·Basic Research·

Promotion of proliferation and migration of Müller cells by RPE cells in a co-culture system

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Abstract

• AIM: To investigate the role of retinal pigment epithelium (RPE) in the growth of Müller cells using a co-culture system *in vitro*.

• METHODS: Müller cells were co-cultured with RPE cells under both normoxic and hypoxic conditions in Transwell chamber culture system. Müller cell proliferation was evaluated by MTT assay. The number of cells that migrate through micropores and stay on the outer bottom side of insert systems were observed and counted.

• RESULTS: The activities of proliferation and migration of Müller cells when co-cultured with RPE cells were significantly higher than those of the Müller cells when cultured alone at all time points under both normoxic and hypoxic conditions. However, for both the co-culture and control groups, there was no significant difference between the measurements at 3 and 6 hours.

• CONCLUSION: Evidence suggests that RPE, when co-cultured with Müller cells, can stimulate migration and proliferation of Müller cells under both hypoxic and normoxic conditions in a time-dependent manner; however, there is no evidence to support the synergetic interaction of RPE and Müller cells co-cultured under hypoxic conditions.

• KEYWORDS: Müller cells; retinal pigment epithelium; co- culture; hypoxia; normoxia; Transwell system

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INTRODUCTION

Müller cells and retinal pigment epithelium cells (RPE) play very important roles in the maintenance of visual function, and they are involved in many pathological processes. For example, experiments conducted to examine the effects of proliferative vitreoretinopathy (PVR) showed that the proliferation of Müller cells and RPE resulted in the formation of proliferative membrane. In the present study, Müller cells were co-cultured with RPE under both normoxic and hypoxic conditions to observe the effect of RPE on the proliferation and migration of Müller cells.

MATERIALS AND METHODS

Cell Culture Newborn pigmented rabbits (8-10 days old, provided by Experimental Center of Harbin Medical University) were used for primary culture. Five rabbits were anesthetized and sacrificed, after which the eyeballs were aseptically removed.

Müller cells culture The eyeballs were dissected along the ora serrata. Lens and vitreous body were removed. Then the pigment epithelium was removed from the neurosensory retina, and the latter was cut into small pieces. The retinal tissue was cultured in a dish with 5mL Dulbecco's Modified Eagle Medium (DMEM), supplemented with 200mL/L fetal bovine serum (FBS), at 37℃ with 50mL/L CO_2. About 7 days later, the tissue block adhered to the wall, and cells began to migrate out of the margin of the block. The obtained cells were cultured as previously described [1]; subsequently they were immunocytochemically identified by two specific markers, GFAP antibody and S-100 antibody. The second-to-fourth-passage RPE cells were used in these studies.

RPE culture The anterior segment and lens were separated. Afterwards, the neural retina and any remaining vitreous body were removed. The remaining optic cup was washed with phosphate-buffered saline (PBS), and a solution of trypsin (2.5g/L) was applied to the cup for 30 minutes at 37℃. The cells were then gently scraped off the posterior layer of the optic cup into the trypsin solution, and an equal volume of DMEM supplemented with 100mL/L FBS was added to neutralize enzyme activity. The final cell suspension was transferred to a 10mL centrifuge tube and spun at 1 000r/min for 5 minutes. The supernatant was

discarded and the cells were resuspended in DMEM, supplemented with 100mL/L FBS, then transferred into culture dishes at 37℃ with 50mL/L CO_2[2]. The homogeneity of cultured RPE cells was confirmed by mAb to cytokeratins. The second-generation cells were used for experiment.

Assay for Proliferation of Müller Cells Two assays were performed to observe the proliferation of Müller cells. One detected the change of Müller cells at various time points; another detected the change of Müller cells under various conditions. Hypoxia was achieved using $CoCl_2$ at a concentration of 200μmol/L.

Assay for various time points Transwell system (0.4μm) was used to develop a co-culture system [3]. In the co-culture group, the RPE (200μL, 5×10^4/mL) were cultured in the upper wells(insert), while the Müller cells(500μL, 5×10^4/mL) were cultured in the lower wells. As controls, there were no cells cultured in the lower wells [4]. In both the co-culture group and control group, Müller cells were measured by MTT at various time points (3, 6, 24 and 48 hours). Absorption was measured by a scanning multiwell spectrophotometer at 570nm, recorded as A value. There were five separate experiments, each performed in duplicate at each time point; therefore, 20 wells were used for each group.

Assay for various conditions Transwell system (0.4μm) was used to develop a co-culture system. In the co-culture group, the RPE cells (200μL, 5×10^4/mL) were cultured in the upper wells (insert), while the Müller cells (500μL, 5×10^4/mL) were cultured in the lower wells. There were no cells cultured in the upper wells in the Müller only group. The proliferation of Müller cells was measured by MTT under various conditions at 24 hour. There were five separate experiments carried out in duplicate under different conditions.

Assay for Migration of Müller Cells Two assays were performed to observe the migration of Müller cells. One was conducted to detect the change of Müller cells at various time points, and the other was conducted to observe any change of Müller cells under various conditions. Hypoxia was achieved by use of $CoCl_2$, with a concentration of 200μmol/L.

Assay for various time points Transwell system (8μm) was used to develop a co-culture system. In the co-culture group, the Müller cells (200μL, 5×10^4/mL) were cultured in the upper wells(insert), while the RPE cells(500μL, 5×10^4/mL) were cultured in the lower wells. As controls, there were no cells cultured in the lower wells. The average number of cells under three observed fields was calculated under a phase-contrast microscope ×400. There were five separate experiments carried out in duplicate at each time point; therefore, there were 20 wells for each group.

Assay for various conditions Transwell system (8μm) was used to develop a co-culture system. In the co-culture group, the Müller cells (200μL, 5×10^4/mL) were cultured in the upper wells (insert), while the RPE cells (500μL, 5×10^4/mL) were cultured in the lower wells. There were no cells cultured in the lower wells in the Müller only group. The number of cells that migrated through micropores and stayed on the outer bottom side of the insert system were calculated at 24 hour under a phase-contrast microscope ×400. In addition, the average number of cells under three observed fields was calculated under a phase-contrast microscope × 400. A total of five separate experiments were carried out in duplicate under different conditions.

Statistical Analysis Statistical analysis was performed using SPSS13.0 statistical software. Repeated ANOVA was applied to data collected at various time points.

Time was treated as the within-subject factor and the group as the between-subject factor. Factorial experiments were employed to analyze the data of various conditions. The level of significance was set at P=0.05.

RESULTS

Cell Culture On day seven of primary culture, the tissue blocks were observed to be sticking to the wall. Several pseudopodiums had developed and gradually crept out of the tissue block to form a single cell. On day 14, more than 80% fusion cells were found. More than 95% subcultured cells showed positive staining results using GFAP or S-100 immunohistochemistry. Under transmission electron microscope (TEM), cells presented enriched cytoplasm, large nuclei and 8-10nm characteristic mid-silks. RPE cells displayed intense pigmentation and presented cobblestone morphology. More than 95% of the cells showed positive staining by cytokeratin immunocytochemistry. On the basis of morphologic appearance and the expression of yellow-brown through cytokeratin immunocytochemistry, the RPE cells were confirmed.

Proliferation of Müller Cells Proliferation of Müller cells in the control group and the co-culture group was detected by MTT at various time points. The MTT results are shown by A values in Table 1. Neither the difference between 3 and 6 hours nor between 24 and 48 hours was statistically significant. Nevertheless, there was statistically significant difference among other time points. Statistically significant difference between the control group and the co-culture

Table 1 *A* **values of the control group and the co-culture group at various time points** (n=5)

	3h	6h	24h	48h
Control group	0.256±0.023	0.284±0.016	0.52±0.071	0.52±0.066
Co-culture group	0.32±0.045	0.342±0.0626	0.618±0.1379	0.638±0.1184

Note: 3h *vs* 6h and 24h *vs* 48h, $P>0.05$; among other time points, $P<0.05$; control group *vs* co-culture group, $P<0.05$

group was found.

A 2×2 factorial design study (condition [CO]: normoxia, hypoxia; culture [CU]: Müller cells and RPE, Müller cells only) showed that the main effects of both CO and CU were significant for proliferation of Müller cells, whereas the interaction term (CO×CU) was not significant. The MTT results under different culture conditions are shown by *A* values in Table 2 and Figure 1.

Migration of Müller Cells The number of Müller cells migrating through micropores and staying on the outer bottom side of insert systems at different time points are given in Table 3. Statistically significant difference was found among four different time points, except between 3 and 6 hours time points. There was statistically significant difference between the control group and the co-culture group.

A 2×2 factorial design study (culture [CU]: Müller cells and RPE, Müller cells alone; condition [CO]: normoxia, hypoxia) showed that the main effects both of CU and CO were significant for migration of Müller cells, whereas the interaction term (CU ×CO) was not significant. The migration of Müller cells in the factorial design is shown in Table 4 and Figure 2.

DISCUSSION

Müller cells and RPE cells are widely distributed in the retina. When the retina is damaged by mechanical or chemical stimuli, RPE cells migrate across Bruch's membrane to fill the lesion with subsequent scar formation. Müller cells and astrocytes replace the damaged outer nuclear layer of the retina, interdigitating with the migrated RPE cells. Müller cells undergo widespread and long-lasting changes after the lesion, including increased expression of glial fibrillary acidic protein associated with hypertrophy migration and scar tissue formation [5]. The normal ability of RPE cells to secrete growth factors can protect the retina from damage; however, irregular growth factor secretion can also be involved in the pathogenesis of retinal diseases. Interestingly, RPE cells produce various growth factors that may induce the mitogenic effects of RPE cells.

Jaynes *et al*[6] reported that conditioned media of RPE could promote the proliferation of Müller cells under normoxia. In addition, RPE, which is a major source of VEGF, can secret several molecules, including VEGF, basic fibroblast growth factor (bFGF), and angiopoietin-1. Under hypoxia, RPE increases the levels of VEGF mRNA and protein synthesis. In contrast, RPE cells cultured under hypoxic conditions show reduced steady-state levels of bFGF mRNA and decreased bFGF protein synthesis[7].

Since hypoxia is a critical process in most pathogenesis,we

Table 2 *A* **values of Müller cell proliferation in factorial design study** (n=5)

Normoxia		Hypoxia	
Müller&RPE	Müller alone	Müller&RPE	Müller alone
0.718±0.1028	0.60±0.0308	0.618±0.1379	0.52±0.071

Main effects of condition, $P<0.05$; main effects of culture, $P<0.05$; interaction term of condition×culture, $P>0.05$

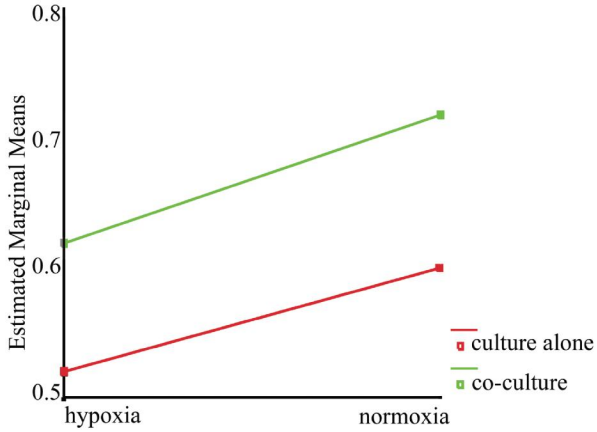

Figure 1 Estimated marginal means of absorbance

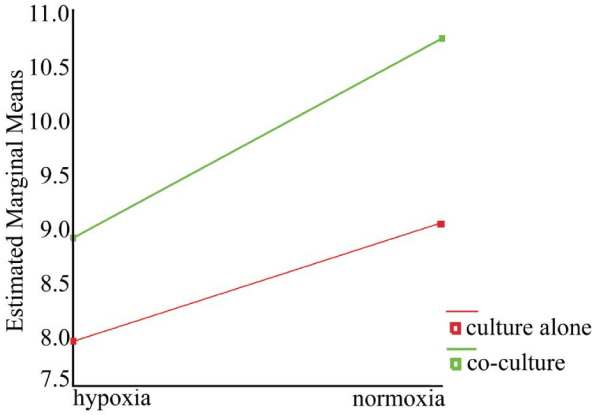

Figure 2 Estimated marginal means of cell counting

Table 3 Number of Müller cells migrating in the control group and the co-culture group at various time points (n=5)

	3h	6h	24h	48h
Control group	4.4±1.181	4.92±1.134	8.52±0.0.5975	10.54±1.899
Co-culture group	3.94±0.251	5.14±0.702	10.08±1.2194	12.92±1.6843

3h *vs* 6h, $P>0.05$; among other time points, $P<0.05$; control group *vs* co-culture group, $P<0.05$

Table 4 Migration of Müller cells in a factorial design (n=5)

Normoxia		Hypoxia	
Müller&RPE	Müller alone	Müller&RPE	Müller alone
10.74±1.09	9±0.7071	8.88±1.107	7.92±1.7138

Main effects of condition, $P<0.05$; main effects of culture, $P<0.05$; the interaction term of condition × culture, $P>0.05$

are interested in knowing whether Müller cells migrate and/or proliferate after being promoted by RPE under both normoxic and hypoxic conditions. In this study, we first used the Transwell chamber system to detect the effect of RPE on Müller cells. Compared to the conditioned media of RPE, the Transwell chamber system can more closely simulate in vivo conditions and thus reveal dynamic changes during the interaction of RPE and Müller cells. Next, we carried out experiments to observe the effect of RPE on Müller cells at various time points and under various culture conditions, as noted above. We expected that co-cultured RPE could stimulate the migration and proliferation of Müller cells under both hypoxic and normoxic conditions in a time-dependent manner. Considering that both RPE and hypoxia are independent factors stimulating the migration and proliferation of Müller cells, it was intriguing to find that these two factors did not combine in a synergetic manner to produce that result.

In summary, we have demonstrated that hypoxia and RPE co-culture are important mediators of migration and proliferation of Müller cells in a time-dependent manner. Previous studies have demonstrated that hypoxia could induce up-regulation of VEGF, which may partially explain the mechanism of these effects on Müller cells. However, the mechanisms underlying this phenomenon are unclear. Many of the effects of VEGF are mediated by other factors, and the hypoxia that induces VEGF production can initiate a cascade of factors. Since there is a significant difference between two time points, we proposed that some growth factor (s) may be released during this period. This study sheds some insights on the proliferative diseases in the retina. Further work is needed to elucidate the exact mechanisms involved in the pathogenesis.

REFERENCES

1 Song E, Yang W, Cui ZH, Wu JX. Effects of high-concentration insulin on expression of vascular endothelial growth factor in cultured Müller cells *in vitro*. *Int J Ophthalmol(Guoji Yanke Zazhi)* 2004;4(2):201-205

2 Feng W, Zheng JJ, Lutz DA, McLaughlin BJ. Loss of RPE phenotype affects phagocytic function. *Graefe's Arch Clin Exp Ophthalmol* 2003;241(3):232-240

3 Wang YL, Hui YN, Guo B, Zhang XG, Hou X, Ma JX. Inhibition of proliferation of retinal microvascular endothelial cells by pericytes through down-regulating KDR/FIK-1 in a co-culture system. *Int J Ophthalmol (Guoji Yanke Zazhi)* 2006;6(2):256-263

4 Allenbach C, Zufferey C, Perez C, Launois P, Mueller C, Tacchini-Cottier F. Macrophages induce neutrophil apoptosis through membrane TNF, a process amplified by Leishmania major. *J Immunol* 2006;176(11):6656-6664

5 Tretiach M, Madigan MC, Gillies MC. Conditioned mediun from mixed retinal pigmented epithelium and Müller cell cultures reduces *in vitro* permeability of retina vascular endothelial cells. *Br J Ophthalmol* 2004;88(7):957-961

6 Jaynes CD, Turner JE. Isolation of a retinal pigment epithelial cell-derived fraction which promotes Müller cell proliferation. *Brain Res Dev Brain Res* 2000;20(2):267-271

7 Strauss O. The retinal pigment epithelium in visual function. *Physiol Rev* 2005;85(3):845-881