



Role of RPE Cells in Pathogenesis of Proliferative Vitreoretinopathy and Age-Related Macular Degeneration: Cell Culture Study of Surgical Excised Pre- and Sub-Retinal Membranes

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Abstract

Objectives: There were few studies on the culture of cells from surgical excised pre- or sub-retinal membranes from Proliferative Vitreoretinopathy (PVR) and Age-Related Macular Degeneration (AMD) patients and all of these studies were restricted to primary cultures only. The purpose of this study was to culture and identify various cellular components of surgically excised membranes from these patients and to explore the role of these cells in the pathogenesis of PVR and AMD by the investigation of the *in vitro* behavior of these cells in and beyond the primary culture.

Methods: Surgically excised membranes were plated in culture dishes by an explants method and cultured with F12 medium containing fetal bovine serum. Immunocytochemical staining was performed to identify cell types. After the confluence of primary culture, cells were subcultured until senescence.

Results: Of the membranes from 41 PVR eyes, 29 cases exhibited cell outgrowth (71%), and 13 cases (32%) grew to confluence. RPE cells, glial cells, fibroblasts and macrophages were the main cell types identified in the outgrowth of these membranes. RPE cells were the predominant and most actively growing cell type and the sole cell type persisted after second subcultures. RPE cells could be cultured up to 12 generations with 30 divisions *in vitro*. Of the membranes from 16 AMD eyes, 5 (31%) demonstrated cell outgrowth, but none of these cultures became confluent.

Conclusions: Cells from PVR membranes exhibited active proliferation and were predominantly RPE cells. Cells from sub-retinal membranes of AMD eyes showed poor viability and growth capacity. These results were consistent with the pathologic and cell biologic changes of RPE cells in the AMD and PVR.

Keywords: Age-related macular degeneration; Cell culture; Pre-retinal membrane; Proliferative vitreoretinopathy

Introduction

Proliferative vitreoretinopathy (PVR) is the most common cause of failure of retinal reattachment surgery. Refinement of surgical techniques has improved the reattachment rate in severe PVR, but failure is still encountered in a significant percentage of eyes, due to re-proliferation of cells after surgery. Study of cells isolated from the pre-retinal membranes or sub-retinal membranes excised during PVR surgery may be helpful to better understand the factors involved in the pathogenesis of PVR. Tissue culture can provide useful information about the growth capacity of cells in such membranes, and may have prognostic significance in eyes with PVR. The effect of anti-proliferative drugs on the cultured cells may provide insight on developing methods for the prevention and possible pharmacological treatment of PVR [1]. There have been limited studies on cell cultures from membranes excised from PVR patients [2-7]. All of these studies based on a series of patients were restricted to primary cultures only [2-5]. Only one paper mentioned that the RPE cells migrated from the membrane from a single case of PVR could grow to beyond the primary culture [6]. Age-related Macular Degeneration (AMD) is the major cause of blindness

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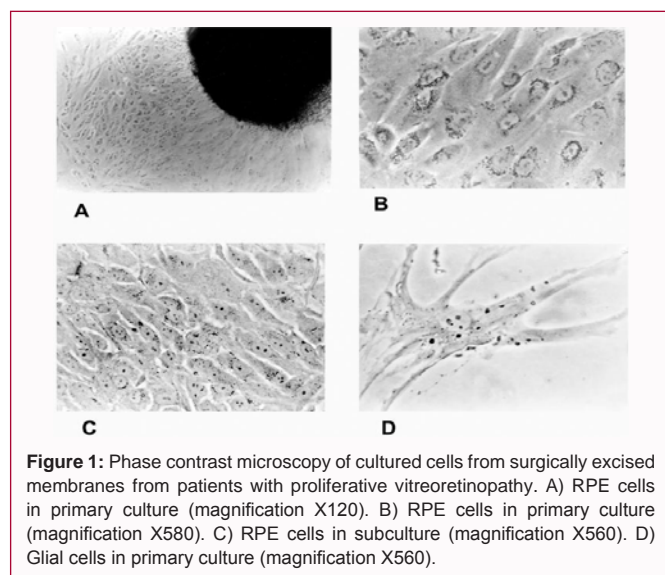


Figure 1: Phase contrast microscopy of cultured cells from surgically excised membranes from patients with proliferative vitreoretinopathy. A) RPE cells in primary culture (magnification X120). B) RPE cells in primary culture (magnification X580). C) RPE cells in subculture (magnification X560). D) Glial cells in primary culture (magnification X560).

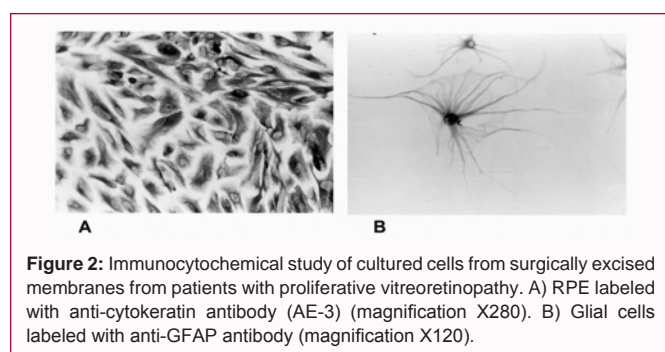


Figure 2: Immunocytochemical study of cultured cells from surgically excised membranes from patients with proliferative vitreoretinopathy. A) RPE labeled with anti-cytokeratin antibody (AE-3) (magnification X280). B) Glial cells labeled with anti-GFAP antibody (magnification X120).

in the United States. In wet type of AMD, Choroidal Neovascular Membranes (CNV) are predominate pathologic changes. Surgical excision of CNV has been used for the treatment of wet type of AMD. Some authors have reported the results of pathologic examination of these surgically excised specimens [8-15]. Culture of surgical excised CNV may provide important information about the pathobiology of AMD. However, cell culture of surgically excised CNV has not been reported previously. We have developed a method for isolation and cultivation of cells from surgically excised membranes from patients with PVR and AMD. Herein, we describe the results of tissue culture of surgically excised membranes, and discuss their clinical significance.

Materials and Methods

Cell culture

Surgically excised retinal membranes were obtained from 57 eyes undergoing vitreoretinal surgery at The New York Eye and Ear Infirmary (45 cases, 29 PVR and 16 AMD) and Show Chwan Memorial Hospital (12 cases, all PVR).

Membranes were placed in a culture dish, washed with Ca^{++} - and Mg^{++} -free Hank's solution, cut into small pieces (0.5 mm × 0.5 mm) and plated into 35 mm Falcon dishes (Becton Dickinson, Oxnard, CA, USA) with 0.5 ml of culture medium (Ham's F12 nutrient mixture supplemented with 30% fetal bovine serum and 2 mM glutamine, all from Gibco, Gaithersburg, MD, USA). Additional culture medium (0.5 ml) was added 1-2 hours and 24 hours later. The culture dishes were incubated at 37°C in a humidified 5% CO_2 atmosphere and observed daily with an inverted phase-contrast microscope. The

culture medium was changed twice weekly. If no cell outgrowth appeared within 4 weeks, the culture was discarded and considered to have no growth. After primary cultures became confluent, the cells were detached by 0.05% trypsin/0.02% EDTA solution, diluted to a ratio 1:2-1:3, and seeded for subculture. The subculture medium was similar to that used in primary culture except that a lower concentration of serum (10%) was used. Cell lines were passaged routinely at a dilution of 1:3 every 5-10 days.

Immunocytochemical studies

Immunocytochemical staining was performed on some of the primary cultures and sub-cultures. Cells were fixed in methanol and acetone (1:1) and stained by peroxidase-streptavidin method (reagents used for immunostaining were obtained from Dako Copenhagen, Denmark). Primary antibodies consisted of various monoclonal antibodies to cytokeratin, including AE-1 (for cytokeratins 10, 14, 15, 16, 19), AE-3 (for cytokeratins 1, 2, 3, 4, 5, 6, 7, 8; both AE-1 and AE-3 were gifts from Dr. T.T. Sun, New York University, New York, NY, USA), anti-cytokeratin 7, 14, and 19 (1:100, all 3 antibodies from Sigma, St. Louis, MO, USA) and Dako's anti-cytokeratin (for cytokeratin 6 and 18, 1:100). Antibodies to glial fibrillary acidic protein (GFAP, 1:100, Dako), to Factor VIII-related antigen (VIII:rAg 1:50, Dako), antibodies for desmin (for smooth muscle cells, 1:50, Dako) and to leukocyte marker CD58 (for macrophages, 1:100 Dako) were also used in this study.

Results were analyzed statistically using χ^2 test.

Results

In the present study, growth patterns observed in tissue culture were divided into three groups: Group 1) No cell migration from the explants. Group 2) Cell migration from the explants with no or very limited growth capacity, so that confluence was not seen. The number of cells usually remains below 100. These cells became stationary for a long period, gradually degenerated and then disappeared. Group 3) Cell migration from the explants, proliferated actively and rapidly to confluence. Confluence rate was defined as the percentage of Group 3 cases over the total number of cases, and the outgrowth rate was defined as the percentage of Groups 2 and 3 cases over total cases.

In 41 PVR patients, the number of cases in Groups 1, 2, and 3 were 12, 16 and 13, respectively (Table 1). Cell outgrew in 29 cases (Groups 2 and 3; outgrowth rate: 70.7%). The mean latent period (time between explanation and the migration of cells from the explants) was 3.18 days. In 13 cases (Group 3, confluence rate: 31.7%), cells grew rapidly, the primary cultures reached confluence and were passaged in subcultures for many generations. The longest-lived cell lines (only containing the RPE cells) have been passaged for more than 12 subcultures with 30 divisions *in vitro*, which indicating that one cell derived from the membrane can grow up to 1 billion cells (1: 1,000,000,000). In the 16 AMD patients, 11 were in Group 1, 5 were in Group 2 and none in Group 3 (Table 1). Cells outgrew from 5 cases only (31.3%), and none of the cultures grew to confluence. Both outgrowth rate and confluence rate in specimens from AMD patients was significantly lower than that of PVR patients (outgrowth rate, $P < 0.01$; confluence rate, $0.01 < P < 0.05$). The cellular outgrowths from the membranes varied greatly in morphology and also in immunocytochemical characters. The following cell types were seen:

Retinal pigment epithelial cells (RPE)

RPE appeared as polygonal or spindle shaped cells with varying

Table 1: Tissue culture of membranes excised from proliferative vitreoretinopathy and age-related macular degeneration patients.

Disease	Group 1 (No migration)	Group 2 (Migration Poor growth)	Group 3 (Active growth)	Total
PVR	12 (29%)	16 (39%)	13 (32%)	41 (100%)
AMD	11 (69%)	5 (31%)	0 (0%)	16 (100%)

Table 2: Cytokeratin expression in primary culture of membranes excised from proliferative vitreoretinopathy and age-related macular degeneration patients.

Antibody	Cytokeratin detected	Cell stained positively	
		Mean	Range
AE-3	1,2,3,4,5,6,7,8	68.8%	30% to 100%
Anti-CK6,18	6,18	58.3%	30% to 80%
Anti-CK7	7	45.8%	30% to 100%
AE-1	10,14,15,16,19	40.0%	10% to 80%
Anti-CK19	19	24.0%	0% to 30%
Anti-CK14	14	0%	0%

degrees of pigmentation (Figure 1A and 1B) [2,3,16]. The pigment granules were diluted gradually after growth. RPE cells tend to aggregate and contact each other to form a monolayer. The outgrowth rate and confluence rate varied among the different specimens. In some specimens, cells grew rapidly, reached confluence within 10-20 days after plating, and could be passaged for many generations in subculture (Figure 1C). Both polygonal and spindle shaped RPE stained positively with anti-cytokeratin antibodies (Figure 2A). All anti-cytokeratin antibodies stained part or all cells in cultures from PVR and CNV except antibodies to cytokeratin 14, which showed negative results in all 7 cultures tested. The positive rate for cytokeratin (positively stained cells among the total cells in culture) in primary culture from PVR patients is summarized in Table 2. In the subcultures from PVR patients, positive rates for various cytokeratins were higher: 3 out of 5 cultures of the first generation of subculture and all 3 cultures of two or more generations revealed staining of all cells with anti-cytokeratin antibodies. These results indicate that RPE are the prominent cell type and the most actively growing cells in the cell cultures from PVR patients. In 4 cultures from AMD patients, positively stained cells for cytokeratin were present in all cultures. The positive rates of various cytokeratin antibodies were: AE-3: 50% to 80% (average, 60.0%); AE-1: 30% to 40% (average 35%); and cytokeratin 6 & 18: 20% to 50% (average, 35%).

Glial cells

Glial cells appeared as larger flattened spindle cells or semilunar cells, which sometimes had several long branching processes (Figure 1D). These cells were often isolated, did not grow or showed limited growth capacity. Immunocytochemical studies revealed GFAP antigen in these putative glial cells (Figure 2B). GFAP positive cells were present in 9 out of 12 primary cultures from PVR patients (75%), and accounted for 10% to 50% (average 30%) of the total number of cells. In subcultures from PVR patients, only 2 of 5 cultures of first generation of subcultures (5% to 10% cells from the whole population) stained positively for GFAP and none of 3 subcultures of more than two generations revealed cells staining with anti-GFAP antibodies. In 4 cultures from AMD patients, positively stained cells for GFAP were present in 2 cultures. The percentage of GFAP positive cells among the whole cell population was 5% to 10%.

Macrophages

Immunocytochemical studies of primary cultures from PVR or AMD patients showed some cells staining positively with antibody

CD58, indicating that these cells were most probably macrophages. These cells were small, phase-bright cells, often with debris in the cytoplasm, sometimes had short villous processes [2]. Macrophages were isolated cells, without any tendency for growth. They usually disappeared in subcultures. Macrophages appeared in some cultures from PVR and AMD patients.

Fibroblasts

Some spindle-shaped cells appeared in cultures from PVR or AMD patients, they did not stained with any of the antibodies tested in the present studies. These cells most probably were fibroblasts. They usually did not grow or showed limited growth capacity.

Immunostaining with anti-Factor VIII antibodies revealed negative results in 6 primary cultures from PVR patients, 3 subcultures from PVR patients and 4 primary cultures from AMD patients. Antibodies for desmin also revealed negative results in 6 cultures from PVR patients and in 3 cultures from AMD patients. This indicates that vascular endothelial cells and smooth muscle cells were not the significant components of the cultured cells from retinal membranes in the present study.

Based on the morphological and immunocytochemical studies, the growth pattern of cell cultures from various diseases could be summarized as follows:

- In cultures from PVR patients, both RPE and glial cells were present in most cultures. The major component of outgrowing cells was RPE. RPE were the only actively growing cells in most cultures. In subculture, glial cells disappeared gradually, becoming pure cultures of RPE.
- In cultures from AMD patients, cell outgrew from few cultures. Cell types included RPE, glial cells and cells not stained by any of the antibodies used here. Cells in most cultures did not grow and none of the cultures grew to confluence.

Discussion

PVR occurs in eyes with rhegmatogenous retinal detachments and accounts for a significant number of failures following retinal reattachment surgery. The introduction of various vitreous surgical techniques has improved the success rate of surgery for PVR during the past decades. Despite these advances, a definite proportion of PVR patients still have a poor prognosis [7,17-20]. The main cause of failure is reepithelialization of cells and regrowth of retinal membranes, resulting in recurrent retinal detachment [18-20]. Therefore, studying the pathobiology of the cellular components of pre- and sub-retinal membranes, and subsequently developing methods for preventing the reepithelialization of cells are important goals for improving prognosis in PVR patients. In PVR, pathological studies of surgically excised membranes have demonstrated RPE, glial cells, fibroblasts and macrophages [21-31]. Most authors, based on the pathological studies and experimental animal model studies, suggest that RPE are the main cell component of these membranes and that they play an important role in the pathogenesis of PVR [2,6,7,20,23,26,29-32]. Some authors have stated that glial cells are important in the pathogenesis and constitution of pre- and sub-

retinal membranes [3,4,26,33]. In the culture of cells from surgical excised membranes from PVR or AMD patients, only these cells with good viability and capacity of migration can derive from the explants. In these cells, some cell types can proliferate and increase, some of them only survive but not proliferate and yet others fail to survive in the culture. After confluence is reached in the primary culture, cells should be subcultured. With each subculture, the cell component with the ability to proliferate most actively and rapidly will gradually predominant, and none or slowly proliferate cell types will be diluted and eventually being eliminated. After several subcultures, the culture becomes more stable, and contains only the rapid proliferate cells – a pure culture. Therefore, the continuous subculture of cells derived from the specimens is the appropriate procedure that able to find the most important proliferated cell types. Previous studies in this field only restricted to the primary culture and the present study is the first report that subcultured various cell types until senescence to find the predominate cell types in the PVR patients. In the present study, both RPE and glial cells outgrew from the explanted membranes. Based on morphologic and immunocytochemistry studies, RPE appeared in all cultures and accounted for 50% to 100% cells among the whole cell populations. Glial cells appeared in 75% cultures and accounted for about 30% of whole cell populations. However, the growth capacity of these two cell types was quite different. RPE grew very actively and constituted the main cell type in both primary culture and subculture. Actually, RPE were the sole cell type in subcultures passaged more than two generations. Glial cells did not grow or showed very limited growth capacity, and usually disappeared in subculture. Therefore, our study suggested that the main proliferating cell type in PVR is RPE cells rather than glial cells. This result is consistent with many studies on the pathogenesis of PVR both *in vivo* and *in vitro* [2,6,7,18-20,23,26,29-32]. Development of a minimally cytotoxic and powerful growth inhibiting substance for RPE in a drug-delivery system that allowed constant release over a long time period would be helpful for improving prognosis in PVR patients. Our *in vitro* study of RPE cells isolated from excised membrane for PVR patients revealed that retinoic acid effectively inhibits the proliferation of RPE cells from PVR patients. The following clinical study found that oral 13-cis-retinoic acid had significant effects in the prevention and treatment of PVR [34]. Cytokeratins represents a family of proteins that form intermediate filaments in epithelial cells. Composition of cytokeratin is highly heterogeneous and varies depend on epithelial cell type, stage of differentiation, disease state, and cellular growth environment [35]. The RPE express cytokeratin both *in vivo* and *in vitro*; the cytokeratins expressed by RPE include 5, 6, 7, 8, 14, 15, 16, 17, 18, and 19 [36,37]. Some authors report expression of cytokeratin 18 may be related to RPE proliferation [36] while others link cytokeratin 7 and 19 with RPE proliferation [37]. In the present study, RPE stained positively with various anti-cytokeratin antibodies; positivity rates decreased thusly: AE-3 (cytokeratin 1, 2, 3, 4, 5, 6, 7, 8) > cytokeratin 6 & 18 > cytokeratin 7 > AE-1 (cytokeratin 10, 14, 15, 16, 19) > cytokeratin 19. Cytokeratin 14 stains were always negative. This indicates that for identification of cultured RPE from excised retinal membranes with anti-cytokeratin antibodies, AE-3 and antibodies for cytokeratin 6 & 18 have the highest sensitivity. In the present study, no relationship could be established in the types of cytokeratin expressed and the confluence rate. AMD is the leading cause of irreversible central vision loss in North America in people over 65 years of age. Clinical and pathological studies, combined with experimental animal studies, indicate that damage to or senescence of the RPE plays an important role in the pathogenesis of AMD [38,39].

RPE phagocytose outer segments of photoreceptor discs throughout life. The remnants of incompletely degraded materials accumulate in the RPE as lipofuscin. Progressive engorgement of these residues is associated with dysfunction of RPE, which may lead to the occurrence of AMD. Pathological studies on surgically excised CNV from AMD demonstrated that the principle cellular components of the membrane are RPE, vascular endothelial cells, fibrocytes and macrophages. Less common types of cell include pericytes, myofibroblasts and glial cells. RPE cells in the membranes often displayed intracytoplasmic golden brown, lancet-shaped pigment granules and showed basal laminar deposits between the RPE and its basement membrane. These findings also indicate the RPE in CNV are damaged. Some authors reported proliferative changes of RPE in CNV of AMD, suggesting that this serve as a reparative process to seal off leaking vascular channels. In the present study, RPE in the CNV from AMD showed poor viability and growth capacity; the outgrowth rate was only 31.3%, far less than that of PVR patients (70.7%). In AMD patients, no culture grew to confluence. These results indicate that the pathologic changes of RPE in AMD are degenerative and dysfunction in nature. The presence of RPE in the culture of CNV may be only the result of migration rather than active proliferation. This is also consistent with the fact that the dense pigmentation present in the RPE from CNV is in distinct contrast with the actively growing, less pigmented RPE from PVR patients. Because degeneration and dysfunction of RPE are the prominent features in AMD, various treatments that might protect RPE from aging processes or reduce damage from the genetic, epigenetic or environmental factors may be rational approaches in the treatment of AMD. Furthermore, approaches to restore RPE function in the macular region (e.g., macular translocation, implantation of homogenous RPE cells [40], autologous RPE cells or iris pigment epithelial cells [41,42], RPE cells derived from human embryonic stem cells [43], or autologous induced pluripotent stem-cells [44]) have been studied and used clinically for the treatment of AMD with various therapeutic effects [18-20,40-44].

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