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Full Length Research Paper

Ultrasound-targeted microbubble destruction promotes proliferative vitreoretinopathy induced by RPE-J cells and platelet-rich plasma

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Ultrasound-targeted microbubble destruction (UTMD) has been considered as a new approach to the gene therapy of proliferative vitreoretinopathy (PVR), but whether it affect the *in vivo* PVR model induction remains unknown. In this study, 90 Wistar rats were averagely divided into three groups according to the content of intravitreal injection: normal saline (Group 1), retinal pigmented epithelial -J cells and PRP (Groups 2 and 3). In Group 3, a condition of UTMD was used additionally on days 3 and 7 after injection. On days 14 and 28, the pathological changes of eye grounds were assessed, and the expression levels of transforming growth factor (TGF)- 2 and platelet -derived growth factor (PDGF)-BB were tested. In Group 3, proliferation in the eyes was significantly stronger and faster than that of Group 2 and the ratio of PVR was significantly higher than that of Group 2. The expression levels of TGF- 2 and PDGF-BB were significantly higher in Group 3 than in Group 2. These data suggested that UTMD promotes PVR induced by RPE- J cells and PRP which provide a new method for the development of rat PVR model. This also reminds us that the effects of UTMD should be taken into consideration when using UTMD as an approach to attenuate PVR.

Key words: Proliferative vitreoretinopathy, rat model, ultrasound-targeted microbubble destruction, retinal pigmented epithelial -J cells, platelet-rich plasma.

INTRODUCTION

Proliferative vitreoretinopathy (PVR) is a serious complication of retinal detachment surgery or ocular trauma (Agrawal et al., 2007). In our previous research, we have confirmed that intravitreal co-injection of retinal pigmented epithelial (RPE) -J cells and platelet-rich plasma (PRP) in Wistar rat eyes can effectively induce a rat model of PVR which is characterized by sequential appearance of: 1) inflammatory cell infiltration, 2) extracellular collagen production and 3) formation of epiretinal membranes with or without retinal folds or detachment (Zheng et al., 2009a). Ultrasound-targeted microbubble destruction (UTMD) has been used to enhance the transgene expression in the eyes (Yamashita et al., 2007; Sanders et al., 2007; Zheng et al., 2009b, c, d) and it is considered as a new approach to the gene therapy of eye diseases such as PVR. Our previous studies demonstrate that UTMD can significantly enhance the transgene expression in the retina without tissue damage in normal eyes (Li et al., 2009; Zheng et al., 2009d). But whether it affect the *in vivo* PVR model induction remains unknown.

In the present study, we used a condition of UTMD in the process of *in vivo* PVR model induction in order to clarify the effects of UTMD on the PVR model induction and their possible mechanisms.

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MATERIALS AND METHODS

Microbubble contrast agents

SonoVue® microbubble contrast agent (MBs) (Bracco, Milan, Italy) was reconstituted in saline solution according to the manufacturer's protocol and yielded a preparation containing 2 to 5 × 10^8 micro-bubbles (MBs)/ml by inversion/agitation of the unit. This microbubble contrast agent is a composition of a core of sulfur hexafluoride gas and an envelope of phospholipids. The average diameter of the MBs was 2.5 to 6.0 m.

Animal grouping and Induction of PVR

Ninety Wistar rats used in this study were treated in accordance with the tenets of the ARVO statement for the Use of Animals in Ophthalmic and Vision Research who were averagely divided into three groups (n = 30): Group 1 (G1) received 8 l sterile pyrogen-free normal saline (NS); Group 2 (G2) received 8 l platelet-rich plasma containing RPE-J cells (2.4 × 10^6) and platelets (2 × 10^{10}); Group 3 (G3) not only received 8 l platelet-rich plasma containing RPE-J cells (2.4 × 10^6) and platelets (2 × 10^{10}), but also received a condition of UTMD (1 MHz, 2 W/cm^2, 5 min, 50% DC, 100 Hz, 2 l SonoVue® MBs). Experimental PVR was induced by a method previously described by Zheng et al. (2009a; 2010). Briefly, adult Wistar rats (male, 10 weeks old, 200 g, SLACCAS, Shanghai, China) were anesthetized by intraperitoneal injection of 10% chloral hydrate (350 mg/kg body weight). The pupils were dilated with one drop of 1% atropine sulfate and tropicamide. The eyes were gently protruded using a rubber circle and subsequently covered with 0. 3% ofloxacin eye ointment (Xingqi, Shenyang, China) to simulate a preset lens.

Under a surgical microscope (SM-2000J, Eder, Shanghai, China), 8 l platelet-rich plasma containing RPE- J cells (2.4 × 10^6) and platelets (2 × 10^{10}) were injected into the left eye using a blunt 32-gauge Hamilton syringe. The right eyes served as a control eye and were injected with 8 l NS.

Ultrasound exposure using microbubbles

In Group 3, the injected eyes received 2 l SonoVue® MBs and ultrasound exposure on days 3 and 7 after PVR induction. A therapeutic ultrasound machine (Topteam161, Chattanooga, TN, USA) were applied in this study. Immediately after intravitreal injection of 2 l SonoVue® MBs, a 2-cm^2 US probe placed directly onto the conjunctival surface after smearing a small amount of coupling medium on its face and then the insonation was performed. The parameters of ultrasound exposure were as follows: frequency, 1 MHz, power, 2 W/cm^2, duty cycle, 50%, pulse recurrent frequency, 100 Hz, duration, 300 s.

Ophthalmoscopic examination of eye fundus

Ten injected eyes of Wistar rats of each group were selected randomly and ophthalmoscopically examined by two masked observers on days 14, 28 after PVR induction. Fundus photographs were taken using a surgical microscope (SM-2000J, Eder, Shanghai, China). PVR was classified five grade scales using the clinical criteria described by Zheng et al. (2009a): 0, no proliferative response; 1, vitreous haze, vitreous strands; 2, retinal folds in a single or more quadrants; 3, epiretinal membrane formation in a single quadrant; 4, epiretinal membrane formation in two or more quadrants (Figure 1).

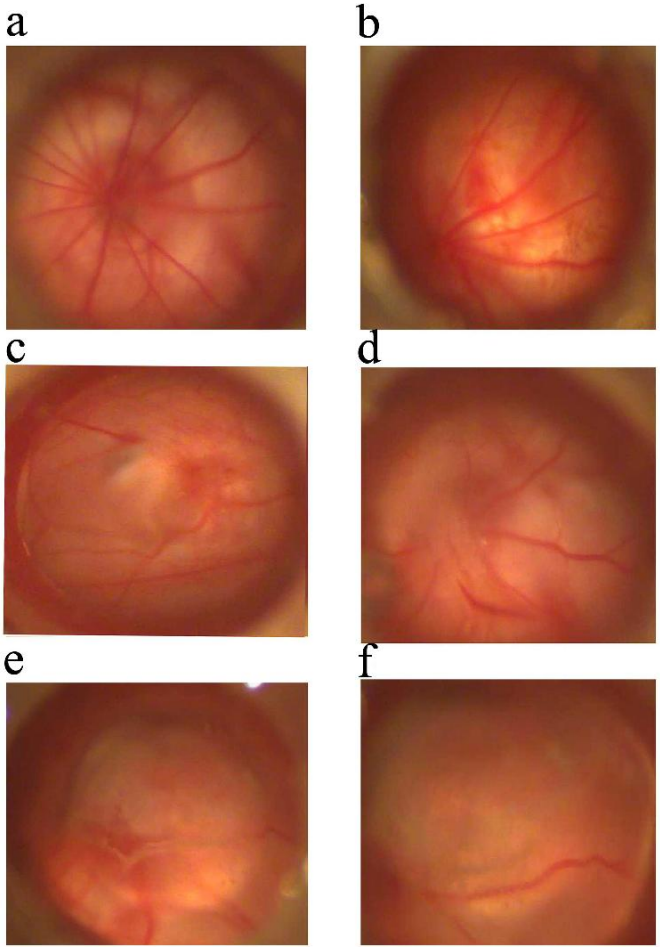

Figure 1. Eye ground photomicrographs showing the level of proliferation. a: 0, no proliferative response; b: 1, vitreous haze, vitreous strands; c: 2, retinal folds in a single or more quadrants; d: 3, epiretinal membrane formation in a single quadrant; e and f: 4, epiretinal membrane formation in two or more quadrants. Magnification, ×25.

Histopathologic examination

On days 14, 28 after PVR induction, the Wistar rats (four per time-point) of each group were sacrificed with a fatal dose of 10% chloral hydrate. The eyes were enucleated and fixed in 10% formaldehyde solution at a room temperature. Thereafter, they were embedded in paraffin and cut into 5 m-thick sections. Subsequently, the sections were stained with hematoxylin-eosin to observe retinal architecture, inflammatory cell infiltration and proliferative membrane using light microscopy (Zeiss Axiovert S 100, Jena, Germany) . All the results of histopathologic examination were con-firmed by two masked experts with experience in ophthalmological pathology.

Enzyme-linked immunosorbent assay (ELISA) analysis of TGF-2 and PDGF-BB expression

On days 14, 28 after PVR induction, the Wistar rats (four per time-point) were sacrificed with an overdose of 10% chloral hydrate. The retinas and the proliferative membranes were extracted and grinded

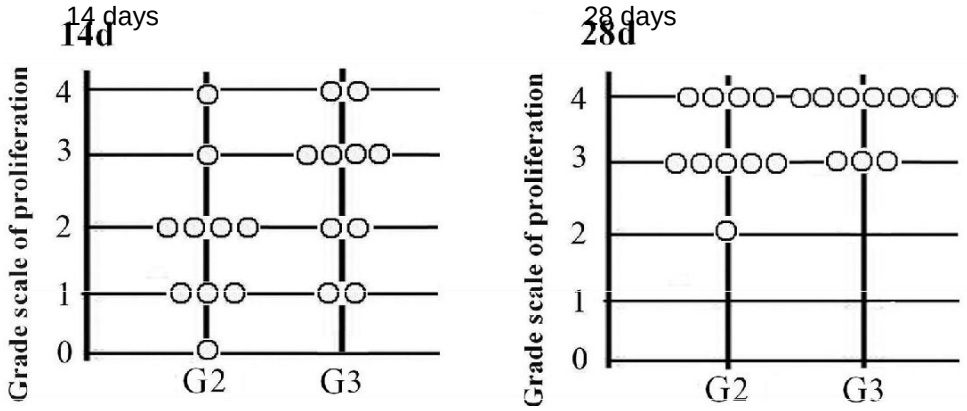

Figure 2. Results of ophthalmoscopic examination of eye ground in different treatment groups on days 14 and 28 after PVR induction.

into homogenate after globe enucleation. TGF- 2 and PDGF-BB were measured using a capture sandwich kit with biotinylated affinity purified mouse monoclonal antibodies to rat TGF- 2 and PDGF-BB (Senxiong, Shanghai, China) . Briefly, a flat-bottom ELISA plate (Costar 96-well) was coated with mouse anti -rat TGF- 2 and PDGF-BB antibody, 100 l of standard preparation (or sample) was added in the wells and incubated at 37°C for 2 h. After washing six times, 100 l biotinylated mouse anti-rat TGF- 2 and PDGF-BB was added and incubated at 37°C in the dark for 1 h, washed, and 100 l horseradish peroxidase labeled streptavidin was added and incubated at 37°C for 1 h. The wells were washed six times again and incubated with 100 l substrate solution for 5 to 10 min. Finally, 50 l stop buffer was added to each well.

Absorbance at 492 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, USA). The samples for the detection of TGF- 2 needed activation using HCl and NaOH just before the conventional procedure. Sensitivity by ELISA was 16 pg/ml with an intra-assay variability of 10%.

Statistical analysis

Data from experimental PVR were compared by using unpaired student t-test. Data from ELISA were presented as mean ± standard deviation (SD). One way analysis of variance (one-way ANOVA) was used to determine the significance of the difference in a multiple comparison. The differences were considered significant when the P values were less than 0.05. The software packages, SPSS (version 13, Chicago, USA) were used in this study.

RESULTS

Ophthalmoscopic observation of eye ground

As shown in Figure 2, the proliferation in the eyes in G3 was significantly stronger and faster than that of G2 and the ratio of PVR was significantly higher than that of G2. On days 14 and 28 after PVR induction, the average grade scales of proliferation in the eyes in G3 were significantly higher than those of G2 (2.6 vs.1.8 for day 14, $p<0.01$; 3.7 vs.3.3 for day 28, $p<0.05$). On day 28, 7 eyes (70%) and 3 eyes (30%) every 10 eyes in G3 had grades -4 and -3 PVR, respectively, with an epiretinal membrane formation and extensive retinal detachment.

Histopathologic examination

The histopathologic changes of eye in the different treatment groups are presented in Figure 3. In the eyes of G1, all the eye grounds were well preserved without inflammation, proliferative membrane formation, photoreceptor loss and nuclear layer vacuolation. In the eyes of G2 and G3, all the eye fundus had gone through a destructive damage from days 14 to 28 after PVR induction which included large number of inflammatory cell infiltration, retinal folds, retinal detachments and proliferative membrane formation with or without extensive retinal detachment. But, in G3, the histopathologic changes of eye progressed quickly which were more serious than those of G2. On days 14 and 28 after PVR induction, the numbers of effector cells such as RPE cells, glial cells, fibroblasts and macrophages, the incidence of inflammation, proliferative membrane formation and retinal detachments in G3 were significantly more than those of G2. In this group, most architectures of eye grounds were completely disrupted so that they could not be discriminated.

TGF- 2 and PDGF-BB protein expression in different treatment groups

Figure 4 shows the protein expression levels of TGF - 2 and PDGF-BB in different treatment groups. On days 14 and 28 after PVR induction, the protein expression levels of TGF- 2 in G2 and G3 were far higher than those of G1 and the protein expression levels of TGF- 2 in G3 were significantly higher than that of G2 (1839.97±196.25 vs. 1514.45±104.68 pg/ml for day 14; 2078.09±213.27 vs. 1309.45±184.77 pg/ml for day 28) . Moreover, the protein expression levels of TGF- 2 in G2 slightly decreased on

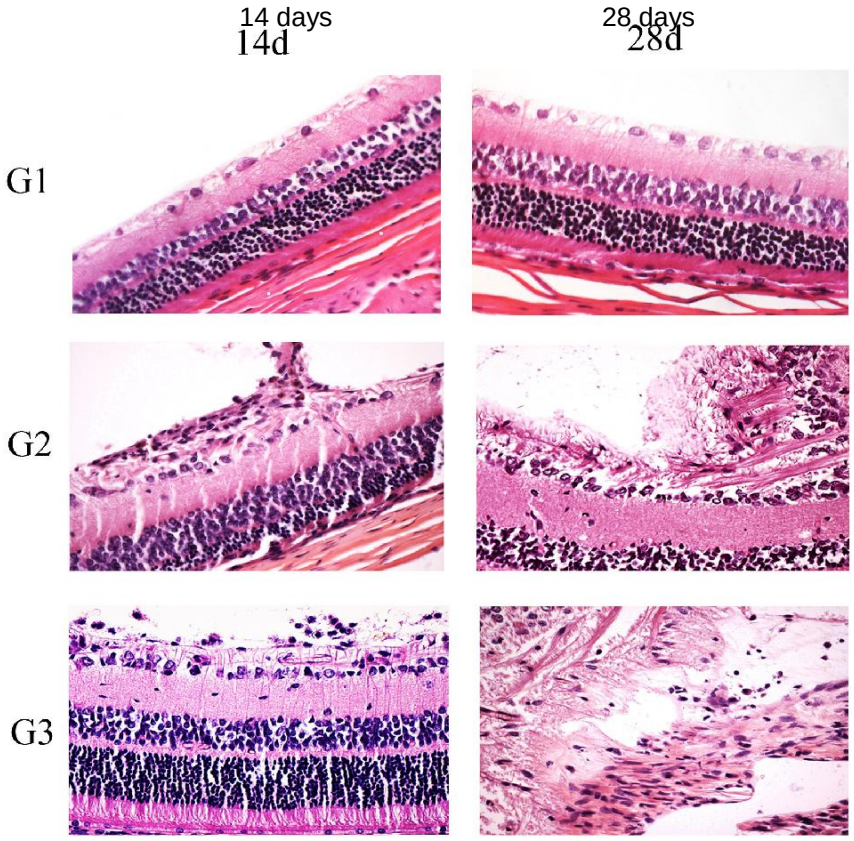

Figure 3. Results of histopathologic examination of eye in different treatment groups on days 14 and 28 after PVR induction. Magnification, ×400.

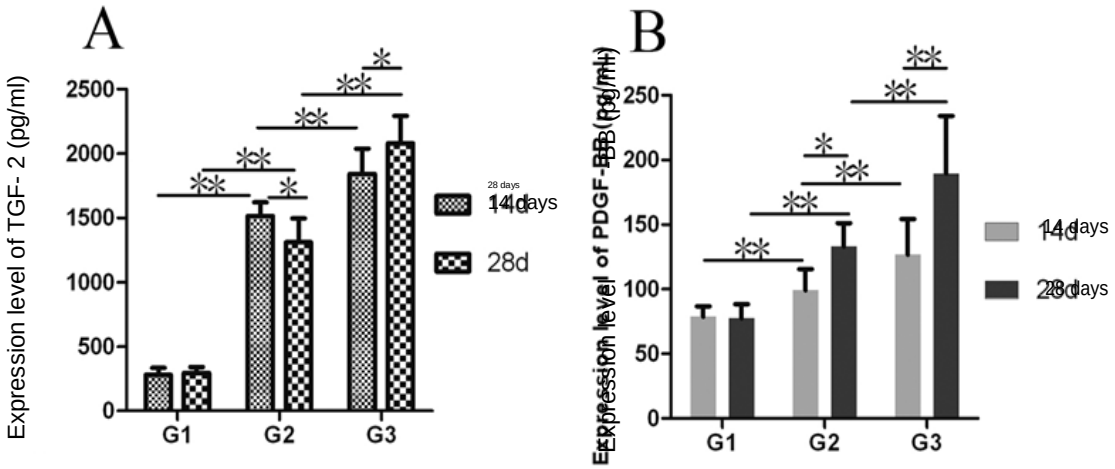

Figure 4. The protein expression levels of TGF- 2 (A) and PDGF-BB (B) analyzed by enzyme-linked immunosorbent assay on days 14 and 28 after PVR induction (*p<0.05, **p<0.01).

day 28, while that of G3 still increased at the same time. On days 14 and 28 after PVR induction, the protein expression levels of PDGF-BB in G2 and G3 were significantly higher than those of G1 and the protein expression levels of PDGF-BB in G3 were significantly higher than that of G2, too (126.63±27.62 vs. 99.02±16.57pg/ml for day 14; 189.48± 44.56 vs. 132.71±18.34 pg/ml for day 28).

DISCUSSION

The present study indicates that UTMD can exert a significant enhancing and improving effect on the induction of PVR, and the changes of the expression levels of TGF- 2 and PDGF-BB partially explained this result. PVR results from the migration and proliferation of cells of different origins such as RPE cells, retinal Müller glial cells, fibroblasts and macrophages. Growth factors such as TGF - 2, PDGF, hepatocyte growth factor, basic fibroblast growth factor or interleukin-6 are believed to play an important role in promoting the events that contribute to PVR (Andrews et al., 1999; Ikuno et al., 2000; Nagineni et al., 2005; Gamulescu et al., 2006; Zheng et al., 2009a). In the rat model of PVR induced by RPE -J cells and PRP, we had confirmed that RPE cells, glial cells, fibroblasts and macrophages took part in the pathogenesis (Zheng et al., 2010), and TGF-β2 and PDGF-BB played an important role in the proliferative process (Zheng et al., 2009a, 2010). Up till date, except in cases were surgical operations are applied, there are no satisfactory treatments available for PVR. Gene therapy for PVR seems to be a good idea. As a potential gene transfer modality, UTMD has been used in gene therapy of eye diseases. In our previous study, we had confirmed that a condition of UTMD ((1 MHz, 2 W/cm^2, 5 min, 50% DC, 100 Hz, 2 l SonoVue® MBs) had no obvious tissue damage to the normal eyes, so we assume that this condition should be used for the treatment of PVR. But how it affects the *in vivo* PVR model induction needs further study.

In this study, the severity of PVR in G3 was significantly higher than that of G2 and the expression levels of TGF- 2, PDGF -BB were significantly higher in Group 3 than in Group 2. These findings indicates that UTMD may promote the release of grow factors and therefore can further promote the proliferation of PVR. We believe that the bioeffects of UTMD such as cavitation, thermal effect, radiation force, chemical effect (Mukherjee et al., 2000; Basta et al., 2003; Juffermans et al., 2006; Murano et al., 2008) together may result in the increase of growth factors released by PRP, RPE -J cells and other effector cells, or some degree cell or tissue damage which further lead to the release of growth factors in the environment of PVR induction. Additionally, the expressions of TGF- 2 and PDGF-BB in the rat model of PVR begin to increase on day 3 and significantly increase on day 7 (Zheng et al., 2009a), so, we consider that the expression pattern of TGF- 2 and PDGF-BB in the rat model of PVR may provide an ideal interference time piont, that is, ultrasound exposure using microbubbles on days 3 and 7 after PVR induction may bring about better effects. Other time points still need further investigation. Although this is a preliminary study on the effects of UTMD on PVR induction, which provide a new method for the development of rat PVR model. This also reminds us that the effects of UTMD should be taken into consideration when using UTMD as an approach to attenuate PVR.

Future studies are needed to investigate the exact mechanisms of the effects of UTMD on the RPE-J cells, PRP and other eye cells, and the cell or tissue damage under the different condition of UTMD in the process of PVR induction.

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