

Effects of ripasudil, a Rho-associated protein kinase inhibitor, on conjunctival scarring in a canine filtration surgery model

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ABSTRACT

The aim of this study was to investigate the effects of ripasudil, a Rho-associated protein kinase (ROCK) inhibitor recently developed for glaucoma therapy in Japan, on intraocular pressure (IOP) and conjunctival scarring in a canine model of glaucoma surgery. Glaucoma surgery models were created in 10 eyes of five beagles. Immediately after surgery, one drop (50 μ L) of 0.4% ripasudil hydrochloride hydrate solution (treated eyes) or phosphate saline alone (control eyes) was instilled twice daily for 4 weeks. IOPs and bleb features were assessed for 4 weeks, followed by histological evaluation. Proliferative cell nuclear antigen (PCNA)-positive cells and transforming growth factor (TGF)- β -positive cells were quantified. IOP was found to be significantly lower in the treated eyes than in the control eyes. The ratio of conjunctival area to scleral area was reduced in the treated eyes. In addition, the bleb score was higher, whereas the adhesion score and density of PCNA-positive and TGF- β -positive cells were lower in the treated eyes. Topical application of ripasudil maintained bleb formation and IOP reduction by suppressing cell proliferation. These results suggest that ripasudil might be useful for maintaining filtering blebs after glaucoma filtration surgery.

KEYWORDS: ROCK inhibitor, ripasudil, glaucoma, filtration surgery, bleb formation, intraocular pressure

INTRODUCTION

Trabeculectomy is a filtering surgery that is widely performed on glaucoma patients to obtain a sufficient reduction of intraocular pressure (IOP). However, filtration bleb dysfunction often occurs owing to excessive wound healing by Tenon's capsule tissue, which leads to subconjunctival fibrosis [1]. The proliferation of Tenon's fibroblasts and its migration to the wound site could in part explain why filtration wounds scar and lead to failure of filtration surgery [2]. It has been shown that application of mitomycin C (MMC) has greatly improved the results of glaucoma surgery by strongly suppressing proliferation of fibroblasts. However, thin blebs resulting from the application of MMC increased complications such as infectious endophthalmitis [3, 4]. Therefore, a safer approach to suppress fibroblast proliferation and maintain filtration blebs is needed. There are several candidates as potential agents, such as the antibody against transforming growth factor (TGF)- β 2, for regulating fibroblast activities [5]. Nevertheless, no agent other than MMC is available yet for clinical use.

The Rho GTPase and Rho-associated protein kinase (ROCK) have emerged as regulators of tissue fibrosis [6]. Fasudil, a ROCK inhibitor, has been

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demonstrated to have antifibrotic effects in models of renal, cardiac, pulmonary, and hepatic fibrosis [7, 8, 9, 10]. It has also been reported that fasudil hinders fibroblast contractility and may be beneficial in preventing scar contracture [11]. Another ROCK inhibitor Y-27632 has been reported to reduce keratocyte-to-myofibroblast transition and to alter wound healing response in the corneal stroma [12]. In addition, the other ROCK inhibitor AMA0526 prevented angiogenesis *in vitro* (human vascular endothelial cells) and controlled the complete process of wound healing in an experimental model of corneal opacity and neovascularization [13]. Moreover, ROCK inhibitors reportedly release a TGF- β -induced contractile response in human tenon fibrosis and block subsequent myofibroblast transdifferentiation, thereby modulating postoperative scarring after glaucoma filtration surgery [14]. Further, it was also reported that Y-27632 affected activities of human tenon fibroblasts and prevented fibroproliferation and scar formation in a rabbit model of glaucoma filtration surgery [15].

Recently ripasudil, a selective ROCK inhibitor, was approved in Japan for the treatment of glaucoma and ocular hypertension [16]. One-year application of 0.4% ripasudil exhibited IOP-lowering effects and an acceptable safety in patients with open-angle glaucoma and ocular hypertension [17]. Although ripasudil might have the similar effect on tenon fibroblasts and scar formation in glaucoma filtration surgery as Y-27632, it has not been verified yet to the best of our knowledge.

The aim of the present study was to test the effects of ripasudil on IOP reduction, filtration bleb formation, and cell proliferation in a canine model of glaucoma surgery.

MATERIALS AND METHODS

Drugs

Ripasudil hydrochloride hydrate was obtained from Kowa Company, Ltd. (Nagoya, Japan). A solution of 0.4% ripasudil in physiological saline was prepared for instillation in the current study.

Animals and IOP measurement

Ten beagles weighing 9.0-10.0 kg were purchased from Japan SLC (Shizuoka, Japan). Beagles were

fed with regular chow, had free access to tap water, and were housed in an air-conditioned room at approximately 23 °C and 60% humidity with a 12-hour light-dark cycle. The experimental procedures for animals were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. IOP was measured using a calibrated pneumatonometer (Model 30 Classic; Medtronic Solan, Jacksonville, FL) under general anesthesia with intravenous injection of pentobarbital sodium (35 mg/kg body weight) in a face-front position.

Glaucoma filtration surgery model

Beagles were anesthetized using pentobarbital sodium as mentioned above. A 10-mm fornix-based flap of conjunctiva and the Tenon's capsule (length, 5 mm) was prepared as described previously [18]. After a 3 × 1 mm scleral portion was removed at the limbus, peripheral iridectomy was performed, and the conjunctiva was closed with a 10-0 nylon suture. After surgery, 1 cm of 3 mg/g ofloxacin ointment was applied to the eye.

Experimental protocol

In 5 beagles, one of the eyes was treated with a 1-drop (50 μ L) instillation of 0.4% ripasudil hydrochloride hydrate solution (treated eyes) and the other eye was treated with physiological saline (control eyes), twice daily for 4 weeks after surgery.

IOPs as well as bleb scores were assessed every 2 weeks for 4 weeks, followed by histological evaluation of the eye after the dogs were killed by injecting a lethal dose of pentobarbital sodium. Conjunctival and scleral areas of the lesion were then measured, as described below. Density of fibroblasts and proliferative cell nuclear antigen (PCNA)-positive cells were quantified.

Bleb scores and adhesion scores

Blebs were examined via a slit lamp and graded according to the definition by Perkins *et al.* [19], as follows: 1, minimally high conjunctiva thickening without swelling; 2, mild swelling present; 3, elevated bleb covering 2 to 3 clock hours of the eye; and 4, greatly elevated bleb covering more than 4 clock hours. A score of 0 indicated no observable bleb.

Adhesion degrees were determined by comparing the percentage of the area of adhesion to the whole flap area ($5 \times 10 \text{ mm}^2$) as an index [20]. Scores were determined as follows: Score 1, the flap could be detached by lightly lifting it, or the area of adhesion was not larger than 20%; score 2, the area of adhesion was between 20% and 40%; score 3, the area of adhesion was between 40% and 60%; and score 4, the area of adhesion was between 60% and 100%.

Histology and immunohistochemistry

Conjunctival and scleral tissue specimens were fixed with Carnoy Solution (MUTO PURE CHEMICALS Co., LTD., Tokyo, Japan) and embedded in paraffin. Five-micrometer-thick sections were cut, mounted on silanized slides (Dako Japan, Kyoto, Japan), and deparaffinized with xylene and a series of graded ethanol. Each section was stained with azan to identify collagen fibers. The conjunctival and scleral areas of the lesion, where the flap was created, were assessed using a computerized morphometry system, MacSCOPE Ver. 2.2 (Mitani Co., Fukui, Japan), and then, the ratio of the conjunctival area to the scleral area was calculated.

To retrieve the antigen, sections were pretreated with 10 mM citrate buffer at pH 6.0 and autoclaved for 5 min at 121 °C before immunohistochemical staining. Sections were soaked in absolute methanol containing 3% hydrogen peroxide for 5 min at room temperature to remove endogenous peroxidase activity. To suppress non-specific binding of the primary specific antibodies, sections were incubated with a solution of Protein Block Serum-Free (Ready-to use, X0909, Dako, Japan) for 5 min. The procedures of immunohistochemical staining with mouse monoclonal antibody against human PCNA (PC10) (M0879; Dako Japan) and chicken polyclonal antibody against human TGF- β 1 (RnD Systems, USA) are described in our previous report [18]. We counted PCNA- and TGF- β 1-positive cells at the sites where they accumulated in the conjunctival and scleral lesions by observing under a light microscope (number per 100x field). The average number of each type of cells or vessels in five randomly selected fields was calculated.

Statistical analysis

Each measurement is expressed as the mean \pm standard deviation (SD). For statistical comparisons

of IOP and histological evaluation, Student's *t*-test for paired data was used. Bleb scores and adhesion scores were statistically analyzed using Mann-Whitney *U* test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Effects of ripasudil on IOP, bleb score, and adhesion score in a canine filtration surgery model

The initial values of IOP (mean \pm SD) were $16.0 \pm 4.4 \text{ mmHg}$ in the treated eyes and $16.4 \pm 4.5 \text{ mmHg}$ in the control eyes. IOP was significantly lower in the treated eyes at 2 and 4 weeks postoperative than in the control eyes (Figure 1).

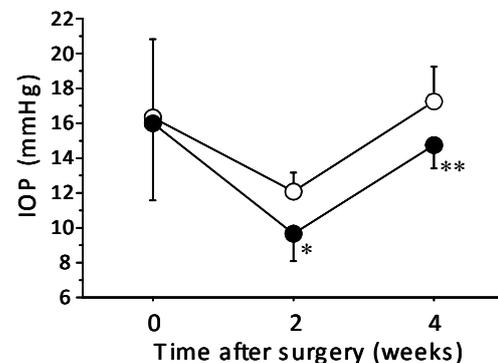


Figure 1. IOP changes in the treated eyes (●) and control eyes (○). Data are shown as mean \pm SD for 5 dogs. * $P < 0.05$, ** $P < 0.01$ versus control eyes (paired *t*-test).

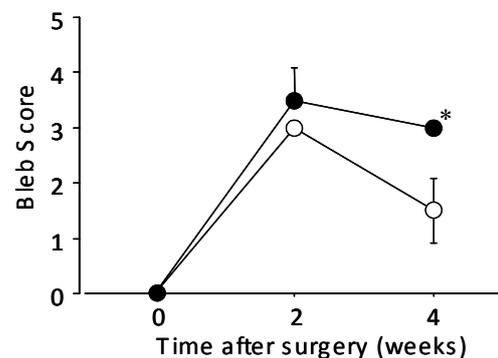


Figure 2. Bleb score changes in the treated eyes (●) and control eyes (○). Data are shown as mean \pm SD for 5 dogs. * $P < 0.05$ versus control eyes (Mann-Whitney *U* test).

Bleb scores were high at 4 weeks postoperative in the treated eyes (Figure 2). Adhesion scores were significantly lower in the treated eyes than in the control eyes (Figure 3).

Histological changes induced by ripasudil in a canine filtration surgery model

As shown in figure 4, the conjunctival surface was thickened in the control eye, whereas in the treated eye, thickening of the conjunctiva seemed inhibited. The ratio of conjunctival area to scleral area was significantly reduced in the treated eyes (Table 1).

Distributions of PCNA-positive cells and TGF- β -positive cells are shown in figures 5 and 6. Their density seemed to be statistically lower in the treated eyes than in the control eyes (Table 1).

DISCUSSION

The current study demonstrated, for the first time, that multiple application of ripasudil, a selective

ROCK inhibitor, maintained IOP reduction and bleb formation by suppressing cell proliferation and TGF- β activity in a canine glaucoma surgery model.

Previously Honjo *et al.* [15] reported the effects of another ROCK inhibitor Y-27632 on fibroproliferation and scar formation in a rabbit model of glaucoma filtering surgery, although this agent is yet to be clinically applied. On the other hand, ripasudil, the ROCK inhibitor we used in this study, has already been approved for the treatment of glaucoma. One-year clinical application of ripasudil revealed that all of the adverse events including conjunctival hyperemia, blepharitis, and allergic conjunctivitis were resolved after discontinuation of ripasudil administration. [17]. The validated safety profile of ripasudil was important for its clinical use, in contrast to Y-27632.

We observed the postoperative course for a longer duration (4 weeks) than in the above-mentioned report by Honjo *et al.* (1 week) [15]. As it was reported that 58% of blebs had collapsed by 10 days and 94% by 17 days in a rabbit model of glaucoma surgery [21], we considered that a postoperative observation period of more than 2 weeks was appropriate. Since ripasudil has IOP-lowering effects by itself [16], maintenance of IOP reduction after glaucoma surgery in this model might be partly owing to this effect. However, we also found that bleb formation was maintained for longer duration and the density of PCNA-positive cells and TGF- β -positive cells was lower in the treated eyes than in the control eyes. Particularly, TGF- β has been implicated in

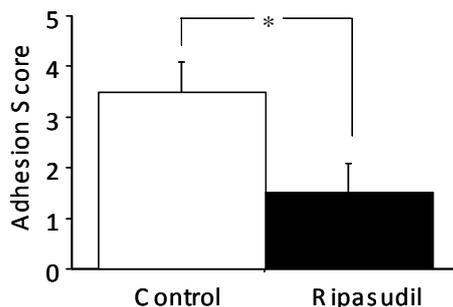


Figure 3. Adhesion score in the treated eyes and control eyes. Data are shown as mean \pm SD for 5 dogs. * $P < 0.05$ versus control eyes (Mann-Whitney U test).

Table 1. Comparison of the ratio of the conjunctival area to the scleral area, and densities of PCNA-positive cells and TGF- β -positive cells in the treated and control eyes.

	Treated	Control	P-value
Ratio of the conjunctival area to the scleral area	0.7 \pm 0.3	1.6 \pm 0.5	0.03
Density of PCNA-positive cells (per mm ²)	1.4 \pm 1.0	11.4 \pm 4.1	0.049
Density of TGF- β -positive cells (per mm ²)	1.8 \pm 2.4	23.8 \pm 1.8	0.045

Data are shown as mean \pm SD for five dogs. P-values were obtained using the paired t -test.

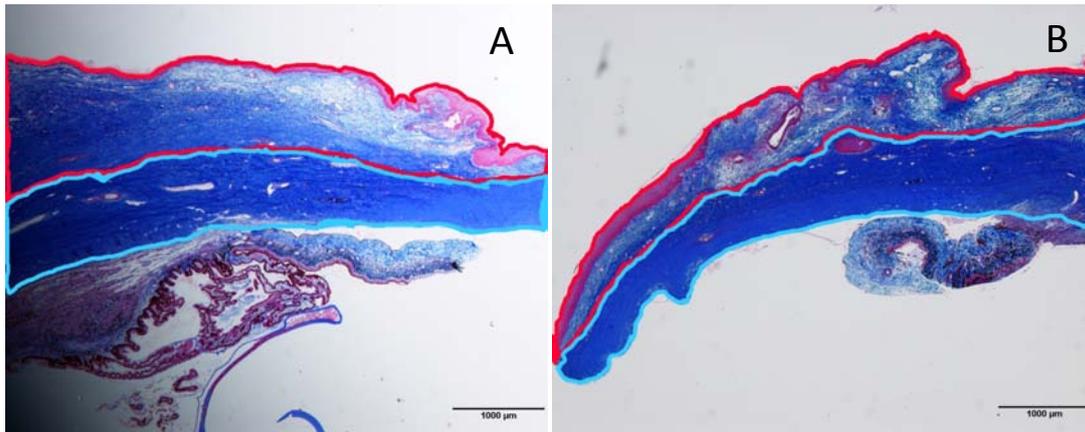


Figure 4. Representative photomicrographs of the sections obtained from the control eye (**A**) and the treated eye (**B**) at 4 weeks postoperative and stained with azan stain. The conjunctiva and the sclera are surrounded by red and blue lines, respectively. Scale bars, 1 mm.

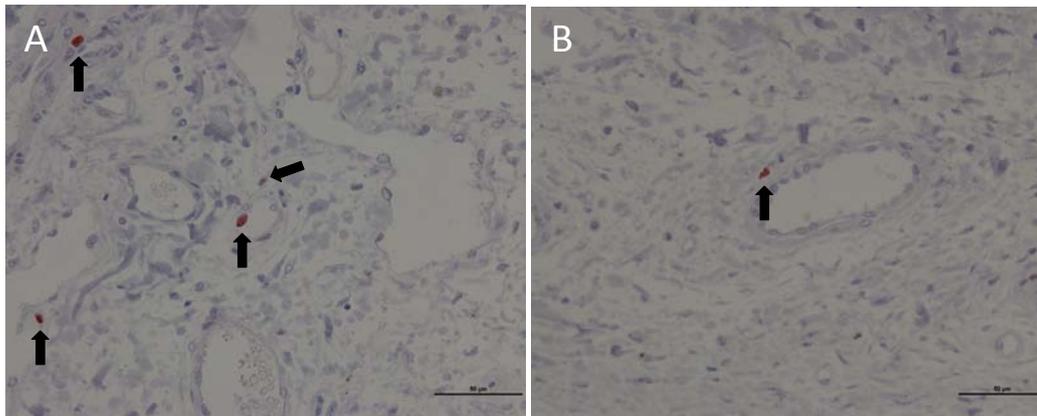


Figure 5. Representative immunohistochemical staining of the section for proliferative cell nuclear antigen (PCNA) in the control eye (**A**) and the treated eye (**B**) at 4 weeks postoperative. Arrows indicate positive cells. Scale bars, 50 µm.

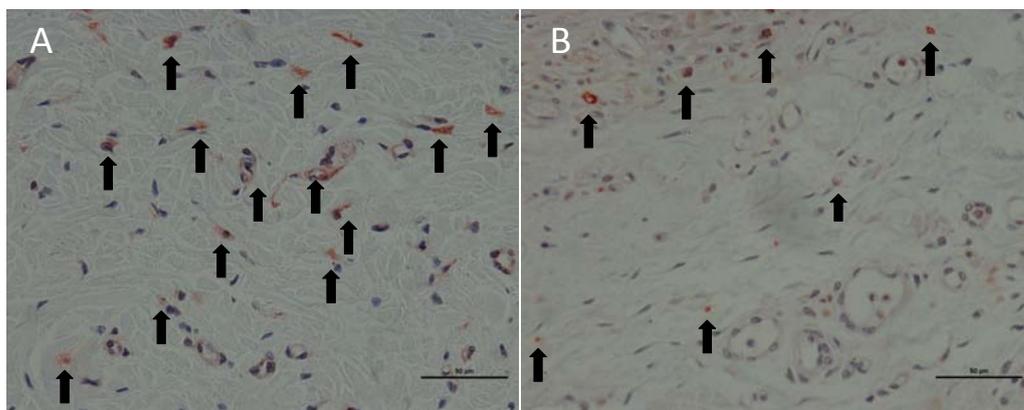


Figure 6. Representative immunohistochemical staining of the section for transforming growth factor (TGF)- β in the control eye (**A**) and the treated eye (**B**) at 4 weeks postoperative. Arrows indicate positive cells. Scale bars, 50 µm.

conjunctival wound healing following intraocular surgery including glaucoma filtration surgery [22]. Taken together, our present results suggest that anti-scarring effects of ripasudil might affect continuous IOP reduction after surgery.

There are still several limitations in the present study. First, the most suitable dosage of ripasudil, i.e. the number of instillation per day and its duration, needs to be investigated further. Moreover, the effect of instillation of ripasudil should be compared to other ways of application, i.e. subconjunctival injection and recently developed drug delivery systems including gelatin hydrogel [18]. However, the instillation of ripasudil, used in the current study seems to be the most convenient method for clinical use. Anyway, the topical application of ripasudil is promising for improvement in the results of glaucoma surgery.

CONCLUSION

Our present results suggested that topical application of ripasudil, a ROCK inhibitor recently developed in Japan, might be effective for maintaining filtration bleb formation and IOP reduction after glaucoma surgery. The application of this agent is probably worthy of further investigation for improving the outcomes of glaucoma surgery.

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CONFLICT OF INTEREST STATEMENT

None of the authors has any conflict of interest.

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