

Platelet-derived Growth Factor Receptor Kinase Inhibitor AG1295 and Inhibition of Experimental Proliferative Vitreoretinopathy

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Purpose: Receptor tyrosine kinase (RTK) activation is critical for growth factor-mediated cell proliferation. The present study was designed to determine the effect of tyrphostin AG1295, a selective blocker of platelet-derived growth factor (PDGF) RTK, on proliferative vitreoretinopathy (PVR) development.

Methods: Rabbit conjunctival fibroblasts cells (1×10^4) were seeded into 96-well plates and maintained in Dulbecco's modified essential medium (DMEM) with 0.5% fetal bovine serum. The cells were exposed to 50 ng/mL PDGF-AA or PDGF-BB or phosphate-buffered saline with or without AG1295 (1 μ M, 10 μ M, and 100 μ M). After 3 days, the viable cells in each well were measured by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Homologous rabbit conjunctival fibroblasts were injected intravitreally, followed by injection of 100 μ M of AG1295. The development of tractional retinal detachment (TRD) was assessed to evaluate the effect of AG1295 *in vivo*. Electroretinography and histologic studies were performed after intravitreal injection of AG1295 into untreated eyes to evaluate toxicity.

Results: Two concentrations of AG1295 (10 and 100 μ M) significantly inhibited rabbit conjunctival fibroblast cell growth stimulated by PDGF-AA or PDGF-BB *in vitro*. Development of TRD was significantly attenuated ($P < .01$) with 100 μ M of AG1295 until day 21. No significant histologic or retinal functional damage was found in the AG1295-treated group.

Conclusions: PDGF receptor specific inhibitor AG1295 attenuated PVR without significant side effects in rabbits. This reagent could be a useful treatment to prevent PVR. **Jpn J Ophthalmol** 2003;47:158–165 © 2003 Japanese Ophthalmological Society

Key Words: Kinase inhibitor, platelet-derived growth factor, proliferative vitreoretinopathy.

Introduction

Proliferative vitreoretinopathy (PVR) is a serious complication of rhegmatogenous retinal detachment and severe ocular trauma and is a leading cause of surgical failure. The events in PVR pathogenesis include fibrin deposition, cellular accumulation and proliferation, production of extracellular matrix, and contraction of fibrotic membranes.¹ PVR can be viewed as an exaggerated wound-healing process in

the retina. Growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF- β), epithelial growth factor (EGF), and insulin-like growth factor-1 (IGF-1) promote PVR.² PDGF, expressed by platelets, macrophages, fibroblasts, retinal pigment epithelial (RPE) cells, and many other cell types^{3,4} plays an important role in the progression of PVR.^{5–8} The expression of PDGF ligands and receptors in the epiretinal membranes^{9–11} and an elevated intravitreal PDGF level^{12,13} were found in patients with PVR. PDGF is a potent chemoattractant and mitogen for both fibroblasts and RPE cells.^{14–16} PDGF promotes dedifferentiation of RPE cells¹⁷ and also enhances contraction of RPE cells and fibroblasts in collagen gel.^{18,19} Fur-

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thermore, fibroblasts expressing PDGF receptors, especially the α receptor, have a strong intrinsic ability to induce PVR in animal models, while cells that do not have PDGF receptors cannot induce PVR.⁵ Because PDGF and its receptors play a pivotal and versatile role in the progression of PVR, the application of an anti-PDGF system in the eye might be a therapeutic intervention in this disease.

To evaluate the possibility of preventing PVR pharmacologically, we investigated the effect of AG1295, a novel class of tyrosine kinase inhibitor that selectively inhibits tyrosine phosphorylation of the PDGF receptor and its downstream signaling pathway,²⁰ and thus inhibits many malignant and fibrotic diseases.²¹⁻²⁷ In this study, we investigated if the injection of AG1295 into the vitreous cavity could prevent PVR in an animal model.

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. Fifty-five pigmented rabbits of either sex weighing 1.5–2.0 kg were included in the study. Before each procedure, the rabbits were anesthetized with intramuscular injections of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg). The pupils of experimental eyes were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride, and the cornea was anesthetized with 0.4% oxybuprocaine hydrochloride eye drops. After performing a 0.2-mL anterior chamber paracentesis, 0.4 mL of pure perfluoropropane (C_3F_8) gas was injected into the vitreous cavity with a 30-gauge needle to induce vitreous liquefaction. Cryopexy and fluid-gas exchange were not performed.

Cytotoxic Studies

To confirm the safety of the drug, the retinas of a separate group of rabbits were examined after injection of AG1295 (Calbiochem, San Diego, CA, USA).

The AG1295 was prepared at a concentration of 100 mM in dimethyl sulfoxide (DMSO) as a working solution by the following procedure: 74 μ g of AG1295, diluted by 3 μ L of DMSO, was dissolved in 0.1 mL of balanced saline solution (BSS plus; Santen, Osaka) and injected into the rabbit vitreous, resulting in a final concentration of approximately 100 μ M in vivo. The 7.4 μ g and 0.74 μ g injections of AG1295 were

treated similarly, with an estimated final concentration of 1 μ M and 10 μ M in the vitreous, respectively. 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 0.1 mL BSS. The concentration of DMSO in all eyes was estimated as 0.1% of the vitreous. The 100 μ M concentration of AG1295 was injected into 10 rabbits, and the 1 and 10 μ M concentrations were injected into 5 rabbits each. The eyes were examined by indirect ophthalmoscopy and fundus videography (Topcon, Tokyo). Scotopic electroretinograms (ERG) were recorded just before AG1295 was injected and at 3, 7, 14, and 28 days after the first injection of AG1295. 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) was placed on the anesthetized cornea (oxybuprocaine hydrochloride 0.4%). Topical methylcellulose 1% was used as the conducting medium. The reference and ground electrodes were attached to both ears of the animals. The ERG then was recorded (Neuropack 2; Nihon Koden, Tokyo). The light stimulator was 20 cm above the cornea. The dark-adapted responses were evoked using a conventional full-field flash unit that produced flashes of 0.5-second duration and 20-Joule intensity. 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.^{28,29}

Histologic Studies

The animals were sacrificed and the eyes were enucleated on days 14, 21, and 28. The eyes were cut circumferentially at the limbus to make posterior cups and then fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) at 4°C overnight. The specimens were rinsed with distilled water for 30 minutes, dehydrated in a graded ethanol and xylene series, and embedded in paraffin. Sections 2–4- μ m thick were stained with hematoxylin and eosin.

Rabbit Conjunctival Fibroblast Cell Culture

Primary cultures of rabbit conjunctival fibroblasts (RCFs) were obtained as described previously.⁶ Cul-

tures were incubated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) supplemented with 1% antibiotics including penicillin G, streptomycin sulfate, and amphotericin B in 5% CO₂ at 37°C. The cells were trypsinized and passaged every week.

Cell Proliferation Assay

Proliferation of RCF was measured by MTT [3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] using a commercially available kit purchased from Nacalai Tesque (Kyoto). Cells were plated in DMEM containing 10% FBS at a density of 1×10^4 cells/well in 96-well plates and allowed to adhere for 18–24 hours. Cultures were then washed once with DMEM and fed with DMEM with 0.5% FBS. After 48 hours, the medium was aspirated and fresh DMEM with 0.5% FBS containing dilutions of PDGF-AA (50 ng/mL; R & D Systems, Minneapolis, MN, USA) and PDGF-BB (50 ng/mL; R&D Systems) with or without AG1295 (10 and 100 μ M). The cells were incubated for another 72 hours, and finally the cells were treated with 5 mg/mL of MTT for 4

hours at 37°C. The 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 570 nm using a control wavelength of 655 nm measured in an automatic plate reader (Bio-Rad Model 450; Bio-Rad Laboratories, Hercules, CA, USA). In order to evaluate the effect of DMSO on fibroblast proliferation, 0.1% of DMSO was added to the culture media of the cells stimulated with PDGF-AA or PDGF-BB, and a cell proliferation assay was also performed using this method.

Experimental PVR Animal Model

PVR was induced in the left eyes of 35 pigmented rabbits as described previously.⁶ Briefly, gas vitrectomy was performed by injecting 0.4 mL of C₃F₈ into the vitreous cavity 4 mm posterior to the corneal limbus after anesthesia was induced. Three days later, 0.1 mL of DMEM containing 1×10^5 of RCFs was injected into the vitreous cavity together with 0.1 mL of platelet rich plasma (PRP), using a 30-gauge needle.

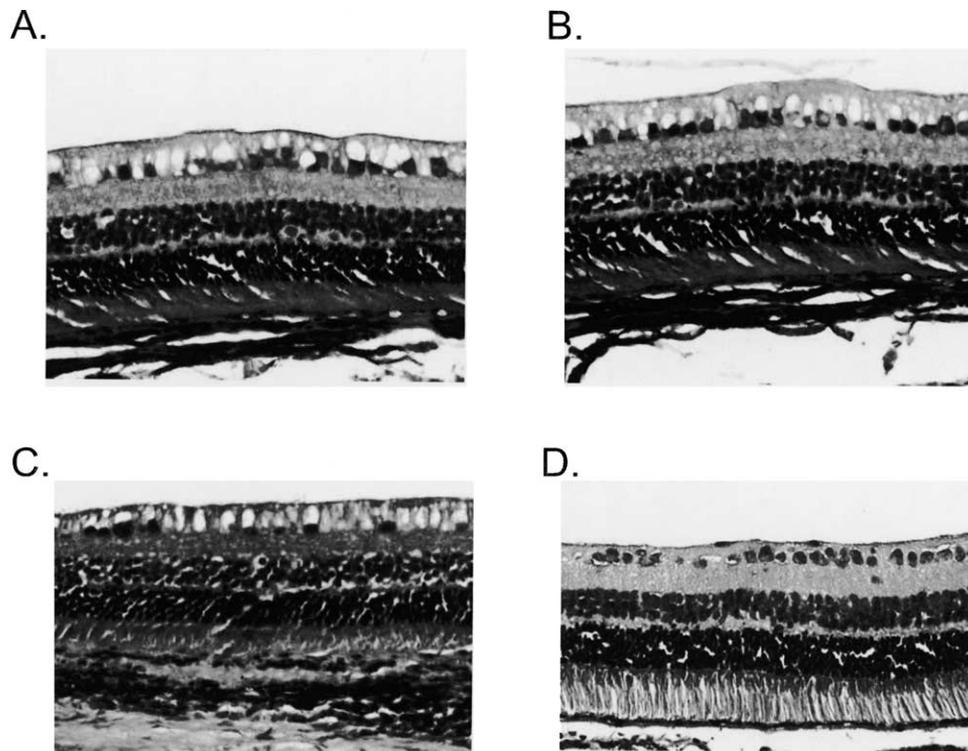


Figure 1. AG1295 induced no significant morphological changes in the retina after injection. (A) Normal untreated control retina. (B–D) AG1295 (final in vivo concentration 100 μ M) was injected into the rabbit vitreous cavity and the injections were repeated weekly for 28 days. The rabbits were sacrificed at 14 days (B), 21 days (C), or 28 days (D) after the first injection. Hematoxylin and eosin staining was performed to evaluate architectural damage in the retina. No significant histologic damage was seen at any time point.

In the treated group, the experimental eye of each rabbit ($n = 18$) was injected with $74 \mu\text{g}$ AG1295 diluted by $3 \mu\text{L}$ DMSO in 0.1 mL BSS immediately after fibroblast injection to achieve a final intraocular concentration of $100 \mu\text{M}$ AG1295. Because preliminary experiments showed no cytotoxicity in any rabbit retina that received 100 , 50 , or $5 \mu\text{M}$ of AG1295 intravitreal injection (data not shown), we tested only the $100 \mu\text{M}$ injection in a PVR experiment. In the control group ($n = 17$), $3 \mu\text{L}$ DMSO in 0.1 mL BSS was injected. On days 7, 14, and 21, the treated rabbits continuously received the same volume of AG1295 injection, while rabbits in the control group received the sham treatment.

Each eye was examined by indirect ophthalmoscopy, and fundus video photos were taken 3, 7, 14, 21, and 28 days after the RCF injection. The development of PVR was evaluated on videography in a masked fashion, and the PVR was graded according to the scale of Fastenberg et al.³⁰

Statistical Analysis

The Student *t*-test and the Fisher exact test were used for statistical analysis. A *P*-value $< .05$ was considered to be statistically significant.

Results

Retinal Architecture and Function

Unaffected by AG1295 in Rabbit Eyes

To assess AG1295 as a preventive drug for PVR in vivo, we investigated if intravitreal injection of this drug affects the retinal morphology or produces functional changes. We evaluated the retinal architecture histologically 14, 21, and 28 days after the first injection of $100 \mu\text{M}$ of AG1295 (Figure 1). Compared with control eyes, no significant morphologic damages were observed at any time point. The results with the 1 and $10 \mu\text{M}$ concentrations of the drug were also examined, and no significant morphologic changes were observed (data not shown).

The ERG data are shown in Figure 2. The b-wave ratio of the rabbits treated with concentrations of 1 , 10 , or $100 \mu\text{M}$ was well preserved even with repeated injections for 28 days, indicating that AG1295 had no adverse effects on retinal function and architecture.

AG1295 Inhibition

of Fibroblast Proliferation

Induced by PDGF-AA and PDGF-BB

Proliferation of RCFs after stimulation either with PDGF-AA, PDGF-BB, or PBS is shown in Figure 3.

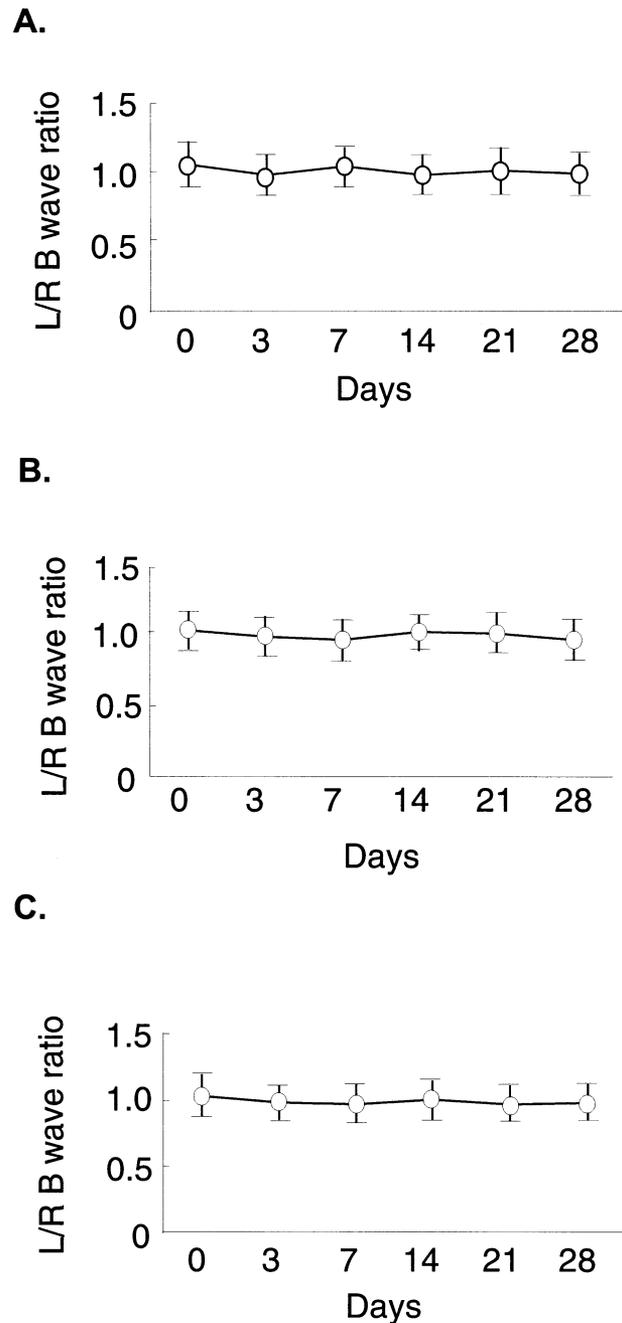


Figure 2. The β -wave ratio and SD in the rabbits treated with AG1295 in the following concentrations in the vitreous cavity of the left eye and 0.1 mL balanced saline solution in the right (control) eye: (A) $1 \mu\text{M}$, (B) $10 \mu\text{M}$, (C) $100 \mu\text{M}$. Electrophysiological examinations were performed on days 0, 3, 7, 14, 21, and 28. AG1295 induced no significant electrophysiological retinal changes. The mean of the β -wave amplitude ratio was obtained by dividing the value in the treated eyes by the value in the right (control) eyes. The β -wave amplitude was well preserved at all concentrations, indicating preserved retinal function.

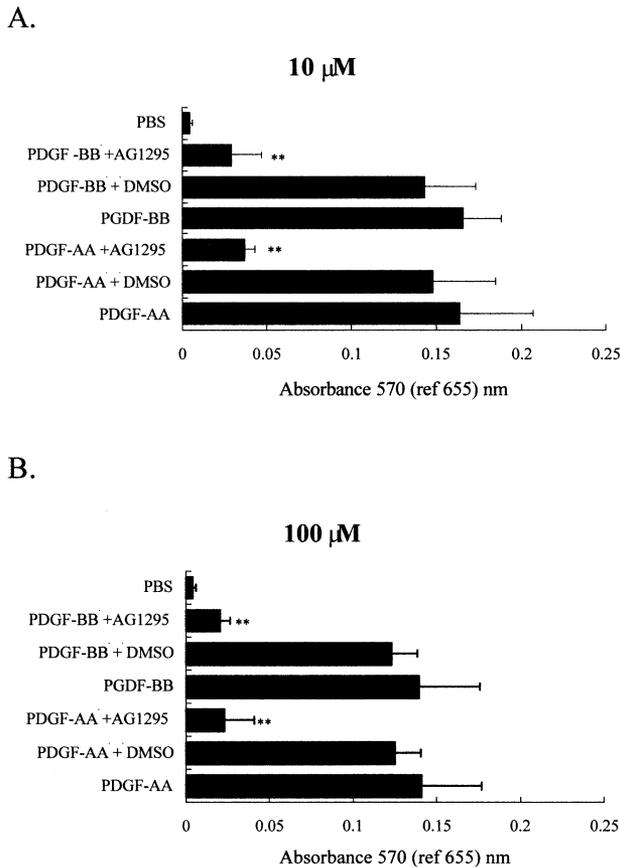


Figure 3. AG1295 inhibits platelet-derived growth factor (PDGF)-AA and PDGF-BB induced cell proliferation. Rabbit conjunctival fibroblasts (RCFs) were harvested into 96-well plates and an MTT assay was performed. AG1295 inhibited PDGF-AA and PDGF-BB induced fibroblast cell proliferation. **(A)** The 10- μ M concentration of AG1295 inhibited PDGF-AA and PDGF-BB induced cell proliferation by 75% and 80% ($P < .01$ for both, two-tailed t -test). **(B)** The 100- μ M concentration of AG1295 inhibited proliferation by 82% and 83% ($P < .01$ for both, two-tailed t -test). The 0.1% of in dimethyl sulfoxide (DMSO) slightly inhibited PDGF-AA and PDGF-BB-induced cell proliferation by 10% and 14% ($P > .05$ for both, two-tailed t -test) in the 10- μ M concentration of AG1295 **(A)**, and the cell proliferation was also inhibited by 11% and 12% ($P > .05$ for both, two-tailed t -test) in the 100- μ M concentration of AG1295 **(B)**. PBS: phosphate-buffered saline.

The 10- μ M concentration of AG1295 inhibited PDGF-AA and PDGF-BB induced cell proliferation by 75% and 80%, respectively ($P < .01$ for both, two-tailed t -test) (Figure 3A), and the 100 μ M concentration of AG1295, by 82% and 83%, respectively ($P < .01$ for both, two-tailed t -test) (Figure 3B). The 0.1% of DMSO slightly inhibited PDGF-AA and PDGF-BB induced cell proliferation by 10% and 14%, respec-

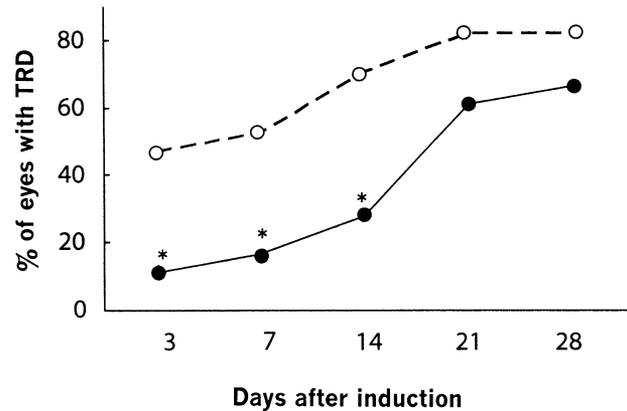


Figure 4. AG1295 inhibits the incidence of tractional retinal detachment (TRD) in the rabbit proliferative vitreoretinopathy (PVR) model. PVR was induced in the rabbit eye by rabbit conjunctival fibroblasts (RCFs) and platelet-rich plasma after gas vitrectomy. At the same time, either AG1295 or sham treatment was administered to a separate group of animals. Rabbit eyes were observed by indirect ophthalmoscopy and PVR was evaluated according to the scale of Fastenberg et al. Closed circles indicate the % of TRD in AG1295-treated rabbits and open circles indicate that in the control group. The differences in the incidence of TRD between the two groups at the early time points were significant ($P < .05$). No significant inhibition of TRD is observed in the late phase. * $P < .05$.

tively ($P > .05$ for both, two-tailed t -test) with the 10- μ M concentration of AG1295 (Figure 3A), and cell proliferation was also inhibited by 11% and 12% ($P > .05$ for both, two-tailed t -test) with the 100- μ M concentration of AG1295 (Figure 3B). Thus, the 0.1% concentration of DMSO exhibited minimal inhibition to PDGF-AA and PDGF-BB induced cell proliferation, and there was no significant difference between the effects.

AG1295 Attenuated PVR in an Animal Model

Figure 4 shows the percentage of tractional retinal detachment (TRD) in the rabbits that received RCF and PRP, followed by either AG1295 or the control DMSO injection every week. The PVR score was lower in the rabbits given AG1295 compared to controls. On day 3, 2 of 18 (11%) treated eyes and 8 of 17 (47%) control eyes developed TRD. On day 7, 3 of 18 (17%) treated eyes and 9 of 17 (53%) control eyes had TRD. On day 14, TRD developed in 5 of 18 (28%) and 12 of 17 (71%) treated and control eyes, respectively. The difference in the incidence of TRD between the two groups within 14 days was significant ($P < 0.05$). In the late phase, the treated group tended to develop PVR. Eleven of 18 (61%) rabbits

in the treated group developed TRD on day 21 and 12 (67%) on day 28, while in the control group, 14 of 17 (82%) developed TRD at both of those time points. There was no statistically significant difference between the two groups on days 21 and 28.

Discussion

PDGF has five isoforms, PDGF-AA, -BB, -AB, -CC, and -DD,^{31,32} that 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.³³⁻³⁵ 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 or in a variety of pathologic disorders.³⁶ The approaches for interference with PDGF-induced signaling include (1) peptide competing with PDGF for receptor binding³⁷; (2) dominant negative mutants of PDGF,^{38,39} or PDGF receptor^{6,8,40}; or (3) low-molecular-weight blockers of the receptor tyrosine kinase activity known as typhostin.⁴¹ Only the dominant negative PDGF receptor has been studied *in vivo*,^{6,8} however, a successful gene delivery system also needs to be developed to deliver dominant negative receptors.

Low-molecular-weight tyrosine kinase inhibitors of the typhostin type suppress the development of PVR.⁴² However, immediate clinical use of this kind of drug in human eyes is not practical because of the relatively narrow safety margins. Protein tyrosine phosphorylation, induced by many peptide growth factors and cytokines, plays an important role in cell proliferation and differentiation. Nonselective tyrosine kinase inhibitor may affect normal cell proliferation and other functions and thus could be potentially harmful to the retina and choroid. The ideal drug would kill pathologic proliferating cells without damaging nonproliferating cells in the normal tissue such as neural retina.

The present study demonstrated that a novel class of selective PDGF receptor tyrosine kinase blocker, AG1295, completely inhibited PDGF-induced fibroblast proliferation *in vitro*; it is also effective in preventing experimental PVR in a rabbit model. In addition, no retinal toxicity was observed in the ERG and histologic sections of normal rabbit eyes following intravitreal injection.

Other cell proliferation inhibitory drugs such as corticosteroid,⁴³ 5-fluorouracil,⁴⁴ cyclosporine A,⁴⁵

and daunomycin⁴⁶ decrease the severity of membrane formation and the incidence of retinal detachment by controlling cell proliferation in rabbit eyes. These drugs were not fully satisfactory either because they inhibited membrane formation only transiently or had too small a safety margin because of less selectivity. AG1295 is a highly selective inhibitor, in that its IC_{50} for PDGF receptor tyrosine kinase inhibition is less than 1 μ M.⁴⁷ To obtain optimal therapeutic results, we used a much higher concentration in an animal model, but we did not find any obvious architectural or functional damage in the treated eyes even when the rabbits were repeatedly injected every week.

Our results indicated that the inhibitory effect of the drug was not maintained in the late phase (days 21 and 28) even when administered weekly. In other words, the drug only postpones TRD in PVR. The concentration of AG1295 (100 μ M final in the vitreous) *in vivo* seemed to be sufficient based on the finding that 10 μ M AG1295 can completely inhibit PDGF-AA and PDGF-BB induced fibroblast proliferation *in vitro*. One explanation for this observation is that PDGF is not the main contributor in all the stages of PVR. The PVR produced in this model is induced by the abundance of growth factors in the PRP that enhances fibroblast proliferation. Other growth factors than PDGF, including TGF- β , EGF, and IGF-1 from plasma, are also known to enhance the fibroblast proliferation or contraction of the vitreous fibers and retina, which could induce PVR.^{48,49} Thus, different growth factors may provide the most critical contribution to the disease progression in different stages of PVR.

In the present study, we only examined the effect of this drug on fibroblast proliferation in an animal model; however, RPE cells apparently play a critical role in clinical PVR. Although dedifferentiated RPE cells possess a PDGF autocrine loop,¹⁵ which could be a target of AG1295, it is necessary to confirm whether this drug could be useful for RPE cells in a human setting. Glial cells are believed to be another contributor to PVR development. AG1295 can also target not only RPE cells but also retinal glial cells, because PDGF has a mitogenic effect on glial cells *in vitro*.⁵⁰ Therefore, AG1295 may prevent PVR partly by inhibiting the chemotaxis of retinal glial cells.

Finally, we found that the PDGF receptor-specific inhibitor, AG1295, attenuated PVR without any significant side effect in rabbits. Although more study is needed to understand the pathogenesis of PVR and to confirm the safety of the drug, this reagent could be a useful preventive treatment for PVR.

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