Expression of IGFBP−6 in a proliferative vitreoretinopathy rat model and its effects on retinal pigment epithelial cell proliferation and migration

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Abstract

• **AIM: To investigate the expression of insulin –like growth factor binding protein –6 (IGFBP –6) in a proliferative vitreoretinopathy (PVR) model and its effects on proliferation and migration in retinal pigment epithelial (RPE) cells.**

• **METHODS: A PVR Wistar rat model was established by the intravitreal injection of RPE –J cells combined with platelet –rich plasma (PRP). The expression levels of IGFBP –6 were tested by ELISA. ARPE –19 cell proliferation was evaluated by the MTS method, and cell migration was evaluated by wound healing assays.**

• **RESULTS: The success rate of the PVR model was 89.3% (25/28). IGFBP–6 was expressed at higher levels in the vitreous, serum and retina of rats experiencing advanced PVR (grade 3) than in the control group (vitreous: 152.80±15.08ng/mL *vs* 105.44±24.81ng/mL, *P*> 0.05; serum: 93.48 ±9.27ng/mL *vs* 80.59 ±5.20ng/mL, *P* < 0.05; retina: 3.02±0.38ng/mg *vs* 2.05±0.53ng/mg, *P*<0.05). *In vitro* IGFBP–6(500ng/mL) inhibited the IGF–II (50ng/mL) induced ARPE –19 cell proliferation (OD value at 24h: from 1.38±0.05 to 1.30±0.02; 48h: from 1.44±0.06 to 1.35± 0.05). However, it did not affect basal or VEGF–, TGF–β– and PDGF–induced cell proliferation. IGFBP–6 (500ng/ml) reduced the IGF–II (50ng/mL)–induced would healing rate [24h: from (43.91±3.85)% to (29.76 ±2.49)%; 48h: from (66.09±1.67)% to (59.88±3.43)%].**

• **CONCLUSION: Concentrations of IGFBP –6 increased in the vitreous, serum, and retinas only in advanced PVR *in viva* IGFBP –6 also inhibited IGF –II –induced cell proliferation in a not dose or time dependent manner and migration. IGFBP –6 participates in the development of PVR and might play a protective role in PVR.**

• **KEYWORDS:** insulin-like growth factor binding protein-6; proliferative vitreoretinopathy; retinal pigment epithelial cells
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INTRODUCTION

Proliferative vitreoretinopathy (PVR) is a tissue response characterized by the proliferation of many types of cells within the vitreous gel and on the surface of the retina, resulting in membrane formation and traction on the retina. It is the major cause of failed retinal detachment surgery, developing in 5% to 10% of rhegmatogenous retinal detachments [1-4]. The management of this condition is further complicated because PVR can result in the detachment of otherwise successfully reattached retinas or even cause new breaks, necessitating additional corrective surgeries. This issue has led researcher to search for an ideal PVR treatment. Although significant progress in vitreoretinal microsurgical techniques, including internal limiting membrane (ILM) peeling, 25-gauge vitrectomy, and a silicone oil/acetyl-salicylic acid suspension tamponade, the incidence of PVR in primary retinal detachments has failed to decline over the past 20 years and remains a challenge in the clinical setting[5-7]. To facilitate the study of the pathogenesis of PVR and to find new clues to develop new therapeutic agents, a vitreous humor database of the proteins expressed in the eye was created. In our previous proteomic study, 102 PVR-specific proteins were identified in the vitreous humors of PVR patients by two-dimensional-nano-liquid chromatography coupled with tandem mass spectrometry [8]. Of these proteins, IGFBP-6 aroused our interest [9]. Our current study showed that IGFBP-6 is a special protein in the vitreous and serum of the PVR patients. The concentration of IGFBP-6 is correlated with the severity and prognosis of PVR. Therefore, IGFBP-6 is a candidate serum biomarker for PVR [10,11]. To investigate the expression of IGFBP-6 in PVR model and its effects on proliferation and migration in RPE cells, we designed the following experiments.

MATERIALS AND METHODS

Materials

RPE –J cells preparation The RPE-J cells (CRL-2240) were a generous gift from Professor Lian-Fang Du (Department of Medical Ultrasound, Shanghai Jiaotong

University Affiliated First People's Hospital, China) and were cultured in dulbecco modified eagle medium (DMEM) (Gibco, Grand Island, NY, USA) with 4% fetal bovine serum (FBS) (Gibco), MEM nonessential amino acids, and antibiotic/antimycotic (Invitrogen, Grand Island, NY, USA) at 33℃ with 5% CO_2. Cells from confluent cultures were detached by 0.25% trypsin digestion (Invitrogen), collected, centrifuged at 1 000r/min for 10min, and resuspended in sterile pyrogen-free normal saline at a concentration of 10^8/mL.

Preparation of platelet –rich plasma Wistar rats were anesthetized with an intraperitoneal injection of pentobarbital (50mg/kg, SIGMA-ALDRICH, Selma, Carlsbad, USA). Blood was collected into ethylene diamine tetraacetic acid vacuum tubes from the abdominal aortic vein. The samples were centrifuged at 200g for 10min to separate the platelet-rich plasma (PRP) from the erythrocytes and leukocytes. The PRP was transferred to a clean tube and centrifuged at 200g for 10min to separate the platelets. The platelet number in the PRP was counted by an automatic hemocytometer and adjusted to 2.5×10^8/mL with phosphate buffered saline (PBS).

Animals In total, 60 male adult Wistar rats were included in the study and were divided into the PVR (n =30) and control (n =30) groups. All experimental procedures adhered to the ARVO Resolution on the Treatment of Animals in Research.

Methods

Intravitreal injection Before the intravitreal injection (IVit), the rats were anesthetized as before. The pupil was dilated with tropicamide (Santen, Osaka, Japan), and the eye was topically anesthetized with 0.4% oxybuprocaine hydrochloride eye drops (Eisai Co. Ltd., Tokyo, Japan) to reduce the animals' discomfort. Under the operating microscope, the right eye of the rats in the PVR group received an IVit of RPE-J cells (4μL) with PRP (6μL) with a 30-gauge, 0.5-inch needle (BD Biosciences, Franklin Lakes, NJ, USA) on a microsyringe (Hamilton, Reno, NV, USA). The injection location was approximately 1mm posterior to the limbus in the superotemporal quadrant. A 5% povidone-iodine solution was applied before and immediately after the IVit. A cover slip was adapted on the corneal surface to control the fundus during the injection. Similarly, 10μL PBS was injected into the vitreous cavity of the eyes of the control group.

Slit lamp and fundus examination The animals were examined by slit lamp and indirect ophthalmoscopy 1, 3, 7, 14, 21, and 28d after IVit. Retinal photographs were taken using a surgical microscope (SM-2000J, Eder, Shanghai, China). Any vitreous hemorrhage or cataract excluded the rats from the protocol. The proliferative response was evaluated according to the following grade scale: 0, no proliferative response; 1, vitreous haze, vitreous strands; 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[12].

Histopathological examination The rats (three rats per time point, 2 in control group and 1 in PVR group) were sacrificed with cervical dislocation 7, 14, 21 and 28d after the IVit. The eyes were enucleated and fixed in a 10% formaldehyde solution at room temperature. Thereafter, they were embedded in paraffin and cut into 5-μm-thick sections. Subsequently, the sections were stained with hematoxylin-eosin to observe the retinal architecture using light microscopy (Zeiss Axiovert S 100, Jena, Germany). All the results of the histopathological examination were confirmed by two blinded experts experienced in ophthalmological pathology, including PVR.

Enzyme –linked immunosorbent assay (ELISA) for IGFBP –6 measurements Blood samples were collected, and then serum samples were prepared. The vitreous and retinas were carefully dissected from the posterior section of the eyes under a dissecting microscope and frozen at -80℃. The vitreous and retina protein concentrations were measured and adjusted to the same protein concentration using a BCA protein assay kit (Pierce, IL, USA). Briefly, the vitreous and retinas were homogenized in ice-cold radioimmune precipitation assay (RIPA) buffer supplemented with a protease inhibitor, phenylmethanesulfonyl fluoride (Shenergy Bicolor Bioscience Technology Company, Shanghai, China), and sonicated at 0.5Hz for 40s (50-watt sonicator, Sonics & Materials, Danbury, CT, USA). The lysate was centrifuged at 20 000g for 15min at 4℃. The supernatants were collected independently and frozen at -80℃. The IGFBP-6 concentrations in the serum, vitreous and retina were determined using a capture sandwich kit with a biotinylated affinity-purified rabbit polyclonal antibody to rat IGFBP-6 ELISA kit (Shanghai Lengton Bioscience Co. Ltd., Shanghai, China) following the manufacturer's protocol. The absorbance at 450nm and reference at 600nm were measured using a micro plate reader (Safire2, Tecan Group Ltd., Maennedorf, Switzerland).

Cell cultures The adult human RPE cells (ARPE-19) were a generous gift from Professor Guo-Tong Xu (Medical School, Tongji University, China). They were maintained in DMEM/F12 (Gibco) supplemented with 10% FBS, 100U/mL penicillin and 100mg/mL streptomycin (Gibco). The cells were cultured in a humidified incubator at 37℃ and 5% CO_2.

MTS proliferation assay The number of viable cells was measured using the MTS assay, which relies on the formation of a colored substrate by mitochondrial enzyme activity in viable cells. ARPE-19 cells were plated into wells of a 96-well plate in growth medium and allowed to attach overnight (2 000 per well). After washing twice with PBS, the cells were changed into serum-free media and left
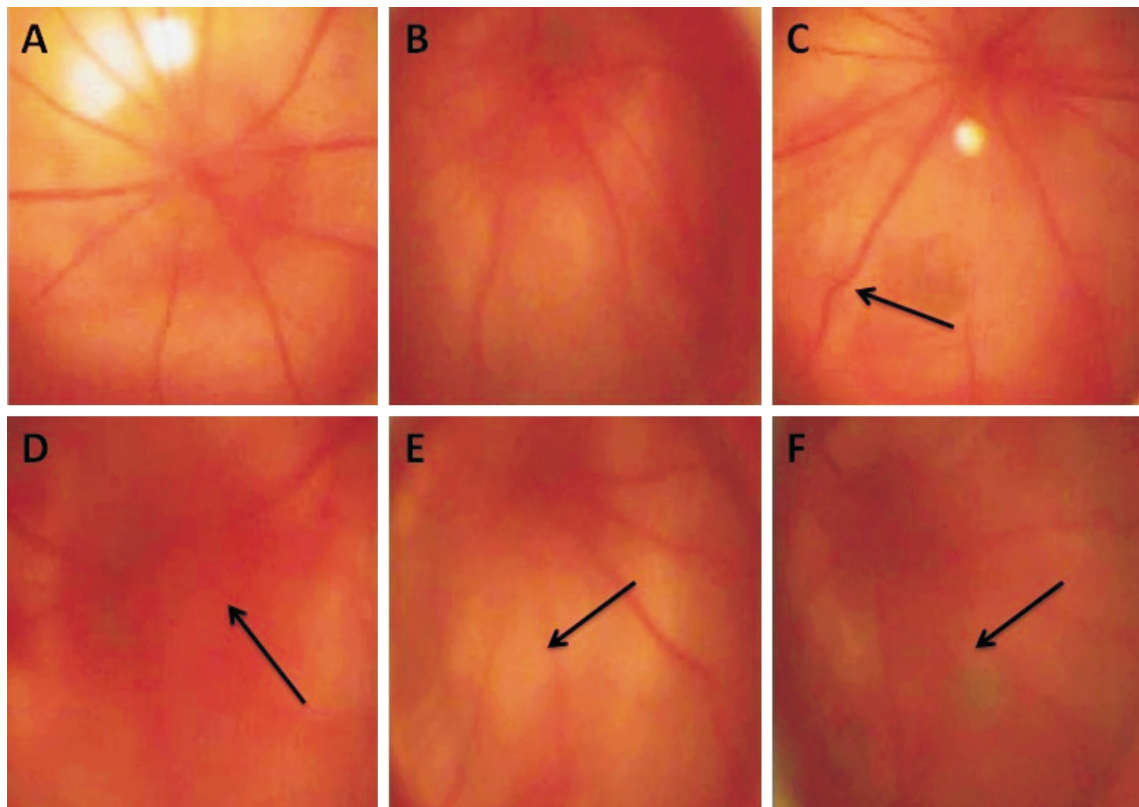
Figure 1 Retinal photographs of rats in the PVR and control groups (×25) A: Control group, no proliferation; B: PVR group, 7d after IVit, grade 1, intravitreal proliferation; C and D: PVR group, 14d after IVit, grade 2, tortuous retinal vessels (arrow in C) and retinal membrane formation with retinal folds (arrow in D); E and F: PVR group, 28d after IVit, grade 3, white dense membrane (arrow) covering the retina with retinal folds and localized retinal detachments (arrow).

overnight at 37℃ . The cells were incubated in serum-free medium , serum-free medium with IGF-II (50ng/mL; R&D, Minneapolis, USA), vascular endothelial growth factor (VEGF, 40ng/mL; R&D), platelet-derived growth factor (PDGF, 20ng/mL; R&D) or transforming growth factor-β (TGF-β, 4ng/mL; R&D) at 37℃ for 24 or 48h. The cells were treated with variable concentrations of recombinant human IGFBP-6 (1, 10, 100, 500, and 1 000ng/mL; R&D). MTS (20μL per well) was then added for 3h. The absorbance was counted in a plate reader (Molecular Devices, Sunnyvale, USA) at 490nm.

Migration assay For the migration assay, the ARPE-19 cells were grown to 80% confluence in 12-well tissue culture plates and wounded with a sterile 10-μL pipette tip (Eppendorf, Hamburg, Germany) to remove cells by two perpendicular linear scrapes. The debris of the damaged cells was removed by washing, and the cells were incubated with DMEM in different culture media, such as 10% FBS alone, serum-free medium with IGF-II (50ng/mL) and serum-free medium with IGF-II (50ng/mL) and IGFBP-6 (500ng/mL). The progression of migration was photographed immediately at 24 or 48h after wounding in the same field near the crossing point with an inverted microscope equipped with a digital camera (Zeiss, Oberkochen, Germany). The extent of healing is defined as the ratio of the area difference between

the original wound and the remaining wound 24 or 48h after the injury compared with that of the original wound [13]. The wound area was determined by the number of pixels in a histogram (Image J, NIH, USA).

Statistical Analysis All data were expressed as the means± SD. The analysis of variance (ANOVA) test was used to determine the significance of the difference with multiple comparisons. The differences were considered significant at P-values less than 0.05. The software package used was SPSS (version 17.0, Chicago, USA).

RESULTS

Slit Lamp and Fundus Examination Clinically, the findings of the fundus examination were normal in the control group (Figure 1A). Two rats in the PVR group were excluded because of cataracts 7d after the IVit. Overall, different grades of PVR were observed in 25 rats 28d after the IVit. The success rate of the PVR model was 89.3% (25/28). Specifically, vitreous haze and vitreous strands could be observed, but there were no retinal folds 7d after the IVit (Figure 1B). Tortuous retinal vessels with retinal folds (Figure 1C) and epiretinal membrane formation (Figure 1D) could be observed 14d after the IVit. A white dense membrane covering the retinas (Figure 1E) with retinal folds and localized retinal detachments (Figure 1F) could be observed 28d after the IVit.
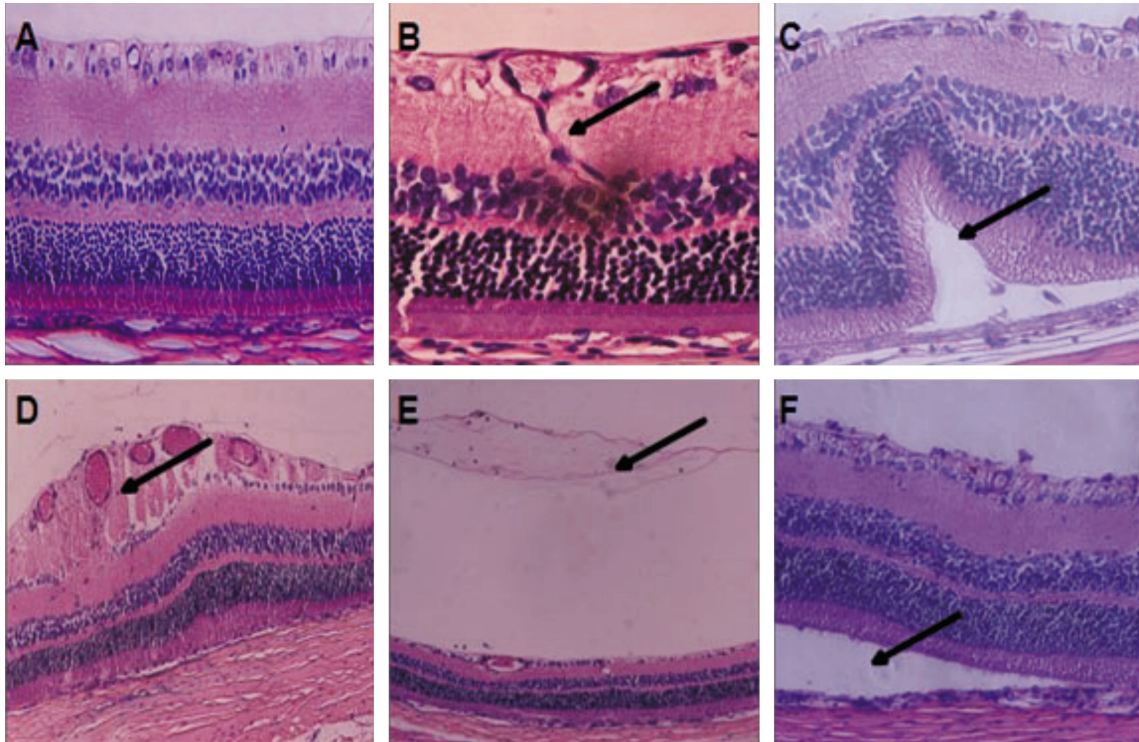
Figure 2 Histologic images of eyes in the control and PVR groups (5μm−thick, stained with hematoxylin−eosin, ×400) A: Control group, 28d after IVit, the retinal structure was normal without proliferation or retinal folds or retinal detachment; B: PVR group, 7d after IVit, the inflammatory cells infiltrated the inner retina (arrow); C: PVR group, 14d after IVit, retinal folds and localized retinal detachment (arrow) appeared; D: PVR group, 21d after IVit, proliferative membrane before the retina; E: PVR group, 21d after IVit, proliferative membrane in the vitreous (arrow); F: PVR group, 28d after IVit, retinal detachment (arrow) appeared.

Histological Observation The retinal structure was normal, and no proliferation was seen in the control group (Figure 2A). Numerous cell phenotypes experienced a series of time-dependent changes in the PVR group 7d after the IVit, which had established connections with the inner retina (Figure 2B). As time progressed, retinal folds (Figure 2C), a white dense membrane covering the retina (Figure 2D, E) with retinal folds, and localized retinal detachments (Figure 2F) appeared.

Enzyme−linked Immunosorbent Assay Twenty-eight days after the IVit, IGFBP-6 was detected in the serum, vitreous, and retinas of all rats in both the PVR and control groups. The vitreous concentration of IGFBP-6 was significantly higher in the PVR grade 3 group (152.80±15.08ng/mL) than that in the control group (105.44±24.81ng/mL) (P =0.010). However, there was no significant difference (P >0.05) between the PVR grade 1 or PVR 2 group and the control group. The concentrations of IGFBP-6 in the serum and retina in the PVR grade 3 group were significantly higher than that in control group (serum: 93.48 ±9.27ng/mL vs 80.59±5.20ng/mL; retina:3.02±0.38ng/mg vs 2.05±0.53ng/mg) (serum: P=0.038, retina: P=0.043) (Table 1).

MTS Proliferation Assay After the cells were incubated with exogenous IGF-II (50ng/mL) for 24h, the OD value, which reflects cell number, significantly increased (24h: from
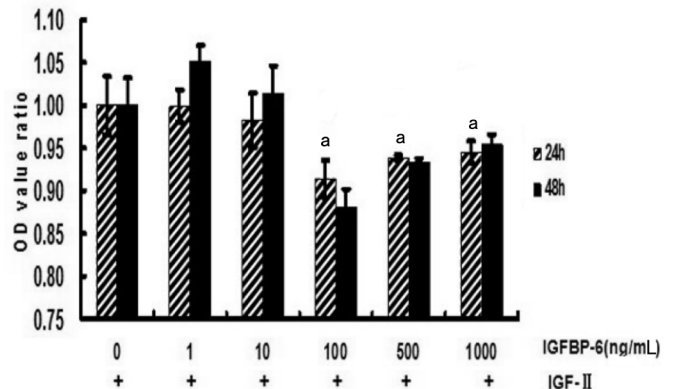
Figure 3 Effects of IGFBP−6 on the proliferation of ARPE−19 cell induced by IGF− II OD value ratio=OD value in the PVR group/OD value in the control group. The ARPE-19 cells in all groups were treated with 50ng/mL IGF- II for 6h to induce cell proliferation, and then different doses of IGFBP-6 were added to the media, which was incubated for 24 or 48h. The ARPE-19 cells were treated without IGFBP-6 in the control group ([a] P <0.05 vs control group, n =6).

1.26±0.05 to 1.38±0.05; 48h: 1.14±0.05 to 1.44±0.06) (n =6, 24h P=0.023, 48h P=0.009). When IGFBP-6 (500ng/mL) was added to the DMEM plates, the OD value was significantly reduced from 1.38±0.05 to 1.30±0.02 at 24h and from 1.44 ±0.06 to 1.35 ±0.05 at 48h (Figure 3). However, there was no significant difference after IGFBP-6 treatment in the VEGF, PDGF or TGF-β groups.
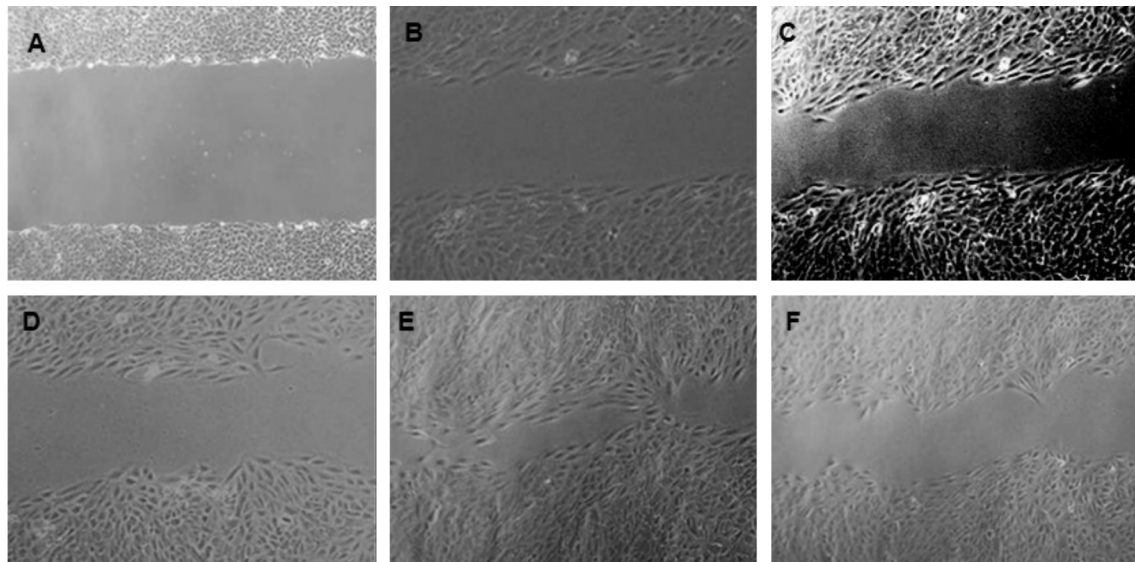
Figure 4 Photomicrographs of migrated ARPE–19 cell following a scratch wound (100×) A: 0h, wound healing rate: 0; B: 24h, serum-free medium, wound healing rate: (26.12±2.33)%; C: 24h, IGF-II (50ng/mL), wound healing rate: (43.91±3.85)%; D: 24h, IGF-II (50ng/mL) with IGFBP-6 (500ng/mL), wound healing rate: (29.76±2.49)%; E: 48h, IGF-II (50ng/mL), wound healing rate: (66.09±1.67)%; F: 48h, IGF-II (50ng/mL) with IGFBP-6 (500ng/mL), wound healing rate: (59.88±3.43)%.

Table 1 Concentrations of IGFBP-6 in control and PVR groups

Groups	n	Vitreous (ng/mL)	Serum (ng/mL)	Retina (ng/mg)
Control group	21	105.44±24.81	80.59±5.20	2.05±0.53
PVR grade 1	7	133.82±21.83	84.15±9.42	2.09±0.44
PVR grade 2	7	129.96±49.58	76.57±7.71	2.40±0.63
PVR grade 3	7	152.80±15.08[a]	93.48±9.27[a]	3.02±0.38[a]

The concentrations of IGFBP-6 in the vitreous, serum and retina in the PVR grade 3 group was significantly higher than that in control group ([a]$P<0.05$ *vs* control group).

Migration Assay To study the effect of IGFBP-6 on RPE wound healing, we adopted a scratch wound model. ARPE-19 cells were able to heal a scratch wound without exogenously added growth factors in serum-free DMEM, suggesting that these injured cells can generate autocrine factors for wound healing. Various concentrations of IGF-II with or without IGFBP-6 were added to the wound models. IGF-II greatly accelerated the wound closure of the ARPE-19 cells. In particular, IGF-II (50ng/mL) increased the wound healing rate from (26.12±2.33)% to (43.91±3.85)% at 24h (n=6, P=0.027) and from (57.05±2.49)% to (66.09±1.67)% at 48h (n= 6, P =0.043). IGFBP-6 (500ng/mL) reduced the IGF-II (50ng/mL)-induced wound healing rate from (43.91± 3.85)% to (29.76±2.49)% at 24h (n =6, P =0.025) and from (66.09±1.67)% to (59.88±3.43)% at 48h (n=6, P=0.039). Therefore, IGFBP-6 inhibits the migration induced by IGF-II (Figure 4).

DISCUSSION

Concentrations of IGFBP-6 increased in the vitreous, serum, and retinas in advanced PVR *in vivo. In vitro,* IGFBP-6 could inhibit IGF-II-induced cell proliferation and migration. Both of which suggest that IGFBP-6 participates in the development of PVR and might play a protective role in PVR. As we all know, PVR is still the leading cause of failure of rhegmatogenous retinal detachment surgery. And the anatomic and functional results are still unsatisfactory[14]. This procedure is still a challenge in the clinical setting. Experimental PVR models help us understand the pathogenesis of the disease and identify new therapeutic interventions. There are more than 25 methods to establish PVR models [15]. Most commonly, models have relied on the addition of cells or factors reported to be found in PVR. Rabbit models that involve the intravitreal injection of fibroblasts or RPE cells have been widely used because of the ease of working in rabbit eyes, which is due to such factors as the smaller size of the lens compared with the eyeball. The Wistar rat PVR model is a well-established model that could mimic the pathological changes observed in human PVR[15]. However, the rat has some disadvantages as an animal model for PVR due to its large relative lens size and smaller vitreous volume. In the current study, we selected a Wistar rat PVR model because of the lack of an antibody against IGFBP-6 for rabbits.

IGFBP-6 is a relatively new member of the IGFBP family because its affinity for IGF-II is approximately 50-fold higher than for IGF-I[16]. This feature makes it a very potent inhibitor of IGF-II activity, which is of particular interest for the growth inhibition of IGF-II-dependent tumors [17,18], such as neuroblastomas, rhabdomyosarcomas and colon cancer [19-22]. Meanwhile, IGFBP-6 could modulate cell differentiation and delay replicative senescence of human fibroblasts [23,24]. Several previous studies showed that IGFBP-6 is

accompanied mainly with an antiproliferative phenotype due to its binding of IGF-II and blocking of IGF/insulin signaling. IGFBP-6 also has IGF-independent functions[25-27].

The present study demonstrates that the concentrations of IGFBP-6 in the serum, vitreous, and retina increased in advanced PVR Wistar rats, which were consistent with previous findings in humans [11]. But the source of IGFBP-6 was unclear. The concentrations of IGFBP-6 in the vitreous were higher than the serum one, which suggested that it might come from the retina. The *in vivo* study suggests that IGFBP-6 may participate in the development of PVR. Because the contributions of IGFBP-6 to PVR were not clear, these in vitro experiments were performed.

During PVR development, RPE cells, inflammatory blood cells and pro-inflammatory serum elements are known to disperse into the vitreous space. Some cytokines, such as PDGF, VEGF, TGF-β and IGF-II, have been detected in the vitreous humor. These cytokines could induce RPE cell proliferation and migration, which are two important stages for PVR and proliferative diabetic retinopathies pathogenesis [28-31]. PVR can be alleviated if we arrest the proliferation and migration of the RPE cells. In this study, IGFBP-6 inhibited the IGF-II-stimulated RPE cell proliferation in a no dose- and time dependent manner. At the same time, IGFBP-6 could only inhibit IGF-II-stimulated but not PDGF-, VEGF-, and TGF-β-stimulated RPE cell migration, suggesting that IGFBP-6 is a potent anti-proliferative agent and that its anti-migration effects depend on its combination with IGF-II. Furthermore, IGFBP-6 could inhibit IGF-II-induced cell migration.

The *in vivo* study, however, showed that IGFBP-6 concentration was elevated in advanced PVR, and it may be speculated, in an attempt to ameliorate tissue damage. This finding argues against an impact of IGFBP-6 on the development, but rather a response to PVR. There are several possible explanations for this outcome. First, the recombinant IGFBP-6 used in the study may differ from that seen under actual physiological conditions. Second, there may be an imbalance between the proliferative and anti-proliferative effects *in vivo*. In advanced PVR, the promoted angiogenic factors, such as VEGF, PDGF, IGF-II and TGF-β, increased significantly. However, IGFBP-6 could only inhibit IGF-II-stimulated cell proliferation. Therefore, the anti-proliferative effects were less than the proliferative effects. Although the preventive effects of various pharmacologic agents in adjuvant treatment for PVR have been studied, none of them has been used as a routine treatment because of different reasons, so the treatment is a problem that needs to be settled. IGFBP-6 can be a potential reliable adjunct for the prevention or treatment of PVR[32-34]. Further experimental and clinical studies are necessary to elucidate its feasibility.

There are some limitations in our study. The sample number of the rat models was not large. *In vitro*, the experiment of whether IGFBP-6 can inhibit the VEGF-, PDGF-, and TGF-β-induced cell migration was not performed.

In conclusion, we found that concentrations of IGFBP-6 increased in the vitreous, serum and retinas in experimental PVR model *in vivo*. IGFBP-6 also inhibited IGF-II-induced cell proliferation and migration. IGFBP-6 participates in the development of PVR and may be a more reliable and effective alternative.

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