A IMMUNOHISTOCHEMICAL ANALYSIS OF A RAT MODEL OF PROLIFERATIVE VITREORETINOPATHY AND A COMPARISON OF THE EXPRESSION OF TGF-β AND PDGF AMONG THE INDUCTION METHODS

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Abstract

Proliferative vitreoretinopathy (PVR) is a serious complication of retinal detachment surgery or ocular trauma. Our previous study indicated that intravitreal co-injection of retinal pigmented epithelial (RPE) -J cells and platelet-rich plasma (PRP) (not RPE-J cells or PRP alone) in Wistar rat eyes can successfully induce a model of PVR. But which cells are involved in this process and why different induction methods, intravitreal injection of RPE-J cells or/and PRP, induced a different situation remain to be unknown. In this study, immunohistochemistry was performed to identify the main cell types involved in this process. The expression levels of transforming growth factor (TGF) -β2, platelet-derived growth factor (PDGF)-AA and PDGF-BB were tested using enzyme-linked immunosorbent assay (ELISA).The results showed that RPE cells, glial cells, fibroblasts and macrophages took part in the pathogenesis of this model. The expression levels and durations of TGF-β2 and PDGF-BB partially explained the different results induced by the different induction methods. This provides an experimental proof for attenuation of the experimental PVR by targeting at a specific cells or growth factor.

KEY WORDS: proliferative vitreoretinopathy, immunohistochemistry, TGF-β2, PDGF, induction methods

Introduction

Proliferative vitreoretinopathy (PVR) is a serious complication of retinal detachment surgery or ocular trauma (1). To study the pathogenesis of PVR and the therapies for this disease, various in vivo models have been developed. However, as one of the most studied animal, rat, their models of PVR are still seldom reported. In our previous research, we have confirmed that intravitreal co-injection of retinal pigmented epithelial (RPE) -J cells and platelet-rich plasma (PRP) (not RPE-J cells or PRP alone) in Wistar rat eyes could effectively induce a PVR-like condition characterized by the sequential appearance of: 1)inflammatory cell infiltration, 2) extracellular collagen production, and 3) formation of epiretinal membranes with or without retinal folds or detachment(2). Quantitative detection of the expression level of TGF-β2, PDGF-AA, and PDGF-BB in this model indicates that TGF-β2 and PDGF-BB played an important role in the pathogenesis. But two questions arise. 1. Which cells involved in this process? 2.Why the induction methods, intravitreal injection of RPE-J cells or/and PRP, induced a different situation ?

In this study, in order to clarify the questions mentioned above, immunohistochemistry was performed to identify the main cell types involved in this process, and a comparative analysis was done among the different induction methods from the angle of the expression levels and durations of growth factors, TGF-β2, PDGF-AA and PDGF-BB.

Material and Methods

RPE-J cells preparation

RPE-J cells (CRL- 2240, ATCC, Rockville, USA) were defrosted from -80 °C liquid nitrogen and suspended in Dulbecco's modified Eagle's medium (Gibco, Grand Island, USA). Cell viability was controlled by trypan-blue counting. After defrosting, an average of 19% of RPE-J cells was observed dead. Then, the surviving RPE-J cells were seeded at a density of 2×10^4 cells/cm^2 in six-well culture plates, and maintained in DMEM with 4,5g/L glucose, 2 mM L-glutamine, and 0.1mM non-essential amino acids supplemented with 4% (v/v) fetal bovine serum (Gibco, Grand Island, USA) at 33°C, 5% CO2/95% air (3,4). For subsequent passages, cells from confluent cultures were detached by 0,25% trypsin digestion and seeded as described above. Just before intravitreal injection, RPE-J cells were collected from six-well culture plates, centrifuged at 1000 rpm for 10 minutes, and resuspended in sterile pyrogen-free normal saline (NS) or platelet poor plasma (PPP) at a concentration of 3×10^8/mL.

Preparation of PRP

Wistar rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (350 mg/kg body weight). Blood was collected into ethylene diamine tetraacetie acid vacuum tubes from the tail vein. The samples were centrifuged at 180g for five minutes to separate PRP from erythrocytes and leukocytes. PRP was transferred to a clean tube and centrifuged at 600g for 15 minutes to separate platelets from PPP(2,5). The platelet number in PRP was counted by an automatic hemocytometer and adjusted to $2,5\times10^{12}$/mL with PPP. The PRP was conserved at 20-24°C for about 10 minutes until intravitreal injection.

Animal preparation

After obtaining the approval of the local ethics committee, 88 normal adult Wistar rats (male or female, age=8-10 weeks, weight=180-200g, SLACCAS, Shanghai, China) were enrolled in this experiment. All animals were bred, maintained, and sacrificed humanely in strict compliance with the policies stated in the statement of Association for Research in Vision and Ophthalmology for the use of animals in ophthalmic and vision research.

Intravitreal injection of RPE-J cells and PRP

All Wistar rats were divided into four groups (20 rats per group in Group 1, 2, 3, and 28 rats in Group 4). Group 1, 2, 3 and Group 4 received an intravitreal injection of NS, RPE-J cells, PRP, and RPE-J cells +PRP, respectively. Before intravitreal injection, Wistar rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (350mg/kg body weight). Surgical procedures and post-surgery care were performed as described previously(6). Briefly, 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. Then, a self-sealing wound tunnel was constructed using a 1,5-cm, 28-gauge needle one mm posterior to the corneal limbus. After the vitreous cavity collapsed because of the outflow of vitreous fluid, a blunt 32-gauge Hamilton syringe was introduced through the sclera into the vitreous cavity under a surgical microscope (SM-2000J, Eder, Shanghai, China), 8μL NS containing RPE-J cells ($2,4\times10^6$) or 8μL PRP containing platelets (2×10^{10}) and 8μL PPP containing RPEJ cells ($2,4\times10^6$) and platelets (2×10^{10}) was injected into the left eye. The right eyes served as a control eye, and were injected with 8μL NS.

Immunohistochemical staining

Fourteen and 28 days after intravitreal injection, the Wistar rats (four rats per time-point) in Group 4 were sacrificed with a fatal dose of 10% chloral hydrate. Their eyes were enucleated, and fixed in 10% formaldehyde solution at a room temperature. Thereafter, they were embedded in paraffin, and cut into 5μm-thick sections. After deparaffinisation, endogenous peroxidase was abolished with 2% hydrogen peroxide in methanol for 10 min, and non-specific background staining was blocked by incubating the sections for 5 min in normal goat serum. After microwave antigen retrieval, the sections were incubated with the monoclonal mouse anti-rat antibodies listed in Table 1: Cytokeratin is expressed in all epithelial tissue, and cytokeratin-18 antibody was used to specifically identify proliferating RPE here. Glial fibrillary acidic protein (GFAP) Ab-1 directed at GFAP was used to identify glial cells. Vimentin is a marker of cytoskeletal intermediate filaments, while vimentin Ab-2 was used to identify fibroblasts in this study. ED1 antibody is a marker for monocytes, macrophages, and some dendritic subpopulations which was used to identify macrophages here. Optimal working concentration and incubation time for the antibodies were determined earlier in pilot experiments. All the sections were incubated for 60 min with the biotinylated anti-mouse secondary antibody and reacted with the avidin-biotinylated peroxidase complex (mrbiotech, Shanghai, China). The reaction product was visualised by the addition of 3, 39-diaminobenzidine (mrbiotech, Shanghai, China) and hydrogen peroxide, resulting in brown immunoreactive sites. The slides were faintly counterstained with Harris haematoxylin. Finally, the sections were rinsed with distilled water and coverslipped with glycerol. Control sections were incubated with Tris-buffered saline (pH 7,2–7,4) replacing the primary antibody. A upright microscope (Zeiss Axioplan 2 imaging, Carl Zeiss, Germany) was used to study the stained sections.

Primary antibody	Dilution	Incubation time	Source
Cytokeratin 18 (C-04)	1:50	60mitues	Santa Cruz, USA
GFAP Ab-1 (Clone GA-5)	1:100	60mitues	NeoMarkers,USA
Vimentin Ab-2(Clone V9)	1:100	60mitues	NeoMarke USA
ED1	1:100	60mitues	Santa Cruz, USA

TABLE 1. Monoclonal antibodies used in this study

Enzyme-linked immunosorbent assay (ELISA) Three, 7, 14, 21, and 28 days after intravitreal injection, the Wistar rats (four rats per time-point) were sacrificed with an overdose of 10% chloral hydrate. The retinas were extracted and grinded into homogenate after enucleation of the globe by removing the anterior segment with eye scissors. TGF-β2, PDGF-AA and PDGF-BB were measured using a capture sandwich kit with biotinylated affinity purified mouse monoclonal antibodies to rat TGF-β2, PDGF-AA or PDGF-BB (Senxiong, Shanghai, China). Briefly, a flat-bottom ELISA plate (Costar 96-well) was coated with mouse anti-rat TGF-β2, PDGF-AA or PDGF-BB antibody, 100μL of standard preparation (or sample) was added in the wells and incubated at 37°C for two hours. After washing six times, 100μL of biotinylated mouse anti-rat TGF-β2, PDGF-AA or PDGF-BB was added and incubated at 37°C in the dark for one hour, washed, and 100μL of horseradish peroxidase labeled streptavidin was added and incubated at 37°C for one hour. The wells were washed six times again, and incubated with 100μL of substrate solution for 5-10 minutes. Finally, 50μL of 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-β2 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

All data were expressed as mean±SD. Analysis of variance (ANOVA) test was used to determine the significance of the difference in a multiple comparison. The differences were considered significant at p-values less than 0,05. The software packages used were SPSS (version 13, Chicago, USA).

Results

Immunohistochemical analysis of eyes 14 and 28 days after co-injection of RPE -J cells and PRP As shown in Figure 1., there was no staining in the negative control slides (Figure 1A). At 14 days, a small amount of RPE cells expressing cytokeratin-18 migrated from pigment epithelium layer to the ganglion cell layer, and began to proliferate (Figure 1B). At 28 days, numerous proliferating RPE had distributed in the detached retina and proliferative membrane in vitreous cavity (Figure 1C). At 14 days, there were a number of glial cells expressing GFAP distributing in the epiretinal membrance, and they significantly reduced at 28 days (Figure 1D and 1E). The same tendency was seen in the fibroblasts which express the vimentin. At 14 days, a great quantity of fibroblasts distributed at the retinal surface and most of them had established con-

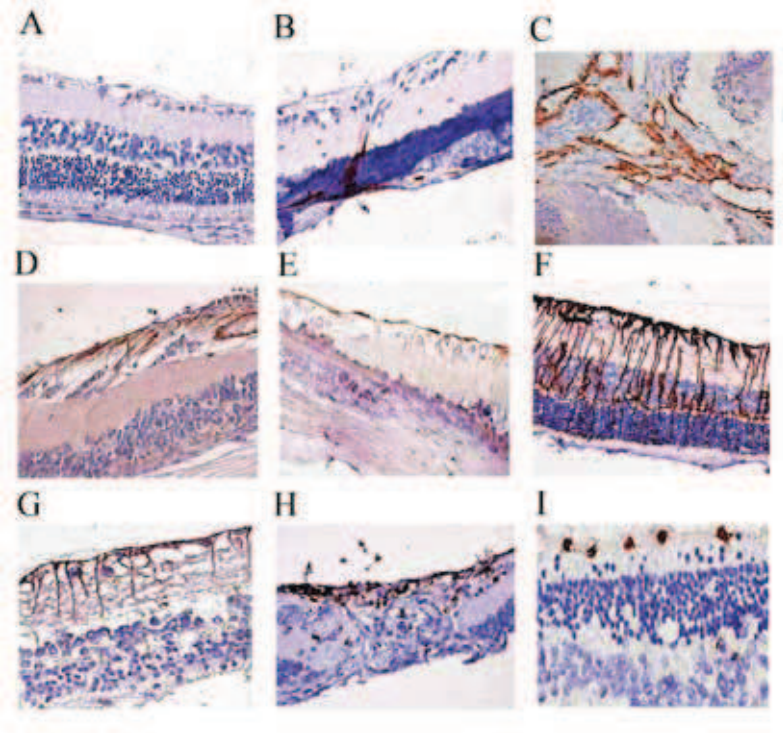

FIGURE 1. Immunohistochemical staining of eyes 14(B, D, F, H) and 28(C, E, G, I) days after co-injection of PRP and RPE-J cells. (A) negative control; (B) and (C) immunohistochemical staining for cytokeratin-18 showing proliferating RPE cells expressing cytokeratin-18; (D) and (E) immunohistochemical staining for glial fibrillary acidic protein (GFAP) showing glial cells expressing GFAP; (F) and (G) immunohistochemical staining for vimentin showing fibroblasts expressing vimentin; (H) and (I) immunohistochemical staining for ED1 showing macrophages expressing ED1 (A, B, C, D, E, F,G, H and I: 400× magnification)

nections with the inner retina (Figure 1F), however, at 28 days, only a few of fibroblasts still distributed at the retinal surface, and the connections with the inner retina was seldom seen (Figure 1G). In addition, a lot of ED1-positive cells, i.e., macrophages, distributed in the epiretinal membrance at 14 days(Figure 1H), and they were still present at the retinal surface at 28 day, despite the small quantity (Figure 1I). The expression levels and durations of TGF-β2, PDGF-AA or PDGF-BB in different treatment groups Figure 2. shows the growth factors, TGF-β2, PDGF-AA and PDGF-BB expressed in the eyes of Wistar rats received intravitreal injection of NS, PRP or/ and RPE-J cells. It was found that the expression levels of TGF-β2 were far higher than that of PDGF-AA and PDGF-BB, and the expression levels of PDGF-BB were significantly higher than that of PDGF-AA. The expression levels of TGF-β2 in the RPE -J cells and PRP co-injection group were significantly higher than that of NS, PRP or RPE-J cells injection groups at 7,14, 21, and 28 days (about 1,5-2,8 times). In this co-injection group, the peak of expression level of TGF-β2 appeared at 21 days (1206,73±50,52pg/ML) and there was a significant decrease at 28 days. In PRP injection group, the expression level of TGF-β2 presented a downward trend as a whole, although there was a transient increase at 7 days. As for the expression level of TGF-β2 of NS and RPE-J cells injection group, they were little changed with time (Figure 2A and 2B). Interestingly, there was no significant difference of the expression levels of PDGF-AA among the different treatment groups, they all gradually decreased from 3 days to 28 days(Figure 2C and 2D). The expression level of PDGF-BB was another indicator of concern. At 7, 14, 21, and 28 days after intravitreal injection, the expression levels of PDGF-BB of the RPE-J cells and PRP co-injection group were also significantly higher than that of NS, PRP or RPE-J cells injection groups (about 1,1-1,7 times). As TGF-β2, a similar trend was seen in PDGF-BB. It decreased at 28 days after achieving its peak at 21 days, while in the NS, PRP or RPE-J cells injection groups, the trends of PDGF-BB were overall decline, too.

Discussion

The present study indicates that RPE cells, glial cells, fibroblasts and macrophages took part in the pathogenesis of this rat model of PVR. Meanwhile, the expression levels and durations of TGF-β2 and PDGF-BB partially explained the different results induced by the different induction meth-

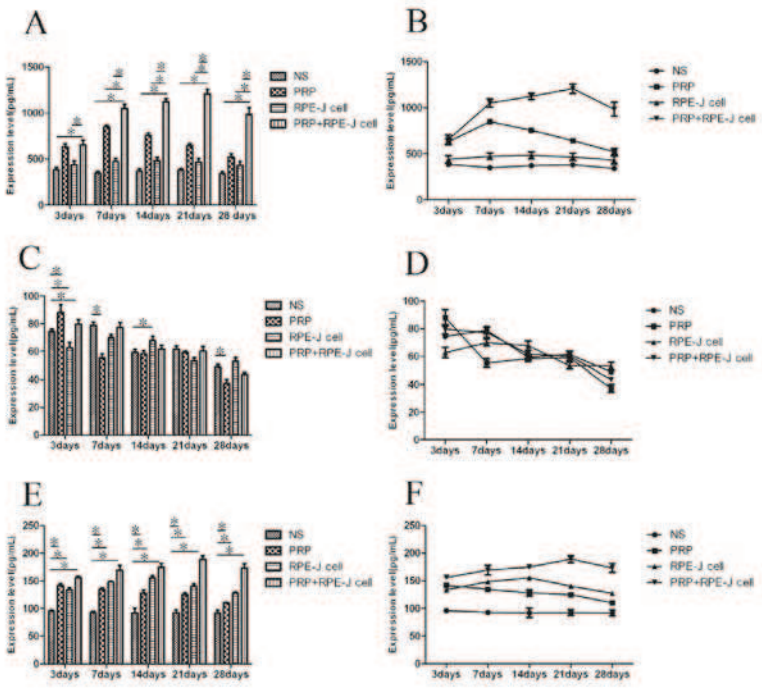

FIGURE 2. Histogram and curve chart showing the levels of TGF-β2(A,B), PDGF-AA(C,D) and PDGF-BB (E,F) expression and their variation with time in the eyes after intravitreal injection of NS, PRP or/and RPE-J cells (*p<0,05) by the detections of growth factors of every treatment group.

ods, intravitreal injection of RPE-J cells or/and PRP. Our clinical and histopathologic observations show that intravitreal co-injection of PRP and RPE-J cells in Wistar rats can successfully induced a PVR-like condition, but the cellular and molecular mechanisims underlying this process were unknown. It is known that RPE cells, fibrous astrocytes, fibroblasts, myofibroblasts and macrophages are the principal cells involved in periretinal proliferation(7,8). Then, are these cells involved in this PVR-like condition, too? Our results of immunohistochemistry showed that RPE cells, glial cells, fibroblasts and macrophages are all the contributors to this pathogenesis, which is more relevant to the human disease, especially human PVR associated with intraocular bleeding. Furthermore, these cells appeared with certain regularity: gradually increased for RPE cells, first increased and then decreased for other cells.This provides an experimental proof for attenuation of the experimental PVR by targeting at a specific cells at a particular time point. Our previous study also indicates that PRP alone could induce a PVR-like condition characterized by low ratio, slow advancement, and low severity, while RPE-J cells alone could never induce a Wistar rat model of PVR. How to explain this phenomenon? We hypothesize that the different expression levels and durations of growth factors played a key role, because PRP has been shown to be rich in growth factors, which are able to enhance endothelial cell migration and proliferation (5, 9). PRP and RPE-J cells co-injection may have synergistic effects. This view was confirmed by the fact that the expression levels of TGF-β2 and PDGF-BB in PRP and RPE-J cells co-injection group were higher than that of other groups, and their durations were also longer, which ensured higher rates of adult model. However, the low expression levels and short durations of TGF-β2 and PDGF-BB in PRP or RPE-J cells injection group resulted in an opposite outcome. Growth factors, such as TGF-β2, 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(10-12). Moreover, there is a cooperative interaction between TGF-β2 and PDGF. The increase of growth factors favor the recruitment of effector cells, such as RPE cells, glial cells, macrophages, and fibroblast, so as to promote the inflammatory reaction during the first phases of PVR. This is followed by a proliferative process (13,14). In such a process, PDGF-AA seemes not to play any role. This is because PDGF-AA is selective more than PDGF-BB, which binds PDGF β receptor only. The expression of PDGF β receptor in this model may be low, and further characterization is required to do. Nevertheless, this maybe provides another experimental proof for attenuation of the experimental PVR by targeting at a specific growth factor or its receptor.

Conclusion

RPE cells, glial cells, fibroblasts and macrophages took part in the pathogenesis of this rat model of PVR. The differences of expression levels and durations of TGF-β2 and PDGF-BB partially explain the different results induced by the different induction methods, intravitreal injection of RPE-J cells or/and PRP. Intravitreal co-injection of RPE-J cells and PRP in Wistar rat eyes could effectively induce a model of PVR which offers a foundation for the study of the pathogenesis and therapies for PVR.

List of Abbreviations

ELISA	-	enzyme-linked immunosorbent assay
GFAP	-	Glial fibrillary acidic protein
NS	-	normal saline
PDGF	-	platelet-derived growth factor
PPP	-	platelet poor plasma
PRP	-	platelet-rich plasma
PVR	-	proliferative vitreoretinopathy
RPE	-	retinal pigmented epithelial
TGF	-	transforming growth factor

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