

Expression of IGFBP-6 in proliferative vitreoretinopathy rat models and its effects on retinal pigment epithelial-J cells

WEI ZHU^{1,2*}, YAN WU^{1,2*}, CHEN CUI³, HONG-MEI ZHAO¹, JUN BA¹, HUI CHEN³ and JING YU¹

¹Department of Ophthalmology, Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai 200072;

²Department of the First Clinical Medical College, Nanjing Medical University, Nanjing, Jiangsu 210029;

³Department of Ophthalmology, Affiliated Hospital of Nantong University, Nantong, Jiangsu 226001, P.R. China

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Abstract. Proliferative vitreoretinopathy (PVR) is one of the most common causes for failed retinal detachment surgeries. The aim of the present study was to investigate the role of insulin-like growth factor-binding protein-6 (IGFBP-6) in PVR using rat models and its effects on retinal pigment epithelial-J (RPE-J) cells. PVR Wistar rat models were administered intravitreal injection of RPE-J cells ($1 \times 10^6/5 \mu\text{l}$) combined with platelet-rich plasma ($1 \times 10^7/5 \mu\text{l}$). The concentration of IGFBP-6 in the vitreous and serum of rats was tested by an enzyme-linked immunosorbent assay and the expression of IGFBP-6 mRNA in the liver and retina of rats was determined by quantitative polymerase chain reaction (qPCR). The expression of IGFBP-6 mRNA in the RPE-J cells stimulated by vitreous or serum from PVR patients or normal volunteers was also determined by qPCR. The proliferation of RPE-J cells was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) method. The success rate of PVR rat model induction at the 8th week was 89.5% (34/38). The concentration of IGFBP-6 in the vitreous and serum of PVR rats was significantly higher than that of the control group ($P < 0.05$). The expression of IGFBP-6 mRNA in the retina of PVR rats was also significantly higher compared with the control group ($P < 0.05$). The vitreous from PVR patients and donors significantly stimulated the expression of IGFBP-6 mRNA in the RPE-J cells ($P < 0.05$). IGFBP-6 only inhibited IGF-II-stimulated proliferation but not the basal level of proliferation or the PDGF/VEGF-stimulated RPE-J cell proliferation. Thus, the trends and effects of IGFBP-6 provide the possibility of PVR therapeutic targets, with the

vitreous representing a significant environmental factor in the progression of PVR.

Introduction

Proliferative vitreoretinopathy (PVR) is essentially an excessive wound-healing response mediated by the proliferation of many types of cells inside the vitreous cavity and on the surface of the retina, resulting in membrane formation and traction on the retina (1,2). It is one of the most common causes for failed retinal detachment surgeries, and it develops in 5-10% of all retinal detachments (3,4). The management of this situation is complicated further due to the capability of PVR to result in the detachment of otherwise successfully reattached retinas or even cause new breaks, necessitating additional corrective surgeries. The pathogenesis of PVR is not completely understood, but it is widely accepted that PVR is a phenomenon involving the migration, proliferation, and connective tissue production by a variety of cells that gain access to the vitreous cavity (5,6). In addition, the various types of cells involved in PVR, immunohistochemical and ultrastructural studies have consistently reported the presence of retinal pigment epithelial (RPE) cells in fibrocellular scars (7,8). Animal model experiments have demonstrated the ability of RPE cells to cause tractional retinal detachment, which supports the pathogenic role of RPE cells (9,10). Previous studies on RPE cell behavior *in vitro* suggested numerous growth factors (GF), including platelet-derived GF (PDGF), vascular endothelial GF (VEGF), transforming GF, insulin-like GF (IGF), fibroblast GF and epidermal GF, as promoters of key cellular activities (11-15).

In a previous proteomic study, insulin-like growth factor-binding protein-6 (IGFBP-6) was one of 24 specific vitreous proteins shared between moderate and severe PVR samples (16). Experiments were designed to verify whether a similar correlation could be observed in the PVR rat models and to investigate the mechanisms of IGFBP-6 expression in PVR.

Materials and methods

The present study was conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animal experiments were performed

Correspondence to: Professor Jing Yu, Department of Ophthalmology, Affiliated Tenth People's Hospital of Tongji University, 301 Middle Yanchang Road, Shanghai 200072, P.R. China
E-mail: dryujing@yahoo.com.cn

*Contributed equally

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under the protocols approved by the National Eye Institute Institutional Animal Care and Use Committee. The present study also followed the tenets of the Declaration of Helsinki for the use of human subjects.

RPE-J cell preparation and culture. RPE-J cells (CRL-2240, ATCC, Rockville, MD, USA) were a generous gift from Lian-Fang Du (Department of Medical Ultrasound, Shanghai Jiaotong University Affiliated First People's Hospital, Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium with 4% fetal bovine serum and 1% antibiotic/antimycotic (all from Gibco, Grand Island, NE, USA) at 37°C with 5% CO₂. The cells used in the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) proliferation assay were cultured in a humidified incubator.

The cells used for rat model induction were inoculated in a six-well plate at a concentration of 2x10⁴/cm² and maintained in complete medium for 24 h. Following adhesion of the cells, the medium was switched to serum-free medium for 24 h.

Induction of rat models. Seventy-six male adult Wistar rats (Shanghai Slac laboratory animal Co. Ltd., Shanghai, China; weight, 200±10 g; age, 7 weeks; specific pathogen-free) were included in the present study, and they were divided into a PVR (n=40) and a control group (n=36). The PVR Wistar rat models were established as previously described (17). In brief, hyaluronidase (1 U) was injected into the vitreous cavity to liquefy the vitreous, and RPE-J cells (1x10⁶/5 μl) and platelet-rich plasma (PRP) (1x10⁷/5 μl) were injected into the vitreous cavity of rats to induce PVR. Sterile pyrogen-free normal saline was injected into the vitreous cavity in the control group.

The rats were observed by slit lamp, fundus examination and photography at 1, 2, 4 and 8 weeks after intravitreal injection. Rats with any anterior segment inflammation, vitreous hemorrhage or cataracts were excluded from the present study. Tissues of the PVR (n=10, 8, 10 and 6) and control (n=10, 10, 10 and 6) groups were collected at 1, 2, 4 and 8 weeks after model induction, respectively. The proliferative response was evaluated according to the following grading scale (18): 0, no proliferative response; 1, intravitreal proliferation; 2, epiretinal membrane formation with retinal folds and 3, white dense membrane covering the retina, with retinal folds and localized retinal detachments with or without a localized posterior capsular cataract.

Collection of tissues and sample preparation

Liver. The livers of Wistar rats were isolated immediately after anesthesia with Napental. In total, 200 mg liver tissue was stored in a 1.5 ml centrifuge tube at -80°C.

Serum. Venous blood (2 ml) was collected from the rats from the abdomen cardinal vein and placed in a procoagulant tube at room temperature for 1 h. The blood was centrifuged at 4°C at 7,000 x g for 5 min. The supernatant was stored at -80°C.

Vitreous. The vitreous was placed in a 1.5 ml centrifuge tube following isolation and centrifuged at 4°C at 13,400 x g for 5 min. The supernatant was stored at -80°C.

Retina. The retina was placed in a 1.5 ml centrifuge tube at -80°C.

Effect of cytokines on IGFBP-6 mRNA expression in RPE-J cells. The groups were divided as follows: the control, which contained no growth factors; the IGF-II, which contained 10, 20 or 50 ng/ml IGF-II; the VEGF, which contained 10, 20 or 40 ng/ml VEGF and the PDGF, which contained 10, 20 or 40 ng/ml PDGF.

Effect of vitreous and serum on IGFBP-6 mRNA expression in RPE-J cells. Vitreous and serum samples of PVR patients with primary rhegmatogenous retinal detachment from the Department of Ophthalmology, Nantong University Affiliated Hospital, were used in the present study. Patients with ocular trauma, age-related macular degeneration, uveitis, glaucoma, diabetes mellitus, a history of ocular surgery or other systemic diseases were excluded. Informed consent was obtained from all the patients following verbal and written explanation of the nature and possible consequences of the present study. PVR was graded in accordance with the standards of the Committee TRST in 1983 (19) and evaluated by at least three associate chief or chief surgeons. Severe PVR (grade C or D, n=5) and moderate PVR (grade B, n=5) (20) were included in the present study. Undiluted 0.3-1.0 ml vitreous humor samples were obtained from patients with a syringe by aspirating liquefied vitreous from the center of the vitreous cavity prior to the vitrectomy infusion. The corresponding serum samples were obtained prior to surgery.

The control group of normal human eyes without any known ocular diseases (n=5), which were donated for corneal transplant in accordance with the standardized rules for the development and applications of organ transplants, was obtained from the Organ Transplant Center in Shanghai (Shanghai, China). In total, 0.8-1.0 ml of normal vitreous samples were aspirated with a syringe from the pars plana. The normal serum samples were obtained from five healthy volunteers who underwent a physical examination at the Shanghai Tenth People's Hospital; these volunteers had no ocular or systemic diseases.

Harvested vitreous humor samples were collected in Eppendorf tubes (Axygen, Union City, CA, USA), immediately placed on ice, centrifuged for 15 min at 12,000 rpm to separate the cell contents and stored at -80°C until use. The serum samples were placed at room temperature for 1 h, centrifuged for 15 min at -4°C and stored at -80°C. The demographic characteristics of the samples obtained from the donors are shown in Table I. There was no significant difference among the groups (P>0.05).

Quantitative polymerase chain reaction (qPCR). Total RNA was isolated from the retina of PVR rats and RPE-J cells as per the manufacturer's instructions (TRIzol[®]; Invitrogen, Carlsbad, CA, USA). In total, 2 μg RNA was converted into cDNA. The primer sequences (5'-3') were: IGFBP-6 (NM_013104): forward, 5'-GAAGAGACTACCAAGGAGAGCAAAC-3' and reverse, 3'-CTGCAGTACTGAATCCAAGTGTCT-5'; β-actin (NM_031144): forward, 5'-CCCATCTATGAGGGTTA CGC-3' and reverse, 3'-TTTAATGTCACGCAGATTTC-5'. The qPCR assays were performed according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay for IGFBP-6 measurement in rats. The IGFBP-6 concentration was measured

Table I. Demographic characteristics of the samples.

Characteristics	N	Vitreous	Serum	Age, years	Gender (male/female)	Eye (right/left)
Moderate PVR	5	+	+	57.8±5.8	2/3	2/3
Severe PVR	5	+	+	56.4±5.4	3/2	3/2
Normal donors	5	+	-	58.2±8.3	3/2	2/3
Healthy volunteers	5	-	+	56.2±4.3	2/3	-

PVR, proliferative vitreoretinopathy.

Table II. Results of the PVR rat model induction.

Variables	1 weeks	2 weeks	4 weeks	8 weeks
Grade 0	20	13	6	4
Grade 1	15	14	5	5
Grade 2	3	9	10	8
Grade 3	0	2	17	21
PVR rate, %	47.4	65.8	84.2	89.5

PVR, proliferative vitreoretinopathy.

in the serum and vitreous of rats with an enzyme-linked immunosorbent assay kit (Millipore, Billerica, MA, USA) at 8 weeks following intravitreal injection. All procedures were conducted according to the manufacturer's instructions.

MTS proliferation assay. The RPE-J cells were counted by the MTS assay, which relies on the formation of a colored substrate by mitochondrial enzyme activity in viable cells. The cells were plated in a 96-well plate in growth medium and allowed to attach overnight (2,000 per well). Following washing twice with phosphate-buffered saline, the cells were switched to serum-free media and left overnight at 37°C. The cells were incubated in serum-free medium with or without 50 ng/ml IGF-II (R&D Systems, MN, USA) at 37°C for 24 or 48 h. Variable concentrations of 1, 10, 100, 500 and 1,000 ng/ml recombinant human IGFBP-6 (R&D Systems) were added to the cells. MTS (20 μ l per well) was then added for 3 h. The absorbance was measured with a plate reader (Molecular Devices, Sunnyvale, CA, USA) at 490 nm.

Statistical analysis. Statistical analysis was performed using SPSS (version 14.0, SPSS, Inc., Chicago, IL, USA). The results were expressed as the mean \pm standard deviation (SD). Multiple comparisons within the experimental groups were performed using a one-way analysis of variance, and comparisons between the two groups were performed using independent group t-tests. $P < 0.05$ was used to indicate a statistically significant difference.

Results

In vivo results of the PVR rat model induction. Two rats were excluded due to cataracts in the PVR group in the 1st week after intravitreal injection. The success rate of the PVR rat

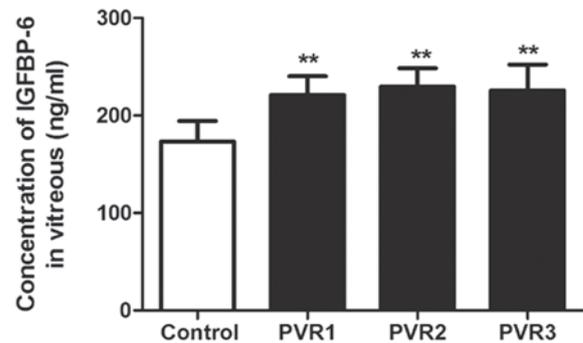


Figure 1. The expression of IGFBP-6 221.00±19.32, 229.63±18.89 and 225.70±26.71 ng/ml in vitreous of PVR grade 1, 2 and 3, respectively, was significantly higher than 173.25±21.11 ng/ml in the control group ($P < 0.05$). (** $P < 0.01$ vs. control group). PVR, proliferative vitreoretinopathy.

models at the 8th week was 89.5% (34/38). In total, 15 grade one and three grade two PVR rat models were observed at the 1st week subsequent to intravitreal injection. At the 2nd week, two grade three PVR rat models were observed. More grade two and three PVR rat models were observed at the 4th week. At the 8th week, there were five, eight and 21 rat models at PVR grades one, two and three, respectively (Table II).

In vivo IGFBP-6 concentration in the vitreous and serum of rat models

IGFBP-6 in general samples. The IGFBP-6 concentration (225.44±19.36 ng/ml) in the vitreous of the PVR rat models was significantly higher than 173.25±21.11 ng/ml concentration in the control group ($P = 0.003$). The concentration of IGFBP-6 in the serum of the PVR group was higher than that in the control group ($P = 0.012$).

IGFBP-6 in the different PVR grades. The IGFBP-6 concentrations 221.00±19.3, 229.63±18.89 and 225.70±26.71 ng/ml in the vitreous of PVR grades 1, 2 and 3, respectively, was significantly higher than that in the control group ($P < 0.01$). However, there was no significant difference among the three PVR grade groups ($P = 0.892$) (Fig. 1).

Comparison of IGFBP-6 between vitreous and serum. In normal rats, the concentration of 173.25±21.11 ng/ml IGFBP-6 was significantly higher in the vitreous compared with 95.96±17.40 ng/ml in the serum ($P = 0.000$). Similarly, in the PVR rat models, the concentration of 225.44±19.36 ng/ml IGFBP-6 in the vitreous was significantly higher compared with 108.48±15.78 ng/ml in the serum ($P = 0.000$).

Table III. The expression of IGFBP-6 mRNA in retina of rats ($2^{-\Delta CT} \times 10^{-4}$) (mean \pm SD).

Variables	1 weeks	2 weeks	4 weeks	8 weeks	In general
Control group	8.85 \pm 2.32	8.37 \pm 2.59	8.32 \pm 2.96	8.18 \pm 1.81	8.32 \pm 2.41
PVR group	10.03 \pm 2.55	11.02 \pm 2.92	11.62 \pm 2.33	11.82 \pm 2.27	11.09 \pm 2.57
P-value	0.293	0.058	0.013	0.009	0.000

IGFBP-6, insulin-like growth factor-binding protein-6; PVR, proliferative vitreoretinopathy; SD, standard deviation.

Table IV. The expression of IGFBP-6 mRNA in retina and liver of different grade PVR rat models ($2^{-\Delta CT} \times 10^{-4}$).

Group	Retina		Liver	
	Mean \pm SD	P-value	Mean \pm SD	P-value
Control	8.32 \pm 2.41		25.01 \pm 12.04	
PVR 1	10.87 \pm 2.77	0.035	27.58 \pm 18.98	0.999
PVR 2	11.35 \pm 2.45	0.003	21.92 \pm 11.94	0.982
PVR 3	11.07 \pm 2.67	0.000	31.41 \pm 11.07	0.477

IGFBP-6, insulin-like growth factor-binding protein-6; PVR, proliferative vitreoretinopathy; SD, standard deviation.

It was increased to a higher extent in the vitreous than in the serum.

In vivo results of qPCR in rats.

After measuring the cycle threshold (CT) value of each sample, the ΔCT was determined by subtracting the CT value of β -actin from IGFBP-6. The relative amount of IGFBP-6 expression was calculated as $2^{-\Delta CT}$ and presented as $2^{-\Delta CT} \times 10^{-4}$ due to the low value.

Expression of IGFBP-6 mRNA in the retina. In general, the expression of IGFBP-6 mRNA in the retina and of IGFBP-6 of each grade of the PVR group was higher compared with that of the control group ($P=0.000$; Table III and $P<0.05$, respectively). However, there was no significant difference among the different grades of PVR ($P>0.05$; Table IV).

Expression of IGFBP-6 mRNA in the liver. In the PVR group, no significant difference was observed in the expression of IGFBP-6 mRNA in the liver at different times ($P>0.05$). No difference was found between the PVR and control groups at any time points ($P=0.443$) and there was no significant difference among the different grades of PVR ($P>0.05$; Table IV).

In vitro effects of IGFBP-6 on RPE-J cell proliferation. After the RPE-J cells were incubated with 50 ng/ml exogenous IGF-II for 24 or 48 h, the OD value, which reflected the cell number, increased significantly (24 h: from 1.26 \pm 0.05 to 1.38 \pm 0.05 and 48 h: from 1.14 \pm 0.05 to 1.44 \pm 0.06; $P<0.05$). When 500 ng/ml IGFBP-6 was added to the DMEM plates for 3 h, the OD value was significantly reduced to 1.23 \pm 0.04 and 1.30 \pm 0.05, respectively ($P<0.01$). However, there was no significant difference following IGFBP-6 treatment in the VEGF or PDGF groups. IGFBP-6 alone had no effect on basal proliferation ($P>0.05$; Fig. 2).

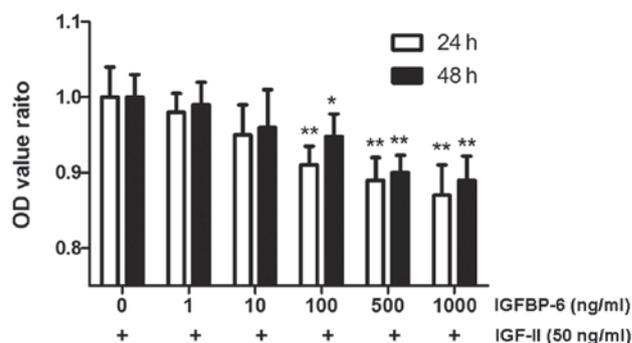


Figure 2. Effects of IGFBP-6 on the proliferation of RPE-J cells were induced by IGF-II. Optical density (OD) value ratio=OD value in the PVR group/OD value in the control group. The RPE-J cells in all the groups were treated with 50 ng/ml IGF-II for 24 or 48 h to induce cell proliferation, then different doses of IGFBP-6 were added to the media incubated for 3 h. RPE-J cell was treated without IGFBP-6 in the control group. ($P<0.05$ and $***P<0.01$ vs. control group). IGFBP-6, insulin-like growth factor-binding protein-6; RPE-J, retinal pigment epithelial-J PVR, proliferative vitreoretinopathy.

In vitro effects of cytokines on IGFBP-6 mRNA expression in RPE-J cells. At 20 and 50 ng/ml, IGF-II significantly stimulated the expression of IGFBP-6 mRNA, which was 1.07 \pm 0.08-fold ($P=0.036$) and 1.08 \pm 0.05-fold ($P=0.020$) higher compared with the control group. There was no significant difference between the IGF-II and control group at 10 ng/ml ($P>0.05$). (Fig. 3)

Different concentrations of PDGF had no significant effect on the expression of IGFBP-6 mRNA. The fold changes were 1.002 \pm 0.061, 0.997 \pm 0.080 and 1.010 \pm 0.051 at 10, 20 and 50 ng/ml, respectively ($P>0.05$).

As with PDGF, there was no significant difference between the VEGF and control group at 10, 20 and 50 ng/ml ($P>0.05$).

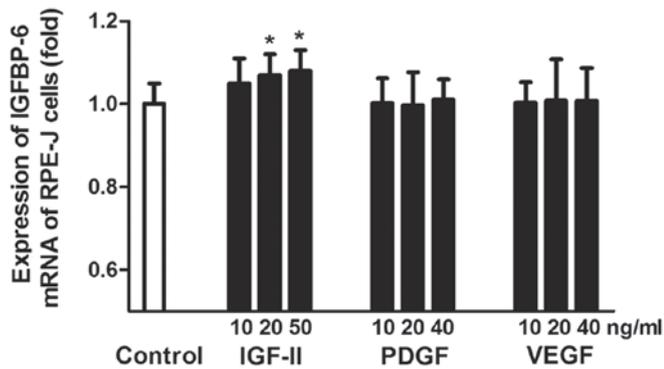


Figure 3. Effect of cytokines on IGFBP-6 mRNA expression of RPE-J cells. The RPE-J cells were cultured with different concentrations of cytokines and the control group was cultured in normal serum-free medium. In total, 20 and 50 ng/ml of IGF-II significantly stimulated the expression of IGFBP-6 mRNA ($P=0.036$ and $P=0.020$, respectively). ($P<0.05$ vs. control group). IGFBP-6, insulin-like growth factor-binding protein-6; RPE-J, retinal pigment epithelial-J; mRNA, messenger RNA.

In vitro effects of vitreous or serum on IGFBP-6 mRNA expression in RPE-J cells. In general, the vitreous from PVR patients and donors significantly stimulated the expression of IGFBP-6 mRNA. The IGFBP-6 mRNA expression level in the RPE-J cells stimulated by vitreous from donors, moderate PVR and severe PVR groups was 1.72 ± 0.33 , 1.83 ± 0.29 and 1.85 ± 0.41 -fold higher, respectively, compared with the control group ($P<0.01$). However, there was no significant difference between the serum and control groups. The IGFBP-6 mRNA level stimulated by serum from healthy volunteers, moderate PVR and severe PVR patient groups was 0.85 ± 0.34 , 0.91 ± 0.22 and 0.88 ± 0.42 -fold higher, respectively, compared with the control group ($P>0.05$) (Fig. 4).

Discussion

In our previous proteomic study, 102 PVR-specific proteins were identified in the vitreous of PVR patients by two-dimensional-nano-liquid chromatography coupled with tandem mass spectrometry (16). Among these, 24 specific vitreous proteins were shared between moderate and severe PVR samples (16). In the previous study, IGFBP-6 was identified as a specific protein in the vitreous and serum of PVR patients. However, the contributions of IGFBP-6 to the PVR process remain unclear.

In the present study, a significantly higher concentration of IGFBP-6 was detected in the vitreous, serum and retina of the PVR rat models compared with the normal control rats. This result demonstrates that IGFBP-6 is a specific protein in the PVR process. The concentration of IGFBP-6 in the vitreous was significantly higher compared with the serum, suggesting that the upregulated IGFBP-6 in the vitreous was not from the serum. No significant difference was found in the expression of IGFBP-6 mRNA in the liver between the PVR and control groups. Due to IGFBP-6 being primarily produced in the liver (21), this result indicates that upregulated IGFBP-6 in the vitreous and serum was not from the liver. Additionally, the upregulated IGFBP-6 was produced in a local autocrine or paracrine manner. Certain studies have

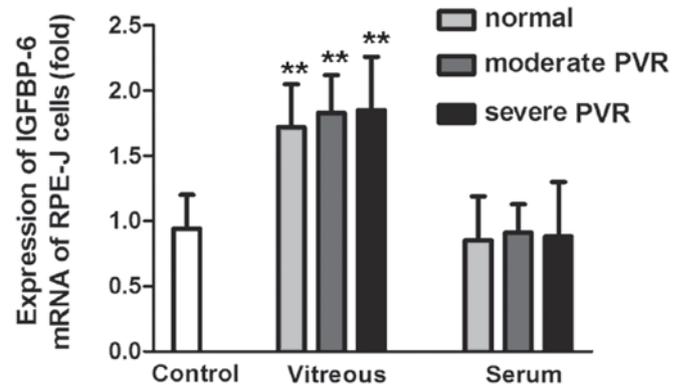


Figure 4. Effect of vitreous and serum on IGFBP-6 mRNA expression of the RPE-J cells. The vitreous significantly stimulated the expression of IGFBP-6 mRNA ($P<0.01$), while the serum did not affect the expression of IGFBP-6 mRNA ($P>0.05$). (** $P<0.01$ vs. control group). IGFBP-6, insulin-like growth factor-binding protein-6; RPE-J, retinal pigment epithelial-J; mRNA, messenger RNA.

indicated that the choroids (22) and ciliary body (23) express IGFBP-6 mRNA, however, further investigations should be performed (23).

IGFBP-6 is a relatively novel member of the IGFBP family that inhibits proliferation and induces apoptosis in rhabdomyosarcoma cells (24) and suppresses striated muscle cell migration (25). Unlike other IGFBPs, the affinity of IGFBP-6 for IGF-II is ~50-fold higher compared with the IGF-I (26). This characteristic makes IGFBP-6 a potent inhibitor of IGF-II, which is significant, in particular in inhibiting the growth of IGF-II-dependent tumors (27,28), including neuroblastoma (29), rhabdomyosarcoma (29) and colon carcinoma (30). IGF-II, an autocrine tumor growth factor, is a potent promoter of RPE cell tractional force generation *in vitro* (11). A previous study confirmed that IGF-II was expressed at higher levels in PVR patients (31). In the present study, the expression of IGFBP-6 mRNA in RPE-J cells was significantly upregulated by IGF-II at 20 and 50 ng/ml, which may have been due to the increased level of IGF-II. Therefore, IGFBP-6 may downregulate RPE-J cell proliferation through inhibiting the actions of IGF-II.

In this study, IGFBP-6 inhibited the IGF-II-stimulated proliferation of RPE-J cells but not basal proliferation, suggesting that the basal growth of RPE-J cells is IGF-II independent under these conditions. The results indicate that IGFBP-6 is a potent anti-proliferative agent, and its anti-proliferative effects depend on its combination with IGF-II. Therefore, IGFBP-6 may be a novel target to control the PVR process.

In addition to IGFBP-6, other growth factors were upregulated in PVR patients, including PDGF (32) and VEGF (33), which are capable of inducing RPE-J cell proliferation and migration (34,35). In the current study, these two growth factors were selected to evaluate the role of IGFBP-6 in the RPE-J cells. The final concentration used was based on previous studies that revealed their effect on the RPE-J cells. Neither PDGF nor VEGF had a significant effect on IGFBP-6 mRNA expression. Additionally, IGFBP-6 only inhibited IGF-II-stimulated but not PDGF- or VEGF-stimulated RPE-J cell proliferation, which indicated that the role of IGFBP-6 in RPE-J cell proliferation was independent of PDGF and VEGF.

In the present study, the vitreous of PVR patients and donated eyes significantly stimulated the expression of IGFBP-6 mRNA in the RPE-J cells, while the serum had no effect on this expression. This result revealed that RPE-J cell proliferation in the PVR progression was dependent on the vitreous environment. The proliferation and migration of RPE-J cells are significant during the development of PVR. RPE-J cells usually remain in the G0 phase, with no proliferative or migratory activity in the normal state, until the retina is broken by trauma or surgery; subsequently, they gain access to the vitreous cavity or subretinal space and begin proliferating and migrating (36). Those data are in agreement with the results of the present study.

In summary, the trends and effects of IGFBP-6 may provide a possibility of a PVR therapeutic target, with the vitreous serving as a significant environmental factor in the progression of PVR.

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