Inhibitory Effects of Plumbagin on Retinal Pigment Epithelial Cell Epithelial-Mesenchymal Transition In Vitro and In Vivo

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Background: This study aimed to explore the effects of plumbagin (PLB) on epithelial-to-mesenchymal transition in retinal pigment epithelial (RPE) cells and in proliferative vitreoretinopathy (PVR) rabbit models.

Material/Methods: Rabbit RPE cells were exposed to various concentrations (0, 5, 15, and 25 µM) of PLB. Motility, migration, and invasion of PLB-treated cells were determined in vitro using Transwell chamber assays and scratch wound assays. The contractile ability was evaluated by cell contraction assay. Expression of matrix metalloproteinases (MMPs) and epithelial-mesenchymal transition (EMT) markers were assessed by western blotting. Furthermore, PLB was injected in rabbit eyes along with RPE cells after gas compression of the vitreous. The presence of PVR was determined by indirect ophthalmoscopy on days 1, 7, 14, and 21 after injection. Also, optical coherence tomography (OCT), ultrasound images, electroretinograms (ERG), and histopathology were used to assess efficacy and toxicity.

Results: PLB significantly inhibited the migration and invasion of RPE cells. The agent also markedly reduced cell contractive ability. Furthermore, PLB treatment resulted in the decreased expression of MMP-1, MMP2, α-SMA, and the protection of ZO-1. In addition, the PLB-treated eyes showed lower PVR grades than the untreated eyes in rabbit models. PLB exhibited a wide safety margin, indicating no evidence of causing retinal toxicity.

Conclusions: PLB effectively inhibited the EMT of rabbit RPE cells in vitro and in the experimental PVR models. The results open new avenues for the use of PLB in prevention and treatment of PVR.

MeSH Keywords: Epithelial-Mesenchymal Transition • Plumbaginaceae • Retinal Pigment Epithelium

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Background

Proliferative vitreoretinopathy (PVR) is a fibrotic disease characterized by the formation and constriction of epiretinal membranes (ERMs) composed of various kinds of cells and their extracellular matrix (ECM), finally leading to tractional retinal detachments and visual impairment [1]. The most important cell type in PVR pathogenesis is retinal pigment epithelial (RPE) cells, which are believed to undergo epithelial-mesenchymal transition (EMT) to a fibroblastic phenotype, then migrate, proliferate, and at last form epiretinal membranes [2]. Nowadays, more efforts have been made to look for prophylactic or therapeutic agents to inhibit the EMT of RPE in order to stop PVR at an early stage [3].

Plumbagin (PLB; 5-hydroxy-2-methyl-1,4-naphthoquinone) is a kind of natural naphthoquinone component isolated from the root of Plumbago zeylanica L, which has an extensive range of effects including anti-inflammation, anti-microbe, anti-cancer, and neuroprotection in multiple cell lines and animal models [4]. Recently, the anti-EMT effect of PLB has been a high interest research topic. It has been proven that this effect of PLB may be the main reason causing cell viability suppression [5,6].

In the present study, we aimed to investigate whether PLB could effectively inhibit the EMT of rabbit RPE cells in vitro and in an experimental model of PVR.

Material and Methods

In vitro study

Rabbit RPE cell culture

The tissue-cultured RPE cells in our study were taken from New Zealand albino rabbits. We enucleated the rabbit eyes, removed the anterior segment and vitreous, and trypsinized the RPE cells with 0.25% trypsin with 0.02% EDTA (Sigma-Aldrich; St. Louis, MO, USA) at 37°C for 30 minutes. Afterwards, the cells were cultured in a DMEM/F12 medium supplemented with 10% FBS and regular antibiotics (1% penicillin and streptomycin) (Gibco®; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere with 5% CO₂, with medium changes every three days, and subcultured if confluence was reached. PLB, Sigma-Aldrich; St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stock at 100 mM, which was diluted into working concentrations with culture medium. The control group received the vehicle (0.05% DMSO) only.

Transwell chamber assays

Transwell chamber assay (Costar, Cambridge, MA, USA) was used to measure the motility of rabbit RPE cells. In brief, the wells of 24-well culture plates were separated into upper and the lower chambers with 8-µm pore size inserts. There was 600 µL DMEM/F12 containing 10% FBS in the lower chamber, while 5x10⁴ RPE cells with various concentration of PLB (0, 5, 15, or 25 µM) were cultured for 24 hours in the upper chamber. The cells which migrated through the pores were displayed by crystal violet and calculated using the microscope (Axiovert 200, Zeiss; Oberkochen, Germany).

Scratch wound assay

RPE cell monolayers were scratched by a 200 µL pipette tip and washed thrice. Then the cells were cultured in serum-free medium with different concentration of PLB (0, 5, 15, or 25 µM) for 24 hours. The migratory ability was assessed by the relative migration rate.

Cell contraction assay

After treatment as described previously, 1x10⁴ RPE cells from each group were suspended in a solution containing 1.5 mg/mL neutralized collagen I (INAMED; Fremont, CA, USA), and transferred into a 24-well plate which had been pre-incubated with 5 mg/mL bovine serum albumin overnight. After incubation at 37°C for 90 minutes, the collagen gel polymerized and the area was calculated to evaluate the contractile ability of RPE cells.

Western blot

The expressions of EMT-related proteins were assessed by western blotting assays. Rabbit RPE cells were incubated with various concentrations of PLB (0, 5, 1, and 25 µM) for 24 hours, or exposed to 15 µM PLB for 12, 24, and 48 hours. The treated cells were lysed with RIPA buffer (Solarbio; Beijing, China) and protein contents were determined by Pierce™ bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc.; MA, USA). Each protein sample at 50 ng and rainbow molecular weight markers (11–245 kDa; Solarbio; Beijing, China) were electrophoresed on 8–10% sodium dodecyl sulfate polyacrylamide gel electrophoresis minigel (SDS-PAGE) after thermal denaturation at 95°C for five minutes. Proteins were electrotransferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore; Billerica, MA, USA) at 100 V at 4°C for two hours, and then stopped with 5% non-fat milk. Subsequently, membranes were probed with indicated primary antibody against MMP1 (ab38929), MMP2 [EPR1184](ab92536), alpha-SMA[EPR5368] (ab124964), and ZO-1(ab96587), (Abcam, Cambridge, UK) at 4°C overnight, then marked by goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (iG; catalog no. 7074S; Cell Signaling Technology Inc.; MA, USA) for one hour at room temperature according to the manufacturer’s protocol. The protein bands were visualized by the Bio-Rad ChemiDoc™ XR5 system (Bio-Rad Laboratories Inc.; CA, USA) and...
the density was measured by ImageJ analytical software (National Institutes of Health, Bethesda, MD, USA). The protein concentrations were calculated based on the value of the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ab128915; Abcam; Cambridge, UK).

**In vivo study**

**Animals**

All animal management in the present study abided by the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and the NIH Principles of Laboratory Animal Care (revised 1985). Thirty-four New Zealand albino rabbits weighing between 2.5 kg and 3 kg were used. The animals were sedated by intravenous injection of pentobarbital (2 mg/kg). The eyes were topically anesthetized with proparacaine hydrochloride eye drops and the pupils were dilated with topical 1% tropicamide and 2.5% phenylephrine. Rabbits were sacrificed by intravenous injection of an overdose of pentobarbital.

**Toxicity study**

We use electroretinograms (ERG) as a criterion to assess the retinal toxicity of PLB. Twelve rabbits were divided into three groups: four eyes accepted intravitreal injections through pars plana with 25 μM PLB, four eyes with 50 μM PLB while four eyes with DMSO (0.5‰) only as the control group. Each right eye was treated with the left eye as the control one. Bilateral ERG (Neuropack-II Plus; Nihon, Kohden, Japan) were operated on the day before the injection, on days 3, 7, 14, and 21 after the injection, and maximal b-wave amplitude was used for evaluation. Three weeks later, the eyeballs were taken out instantly after the rabbits were sacrificed, and fixed with formaldehyde, dehydrated in ethanol series, sliced into epoxy resin sheets, stained with hematoxylin and eosin, then examined by light microscopy.

**Efficacy study**

**Rabbit PVR model induction and observation**

Rabbits were acclimated for one week and then 0.1 mL perfluoropropane gas (C3F8; Alcon; Fort Worth, TX, USA) was injected into the vitreous cavity of each right eye. One week later, the experimental eyes were co-injected with 2 x 10⁵ rabbit RPE cells suspended in 0.1 mL platelet-rich plasma (PRP) and 0.1 mL balanced salt solution (BSS) of either 25 μM PLB in DMSO (0.5‰) or DMSO alone (11 eyes experimental), or DMSO alone (11 eyes control) under aseptic conditions. The retinal status was monitored using indirect ophthalmoscopes (Keeler Instruments, Broomall, PA, USA) on 3, 7, 14, and 21 days after injection. Extent of PVR was evaluated according to Fastenberg classification as follows: stage 0, represented normal; 1, ERM formation; 2, vitreoretinal traction without retinal detachment; 3, localized retinal detachment of one to two quadrants; 4, extensive retinal detachment of two to four quadrants; 5, complete retinal detachment. Retinal function was assessed by ERGs of every week after injection. Intraocular pressures were monitored daily for three days after gas injection and for one day after the co-injections using a tonometer (TONO-PEN XI; Reichert; Depew, USA). Fundus images were obtained using the Spectralis optical coherence tomography (OCT) (Heidelberg, Germany), while ultrasound images were obtained using AVISO ultrasonic diagnostic instrument (QuanTec Medical Ltd., France). Signs of toxicity, including intraocular inflammation and hemorrhages, were assessed during each fundus examination and by histological analysis of retinas after sacrifice of animals. Animals were sacrificed at day 21, and eyes were enucleated for later analysis. All examinations were accomplished by the same observers.

**Statistical analysis**

The in vitro experiments were performed at least three times and data were expressed as mean ± standard deviation (SD). SPSS version 22.0 (SPSS, Inc.; Chicago, USA) was used to perform statistical analyses. Differences between control and treated samples were evaluated by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison procedure. A Pearson test was performed for correlation. For in vivo studies, the Wilcoxon signed-rank test was chosen to compare the degree of PVR between the two groups. A value of p < 0.05 was considered as statistically significant in each experiment.

**Results**

**In vitro studies**

**PLB inhibited migration and invasion of rabbit RPE cells**

To investigate whether PLB could inhibit migratory and invasive properties of rabbit cells, scratch wound assay and Transwell chamber assay were used. When cells were incubated with 0, 5, 15, and 25 μM PLB, the total cell numbers in the scratch area were (184.0±11.1), (127.7±8.6), (92.7±13.8) and (51.3±9.1) respectively (Figure 1A). As shown in Figure 1B, the relative cell migration rate were (0.74±0.09), (0.39±0.08) and (0.08±0.04) respectively, compared to the control group. The results demonstrated that PLB could significantly inhibit
the migration and invasion of rabbit RPE cells dose-dependently (Pearson test; \( p = 0.000 \)).

**PLB suppressed contractile ability of RPE cells**

The formation and contraction of the epiretinal membrane is the hallmark of PVR and plays an important role in tractional retinal detachment during PVR. We have developed an assay to examine the contractile ability in vitro using rabbit RPE cells. As shown in Figure 3, the matrices mixed with RPE cells treated under 0, 5, 15, 25 \( \mu M \) PLB shrank to 26.0%, 40.1%, 56.9% and 77.1% respectively compared to initial area respectively. The collagen matrix contraction was reduced significantly when cells were cultured with 15 and 25 \( \mu M \) PLB (\( p = 0.003, 0.000 \) respectively). Moreover, PLB inhibited the contractile ability of RPE cells in a dose-dependent manner (Pearson test; \( p = 0.000 \)).

**PLB modulated expression of EMT-related proteins and MMPs in RPE cells**

In order to further explore the anti-motive mechanism of PLB on RPE cells, we evaluated the expression of proteins associated with this phenomenon. It was well known that MMPs induced epithelial to mesenchymal transition and thus increased the invasive potential of cells. To determine their involvement in RPE cells, the expression levels of protein MMP-1, MMP-2 were
assessed by western blotting analysis (Figure 4A). When ARPE cells were incubated with PLB at 5, 15, and 25 µM for 24 hours, the level of MMP-1 decreased to 69.4%, 39.7% and 26.7% respectively compared to control group (each \( p = 0.000 \)). Similarly, the content of MMP-2 reduced to 83.5%, 65.2% and 49.9% respectively (\( p = 0.013, 0.000, 0.000; \) Figure 4B). Furthermore, the differences between adjacent groups also had statistical significance (\( p < 0.05 \)) and the changes was dependent on the concentration of PLB (Pearson test; \( p = 0.000 \)). In parallel experiments, we found that PLB regulated these MMP proteins in a time-dependent manner as well (Pearson test; \( p = 0.000 \)). Briefly, RPE cells were exposed to 15 µM PLB for 12, 24, or 48 hours, the expression of MMP-1 decreased to 87.3%, 49.7%, and 48.8% respectively (\( p = 0.033, 0.000, 0.000 \)) and the level of MMP-2 was reduced to 93.8%, 76.3% and 64.4% respectively compared to control cells, respectively (\( p = 0.450, 0.001, 0.000; \) Figure 4C). Together with the data from our previous study, these results demonstrated a downregulating role in the MMPs expression of PLB in ARPE cells.

As is known to all, cells that have undergone EMT usually have reduced epithelial features such as zonula occludens-1 (ZO-1), whereas increase mesenchymal features such as \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA). Herein, we observed the expression of \( \alpha \)-SMA and ZO-1 as EMT symbolic factors using western blotting (Figure 4A). After treatment with 5, 15, and 25 µM PLB for 24 hours, the content of \( \alpha \)-SMA in RPE cells decreased significantly from 0.98 at base level to 0.88, 0.75 and 0.63 respectively, dose-dependently (\( p = 0.104, 0.002, 0.000; \) Pearson test, \( p = 0.000 \)). On contrast, the level of ZO-1 increased slightly from 0.39 at the basal line to 0.42, 0.44, and 0.40 respectively (\( p = 0.067, 0.001, 0.918; \) Pearson test, \( p = 0.672; \) Figure 4D). Furthermore, when RPE cells were exposed to 15 µM PLB for 12, 24, and 48 hours, there was a 11.5%, 24.4%, and 27.4% respective reduction in the level of \( \alpha \)-SMA (\( p = 0.009, 0.000, 0.000; \) Pearson test, \( p = 0.000 \)), while 8.2%, 11.4%, and 4.1% respective augment in the level of ZO-1 (\( p = 0.027, 0.004, 0.324; \) Pearson test, \( p = 0.512 \)), in comparison to the control group (Figure 4E).

**Discussion**

Epithelial-mesenchymal transition (EMT) is a cellular metamorphosis procedure in which epithelial cells abandon their...
Figure 4. Effect of plumbagin (PLB) on the expression of EMT-related proteins in ARPE cells. (* p<0.05 by one-way ANOVA, n=3).
(A) The expression levels of MMP-1, MMP-2, α-SMA and ZO-1 determined by western blotting assay. GAPDH was used as the internal control. (B) Bar graphs show the expression levels of MMP-1 and MMP-2 when ARPE cells were treated with PLB at 0, 5, 15, and 25 µM for 24 hours. (C) Bar graphs show the expression levels of MMP-1 and MMP-2 when ARPE cells were treated with PLB at 15 µM for 0, 12, 24, and 48 hours. (D) Bar graphs show the expression levels of α-SMA and ZO-1 when ARPE cells were treated with PLB at 0, 5, 15, and 25 µM for 24 hours. (E) Bar graphs show the expression levels of α-SMA and ZO-1 when ARPE cells were treated with PLB at 15 µM for 0, 12, 24, and 48 hours.
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Figure 5. Electroretinography and histology of rabbits treated with Plumbagin (PLB). (A) The mean ratio of b-wave amplitudes of dark adaptation electroretinograms of rabbit eyes treated with 25 μM PLB, 50 μM PLB, or DMSO only, on the day before the infection, day 3, 7, 14 and 21; n=4 at each dose. (B) Light microscopy of the rabbit retina 21 days after intravitreous injection of PLB (50 μM). Every layer appeared to be normal with well-preserved photoreceptor outer segments; hematoxylin and eosin staining; magnification 400×.

Figure 5.a. The mean ratio of b-wave amplitudes of dark adaptation electroretinograms of rabbit eyes treated with 25 μM PLB, 50 μM PLB, or DMSO only, on the day before the infection, day 3, 7, 14 and 21; n=4 at each dose.

Figure 5.b. Light microscopy of the rabbit retina 21 days after intravitreous injection of PLB (50 μM). Every layer appeared to be normal with well-preserved photoreceptor outer segments; hematoxylin and eosin staining; magnification 400×.

As everyone knows, RPE is a monolayer of pigmented cells tightly connected and is crucial for the preservation of photoreceptor function. Normally, RPE cells remain quiescent, maintaining original characteristic morphology and action. Under pathological circumstances such as retinal break, the outer blood-retinal barrier (BRB) breaks down, RPE cells disseminate into the vitreous cavity, where they lose RPE cell-cell contact and initiate EMT [11]. During EMT, RPE cells express less epithelial symbols such as zonula occludens-1 (ZO-1), E-cadherin but more mesenchymal symbols such as α-smooth muscle actin (α-SMA), vimentin, and fibronectin [12]. These dedifferentiated RPE cells can then migrate, proliferate, change into fibroblasts, and contract, at last resulting in PVR. Previous studies showed inhibition of proliferative ability of cells may reduce the severity of the illness, but could not completely block the progress of PVR [13]. It has been suggested that perhaps the EMT of RPE deserves more attention since it plays a more crucial role in the development of PVR [10,11]. Therefore, it is imperative to develop drugs for blocking EMT, thus providing another pharmacological target to prevent PVR.

In a previous study, PLB was identified as a proliferative suppressor because of its role on cell apoptosis [14]. Here, we further defined its inhibitory effect on EMT of RPE cells. Our study proved that PLB could downregulate mesenchymal-associated molecules such as α-SMA. At the same time, PLB protected the epithelial junctional protein ZO-1, which is considered to maintain cell-cell adhesion to prevent EMT [11]. Furthermore, PLB is thought to also repress the expression of MMP-1 and MMP-2, which may cleave extracellular matrix components, and thus increase the motility of cells [15–17]. Indeed, the results of our scratch wound assay and Transwell chamber assay indicated that PLB could significantly inhibit the migratory and invasive ability of RPE cells. Herein, we elucidated these suppressive effects of PLB on EMT of PRE cells, leading to weakened motility and contraction. Moreover, these declined abilities were more than the decreased cell numbers as marker ZO-1 were elevated or maintained during the whole experiment.

In the present study, rabbit eyes were chosen as animal models to test the possibility that PLB could prevent and treat of PVR in vivo, because they imitated the human disorder closely, with collapsed vitreous and epiretinal membranes [18,19]. Under control conditions, a dense fibrotic membrane derived from RPE cells formed on the collagen matrix, resulting in retinal detachment. In PLB treatment group, these membranes were not as dense as in the control group and were significantly less contractile, similarly like the cell contraction assay in vitro. Our animal results demonstrated that intravitreous injection of 25 μM PLB could effectively reduce the morbidity and severity of experimental PVR. Moreover, no evidence of toxicity has been observed in rabbit eyes given either 25 or 50 μM PLB injection, suggesting PLB had a wide safety margin.
The present experiment has some limitations: first, RPE cell may be the most important cells involved in the process of PVR but they are not the only factor. The pathogenesis of PVR is extremely complicated involving many cells, such as glial cells or macrophages [1]. We only used RPE cells in this experiment, excluding the action and interference of other cells. Second, in a real retina detached eye, the retina and cells are already impaired and may be more susceptible, which could affect the efficacy and toxicity of PLB. Third, some aspects of toxicity may take longer to present. Further studies on pharmacokinetics of PLB and long-term observation of animal model are needed to perform next. Fourth, since many cell proliferation inhibitor could suppress the development of PVR [18,20], next we may choose some of them as control groups to compare the efficacy of the two therapies or discover a proper combination of drugs to prevent PVR better.

**Conclusions**

In the present study, PLB was proven to have inhibitory effects on migration, invasion, as well as the expression of EMT-related proteins and MMPs in RPE cells. Besides, PLB could attenuate the severity degrees of PVR in rabbit experimental models. These findings in vitro and in vivo suggest that PLB may possess potential efficacy for prophylaxis and treatment of PVR and needs further investigation regarding pharmacokinetics and comparison with analogous drugs.
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Conflicts of interests

None.