·Basic Research·

Optimization of culture medium for primary retinal pigment epithelium cells and investigation of medium effects on growth factor expression

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Abstract

• AIM: To optimize the conditions for *in vitro* culture of retinal pigment epithelium (RPE) cells, we characterized expressions of various growth factors in RPE cells, including tumor necrosis factor (TNF-α), vascular endothelial growth factor (VEGF), β fibroblast growth factor (β FGF), transforming growth factor β 2 (TGFβ 2), and interferon-γ (IFN-γ). We also studied expressions of caspase-3 under different concentrations of fetal bovine serum (FBS) with insulin-transferrin-sodium selenite (ITS) supplement.

• METHODS: First, we investigated if the expressions of TNF-α, VEGF, β FGF, TGFβ 2, IFN-γ, and caspase-3 in FBS and ITS with of concentration. Second, we cultured primary RPE cells from eyes of forty C57 BL/6 mice in standard dulbecco's modified eagle's medium (DMEM) containing 20, 40, 100mL/L FBS and 20, 40, 100mL/L FBS together with 10g/L ITS. Immunohistochemical staining and cell counting were performed to verify the existence and growth condition of RPE cells. The expressions of TNF-α, VEGF, β FGF, TGFβ 2 and IFN-γ were determined using cells and supernatant from passage-3 to -4 primary RPE cell after 48 hours of culture with RT-PCR and enzyme-linked immunosorbent assays (ELISA). The expression of casepase-3 was determined via Western blotting. The major outcome measurement was the expression level of growth factors in cultured RPE cells and the experiment design was to expose the RPE cells to different culture medium.

• RESULTS: TNF-α, VEGF, β FGF, TGFβ 2, but not IFN-γ, were expressed and the expressions increased with concentration. No expression of the aforementioned genes was detected in presence of ITS. The primary cultures of RPE cells were successfully established. TNF-α, VEGF, β FGF, TGFβ 2 (but no IFN-γ) and the active caspase-3 were detected in 20, 40, 100mL/L FBS or 20, 40, 100mL/L FBS combined with 10g/L ITS; the expressions were upregulated with increasing concentration of FBS. There was no significant difference in the expression of growth factors between these groups. However, significant differences were shown among different concentration of FBS ($P < 0.01$). The lowest expression was observed in 20mL/L FBS or 20mL/L FBS combined with 10g/L ITS medium with RPE cells. But RPE cells were shown in better growth condition in 20mL/L FBS combined with 10g/L ITS.

• CONCLUSION: TNF-α, VEGF, β FGF, TGFβ 2 and caspase-3 are expressed in RPE cells and supernatants. The production of above 20mL/L FBS combined with 10g/L ITS in DMEM may be the ideal cell culture medium that supports the normal growth of RPE cells.

• KEYWORDS: retinal pigment epithelial cells; growth factors; caspase-3; culture

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INTRODUCTION

Retinal pigment epithelial (RPE) cells play a fundamental role in many normal biological processes, including absorption of light adjacent to the outer segments of the photoreceptors, storage and conversion of vitamin A esters, formation of an acid mucopolysaccharide complex that surrounds the outer segments of rods and cones, as well as phagocytosis of rod and, to a lesser extent, cone outer segments. There are a number of retinal disorders in which dysfunction of the RPE is the key to the pathogenetic

process [1]. Recent researches on transplantation RPE cells into subretinal space have made great progresses [2-6]. However, problems, such as how to supply healthy RPE cells, are the key to successful transplantation that need to be solved. Recent study has shown that vascular endothelial growth factor (VEGF) is responsible for many ocular pathologies [7], which indicates that growth factors could be one main reason that affects ocular pathophysiological properties. Therefore, we established the primary cultures of RPE cells to investigate if growth factors critical for RPE cell function, such as tumor necrosis factor (TNF-α), vascular endothelial growth factor (VEGF), β fibroblast growth factor (βFGF), transforming growth factor β2 (TGFβ2) and interferon-γ (IFN-γ), as well as casepase-3, are expressed in RPE cells, and the ideal medium for the growth of normal RPEs.

MATERIALS AND METHODS

Detecting the Expression of Growth Factors The expression of TNF-α, VEGF, βFGF, TGFβ2 and IFN-γ as well as caspase-3 in groups of 20, 40, 100mL/L fetal bovine serum (FBS) and 10,20,30g/L insulin-transferrin-sodium selenite media supplement (ITS) without RPE cells and Dulbecco'S modified Eagle's medium (DMEM) were tested in order to exclude the influence of the RPE cells and medium. Saline were taken as control groups. Then, the medium were divided into control (only with DMEM), concentration of 20, 40, 100mL/L groups for the experiment.

Animals and Cell Cultures Forty pathogen-free C57BL/6 mice, aged 2-3 weeks, were used in this study. All animals were treated in accordance with ARVO Statement for the use of Animals in Ophthalmic and Vision Research in U.S. and China. Donor C57BL/6 mice were decapitated and their eyes were immediately enucleated and placed in ice-cold DMEM with 2mmol/L glutamine. The whole eyeballs were incubated for 20 minutes at 37℃ in solutions of 20mL/L disperse concentrations. The eyes were rinsed twice in DMEM and placed in a Petri dish containing fresh DMEM. Under a dissecting microscope, the eyeballs were cut open along the edge of the cornea. After the lenses and irises were removed, the RPE and their attached neural retina were gently separated from the eye cup and transferred to a new Petri dish containing fresh DMEM. After two changes to fresh medium and incubation for 15 minutes at room temperature, the entire RPE sheets were easily separated from the neural retina. Separated RPE sheets were cut into pieces then transferred into culture flasks precoated with 1mg/L fibronectin and grew at 37℃ in a 50mL/L CO_2 atmosphere in DMEM containing 20, 40, 100mL/L FBS and 10g/L ITS. It is the same experiment condition of the FBS and ITS during the whole cell cultures course. Cell counting was used to evaluate RPE cell growth. Passage 3 to 4 RPE cells and their supernatants after being cultured for 48 hours were used and all these cells were routinely stained with antibodies against a broad range of epidermal keratins (AE1/AE3; RDI, Flanders, NJ07836) for determination of their epithelial origins. All cell culture material and chemicals were purchased from Invitrogen (Carlsbad, CA).

Characterization of the Primary Culture by Immun-ocytochemistry RPE cell cultures were washed in PBS and fixed in 40g/L paraformaldehyde (PFA) at room temperature (RT) for 15 minutes. Subsequently, nonspecific binding was blocked with normal sheep serum (Serotec, Raleigh, NC) at RT for 30 minutes. The primary antibody (mouse anti-cytokeratin: RDI, PRO 61835: 1:100) used to characterize the cells was diluted in PBS with 0.1% Tween 20 and incubated for 60 minutes at RT. After the cells were washed three times for 5 minutes each in PBS with 0.1% Tween 20, the secondary antibody (sheep anti-mouse-Cy3; Sigma, C-2181; 1:400) was applied for 30 minutes. Subsequent to repeat washing, numbers of cells in four random visual fields were counted and numbers of positive cells were used to calculate the percent positive. Universal negative control mice (Dako, NP015) were use for negative control: Specific binding were visualized under fluorescence optics (Olympus, Melville, NY). Cell morphologies were characterized by phase-contrast microscopy.

Determination of TNF-α, VEGF, βFGF, TGFβ2 and IFN-γ Expression by RT-PCR Total RNA (1μg) which was isolated from RPE cells in different concentration of FBS using SV. Total RNA Isolation System kits (Promega, Madison, WI, USA) and PCR kit (Applied Biosystems, CA) were used for RT-PCR to detect the mRNA levels of β-actin, TNF-α, VEGF, βFGF, TGFβ2 and IFN-γ. The negative controls consisted omission of RNA or reverse transcripts from the reaction mixture. PCR products were quantified by densitometery, using the Molecular Analyst/PC program (Bio-Rad, Hercules, California). RT-PCR was carried out with the following primers: β-actin (F-5'-GCC ACC AGT TCG CCA TGG ATG A -3', R-5'-GTC AGG CAG CTC ATA GCT CTT C-3');TNF-α (F-5'-TCT CAT

CAG TTC TAT GGC CC-3', R-5'-GGG AGT AGA CAA GGT ACA AC-3')[8]; VEGF (F-5'-GCG GGC TGC CTC GCA GTC-3', R-5'-TCA CCG CCT TGG CTT GTC AC-3'); βFGF (F-5'-AGC GGC TCT ACT GCA AGA AC-3', R-5'-TCG TTT CAG TGC CAC ATA CC-3'); TGFβ2 (F-5'-CCA AAG ACT TAA CAT CTC CCA CC-3', R-5'-GTT CGA TCT TGGGCGTATTTC-3'); IFN-γ (F-5'-GCATCTTGGCTTTGCAGCTC-3'-R-5'-CGACTACT TTTCCGCTTCCT-3')[9]; For TNF-α: The amplification consisted of 50 cycles of denaturation for 1 minute 45 seconds at 95℃, annealing for 45 seconds at 61℃, and extension for 7 minutes at 72℃: For VEGF: annealing for 45 seconds at 60℃, 32 cycles; For βFGF: annealing for 45 seconds at 60℃, 30 cycles; For TGFβ2: annealing for 45 seconds at 60℃, 25 cycles; For IFN-γ: annealing for 45 seconds at 60℃, tried 25-60 cycles. Other amplification conditions were the same as TNF-α.

Determination of TNF-α, VEGF, βFGF, TGFβ2 and IFN-γ Protein by Enzyme-linked Immunosorbent Assay The levels of TNF-α, VEGF, βFGF, TGFβ2 and IFN-γ were measured in supernatant of RPE cells under different concentration of FBS with ITS media supplement using enzyme-linked immunosorbent assay (ELISA) according to manufacturer instructions as follows: the supernatant of RPE cells were collected at passage 3 to 4 primary RPE cell cultures and centrifuged for 10 minutes to remove cells and other large debris, aliquoted, and immediately stored at -20℃ until assay for TNF-α, VEGF, βFGF, TGFβ2 and IFN-γ production by ELISA (Quantikine Kits MTA00 for TNF-α, MMV00 for VEGF, DFB50 for βFGF, DB250 for TGFβ2, MIF00 for IFN-γ respectively, from R&D Systems). Frozen cell culture medium (background for assay) was used as negative control for ELISA measurements. In addition, unspecific color production by plastic was quantified by a control provided by the supplier (control kit component).

Determination of Casepase-3 Protein by Western Blotting Method The RPE cells were homogenized and solubilized in ice cold PBS containing protease inhibitors, phenylmethylsulfonyl fluoride (1mg/L), aprotinin (1mg/L), leupeptin (1mg/L), pepstatin A (1mg/L) and EDTA (1mmol/L). The homogenate was centrifuged at 15 000r/min at 4℃ for 10 minutes. The protein content of the supernatants was determined by the Bradford method[10]. After sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% linear slab gel under reducing conditions, separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using a semidry electrophoretic transfer cell (Trans-blot; Bio-Rad, Richmond, CA). Blot was stained at room temperature with a 1:600 dilution of monoclonal mouse anti-casepase-3 antibody over night at 4℃. After washing and incubation with horseradish peroxidase-conjugated secondary antibody (1:1 000 dilution), blot was developed using the enhanced chemiluminescence Western blotting analysis detection system (ECL Plus; Amersham Pharmacia Biotech, Arlington Heights, IL).

Table 1 Comparison with concentrations of TNF-α, VEGF, βFGF, TGFβ2 among various groups (mean±SD)

	TNF-α	VEGF	βFGF	TGFβ2
Control	3.21±4.86	3.37±4.18	1.01±0.67	2.97±2.61
20	36.27±5.02	21.46±4.12	20.09±0.54	60.31±2.91
40	272.99±4.74	98.63±3.76	115.95±0.78	358.87±3.23
100	518.91±4.38	195.23±3.85	219.68±0.96	800.45±3.10

Statistical Analysis All experiments were performed three times and results are shown as mean ±SD. Statistical significance were determined by one-factor analysis of variance (ANOVA) followed by Fisher post hoc test for multiple comparisons. $P<0.05$ was considered significant.

RESULTS

Expression of Growth Factors without RPE Cells and Medium There were upregulation of TNF-α, VEGF, βFGF, TGFβ2 expression in presence of increasing FBS and no expression of TNF-α, VEGF, βFGF, TGFβ2 among 10, 20, 30g/L ITS groups without RPE cells and medium.

Cell Cultures and Immunocytochemistry Primary cultures of RPE cells were successfully established. Clusters of flattened, polygonal cells in culture RPE cells (5×10^5 cells/well) for 5 days with 20, 40, 100mL/L FBS and 20, 40, 100mL/L FBS combined with 10g/L ITS as well as the cytokeratin positive RPE cells were observed with epithelial shape (Figure 1). There was no definite difference among the shape of RPE cells and number of cytokeratin-positive cells by cell counting in those groups. TNF-α, VEGF, βFGF, TGFβ2 (but no IFN-γ) and caspase-3 activity were expressed and upregulated in increasing concentration of FBS (20, 40, 100mL/L), or increasing FBS combined with 10g/L ITS. There were no distinguishing differences in expression levels of different growth factors between these groups, but distinguishing differences were shown among different groups ($P<0.01$, Table 1). The lowest expression

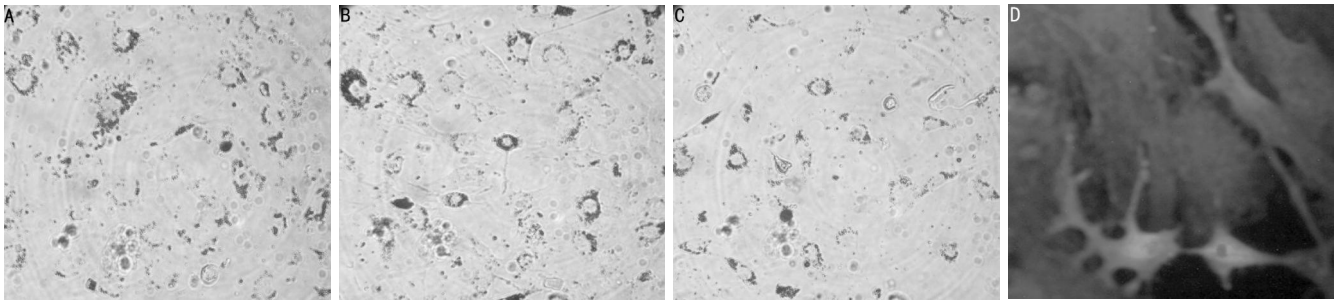

Figure 1 RPE cells cultured for 5 days (×100) A: 20mL/L FBS+ITS; B: 40mL/L FBS+ITS; C: 100mL/L FBS+ITS; D: Cytokeratin

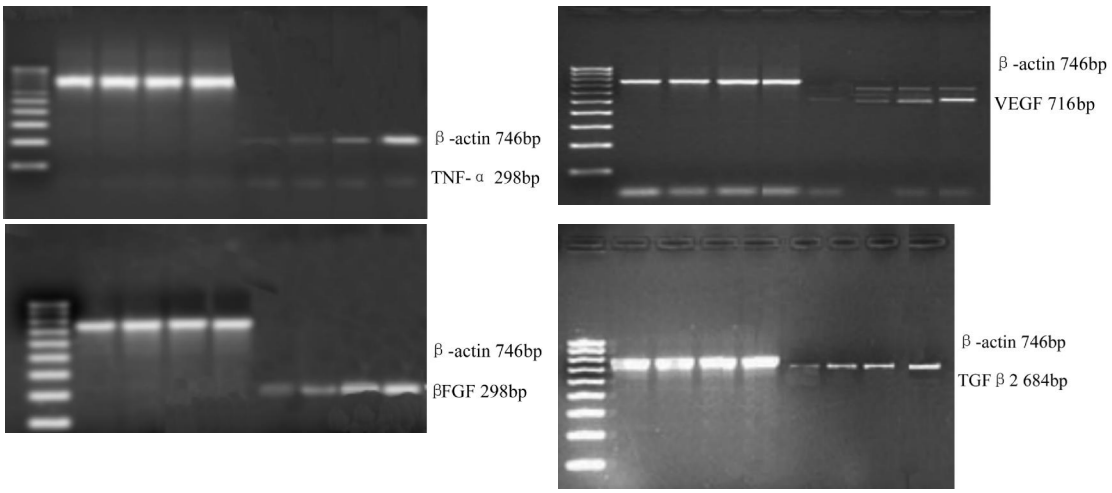

Figure 2 TNF-α, VEGF, βFGF and TGFβ2 levels in RPE cells (RT-PCR)

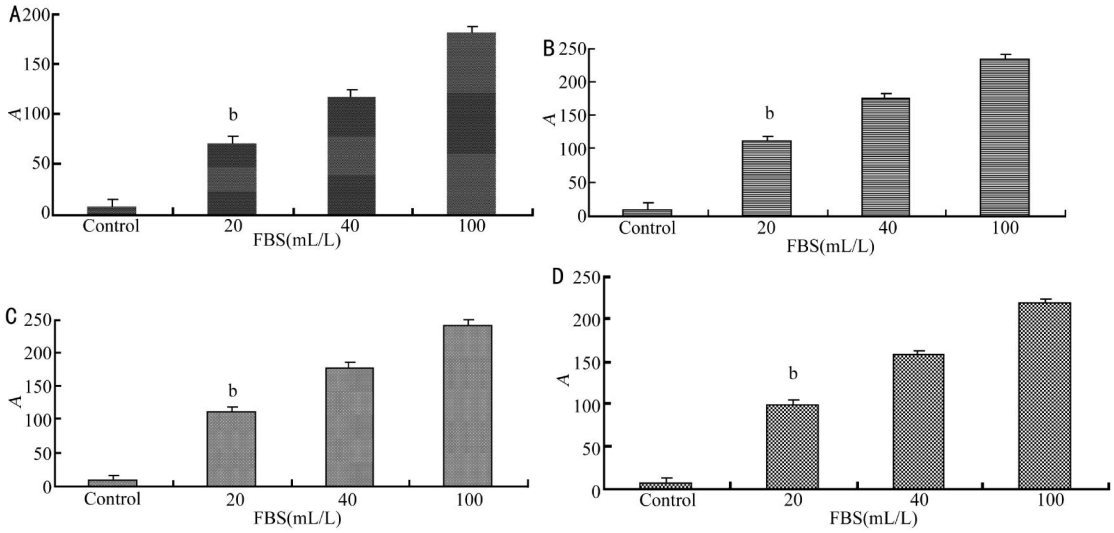

Figure 3 mRNA in RPE cells A:TNF-α; B:VEGF; C:Bfgf; D:TGFβ2. $^{b}P<0.01$ *vs*control 40, 100mL/L FBS

could be seen in RPE cells 20mL/L FBS and 20mL/L FBS combined with ITS medium. In contrast, RPE cells were shown in better growth condition in 20mL/L FBS combined with 10g/L ITS medium RPE.

RT-PCR and ELISART-PCR and ELISA were performed to semi-quantify levels of TNF-α, VEGF, βFGF, TGFβ2 and IFN-γ in the RPE cells and supernatant. With increased concentrations of FBS, the expression of these growth factors showed a progressive increasing (Figure 2,3; $P<0.01$). We also confirmed that there was no compensatory expression of IFN-γ in RPE cells and supernatant.

Western Blotting TNF-α, VEGF, βFGF, TGFβ2 (but no expression of IFN-γ) and caspase-3 were expressed in RPE cells and expressions were upregulated in increasing concentration of FBS (20, 40, 100mL/L) or increasing FBS combined with 10g/L ITS. Western blotting analysis only showed a faint band of casepase-3 in the control group. A weak to strong expression of casepase-3 was observed in

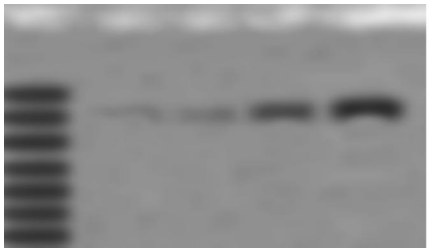

Figure 4 Casepase -3 levels in RPE cells western -blott increasing

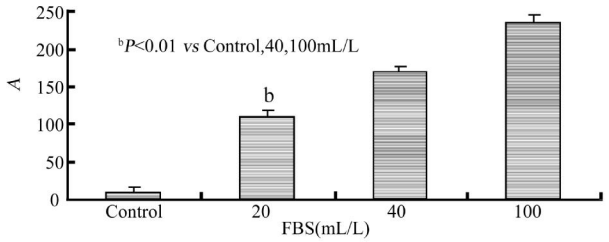

Figure 5 Casepase-3 expression in RPE cells

RPE cells in different concentration of FBS, the production of which are reduced greatly with the medium containing 20mL/L FBS and 20mL/L FBS combined with 10g/L ITS in DMEM (Figure 4). Computer photo analysis indicated that there were significant differences among three groups ($P<$ 0.01, Figure 5; the experiment was performed in triplicate).

DISCUSSION

We found expressions of TNF-α, VEGF, βFGF, TGFβ2, casepase-3 but not IFN-γ in the RPE cells and supernatants as previously reported. The key point for this study is to optimize cell culture medium that supports normal growth of RPE cells. TNF-α is produced mainly by activated macrophages and T cells but may also be made by residual corneal cells [7,8]. In the eye, TNF-α activity is associated with uveitis [9,10] and the corneal response to various type of injury and is related to corneal allograft survival [11]. Recently, researchers reported that exotic TNF-α can induce apoptosis in human RPE cells[12] and play a role in regulation of proliferative vitreoretinopathy (PVR) and choroidal neovascular membranes (CNVMs), as well as regulation of RPE functions [12,13]. VEGF is a multifunctional cytokine that is related to angiogenesis and the accumulation of peritoneal fluid in a variety of physiologic and pathologic conditions. It is produced by a wide range of normal and neoplastic cells, including endothelial cells, smooth muscle cells, fibroblasts, inflammatory cells, and various cancer cells [14]. In the eye, the VEGF is known to be constitutively expressed in the normal corneal epithelium and to be upregulated after trauma. Several studies have demonstrated a role for VEGF in corneal neovascularization (NV) in various animal models of ocular surface inflammation [15-17]. It is related to diabetic macular edema, age-related macular degeneration (AMD)[18,19] and play an important role in the occurrence of PVR[20] and choroidal neovascularization (CNV)[21]. There is close relationship between βFGF and TGFβ2. TGFβ2 has been shown to have numerous diverse biologic effects on βFGF. TGFβ2 is the most potent growth inhibitory polypeptides known for a wide variety of cell types including most epithelial cells, endothelial cells, most lymphoid cells, and many myeloid cells [22]. In the eye, TGFβ2 and βFGF are elevated after laser-induced CNV in C57BL/6 mice [23]. Otherwise, βFGF has been identified as a factor capable of exacerbating the cataractogenic effects of TGFβ2. Thus, βFGF inhibitors, as well as TGFβ2 inhibitors, have the potential to protect the lens against TGFβ2-induced cataractous changes [24] as well as stimulated connective tissue growth factor expression during corneal myofibroblast differentiation, *etc* [25] IFN-γ is a lymphocyte cytokine with broad biologic effects [8]. Like wise, It is also produced in some part of the eyes [26,27], which is not observed in RPE cells and supertanants.

Realizing the detrimental role of most growth factors, more efforts have been made to look for the ideal medium for culturing these cells[28,29]. Our previous study[30] showed that the posterior part of the eye is not absolutely immunologically privileged and that rejection is a serious problem in human retinal transplantation. Many questions concern transplantation except technique. In present study, the expression of TNF-α, VEGF, βFGF, TGFβ2 in RPE cells increases with the increased concentrations of FBS. Given that FBS was the origin of the complement, we can infer that complement in FBS played an important role in the experimental culture conditions critical for the expression of growth factors. Barald *et al* [31] reported no neuron-like cells were found in liver-, notocord-, or neural tube-conditioned media if FBS was used. Douay *et al* [32] showed the same results. Tolnay *et al* [33] found that complement receptor 2 (CR2) participates in the regulation of B cell responses to antigen. The treatment of IM-9 B lymphoblastoid cells or Raji Burkitt's lymphoma cells with 10% heat-inactivated fetal bovine serum for 24 hours increased both the CR2 mRNA level and CR2 surface protein expression more than two-fold. However, no change in the CR2 expression level was observed when cells were cultured in serum-free

medium. Leshem *et al* [34] tested various immune functions of lymphocytes growing in medium containing non-treated and heat-inactivated FBS. The data clearly show that heat inactivation of the serum is not mandatory. In some cases, the addition of untreated FBS resulted in elevated response levels while maintaining immune function specialty. These data strongly support our results. ITS is a serum free media supplement and can substitute for FBS as a media supplement. Compared with 40mL/L and 100mL/L FBS in the DMEM, 20mL/L FBS in the medium showed decreased expression of growth factors. Therefore, the latter maybe the best choice for the RPE cell culture. Gruber *et al* [35] also showed that compared with the average 17.5% colony formation observed in controls, ITS, TGF-beta1 and ITS with IGF-I significantly increased colony formation (28.4%, 30.4%, and 30.4%, respectively). It is of note that there is no expression of IFN-γ in RPE cells and their supernatants. We speculate that the production of many damaged growth factors overwhelmed its expression. Casepase-3 also played a role in RPE cells and showed the same result. As we all know, caspase-3 is expressed in cells as an inactive 32kDa precursor from which the 17kDa and 11kDa subunits are proteolytically generated during apoptosis [36-38]. The upregulation of caspase-3 activity was detected with different concentrations of FBS, and 20mL/L FBS showed lower apoptosis coinciding with the above four growth factors results.

In summary, we infer that 20mL/L FBS with 10g/L ITS medium could decrease the apoptosis during RPE cell culture, and maybe the ideal medium for the growth of normal RPE.

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REFERENCES

1 Thomas DD, EdwardA J. Biomedical foundations of ophthalmology.1st ed. Lippincott U.S 1992;21:1

2 Del Priore LV, Tezel TH, Kaplan HJ. Survival of allogeneic porcine retinal pigment epithelial sheets after subretinal transplantation. *Invest Ophthalmol Vis Sci*2004;45(3):985-992

3 Enzmann V, Howard RM, Yamauchi Y, Whittemore SR, Kaplan HJ. Enhanced induction of RPE lineage markers in pluripotent neural stem cells engrafted into the adult rat subretinal space. *Invest Ophthalmol Vis Sci*2003;44(12):5417-5422

4 Del Priore LV, Ishida O, Johnson EW, Sheng Y, Jacoby DB, Geng L, Tezel TH, Kaplan HJ. Triple immune suppression increases short-term survival of porcine fetal retinal pigment epithelium xenografts. *Invest Ophthalmol Vis Sci*2003;44(9): 4044-4053

5 McLaughlin BJ, Fan W, Zheng JJ, Cai H, Del Priore LV, Bora NS, Kaplan HJ. Novel role for a complement regulatory protein (CD46) in retinal pigment epithelial adhesion. *Invest Ophthalmol Vis Sci*2003;44(8):3669-3674

6 Del Priore LV, Geng L, Tezel TH, Kaplan HJ. Extracellular matrix ligands promote RPE attachment to inner Bruch's membrane. *Curr Eye Res* 2002;25(2): 79-89

7 Andreoli CM, Miller JW. Anti-vascular endothelial growth factor therapy for ocular neovascular disease. *Curr Opin Ophthalmol*2007;18(6):502-508

8 Keadle TL, Usui N, Laycock KA, Miller JK, Pepose JS, Stuart PM. IL-1 and TNF-alpha are important factors in the pathogenesis of murine recurrent herpetic stromal keratitis. *Invest Ophthalmol Vis Sci*2000;41(1):96-102

9 Davis GS, Pfeiffer LM, Hemenway DR. Expansion of interferon-gamma-producing lung lymphocytes in mouse silicosis. *Am J Respir Cell Mol Bio*1999;20 (4):813-824

10 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-254

11 Ferguson TA, Herndon JM, Dube P. The immune response and the eye: a role for TNF alpha in anterior chamber-associated immune deviation. *Invest Ophthalmol Vis Sci* 1994;35(5):2643-2651

12 Niederkorn JY, Mayhew E, Mellon J, Hegde S. Role of tumor necrosis factor receptor expression in anterior chamber-associated immune deviation (ACAID) and corneal allograft survival. *Invest Ophthalmol Vis Sci*2000;45(8):2674-2681

13 Yang P, McKay BS, Allen JB, Jaffe GJ. Effect of NF-kappa B inhibition on TNF-alpha-induced apoptosis in human RPE cells. *Invest Ophthalmol Vis Sci* 2004;45(7):2438-2446

14 Jin M, He S, Wörpel V, Ryan SJ, Hinton DR. Promotion of adhesion and migration of RPE cells to provisional extracellular matrices by TNF-alpha. *Invest Ophthalmol Vis Sci*2004;41(13):4324-4332

15 Stadlmann S, Amberger A, Pollheimer J, Gastl G, Offner FA, Margreiter R, Zeimet AG. Ovarian carcinoma cells and IL-1β-activated human peritoneal mesothelial cells were possible sources of vascular endothelial growth factor in inflammatory and malignant peritoneal effusions. *Gynecol Oncol* 2005;97 (3): 784-789

16 Shoshani Y, Pe'er J, Doviner V, Frucht-Pery J, Solomon A.Increased expression of inflammatory cytokines and matrix metallo-proteinases in pseudophakic corneal edema. *Invest Ophthalmol Vis Sci*2005;46(6):1940-1947

17 Cursiefen C, Ikeda S, Nishina PM, Smith RS, Ikeda A, Jackson D, Mo JS, Chen L, Dana MR, Pytowski B, Kruse FE, Streilein JW. Spontaneous corneal hem- and lymphangiogenesis in mice with destrin-mutation depend on VEGFR3 signaling. *Am J Pathol*2005;166(5):1367-1377

18 Singh N, Amin S, Richter E, Rashid S, Scoglietti V, Jani PD, Wang J, Kaur R, Ambati J, Dong Z, Ambati BK. Flt-1 intraceptors inhibit hypoxia-induced VEGF expression in vitro and corneal neovascularization *in viva Invest Ophthalmol Vis Sci*2005;46(5):1647-1652

19 Funatsu H, Yamashita H, Sakata K, Noma H, Mimura T, Suzuki M, Eguchi S, Hori S. Vitreous levels of vascular endothelial growth factor and intercellular adhesion molecule 1 are related to diabetic macular edema. *Ophthalmology* 2005; 112(5):806-816

20 Hera R, Keramidas M, Peoc'h M, Mouillon M, Romanet JP, Feige JJ. Expression of VEGF and angiopoietins in subfoveal membranes from patients with age-related macular degeneration. *Am J Ophthalmol*2005;139(4):589-596

21 Mitamura Y, Tashimo A, Ohtsuka K, Mizue Y, Nishihira J. Placenta growth factor and vascular endothelial growth factor in the vitreous of patients with proliferative vitreoretinopathy. *Clin Experiment Ophthalmol*2005;33(2):226-232

22 Kinose F, Roscilli G, Lamartina S, Anderson KD, Bonelli F, Spence SG,

Ciliberto G, Vogt TF, Holder DJ, Toniatti C, Thut CJ. Inhibition of retinal and choroidal neovascularization by a novel KDR kinase inhibitor. *Mol Vis* 2005;11: 366-373

23 Sara VR, Hall K, Löw H. Growth factors: From genes to Clinical Applicanttion. 1st ed Raven Press. Ltd. New York 1990;146-147

24 Bora PS, Sohn JH, Cruz JM, Jha P, Nishihori H, Wang Y, Kaliappan S, Kaplan HJ, Bora NS. Role of complement and complement membrane attack complex in laser-induced choroidal neovascularization. *J Immunol* 2005;174(1):491-497

25 Cerra A, Mansfield KJ, Chamberlain CG. Exacerbation of TGF-beta-induced cataract by FGF-2 in cultured rat lenses. *Mol Vis* 2003;9:689-700

26 Folger PA, Zekaria D, Grotendorst G. Transforming growth factor-beta-stimulated connective tissue growth factor expression during corneal myofibroblast differentiation. *Invest Ophthalmol Vis Sci* 2001;42(11):2534-2541

27 Awasthi N, Wagner BJ. Interferon-gamma induces apoptosis of lens alphaTN4-1 cells and proteasome inhibition has an antiapoptotic effect. *Invest Ophthalmol Vis Sci* 2004;45(1):222-229

28 Fukushima A, Ozaki A, Fukata K, Ueno H. Differential expression and signaling of IFN-gamma in the conjunctiva between Lewis and Brown Norway rats. *Microbiol Immunol* 2003;47(10):785-796

29 Yoshida S, Shimmura S, Shimazaki J, Shinozaki N, Tsubota K. Serum-free spheroid culture of mouse corneal keratocytes. *Invest Ophthalmol Vis Sci* 2005;46 (5):1653-1658

30 Hu J, Bok D. A cell culture medium that supports the differentiation of human retinal pigment epithelium into functionally polarized monolayers. *Mol Vis* 2001;7: 14-19

31 Barald KF. Culture conditions affect the cholinergic development of an isolated subpopulation of chick mesencephalic neural crest cells. *Dev Biol* 1989;135(2): 349-366

32 Douay L. Experimental culture conditions were critical for *ex vivo* expansion of hematopoietic cells. *J Hematother Stem Cell Res* 2001;10(3):341-346

33 Tolnay M, Tsokos GC. Serum regulates the expression of complement receptor 2 on human B cell lines. *Immunopharmacol Immunotoxicol* 2000;22(2):205-219

34 Leshem B, Yogev D, Fiorentini D. Heat inactivation of fetal calf serum is not required for *in vitro* measurement of lymphocyte functions. *J Immunol Methods* 1999;223(2):249-254

35 Gruber HE, Leslie K, Ingram J, Hoelscher G, Norton HJ, Hanley EN Jr. Colony formation and matrix production by human anulus cells: modulation in three-dimensional culture. *Spine* 2004;29(13):E267-274

36 Petersen A, Carlsson T, Karlsson JO, Zetterberg M. The proteasome and intracellular redox status: implications for apoptotic regulation in lens epithelial cells. *Curr Eye Res* 2007;32(10):871-882

37 Jha P, Matta B, Lyzogubov V, Tytarenko R, Bora PS, Bora NS. Crucial role of apoptosis in the resolution of experimental autoimmune anterior uveitis. *Invest Ophthalmol Vis Sci* 2007;48(11):5091-5100

38 Pinila I, Cuenca N, Sauvé Y, Wang S, Lund RD. Preservation of outer retina and its synaptic connectivity following subretinal injections of human RPE cells in the Royal College of Surgeons rat. *Exp Eye Res* 2007;85(3):381-392