·Basic Research·

Effects of platelet-derived growth factor α receptor in experimental rabbit PVR

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Abstract

• AIM: To determine the effect of the tyrphostin AG1295 and AG1296, a selective blocker of platelet-derived growth factor (PDGF) β and α RTK, on proliferative vitreoretinopathy (PVR) development.

• METHODS: Rabbit conjunctival fibroblasts (RCF) cells were cultured. The effects of AG1295, AG1296, PDGF-AA and PDGF-BB on RCF proliferation were evaluated by MTT assay. Homologous rabbit conjunctival fibroblasts were injected intravitreally to make animal PVR model, followed by injection of 100μ mol/L of AG1295 or AG1296 respectively. The presence of tractional retinal detachment (TRD) was assessed to evaluate the effect of AG1295 and AG1296 *in viva* Electroretinography and histologic studies were performed after intravitreal injection of AG1295 into untreated eyes to evaluate toxicity.

• RESULTS: Both AG1295 and AG1296 (10μ mol/L) significantly inhibited rabbit conjunctival fibroblast cell growth stimulated by PDGF-AA or -BB *in vitro*. Development of TRD was significantly reduced (P <0.05) with 100μ mol/L of AG1295 or AG1296 *in vivo*, but the effect of AG1295 only present until day 14. Inhibitive effect of AG1296 was longer than that of AG1295. No significant histological or retinal functional damage was found in both drug-treated groups.

• CONCLUSION: PDGF α and β receptor specific inhibitor AG1296 and AG1295 attenuate PVR without significant side effects in rabbits, and AG1296 is better than AG1295. The much longer and stronger therapeutic effect from PDGF α receptor inhibitor indicates that PDGF α receptor is more important in the development of PVR, and inhibition of this pathway can be a useful treatment alternative to prevent PVR.

• KEYWORDS: platelet-derived growth factor; receptor tyrosine kinase; AG1295; AG1296; proliferative vitreoretinopathy

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INTRODUCTION

Proliferative vitreoretinopathy (PVR) is a serious complication of rhegmatogenous retinal detachment and severe ocular trauma and is a leading cause of surgical failure. Platelet-derived growth factor (PDGF), a potent chemoattractant and mitogen for both fibroblasts and RPE cells [1,2], not only promotes dedifferentiation of RPE cells [3] and also enhances contraction of RPE cells and fibroblasts in collagen gel [4,5]. PDGF has five isoforms, PDGF-AA, -BB, -AB, -CC, and DD[6], which interact differentially with structurally related receptors designated α and β receptors; each receptor has an extracellular component with five immunoglobulin-like domains and an intracellular component with a tyrosine kinase domain containing a characteristic insert sequence [7]. Tyrosine kinase is activated following binding of the ligand to its receptor and plays a crucial role in signal transduction pathways that regulate a number of cell functions such as proliferation and differentiation, both during normal physiology and in a variety of pathologic disorders [8]. We had ever tried using a specific inhibitor of PDGF β receptor tyrosine kinase, AG1295, to treat rabbit PVR and found this drug is effective to inhibit PVR in the early stage. Ikuno *et al* [9] found that fibroblasts expressing PDGF α receptors had a stronger intrinsic ability to induce PVR in animal models than those expressing β receptors. Another study showed that TGFβ 1-dependent contraction of fibroblasts was mediated by the PDGF α receptor [10]. Although the detailed mechanism is not clear at present, they indicated that PDGF receptor might be involved in both proliferation and contraction stages of PVR and plays a more important role than that of PDGF-β receptor.

In the present study, we compared the effect of AG1295 and AG1296, specific inhibitor of PDGF β and α receptor RTK respectively, on the progression of rabbit PVR, to try to shed more light on the role of PDGF β and α receptor .

MATERIALS AND METHODS

Animal Preparation All animal experiments were conducted according to the tenets of the Association of Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Experimental manipulations were performed on left eyes only. Seventy-three pigmented rabbits of either sex weighing 1.5-2.0kg were included in the study. Before each procedure, the rabbits were anesthetized with intramuscular injections of ketamine hydrochloride (35mg/kg) and xylazine hydrochloride (5mg/kg). The pupils of experimented eyes were dilated with 5g/L tropicamide and 5g/L phenylephrine hydrochloride, and the cornea was anesthetized with 4g/L oxybuprocaine hydrocholoride eyedrops.

After performing a 0.2mL anterior chamber paracentesis, 0.4mL of pure perfluoropropane (C_3F_8) gas was injected into the vitreous cavity using a 30-gauge needle to induce vitreous liquefaction. Cryopexy and fluid-gas exchange were not performed to avoid associated complications[11].

Cytotoxicity Studies To confirm the safety of the drugs, the retina was examined after injection of AG1295 or AG1296 (Calbiochiem, Germany). The AG1295 and AG1296 were prepared at a concentration of 100mol/L in dimethyl sulfoxide (DMSO) as a working solution. Then 74μg of AG1295 or 68μg of AG1296 which was diluted into 3μL of DMSO respectively, was dissolved in 0.1mL of balanced saline solution (BSS plus; Santen, Osaka, Japan) and injected into the rabbit vitreous, resulting in a final concentration of approximately 100μmol/L *in vivo*. The drug was repeatedly injected every week for 28 days into the left eyes, and the fellow control eyes received the same volume of DMSO in 0.1mL BSS. Thus, for both eyes, the intravetreal concentration of DMSO is 1g/L. Ten rabbits were included in AG1295 and AG1296 trial respectively. The eyes were examined by indirect ophthalmoscopy and fundus videography (Topcon, Tokyo, Japan), and scotopic electroretinograms (ERG) were recorded just before AG1295 or AG1296 was injected and at 3, 7, 14, and 28 days after the first injection of drug. The details of each examination are described below.

ERG Studies The eyes were fully dilated and the rabbits were anesthetized and dark-adapted for 1 hour. A contact lens electrode (Kyoto Contact Lens, Kyoto, Japan) was placed on the anesthetized cornea (oxybuprocaine hydrochloride 4g/L). Topical methylcellulose 10g/L was used as the conducting medium. The reference and ground electrodes were made of stainless-steel surgical needles, which were inserted into the neck-back and one ear respectively. The ERG then was recorded (Neuropack 2; Nihon Koden, Tokyo, Japan). The light stimulator was 20cm above the cornea. The dark-adapted responses were evoked by a conventional full-field flash unit that produced flashes with a maximum intensity of 5.76 cd·s/m^2. Six waves taken every 60 seconds were averaged for both treated and untreated eyes. To overcome individual and daily variances, the B-wave ratios (the amplitude of the wave in the treated eye divided by its amplitude in the control eye) were calculated[12].

Histological Studies The animals were sacrificed and the eyes were enucleated on 14, 21, and 28 days. The eyes were cut circumferentially at the limbus to make posterior cups and then fixed in 40g/L paraformaldehyde and 1g/L glutaraldehyde in 0.1mol/L phosphate-buffered saline (PBS) at 4℃ overnight. The specimens were rinsed with distilled water for 30 minutes, dehydrated in a graded ethanol and xylene series, and embedded in paraffin. Sections 2 to 4μm thick were stained with hematoxylin and eosin (HE).

Rabbit RPE Cell Culture Rabbit RPE cell cultures were obtained from pigmented rabbits by the method of Flood *et al* [13] with slight modification. Briefly, the freshly enucleated eyes were immediately submerged in the RPE medium consisting of Eagle's minimal essential medium (EMEM) (Gibco, Grand Island, NY), 100mL/L fetal bovine serum (Bio-Source International California, USA) and antibiotics (penicillin at 100kU/L, streptomycin at 100mg/L). The globes were opened and cornea, lens and vitreous humor were removed by a circumferential cut just posterior to the ora serrata. The neural retina was carefully washed out by the RPE medium. The eye cups were washed with Hank's balanced salt solution (HBSS), and were digested with 0.12g/L tripsin (Nacalai Tesque, Inc. Kyoto Japan) in 0.05g/L EDTA (Nacalai Tesque, Inc. Kyoto Japan) for 1 hour at 37℃ . The tripsinization was stopped by adding excessive RPE medium. The dissociated RPE cells were carefully washed out without disturbing the underlying choroids. The RPE cells were first cultured in 12-well plates near confluence with the RPE medium, and then were passed to 25cm^2 flasks. The cells were trypsinized and passaged every week.

Cell Proliferation Assay Proliferation of RPE was measured by MTT[3, (4,5-dimethyl-2-thiazolyl)-2, 5-diphenylte -2H-tetrazolium bromide] using a commercially available kit purchased from Nacalai Tesque, Kyoto, Japan. Cells were planted in RPE medium containing 100mL/L FBS at a density of 1×10^4 cells/well in 96 well plates and allowed to adhere for 18 to 24 hours. Cultures were then washed once with PBS and fed with fresh EMEM with 5mL/L FBS containing dilutions of PDGF-AA (50μg/L, R & D Systems, Minneapolis, USA), -BB(50μg/L, R&D Systems, Minneapolis, USA) with or without AG1295 or AG1296 (50μmol/L). The cells were incubated for another 72 hours, and finally the cells were treated with 5g/L of MTT for 4 hours at 37℃. MTT solution was aspirated and the formazan crystals were dissolved in detergent reagent for 10 minutes. Relative cell number was determined based on the optical absorbance of the formazan at 570nm using a control wavelength of 655nm measured in an automatic plate reader (BIO-RAD Model 450, BIO-RAD Laboratories 2000 Alfred Nobel Drive Hercules, California). AG1295 and AG1296 stock solutions were made in dimethylsulfoxide (DMSO) and were diluted in culture medium before adding to the cells. To exclude the effect of DMSO, the concentration of DMSO was decreased to 1g/L, the same as that of *in vivo* experiment, and sham treatments were used as control.

Experimental PVR Animal Model PVR was induced in the left eyes of 35 pigmented rabbits as described previously[14]. Briefly, gas vitrectomy was performed by injecting 0.4mL C_3F_8 into the vitreous cavity at 4mm posterior to the corneal limbus. This was through retina after anesthesia was induced. Ten days later, 0.1mL of RPE medium containing 1×10^5 of RPE cells was injected into the vitreous cavity together with 0.1mL of platelet rich plasma (PRP) using a 30-gauge needle. The 6th passage RPE cells were used in this model.

In the treated group, the experimental eye of each rabbit(n= 18) was injected with 74μg AG1295 or 68μg AG1296 dissolved by 3μL DMSO in 0.1mL BSS immediately after cell injection to achieve a final intraocular concentration of 100μmol/L AG1295 or AG1296. For the control group (n=17), 3μL DMSO in 0.1mL BSS was injected. On 7, 14, and 21 days, the treated rabbits continuously received the same volume of AG1295 or AG1296 injection, while rabbits in the control group received a sham treatment.

Each eye was examined by indirect ophthalmoscopy, and fundus video photos were taken at 3, 7, 14, 21, and 28 days after the RPE injection. The development of PVR was evaluated on videography by masked fashion and the PVR was graded according to the scale of Fastenberg *et al*[15].

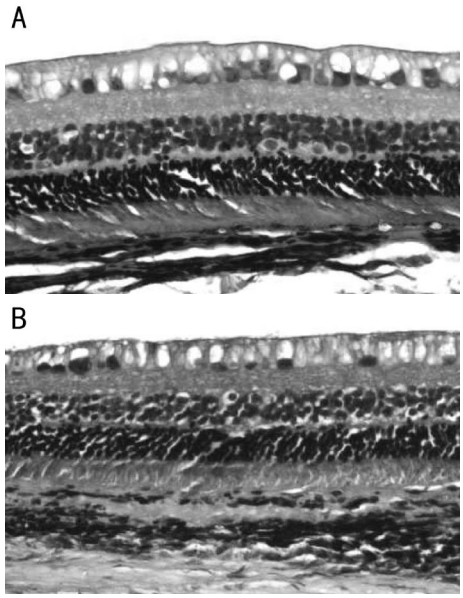

Figure 1 AG1295 (A) and AG1296 (B) induced no significant morphological changes in the retina at 28 th day after injection

Statistical Analysis Student's t-test was used for statistical analysis of RPE proliferation and Fisher's exact test were used for statistical analysis of tractional retinal detachment. A P value less than 0.05 was considered to be statistically significant.

RESULTS

Retinal Architecture and Function Unaffected by AG1295 or AG1296 in Rabbit Eyes To assess AG1295 and AG1296 as a preventive drug for PVR *in vivo*, we evaluated if intravitreal injection of these drugs affects the retinal morphology or produces functional changes. We evaluated the retinal architecture histologically at 14, 21, and 28 days after the first injection of 100μmol/L of AG1295 or AG1296 (Figure 1). Compared with control eyes, no significant morphologic damages were observed at any time points.

The ERG data are shown in Figure 2. The b-wave ratio of the rabbits treated with 100μmol/L of AG1295 or AG1296 was well preserved even with repeated injections for 28 days, indicating that both drugs had no adverse effects on retinal function and architecture.

AG1295 and AG1296 Inhibition of RPE Proliferation from PDGF -AA and -BB Proliferation of RPE after stimulation either with PDGF-AA or -BB is shown in Figure 3. The 50μmol/L concentration of AG1295 inhibited PDGF-AA and -BB-induced cell proliferation by 51% and 63%(P<0.01 for both, two-tailed t-test), and the 50μmol/L concentration of AG1296 by 80% and 52% (P<0.01 for both, two-tailed t-test) respectively. DMSO also inhibited cell proliferation,

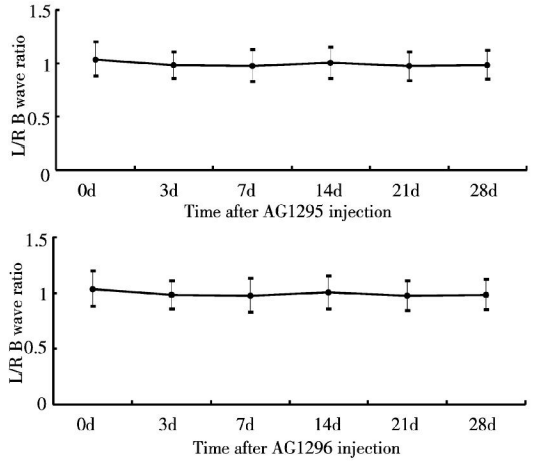

Figure 2 AG1295 and AG1296 induced no significant electrophysiologic retinal changes

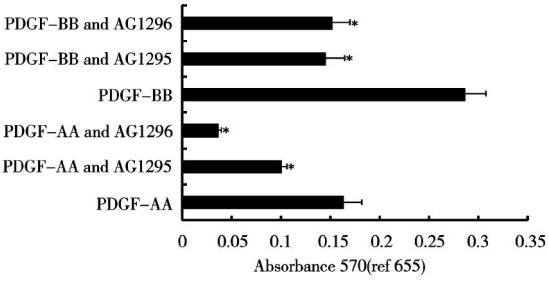

Figure 3 AG1295 and AG1296 inhibits PDGF-AA and -BB-induced cell proliferation

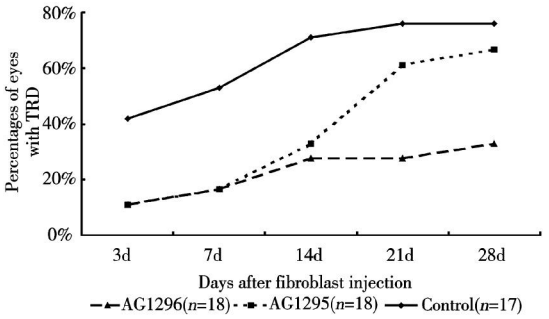

Figure 4 Effect of AG1295 and AG1296 on rabbit PVR

but when its concentration decreased to 1g/L, its negative effect was less than 14% (P>0.05) and could be ignored.

AG1295 and AG1296 Attenuated PVR in an Animal Model The percentage of tractional retinal detachment (TRD) in the rabbits that received RPE and PRP, followed by AG1295 or AG1296 or the control DMSO injection every week are shown in Figure 4. The PVR scores were lower in the rabbits treated with AG1295 or AG1296 compared with controls. On day 3, in both AG1295 and AG1296 group, 2 of 18 (11%) treated eyes developed TRD, while 7 of 17 (41%) eyes developed TRD in control group. On day 7, in both treated groups, 3 of 18 (17%) got TRD, while the number of control group are 9 of 17 (53%). On day 14, TRD developed in 6 of 18 (33%) AG1295 treated eyes, 5 of 18 (28%) AG1296 treated eyes and 12 of 17 (71%) control eyes. After day 14, the AG1295 treated group also tended to develop PVR. The incidence of TRD on day 21 and 28 in AG1295 group was 11 of 18 (61%) and 12 of 18 (67%) respectively, while the percentage of control group was 13 of 17 (76%) and 13 of 17 (76%) respectively. The AG1296 group continually showed inhibitive effect on PVR in the late stage, and its incidence of TRD on day 21 and 28 was 5 of 18 (28%) and 6 of 18 (33%) respectively. Therapeutic effect of AG1295 for rabbit PVR only showed in the early stage, and there is no difference in the incidence of TRD between AG1295 group and control group after day 14. However, compared with AG1295, AG1296 showed longer and stronger inhibitive effect for PVR, there was statistically significance difference in the incidence of TRD between AG1296 group and control group within the observed period.

DISCUSSION

Tyrosine kinase plays an important role in cell proliferation and differentiation. However, inhibitors of tyrosine kinase still cannot be used clinically to treat PVR because non-selective tyrosine kinase inhibitor may affect normal cell proliferation and other functions and thus could be potentially harmful to the retina and choroids. We had found AG1295, a specific inhibitor of PDGF receptor tyrosine kinase could attenuate the development of rabbit PVR without significant adverse effects on retinal function and architecture. Because AG1295 mainly block PDGF β receptor tyrosine kinase, so its inhibitive effect is limited in the pathological proliferation stimulated by PDGF, but its therapeutic effect only showed in the early phase on rabbit PVR. Namely, this drug could prevent the formation of fibrosis membranes, but once the membranes or strands appeared, it could not antagonize their contraction. We postulated that this phenomenon might reflect the role of PDGF in the development of PVR, because animal and clinical studies showed that PDGF has a negative correlation with the severity of PVR, *i.e.* the expression of PDGF decreased with the progression of PVR. In the present study, we found that a specific inhibitor of PDGF receptor could persistently prevent the development of PVR in rabbits. PDGF has two kinds of receptors: α and β, PDGF α receptor can bind with either A or B chain of PDGF ligands, while PDGF receptor only with B chain [2]. AG1296 and AG1295 are specific inhibitors for PDGF α and β receptors respectively. Although both chemicals could restrain RPE proliferation and fibrosis membrane formation at PVR early

stage, AG1296 showed a longer and stronger inhibitive effect than that of AG1295. This phenomenon indicated the following possibilities: (1) PDGF α receptor is related to PVR more closely than PDGF β receptor; PDGF may stimulate PVR by PDGF α receptor mainly. (2) Although the expression peak of PDGF is within the proliferative stage, PDGF α receptor may also intervene the contraction of fibrosis membrane. Many studies have suggested that TGF-β is the principal factor that mediates membrane contraction in PVR. However,Ikuno *et al* [10] proved recently that contraction of fibroblast induced by TGF-β depended on the expression of PDGF α receptor. Our results also showed that once the PDGF α receptors were inhibited, contraction of fibrosis membrane would be prevented and furthermore the incidence of TRD decreased. Although more researches are needed to reveal the mechanism of PVR and the animal model of PVR cannot be identical to human PVR, the present study suggested that inhibition of PDGF α receptor could be an effective therapeutic method for PVR.

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