C for 15 minutes. After fixation, the cultures were rinsed three times with PBS, permeabilized with 1% Triton X-100 for 5 minutes, and then treated with 10% normal horse serum (NHS) diluted with 1% bovine serum albumin in PBS (BSA/PBS). The fixed cultures were then incubated with a primary antibody in BSA/PBS for 1 hour at room temperature. The primary monoclonal antibody was monospecific for human pan-
Figure 3. Primary culture of a human epiretinal membrane explant. Epiretinal membrane cells can be seen migrating away from the membrane. (scale bar = 15μm)
cytokeratin identifying human keratins 1, 4, 5, 6, 8, 10, 13, 18, and 19, (1:200; Sigma-Aldrich, Oakville, Ontario), α-smooth muscle actin (1:50; α-SMA; Dako, CA), or glial fibrillary acidic protein (1:2000; GFAP; Dako, CA). Bound antibody was detected by incubation with a 1:500 dilution in BSA/PBS of biotinylated-labeled horse anti-mouse IgG (Vector, CA), followed by horseradish peroxidase (HRP) conjugated to avidin. Labeled cells were visualized using a standard diaminobenzidine (DAB) reaction. To rule out non-specific staining of the secondary antibody, control cultures were processed in the same way, except that the primary antibody was omitted (i.e. sections were incubated with diluent (1% normal horse serum (NHS) in BSA/PBS) only). In addition, human ovarian adenocarcinoma (NIH:OVCAR-3) cells, which are known to express cytokeratin, were used as a positive control (Wong AS et al., 1999).

Collagen Gel Contraction Assay

A collagen contraction assay was adapted from the method described by Raymond and Thompson (1990). Two groups of RPE cells were investigated: cells at passage 5, and cells at passage 24. The collagen gel was prepared using Vitrogen-100 (3 mg/ml type I bovine dermal collagen; Collagen Corp., Palo Alto, CA) mixed 16:1 v/v with 10x phosphate buffered saline (PBS) and brought to neutral pH with 0.2 M NaOH at 4°C.
The RPE cells were harvested from the culture dishes, centrifuged at 200 g for 3 minutes, and resuspended in 1 ml of F12/10 with 10 mM N-2 hydroxyethyl piperazine N-2-ethanesulfonic acid (HEPES) buffer.

Nine milliliters of the prepared collagen mixture was combined with 1 ml of RPE cells/medium to produce a collagen-RPE cell mixture with a cell concentration of $1.5 \times 10^5$ cells per ml and a collagen density of 2.5-2.7 mg/ml. Five hundred microliters of each collagen-RPE cell mixture were placed in 6 wells of a 24 well plate. Six additional wells for each group served as controls, receiving 500 microliters of collagen gel alone (no cells) at a density of 2.5-2.7 mg/ml. The plates were then placed in a 5% CO$_2$ / 95% air incubator at 37°C for 15 minutes to promote solidification of the gels.

On removal from the incubator, the collagen gels were separated from the side of the well with a 25-gauge needle. Each well then received 1.5 ml of Ham’s F12/10, drop wise down the side of the well. The medium was changed every 2-3 days by aspirating from the edge of each well, taking care not to disrupt the gel.

The gels were observed on days 1, 4, and 8, and their area was determined by measuring the longest and shortest diameters of the collagen gel over a millimeter grid.

**RPE Cell Preparation**

The RPE cells used for all cell injections were of intermediate passage (approximately passage 12). Sub-confluent (and thus still proliferating) RPE cells were dissociated with 0.25% trypsin, and washed twice with BSS. Cell viability was confirmed with negative trypan blue staining (i.e. dead cells turn blue (positively staining) because
trypan can penetrate their cell membrane). Approximately $1 \times 10^6$ live cells were re-suspended in 0.2 ml BSS. The cells were prepared no more than 20 minutes prior to being injected into the eyes.

**RPE Cell Injection**

A total of 24 New Zealand albino rabbits, weighing between 2.5 and 3.5 kg, were used for this study. The right eye of each rabbit was injected with cells, while the left eye served as a control. Following pupillary dilation with two eye drops containing 5% phenylephrine and 1% tropicamide, the rabbits were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg). A 25-gauge needle was passed through the sclera 3 mm posterior to the limbus and 0.2 cc BSS containing approximately $1 \times 10^6$ RPE cells was injected directly over the optic disc of the right eye. In order to prevent an increase in intraocular pressure, 0.2 cc of aqueous was removed simultaneously from the anterior chamber with a 30-gauge needle, with the aid of an assistant. For the left eye, 0.2 cc of BSS not containing RPE cells was injected. Chloramphenicol 1% ointment (Vetcom, Quebec) was applied topically after surgery. Of the 24 rabbits, 23 were sacrificed at day 28, and 1 rabbit at 3 months. The deeply anesthetized animals were sacrificed by injecting 1.5 cc of sodium pentobarbitol (240 mg/ml) into an auricular vein. The eyes were enucleated and prepared for histochemical analysis. All animals were treated according to the Declaration of Helsinki, the guidelines of the Canadian Council on Animal Care, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
Clinical and Histological Evaluation: Inflammatory Response

The rabbits were examined for inflammation by indirect ophthalmoscopy during the first 7 days post-surgery, and then on days 14, 21 and 28. One animal with an epiretinal membrane without retinal traction and/or tractional retinal detachment was observed until 3 months.

Clinical and Histological Evaluation: PVR Development

The 24 rabbits were examined by indirect ophthalmoscopy at days 7, 14, 21, and 28 post-injection. PVR was graded according to the scheme described by Fastenberg. Both eyes of 13 rabbits were enucleated at 28 days, and from a further rabbit at 3 months post-injection. Globes were fixed in 10% neutral buffered formalin for 48 hours and wholly embedded in paraffin wax. Sections were made at a thickness of 12 μm and then stained with hematoxylin and eosin (H&E).

ERM Histochemistry: Antibody and Trichrome Staining

To determine the cellular components of the induced epiretinal membranes, both eyes of the remaining 10 rabbits were enucleated at 28 days post-injection. The anterior segments were removed, and the eyecups were placed in Tissue-Tek OCT (VWR, Ontario) before being immersed in liquid nitrogen. Eight micrometer sections were cut, mounted on glass slides, and placed in an -85°C freezer for storage. After thawing, the sections were air-dried and fixed in acetone for 5 minutes. The cryosections were then
treated with 1% BSA/PBS for 15 minutes to block non-specific binding sites, and immersed in 0.3% hydrogen peroxide. The cryosections were exposed to primary antibody for 30 minutes, and then washed twice in PBS.

Bound antibody was detected by incubation with 1:500 biotinylate-labeled horse anti-mouse IgG (Vector, CA) in PBS/BSA, followed by exposure to the avidin-biotin complex (Vectastain ABC Elite Kit; Vector, CA) and aminoethyl-carbazole (AEC; Vector, CA). The cryosections were also counterstained with Mayer's modified hematoxylin.

Three primary monoclonal antibodies were used on each right and left eye. The antibodies targeted against human pan-cytokeratin, GFAP, and α-SMA. Control cryosections from both right and left eyes were subjected to an immunohistochemical assay in which the primary antibody was substituted with 1% NHS in BSA/PBS alone, in order to rule out non-specific staining. Collagen was stained for in both the right and left eyes using Masson's trichrome. Human cardiac tissue, which is known to contain collagen, served as the positive control.
Chapter III

RESULTS

IN VITRO STUDIES

Cell Culture Immunohistochemistry

All sample cells from passage 4, 17 and 24 cultures stained positively for cytokeratin (Fig. 4). Alpha-SMA and GFAP immunochemistry resulted in weak staining of the cultured cells, which was comparable to staining produced when the primary antibodies were replaced with PBS (negative controls). The positive control for cytokeratin (human ovarian carcinoma (OVCAR-3: NIH) cells) stained for cytokeratin, as expected. These findings suggest that the strong immunoreactivity for cytokeratin was due to specific binding, and that the cells were of RPE origin.

Collagen Gel Contraction Assay

The reduction in collagen gel size due to RPE-mediated contraction for both passage 5 and passage 24 RPE cells is summarized in figures 5 and 6. There was marked gel contraction at day 1: 25% reduction with the passage 5 cells and 49% reduction with the passage 24 cells, when compared to the control gels (no cells added). By day 8, both experimental groups had contracted the collagen to 23% of the size of the control gels.
Figure 4. Cytokeratin Staining of ERM cells. Positive cytokeratin immunoreactivity in cells from passage 4 cultures obtained from the human ERM. These cells showed negative immunoreactivity for α-SMA and GFAP, suggesting that they were pure cultures of RPE cells. (scale bar = 5 μm)
Figure 5. Collagen Contraction Assay: Graphs. Graphs illustrating RPE-mediated collagen gel contraction of the passage 5 (A) and passage 24 (B) cells as a percentage of the control gels over 8 days. Test gels consisted of RPE cells admixed with collagen, whereas control gels consisted of collagen gel alone.
Figure 5

Gel Area (%)

Time (days)

- Passage 5 RPE cells
- Passage 24 RPE cells
Figure 6. Collagen Contraction Assay: P5 Cells. RPE-mediated collagen gel contraction of passage 5 cells at days 1 (A), 4 (B) and 8 (C).
Figure 6
**IN VIVO STUDIES**

**Inflammatory Response**

At day 3, there was minor conjunctivitis in both the experimental and control eyes. The anterior chamber was clear. By day 7, the conjunctivitis had completely cleared in both groups, and the eyes appeared normal.

**Induction of PVR: Clinical Findings**

The degree of PVR observed in all 24 rabbits is summarized in Table 3. By day 7, all eyes developed PVR; the majority (38%) of these eyes developed either ERMs alone or localized tractional retinal detachments (33%) with ERMs present (Fig. 7A). By day 28, 17/24 (71%) of the eyes were diagnosed with localized TRDs, 5/24 (21%) were diagnosed with retinal traction (Fig. 7B), and 1/24 (4%) was diagnosed with an extensive TRD. None progressed to stage 5 (total retinal detachment). All control eyes were normal through 28 days (Fig. 7C). One experimental eye remained at stage 1 (ERM) for three months. The left control eye at 3 months was normal.

**Histopathology**

All eyes that were diagnosed with localized or extensive TRDs by indirect ophthalmoscopy showed the presence of an ERM (Fig. 8), subretinal fluid (Fig. 8), evidence of retinal traction (Fig. 9), and degenerated photoreceptor outer segments (Fig. 10A) upon histological evaluation. All control eyes displayed normal retinal morphology.
(Fig. 10B) with the absence of subretinal fluid (Fig. 11). Artifactual retinal detachment occurred in the control eyes (Fig. 11) after enucleation as a result of the fixation procedure. These histochemical findings confirmed the ophthalmoscopic observations.

**ERM Immunohistochemistry and Trichrome Staining**

All induced ERMs examined displayed cytokeratin (Fig. 12A), α-SMA (Fig. 12B), and GFAP (Fig. 12C) immunoreactivity, indicating that RPE cells, myofibroblasts, and glial cells were present. RPE cells and myofibroblasts were the dominant cell types.

Experimental and control eyes stained for cytokeratin in the RPE layer, and for α-SMA in the choroidal blood vessels. All negative controls, in which PBS replaced the primary antibodies, displayed background levels of immunoreactivity.

Trichrome staining for collagen was seen in our ERMs (Fig. 12D) and in the cardiac tissue control sections.
Table 3. Number of Experimental Eyes* with Induced PVR

<table>
<thead>
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<th>Stages</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
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<td>0</td>
<td>0</td>
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<tr>
<td>1</td>
<td>9</td>
<td>4</td>
<td>2</td>
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<td>2</td>
<td>6</td>
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<td>5</td>
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PVR was graded according to the stages described by Fastenberg (1982) as follows: Stage 1 – intravitreal membrane; Stage 2 – focal traction, localized vascular changes; Stage 3 – localized detachment of medullary ray; Stage 4 – extensive retinal detachment, total medullary ray detachment; Stage 5 – total retinal detachment, retinal folds and holes.

* Control eyes were normal at all time points
Figure 7. Fundus Photographs of Rabbit Eyes with Induced PVR. (A) Experimental eye at day 7 with an ERM and an underlying localized TRD (arrows). (B) An experimental eye at day 28 with an ERM exerting retinal traction (arrows indicate elevation of the medullary ray). The left control eyes were normal at day 28 (C).
Figure 8. *H&E* Stained Section of an Experimental Eye at 28 Days Post-Cell Injection. The presence of subretinal fluid (SF) and an ERM (arrows) reflects provides evidence that a retinal detachment had occurred. (scale bar = 2 mm)
Figure 9. ERM exerting retinal traction. Retina and overlying ERM of an experimental eye stained with H&E shows retinal folds (arrows) providing evidence that membrane-induced retinal traction had occurred. (scale bar = 15 μm)
Figure 10. H&E Staining of the Retina at 28 Days Post-Cell Injection. The experimental eye (A) shows degenerated photoreceptors outer segments (arrows), suggesting that the photoreceptors lost their association with the RPE monolayer before day 28. This provides additional support to the clinical findings that a retinal detachment had occurred in this experimental eye prior to enucleation. Healthy photoreceptor outer segments (arrows) can be seen in the control retina (B). (OS- outer segments; IN-inner segments; ENL-external nuclear layer; EPL-external plexiform layer; INL-internal nuclear layer; IPL-internal plexiform layer; GCL-ganglion cell layer) (scale bar = 5 µm)
Figure 11. *H&E* stained section of a control eye. Subretinal fluid and ERMs were not present in all control eyes at 28 days post-injection. Arrowheads indicate artifactual retinal detachment. (scale bar = 2 mm)
FIGURE 12. Immunohistochemistry and Trichrome Staining of an Induced ERM in a Stage 3 Experimental eye. At day 28, positive immunoreactivity for cytokeratin reflects the presence of RPE cells throughout the ERM (black arrows) (A). The rabbit RPE monolayer (white arrow) also immunoreacted for cytokeratin (A). Positive α-SMA and GFAP immunoreactivity throughout the ERM indicates the presence of myofibroblasts (B) and glial cells (C), respectively. The AEC staining for GFAP is seen as a light AEC reaction product. Positive trichrome staining indicates the presence of collagen throughout the ERM as seen by the blue reaction product (D). Figures 12B & C were counterstained with hematoxylin. (Scale bars = (A) 25 μm; (B) 25 μm; (C) 25 μm; (D) 100 μm)
Figure 12
Our results suggest that all the cells cultured from the single epiretinal membrane obtained at surgery for PVR were RPE in origin. Previous groups have grown mixed cultures of RPE cells, glial cells, fibroblast-like cells, and macrophages, from epiretinal membranes removed from patients with PVR. Not one has isolated a pure culture of RPE cells (Vinores et al., 1990; Jerdan et al., 1989; Shirakawa H et al., 1987; Kwak et al., 1991; Hiscott PS et al., 1983).

After injecting this unique culture of RPE cells into rabbit eyes, there was progression in the clinical manifestations of PVR from the early appearance of a membrane to later signs of retinal traction and detachment. This is consistent with the picture in human PVR, although the time course is shorter than for the development of PVR post retinal detachment surgery.

The short time course of PVR in our model is useful for experimental purposes as it will allow for rapid assessment of potential therapeutic or prophylactic agents against PVR. It may also shed light on the mechanism of PVR. In our model, injection results in a sudden influx of about a million RPE cells into the vitreous, whereas in human PVR, RPE cells migrate more gradually through retinal tears or as a result of surgical procedures employed during retinal detachment surgery (Charteris, 1995; Pastor, 1998). More extensive retinal tears are associated with more rapid onset of PVR (Bonnet, 1984; Scott, 1976).
The shorter time course of PVR in our model also may be explained by differences between RPE cells in PVR membranes from those in the RPE layer in the retina. RPE cells that migrate from their retinal monolayer are known to undergo a number of changes, collectively termed dedifferentiation, metaplasia, or transdifferentiation (Naumann, 1980; Mandelcorn et al., 1975; Müller-Jensen et al., 1975; Vidaurri-Leal et al., 1984; Casaroli-Marano et al., 1999; Kirchhof & Sorgente, 1989).

Firstly, it is thought that RPE cells cultured from the retinal monolayer assume transdifferentiated (myofibroblastic-like) characteristics, and these changes are necessary for increased contractile activities (Vidaurri-Leal et al., 1984, Mandelcorn et al., 1975, Casaroli-Marano, Pagan and Vilaro, 1999; Grisanti and Guidry, 1995). This ability is likely to play a key role in development of tractional retinal detachment due to PVR. In this study, we showed that RPE cells, cultured from the same human PVR membrane used in the intravitreal injections, were able to rapidly contract a collagen matrix. This may help to explain the short time course of development of PVR in our model.

Secondly, RPE cells isolated from PVR membranes are known to express different genes (and thus proteins) from those in the retinal monolayer, for example the cytokine IL-6 (Abe et al., 1996). The genes are likely to code for proteins that are important in the pathogenesis of PVR such as growth factors, cytokines, and their receptors. Cytokines, such as IL-6, may function as chemoattractants for other cell types involved including the glial cells and macrophages seen in our model. Growth factors are also believed to play a vital role in the transdifferentiation of RPE cells, and thus in PVR (Vidaurri-Leal J et al., 1984). Indeed, our group has found that a number of growth factors and their receptors
are expressed in the unique culture of RPE cells used in this study (unpublished data). This also helps explain why our model had a shorter time course than human PVR post retinal detachment surgery, and provides important clues about which therapies to investigate.

In our model of PVR, the clinical findings corresponded accurately with the histopathology, which confirmed PVR and demonstrated the presence of mostly RPE cells, but also glial cells, myofibroblast-like cells, and macrophages. Collagen was also present, which is secreted by RPE cells, promotes their migration, and enables them to contract epiretinal membranes. The composition of membranes in our model was thus similar to that of human PVR membranes.

Previous animal models of PVR have focused either on the injection of cells into the vitreous or on a more physical intervention. Physical interventions have included the injection of particles, gas, albumin, enzymes, and mechanically tearing the retina. Cell injection models have involved administering whole blood, erythrocytes, platelets, leukocytes, fibroblasts, and glial cells. Although some of these models have demonstrated clinical characteristics of PVR, few of the groups published histological data. (Algvere et al., 1988; Fastenberg et al., 1982; Frenzel et al., 1998; Hitchins et al., 1985; Hsu et al., 1984; Hui et al., 1988; Hui et al., 1987; Lean et al., 1984; Mandelcorn et al., 1975; Miller et al., 1986; Peters et al., 1986; Shabo & Maxwell, 1977; Topping et al., 1979).

In 1976, Mandelcorn injected adult human RPE cells into the vitreous of the primate eye. The authors observed metaplasia and proliferation, but were not able to induce tractional retinal detachment. In 1986, Grierson's group injected human RPE cells,
derived from either an adult or fetal cell line, into the monkey eye. They also found that
the adult cells, cultured from the RPE monolayer of a donor eye, produced an
insubstantial epiretinal membrane and no tractional retinal detachment. However, they
observed that fetal RPE cells produced extensive membranes and traction retinal
detachments. In addition, Cui's group (1998) injected adult rabbit RPE cells into the
rabbit eye, and was unable to demonstrate tractional retinal detachment unless platelet-
derived growth factor was also injected.

The previously mentioned studies support the unique nature of the RPE cells
used in our model. It is likely that our RPE cells already possess the characteristics of
transdifferentiation as they were able to induce traction retinal detachments. These
characteristics logically include active proliferation and expression of the necessary
structural proteins, growth factors, and their receptors.

Like the models proposed by Mandelcorn (1976) and Grierson (1986), our model
involves the injection of xenogenic cells (human RPE cells) into the vitreous cavity of the
rabbit. After surgery, we observed minor inflammation in both the experimental and
control eyes that lasted 2-3 days. By day 4, the inflammation in both eyes had
completely resolved, suggesting that the inflammation was due to the surgery itself, not
the human cells. Furthermore, in one eye we allowed an ERM, which did not cause a
TRD, to survive for 3 months. There was no evidence of ocular inflammation throughout
this period. The lack of inflammation is consistent with Anterior Chamber Associated
Immune Deviation (ACAID) - immune privilege in several parts of the eye including the
vitreous cavity. Due to ACAID, a systemic immune response can be induced without
causing ocular inflammation, as the mediators of immunogenic inflammation, delayed hypersensitivity and complement fixation, are quenched. Our data illustrate that in our model, tractional retinal detachment occurred due to ERM contraction, not inflammation.

Artifactual retinal detachments also observed in the control eyes at day 28. When whole eyes are fixed in 10% formalin, the neurosensory retina often detaches from the RPE. Margo and Lee (1995) postulated that these artifactual retinal detachments are likely due to the relatively high osmolarity of the fixative, which causes the retina and other intraocular structures to contract. The control eyes did not show any clinical signs of retinal detachments or tears. Histology revealed healthy photoreceptors in the absence of an ERM. These data confirm that the retinal detachments observed in the control eyes were artifactual.

In our model, the only human cells injected into the vitreous of the rabbit were retinal pigment epithelial cells. Therefore, glial cells found in the ERMs at day 28 must have originated from the rabbit. It is likely that these glial cells invaded the vitreous through the tractional tears in the retina. It is also likely that most of the cells within the developing epiretinal membrane in our model originated from the human RPE cells injected. The injection did not break the inner limiting membrane, thus rabbit cells did not have access to the vitreous at this stage. Rabbit cells only gained access to the vitreous cavity after the tractional retinal tears had occurred. This is an advantage of our model because therapeutic agents that target RPE proliferation and contraction can be tested specifically.
CAUTIONS AND FUTURE DIRECTIONS

There are a few cautionary factors that should be addressed. Firstly, the RPE cells used in our model are unique in that they were derived from a single PVR membrane and are able to complete several population doublings (at least 48) without showing signs of replicative senescence. It is unusual for cells cultured from human PVR membranes to survive more than a few passages. Even human RPE cells cultured de novo from donor eyes tend to become senescent after 15 to 20 passages. It is likely that the RPE cells used in this study had spontaneously transformed. RPE transformation could serve as a benefit in providing an ‘immortalized’ culture of cells for future studies. Conversely, transformed RPE cells may not accurately reflect RPE cells found in other PVR membranes. We previously demonstrated that our RPE cells retained epithelial-like characteristics and contractile properties up to passage 24. However, additional experiments should be performed to further characterize this unique culture of cells. These could include studying the expression of structural proteins, growth factors, and their receptors. Evaluating the stability of both morphology and gene expression in successive population doublings would also help characterize our unique RPE cells.

Secondly, our model relies on the sudden influx of 1 million cells into the vitreous cavity. Other cell-injection models induced tractional retinal detachments with fewer cells - the mean cell number being 500,000, although the concentration of cells in solution was similar to our model. Studies could be performed to examine whether 100,000 – 500,000 of our RPE cells can induce stage 3 or 4 PVR at a similar rate compared with the million cells used in this study. A PVR model involving fewer cells would be advantageous as this
would allow experimental drugs that act on RPE cells to be evaluated at lower doses. Using the minimum number of RPE cells that could rapidly induce stage 3 or 4 PVR, to test potential therapies, would minimize drug side effects whilst retaining an efficient and clinically relevant model.
SUMMARY

In summary, this study demonstrates that human RPE cells cultured from a patient with PVR are capable of inducing the same disease in rabbit eyes. My model is unique and has distinct advantages that make it an excellent tool for studying the pathogenesis of, and potential treatments for proliferative vitreoretinopathy.
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