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Frequency and Risk Factors for Intraocular Pressure Elevation After Posterior Sub-Tenon Capsule Triamcinolone Acetonide Injection

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Purpose: This study investigated the effects of posterior sub-Tenon capsule (PST) injection of triamcinolone acetonide (TA) on intraocular pressure (IOP) in the human eye.

Methods: The study included 115 patients who received PST injections of 40-mg TA to treat macular edema with diabetic retinopathy (n = 57), branch retinal vein occlusion (n = 35), central retinal vein occlusion (n = 13), or other disorders (n = 10). IOP measurements were performed on the day of injection, and 0.5, 1, 2, 3, 6, 9, and 12 months later.

Results: In 26 (22.6%) of the 115 eyes, an IOP of 24 mm Hg or higher was observed during the 12-month follow-up period after PST TA injection. IOP elevation significantly correlated with young age, but not with past history of diabetes mellitus or systemic hypertension, sex, or type of retinal disease with macular edema. In total, 23 eyes were treated with antiglaucoma medications to control elevated IOP (24 mm Hg or higher). External trabeculotomy was performed in 1 case where medications failed to correct elevated IOP.

Conclusions: PST TA injection is associated with high rates of steroid-induced IOP elevation in eyes with previously normal IOP. However, IOP elevation may be less common after PST injection than after intravitreal injection. Our findings indicate that IOP must be carefully monitored after PST TA injection.

Key Words: glaucoma, corticosteroid, trabeculotomy

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Triamcinolone acetonide (TA) is increasingly used for the treatment of numerous macular disorders that are associated with diabetic retinopathy,[1–6] retinal vein occlusion,[6,7] choroidal neovascularization,[8–10] and uveitis.[11,12] TA is injected into either the vitreous or the sub-Tenon capsule to confine the corticosteroid effects to the ocular tissues, while minimizing the side effects associated with systemic steroid therapy. However, intravitreal injections of TA are associated with increased risks of ocular complications, including vitreous hemorrhage, retinal detachment,[12] and bacterial endophthalmitis,[6,13–16] which can often result in serious visual loss. Nonetheless, many research groups, including our own,[17] have shown that posterior sub-Tenon capsule (PST) injections can effectively reduce macular edema in cases of uveitis[18,19] and diabetic macular edema[20,21] without inducing serious ocular complications.

Intraocular pressure (IOP) elevation is a common side effect of corticosteroid therapy. Although the topical, intravitreal, and systemic corticosteroid administration routes have been reported to cause IOP elevation, the effects of PST injection remain unclear. A few case series of uveitis patients showing IOP elevation after PST injection have been reported previously.[18,19,22,23] However, bearing in mind the large number of patients with macular edema, surprisingly little is known about the frequency, time course, duration, and risk factors of IOP elevation after PST injection. To address this deficit, we carried out a retrospective investigation into the predictive factors of IOP elevation following PST injection of TA.

PATIENTS AND METHODS

Our interventional case series included 115 consecutive eyes from patients with macular edema who underwent PST TA injection, after giving informed consent, at the Kumamoto University Hospital in Japan between June 2003 and January 2004. Patients with macular edema due to uveitis were excluded from the analysis, because treatment with additional corticosteroids and inflammation in the anterior chamber can both affect IOP. Eyes with an IOP of 22 mm Hg or higher were also excluded from the analysis. To avoid biases related to host factors, if both of a patient's eyes were treated with TA, only the eye that first received treatment was included in the analysis. The PST injections were performed using the previously described protocol, with minor modifications.[24] After disinfection with

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povidone-iodine and topical anesthesia with xylocaine, the conjunctiva and sub-Tenon capsule in the inferotemporal quadrant were incised with scissors. A 25-gauge curved blunt cannula was inserted into the sub-Tenon space to allow the infusion of 40-mg TA (Kenacort; 40 mg/mL; Bristol Pharmaceutical, YK, Tokyo, Japan). At the end of the procedure, the wound was left unsutured and ofloxacin ointment (Tarivid ophthalmic ointment; Santen Pharmaceutical Co, Ltd, Osaka, Japan) was applied to the eye. Each patient was instructed to use 0.5% levofloxacin (Cravit ophthalmic solution; Santen Pharmaceutical Co, Ltd, Osaka, Japan) 4 times per day for 1 week. In addition to these examinations, optical coherence tomography (Humphrey model 2000; Carl Zeiss Meditec International, Germany) was used to measure the central retinal thickness. The IOP was monitored for at least 6 months after the TA injection.

Information on each subject was obtained from a review of their medical records. The IOP measurements were recorded on the day of injection, and 0.5, 1, 2, 3, 6, 9, and 12 months later. All data are presented as the mean (± the standard deviation; along with the range of values). Multiple regression analysis was used to evaluate the effects on IOP of age, sex, lens state (phakic or pseudophakic), vitreous state (vitreous or nonvitreous), systemic diseases, and the number of injections. A Wilcoxon signed-rank test was used to compare the rise in IOP after the first injection with those after subsequent injections. The Spearman rank correlation was used to analyze the relationship between IOP and the thickness of macular edema after the injection. A probability (P) value less than 0.05 was considered statistically significant.

RESULTS

The study population consisted of 115 eyes from a total of 74 males and 41 females, with a mean age of 62.2 (± 12.6; range = 13 to 84) years. The most common retinal diseases accompanying macular edema were diabetic maculopathy (57 eyes; 49.6%), branch retinal vein occlusion (35 eyes; 30.4%), and central retinal vein occlusion (13 eyes; 11.3%). The other disorders present (10 eyes; 8.7%) included exudative age-related macular degeneration, idiopathic focal subretinal neovascularization, polypoidal choroidal vasculopathy, and idiopathic juxtafoveal retinal telangiectasis. The mean follow-up period was 394.9 (± 145.3; range = 180 to 672) days.

The mean IOP before the TA injection was 13.1 (± 2.8; range = 6 to 20) mm Hg, and the mean maximum IOP after the injection was 19.8 (± 6.3; range = 9 to 42) mm Hg. Thus, the mean rise in IOP was 6.7 mm Hg. The IOPs recorded from 2 weeks to 9 months after the TA injection were significantly higher than those observed before the injection. The mean IOP showed a gradual increase after the injection, peaked at 2 months, and then decreased gradually until reaching a minimum at 12 months (Fig. 1). In addition, 26 (22.6%) of the 115 eyes showed an IOP of 24 mm Hg or higher 1.8 (± 0.8) months after the TA injection. The numbers of eyes with an elevated IOP of 24 mm Hg or higher gradually increased from 2 weeks to 2 months after the TA injection (Table 1), and all such cases were established by 3 months after the injection.

Additional TA injections were performed in 48 (41.7%) of the 115 eyes, because of a recurrence of macular edema after the first injection. For each of the eyes treated with an additional TA injection, we calculated the peak IOP after the injection minus the IOP before the first injection (that is, the ΔIOP). The mean ΔIOP values were 4.1 (± 3.4) mm Hg for the first injection, and 6.4 (± 5.8) mm Hg for the second injection. There was a statistically significant difference between the ΔIOP values for the first and second injections ($P < 0.01$). Interestingly, when additional injections were performed within 6 months of the first, the ΔIOP values for the second injection were significantly higher than those for

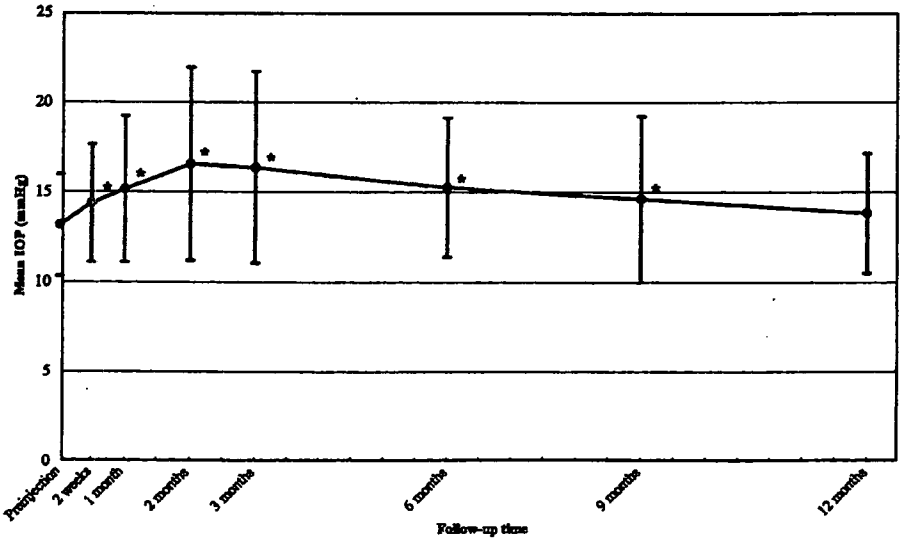

FIGURE 1. The mean IOP at each time point after PST injection of TA. The IOPs at 2 weeks, and 1, 2, 3, 6, and 9 months, were significantly higher than those before the injection. *P<0.05; Mann-Whitney U test.

TABLE 1. Frequency of Cases of Elevated IOP After PST Injections of 40 mg TA

IOP (mm Hg)	No. Cases (%)								
	Baseline	2 wk	1 mo	2 mo	3 mo	6 mo	9 mo	12 mo	Final
≤21	115/115 (100)	108/112 (96.4)	101/110 (91.8)	88/104 (84.6)	97/113 (85.8)	107/114 (93.9)	81/89 (91.0)	61/62 (98.4)	113/115 (98.3)
22-23	0	3/112 (2.7)	5/110 (4.5)	4/104 (3.8)	5/113 (4.4)	3/114 (2.6)	3/89 (3.4)	1/62 (1.6)	1/115 (0.9)
24-29	0	1/112 (0.9)	4/110 (3.6)	9/104 (8.7)	8/113 (7.1)	3/114 (2.6)	4/89 (4.5)	0	1/115 (0.9)
30-34	0	0	0	1/104 (1.0)	2/113 (1.8)	1/114 (0.9)	0	0	0
35-39	0	0	0	1/104 (1.0)	1/113 (0.9)	0	1/89 (1.1)	0	0
≥40	0	0	0	1/104 (1.0)	0	0	0	0	0
>21	0	4/112 (3.6)	9/110 (7.6)	16/104 (15.4)	16/113 (14.2)	7/114 (6.1)	8/89 (9.0)	1/62 (1.6)	2/115 (1.7)
≥30	0	0	0	3/104 (2.9)	3/113 (2.7)	1/114 (0.9)	1/89 (1.1)	0	0
≥40	0	0	0	1/104 (1.0)	0	0	0	0	0

the first ($P < 0.01$). However, there was no statistically significant difference between the ΔIOP values for first and second injections that were performed with more than a 6-month gap between them (Fig. 2).

To investigate the factors affecting IOP elevation after TA injection, multiple regression was used to analyze the relationships between the ΔIOP values (defined as the peak IOP during the total time course minus the preinjection IOP) and the other items. The results demonstrated that age was significantly negatively correlated with ΔIOP. By contrast, there were no significant correlations between ΔIOP and systemic associations of diabetic mellitus or hypertension, or sex (Table 2). In addition, there was no correlation between the ΔIOP and the reduction in relative retinal thickness (RT) calculated using the following formula:

$$RT_{(\text{before the injection})} - RT_{(\text{minimum value after the injection})}/RT_{(\text{before the injection})}$$

Of the 26 eyes that showed an IOP of 24 mm Hg or higher after the TA injection, 19 (73.1%) were administered antiglaucoma ophthalmic drops. The mean maximal number of drops administered during the period of IOP elevation was 1.5 (± 0.7; range = 1 to 3). Oral carbonic anhydrase inhibitors were used in 1 case, which subsequently needed surgical treatment, because the peak IOP reached 35 mm Hg and remained associated with glaucomatous visual field defects, despite treatment with antiglaucoma ophthalmic drops (timolol, latanoprost, and dorzolamide) and oral acetazolamide. External trabeculotomy was performed 10 months after the TA injection in this case, in an attempt to reduce the unresponsive IOP. During the follow-up period after surgical treatment, the IOP in this patient decreased to between 14 mm Hg and 16 mm Hg after treatment with 1 type of antiglaucoma ophthalmic drop (latanoprost).

With regard to TA-related side effects other than IOP elevation, 9 (15.0%) of the 60 phakic eyes showed progression of posterior subcapsular or cortical cataracts. Surgery was performed in 4 of these cases during the follow-up period. An additional complication that was observed in 1 eye (0.9%) after the TA injection was blepharoptosis. Bacterial endophthalmitis, progression of retinopathy, perforation of the eyeball, and orbital fat atrophy were not observed.

DISCUSSION

Although a PST injection of TA delivers a large amount of corticosteroid to the posterior segment of the eye via transscleral absorption, the side effects of this procedure have remained unclear. Our current data show the frequency, time course, duration, and risk factors for IOP elevation after PST TA injection. Several groups

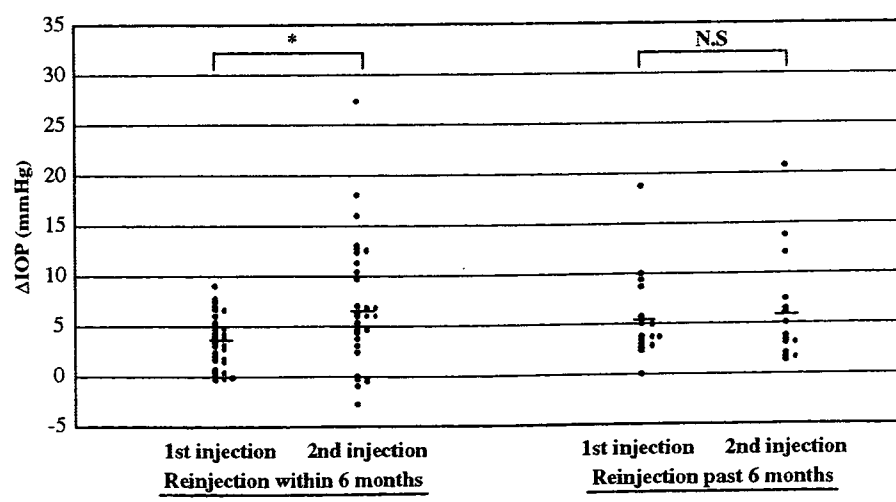

FIGURE 2. IOP elevation augmented by an additional injection of TA after a short time interval. The ΔIOP was calculated for the first and second injections, respectively. When additional injections were performed within 6 months of the first, the ΔIOPs for the second injection were significantly higher than those for the first injection. *$P<0.01$; Wilcoxon singed-rank test.

TABLE 2. Risk Factors of IOP Elevation After PST Injections of 40 mg TA

	t value	(95% CI)	P
Age	−2.32724	(−0.186 to −0.015)	0.022
Sex	−1.41544	(−4.096 to 0.684)	0.160
Laterality of the eye	−0.33899	(−2.431 to 1.721)	0.735
Lens state (phakic or pseudophakic)	0.150151	(−2.448 to 2.849)	0.881
Vitreous state (vitreous or nonvitreous)	−0.35939	(−3.060 to 2.121)	0.720
General disease			
Diabetic mellitus	−1.88658	(−4.460 to 0.111)	0.062
Hypertension	0.54164	(−1.545 to 2.706)	0.589
No. injections	2.193439	(0.170 to 3.363)	0.030

previously reported IOP elevation after PST injection of TA in eyes with uveitis. However, the frequency of IOP elevation seemed to vary significantly between the different studies, with values ranging from 1.7% to 36% being reported.[18,19,22,23,25] Corticosteroids other than TA are often simultaneously administered, both topically and orally, for the treatment of uveitis, and these drugs, along with uveitis-associated ocular inflammation, might affect IOP. We therefore excluded eyes with uveitis from our current study, to evaluate the frequency of TA-induced IOP elevation more accurately. In our series, 26 (22.6%) of the 115 eyes showed an elevated IOP of 24 mm Hg or higher. In this study, we considered 24 mm Hg or higher as a level of abnormal IOP, that has been done in previous study.[26] These data were similar to those reported by Okada et al[19] in eyes with uveitis. By contrast, it was previously found that TA injection into the vitreous induced ocular hypertension in 30% to 40% of eyes,[26 29] indicating that IOP elevations might be more common after intravitreal injection than after PST injection. Jonas et al[27] showed that IOP readings higher than 21, 30, and 40 mm Hg were measured in 41.2%, 11.4%, and 1.8%, respectively, after intravitreal injection of approximately 20 mg TA. It indicated that intravitreal injection of TA induces more drastic and frequent IOP elevation than the PST injection does (Table 1). Additionally, the IOP elevation in the present study peaked 2 months after the injection, and then decreased gradually, reaching a minimum after 12 months. Previous reports on intravitreal injection[27] showed that the TA-induced IOP elevation peaked within 3 months of the injection, demonstrating a similar time course to PST injection. Although the pharmacokinetics of TA after PST injection remain unclear,[30,31] the duration of IOP elevation might correspond to the decay time of TA crystals within the ocular tissues. Interestingly, the amount of IOP elevation after the second injection was significantly greater than that after the first injection when the interval between the 2 was 6 months or less. These data suggest that the accumulation of TA in the sub-Tenon capsule might amplify the side effects on IOP. It is not known whether the TA-induced IOP elevations after PST and intravitreal injections are dose-dependent. Further analyses will therefore be needed to verify whether the IOP elevation depends upon the dosage of TA.

The multiple regression analysis showed that younger age was a significant predictive factor for IOP elevation. Younger patients are also reported to be at a higher risk of developing steroid-induced glaucoma through the use of corticosteroid eye-drops.[32] Although it remains unclear why younger patients should experience steroid-induced ocular hypertension more frequently, it is possible that PST injection of TA might induce IOP elevation in younger patients via the same mechanism. In our current analysis, there were no correlations between the frequency of IOP elevation and any factors other than age. Patients with diabetes have been reported to experience a higher incidence of IOP elevation caused by corticosteroid therapy.[33] By contrast, a recent randomized clinical trial demonstrated that diabetes mellitus was not a major risk factor for glaucoma.[34,35] This supports the present finding of a correlation between TA-induced IOP elevation and diabetes mellitus.

With the exception of cases of IOP elevation, cataracts developed in 15% of the patients in the current study after the PST TA injection. The progression of cataracts was previously reported in 24.2% of patients after intravitreal injection,[29] thereby demonstrating a higher incidence than that observed after PST injection. PST injection has previously been linked to complications such as mis-injection-related embolic occlusion of the central retinal artery,[36,37] orbital abscess,[38] and cutaneous hypopigmentation.[39] However, no such complications were encountered in the present study. Blepharoptosis, which was encountered in the present study, is a known complication of PST injection, because the local effects of triamcinolone are thought to be associated with wasting of the lid muscle, and weakening of the tendon, levator muscle, levator aponeurosis, and orbital septum.[40] The intravitreal injection of TA is reportedly associated with bacterial endophthalmitis[6,13 16] at a frequency of 0.87%.[15] On the other hand, there have been no previous reports on endophthalmitis after PST injection. Because this technique does not penetrate the sclera tissue, the risk of endophtalmitis might be much lower for PST injection than for intravitreal injection. However, bacterial endophthalmitis is rare complication in the field of ophthalmic surgery, so future collaboration and pooling of data from other intervention studies will be useful to clarify the safety of PST injection of TA.

Despite the administration of antiglaucoma medication, prolonged IOP elevation was encountered in 1 eye. We therefore performed an external trabeculotomy to correct this uncontrollable elevated IOP. Several other groups have reported using filtering surgeries, such as trabeculectomy, on eyes with IOP elevation induced by TA.[11,18,22,27,41] We previously demonstrated that external trabeculotomy was effective in 14 out of 14 eyes with steroid-induced glaucoma.[42] It has been hypothesized that corticosteroids promote the abnormal accumulation

of extracellular matrices in the trabecular meshwork, thereby leading to an increased resistance of aqueous outflow.[43 47] Because trabeculotomy reduces the outflow resistance in the trabecular meshwork and the inner wall of Schlemm's canal, we believe that external trabeculotomy is the reasonable surgical choice for controlling elevated IOP in eyes with TA.

In conclusion, PST injection of TA caused an elevated IOP of 24 mm Hg or higher at a frequency of 22.6% within 3 months of the injection. Furthermore, in younger patients, an additional injection within 6 months of the first often caused a further increase in IOP. Our data suggest that IOP should be monitored for at least 3 months after PST TA injection, especially in younger patients or those who are given an additional injection within 6 months.

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CLINICAL INVESTIGATION

Stability of the Central Visual Field After Modern Trabeculectomy Techniques in Eyes with Advanced Glaucoma

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Abstract

Purpose: To review the surgical results and complications of trabeculectomy techniques in patients with advanced glaucoma and threatened fixation.

Methods: Trabeculectomy had been carried out on 49 advanced glaucoma patients (49 eyes) using mitomycin C and postoperative laser suture lysis. The clinical records prior to and 2 months after surgery were reviewed, and the long-term surgical outcomes were determined.

Results: Two months after surgery there were no eyes with fixation loss. Intraocular pressure (IOP) levels were reduced from 22.8 ± 6.0 to 11.7 ± 4.7 mmHg. Kaplan-Meier survival analysis showed that the success rate in achieving IOPs of 15 mmHg or lower 5 years after surgery was 70%. The chance of visual acuity remaining within two lines of the preoperative level was 75%. In 29 of the 49 eyes, visual acuities remained at their preoperative level at the time of the final visit, but had decreased to less than 0.1 in three eyes (cataract progression, $n = 2$; fixation loss, $n = 1$).

Conclusion: The results suggest that laser suture lysis and stepwise management of IOP levels, which are performed as part of the modern postoperative management of trabeculectomy, decrease the frequency of fixation loss during the early postsurgical phase. **Jpn J Ophthalmol** 2007;51:116–120

Key Words: hypotensive maculopathy, hypotony, overfiltration, wipe-out

Introduction

Loss of the central visual field (fixation loss) in glaucomatous eyes with advanced visual field defects has been reported by a number of investigators.[1–12] Sudden visual loss during or immediately after trabeculectomy, which is referred to as "wipe-out"[1,2] or "snuff-off,"[13] is regarded as one of the most serious complications of trabeculectomy, and results in a decreased quality of life for glaucomatous patients. However, the mechanisms behind this problem and the means to prevent it are unknown, although it has been suggested that a hypotonic condition may be one of the risk factors for fixation loss in these advanced glaucoma patients.[14]

Recently, the adjunctive use of antimetabolites such as mitomycin C (MMC) and 5-fluorouracil has dramatically improved filtration efficiency. On the other hand, the occurrence of adverse effects associated with overfiltration, such as shallow anterior chamber, choroidal detachment, and hypotonic maculopathy, have increased. To counter these, several modified intraoperative and postoperative management techniques have been developed to decrease postoperative intraocular pressure (IOP) fluctuations. These include tight suture closure of scleral flaps, postoperative laser suture lysis, and ocular massage. These modifications

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may influence the postoperative complications associated with hypotony, including hypotensive maculopathy and fixation loss. In this study, we reviewed the postoperative surgical results, complications, and visual prognosis after the performance of modern MMC trabeculectomy techniques in eyes with advanced glaucoma.

Subjects and Methods

We retrospectively examined the surgical outcomes of 49 patients (49 eyes) with advanced glaucoma who underwent trabeculectomy at Kumamoto University Hospital, Japan. Written informed consent about the surgical procedure, the predictive merits, and complications were obtained from all patients. The Internal Review Board (IRB) at the Kumamoto University Graduate School of Medical Sciences approved this retrospective chart review.

A previous study devised a generic grading system for glaucomatous visual field defects.[15] Visual fields were assessed by Goldmann perimeter. The classifications were: grade 0 = no visual field defect present; grade I = nasal step or localized paracentral defect; grade II = nasal step and paracentral defect or a single arcuate defect; grade III = two arcuate scotomas or an altitudinal scotoma not encroaching on fixation; grade IV = advanced visual field loss abutting, but not involving, fixation; and grade V = advanced visual field loss with loss of fixation.

To avoid biases related to host factors when both eyes were included in the criteria, the eye that first received trabeculectomy was included in the study. The 49 patients (49 eyes) in this study were classified as having grade IV visual field defects. These visual field defects were subdivided into three categories: grade IV-1 = visual field loss in one or more hemispheres; grade IV-2 = isolated fixation with visual island(s); grade IV-3 = fixation only (Fig. 1). Twenty-three eyes were classified as grade IV-1, eight eyes as grade IV-2, and 18 eyes as grade IV-3.

Patients with grades 0, I, II, III, or V eyes or those without reliable visual field data were excluded from the study. In addition, patients with glaucoma secondary to uveitis, trauma, or ischemic retinal diseases were also excluded. In the current study, there were 27 cases of primary open-angle glaucoma, 10 cases of primary angle-closure glaucoma, and 12 cases of exfoliative glaucoma (Table 1). Previous glaucoma surgeries included trabeculectomy in ten eyes, laser iridotomy in eight eyes, and goniosynechiolysis in one eye.

Patients requiring trabeculectomy were those with progressing visual field damage even with maximally tolerated antiglaucomatous medications, and/or those treated with oral acetazolamide to control IOP. Trabeculectomy was performed after retrobulbar anesthesia. After a limbal-based conjunctival incision, a 4 × 4 mm^2 scleral flap was prepared. Then, 0.04% MMC was applied to the conjunctival scleral flaps for 3–5 min. The operative field was washed immediately with 200 ml of balanced saline solution. Sclerocorneal tissue, including the trabecular meshwork, was then excised. Two to ten 10–0 nylon sutures were placed to close the scleral flap, followed by a shoelace continuous conjunctival suture.

Table 1. Preoperative patient data

Variable	
Age (years)	66.5 ± 9.7
Sex	
Male	24 eyes of 24 patients
Female	25 eyes of 25 patients
Lens	
Phakic	37 eyes
Aphakic	1 eyes
Pseudophakic	11 eyes
Type of glaucoma	
POAG	27 eyes
PACG	10 eyes
XG	12 eyes
Preoperative IOP (mmHg)	22.8 ± 6.0
Grade of visual field defect	
Grade IV-1	23 eyes
Grade IV-2	8 eyes
Grade IV-3	18 eyes

POAG, primary open-angle glaucoma; PACG, primary angle-closure glaucoma; XG, exfoliative glaucoma; IOP, intraocular pressure.

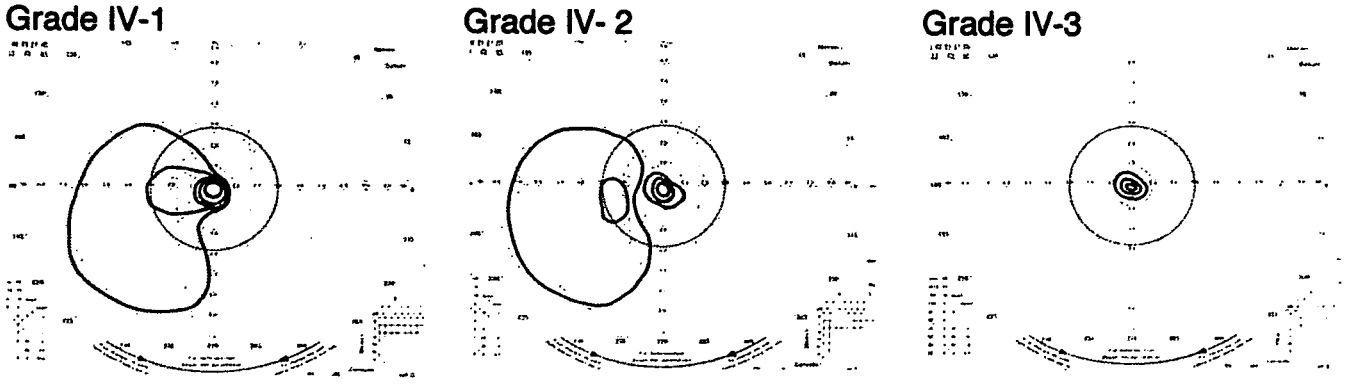

Figure 1. Visual field defects of the 49 eyes in this study as classified by Goldmann perimetry. There were 23 eyes classified as Grade IV-1, 8 eyes as Grade IV-2, and 18 eyes as Grade IV-3.

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After surgery, IOP levels and the formation of filtering blebs were monitored. Postoperative laser suture lysis and associated ocular massage were conducted in a step-by-step manner until the expected target pressure was achieved. Laser suture lysis was conducted postoperatively when the IOP increased and/or the filtering bleb became more localized and flattened. Ocular compression (massage) was added routinely after laser suture lysis. Topical corticosteroid was given as a postoperative medication, and antibiotic drops were administered for 3 months.

Pre- and postoperative (2 months postsurgery) best-corrected visual acuities were compared, and the IOPs and operative complications were evaluated from patient records. Operative complications were defined as choroidal detachment, hyphema, hypotony (IOP ≤ 4 mmHg) for at least 14 days, flattened anterior chamber, hypotensive maculopathy, expulsive hemorrhage, endophthalmitis, cataract progression, and fixation loss. The number of scleral sutures placed during surgery, and the timing and number of laser suture lysis after surgery were also noted. Long-term outcomes of trabeculectomy were evaluated by Kaplan-Meier analysis using target IOPs (20, 15, and 12 mmHg) to determine cumulative success probabilities. Postoperative visual acuity at the long-term follow-up was examined on the final visit.

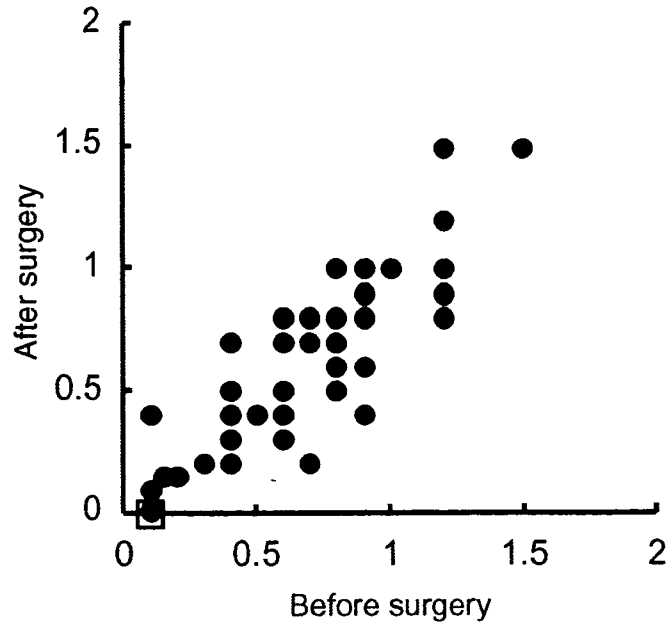

Figure 2. Corrected visual acuity before and 2 months after surgery in the 49 eyes. One outlier (▣) shows an eye with severe hyphema. In this case, a vitreous hemorrhage developed from hyphema as the eye was aphakic. Visual acuity was recovered to presurgery levels when the vitreous hemorrhage disappeared.

Results

Visual Acuity Outcomes and Operative Complications During the Early Posttrabeculectomy Phase

At 2 months postsurgery, there were no eyes with fixation loss. Changes in visual acuity before and after trabeculectomy are shown in Fig. 2. The chance of visual acuity remaining within two lines of the preoperative level at 2 months postsurgery was 75%. Decreased visual acuity at three lines or above was encountered in seven eyes, but this gradually recovered to the preoperative level in two of the seven eyes. The remaining five eyes had associated cataract progression (n = 3), retinal vein occlusion (n = 1), or hyphema (n = 1). In the eye with hyphema, a hemorrhage in the anterior chamber diffused into the vitreous space because of aphakia. However, visual acuity recovered to presurgical levels once the vitreous blood was absorbed.

On the first day after surgery, the mean postoperative IOP was 11.4 ± 7.8 mmHg (range, 1–34). Seven eyes recorded IOP levels of 4 mmHg or lower on the first day after surgery, and no eyes had associated, prolonged hypotony for 14 days or more. Choroidal detachment was seen in 15 eyes, but this disappeared several days postsurgery. Hyphema was observed in 12 eyes between 3 and 37 days after surgery. Hypotensive maculopathy, flattened anterior chamber, endophthalmitis, and expulsive hemorrhage were not seen in the current study. The mean number (± SD) of scleral flap sutures was 4.5 ± 1.3 (range, 2–7), and the mean number of suture lysis procedures performed was 1.6 ± 1.7 (range, 0–6). Laser suture lysis was conducted an average of 7.6 ± 12.1 days (range, 2–65 days) after surgery. IOP levels were reduced from 22.8 ± 6.0 to 11.7 ± 4.7 mmHg by 2 months postsurgery.

Long-term Outcome of Trabeculectomy

At the final visit, IOPs were reduced to 11.8 ± 4.2 mmHg in all 49 eyes. Kaplan-Meier survival analysis revealed that the success rates for achieving target IOPs of 20, 15, and 12 mmHg 5 years postsurgery were 100%, 70%, and 25%, respectively (Fig. 3). The chance of visual acuity remaining within two lines of the preoperative level was 59% (29 eyes) at the time of the final visit.

Three eyes exhibited a decreased postoperative visual acuity of less than 0.1 (9%), caused by cataract progression in two eyes and fixation loss in one eye (Fig. 4). This latter eye with fixation loss had associated primary open-angle glaucoma. Its visual field of Grade IV-2 and visual acuity of 1.0 had regressed by 3.5 years after surgery to fixation loss and hand motion level, respectively, although the presurgery IOP level of 20 mmHg was controlled at 8–11 mmHg until the development of fixation loss.

Discussion

In the present study, no eyes with advanced glaucoma developed sudden postoperative fixation loss during the early posttrabeculectomy phase. This contrasts with the

Table 2. Incidence of fixation loss in our series and previous reports

Series	*n*	Patients	Incidence of fixation loss (%)	Use of antimetabolites
Kolker[1]	101	Advanced glaucoma (POAG)	13.6	None
Aggarwal et al.[2]	26	Advanced glaucoma (POAG)	15	None
Levene[16]	96	Advanced glaucoma	1	None ($n = 76$) or 5-FU ($n = 20$)
Martinez et al.[14]	54	Advanced glaucoma (POAG, CACG, angle-recession glaucoma, XG)	0	None
Costa et al.[13]	508	Any stage of glaucoma (POAG, CACG, XG, etc.)	0.95	None or 5-FU
Jian et al.[17]	37	Advanced glaucoma (POAG, XG)	0	MMC ($n = 37$)
This study	49	Advanced glaucoma (POAG, PACG, XG)	0	MMC ($n = 49$)

n, number of eyes; 5-FU, 5-fluorouracil; MMC, mitomycin C.

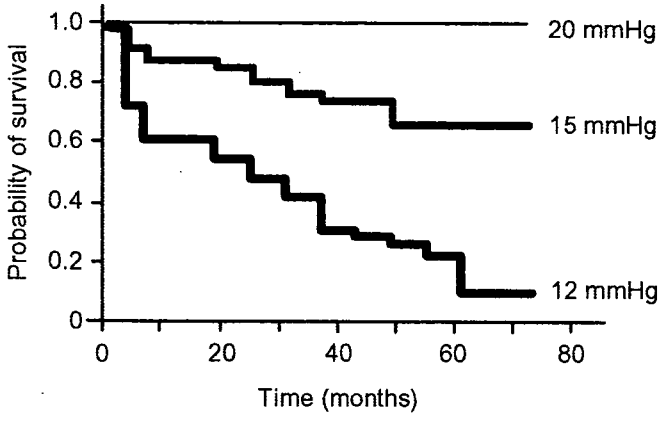

Figure 3. Kaplan-Meier survival curve of intraocular pressure control. Data are for patients both with and without antiglaucoma medication use.

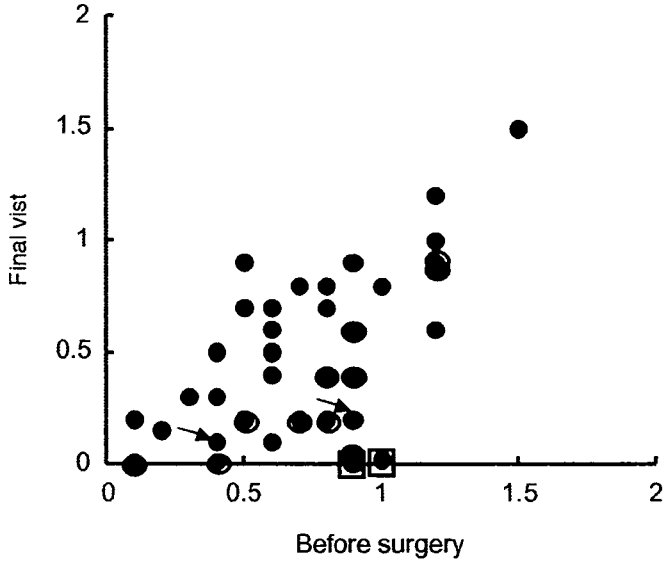

Figure 4. Corrected visual acuity before surgery and at the final visit in the 49 eyes. Eleven outliers (◉) show eyes with a progression of cataracts during the follow-up period. Two outliers (▣) indicate eyes with central visual field loss during the follow-up period. Two outliers (*arrows*) indicate eyes with branch retinal vein occlusion.

findings of several previous studies (Table 2). Kolker et al.[1] and Aggarwal et al.[2] reported that loss of fixation was seen in three of 22 eyes (13.6%) and four of 26 eyes (15.4%), respectively, after trabeculectomy was performed for advanced primary open-angle glaucoma.

However, since the 1990s, the frequency of fixation loss has decreased. Costa et al.,[13] Martinez et al.,[14] and Levene[16] reported that loss of fixation after trabeculectomy was encountered in just four of 508 glaucomatous eyes, in no eyes of 54 advanced glaucomatous eyes, and in one of 96 advanced glaucomatous eyes, respectively. The decreased incidence of sudden visual acuity loss after trabeculectomy in eyes with advanced glaucoma is thought to be due to the postoperative step-by-step IOP reduction obtained by intraoperative application of antimetabolites and postoperative laser suture lysis.

The filtering effect of trabeculectomy has become more severe during the long postoperative period because wound-healing activities can be inhibited by the adjunctive aid of antimetabolites such as 5-fluorouracil and MMC. On the other hand, overfiltration has caused higher incidences of hypotonic maculopathy and prolonged choroidal detachment, and can lead to poorer visual results. Costa et al.[13] reported that hypotonic IOP levels between 0–2 mmHg were observed in three of four eyes that encountered fixation loss after surgery. Thus, postoperative hypotonic conditions may be regarded as a significant risk factor.

With the subsequent introduction of multiple sutures combined with laser suture lysis, it has recently become possible to lower postoperative IOP levels in a step-by-step manner. In our investigation, no eyes encountered prolonged hypotony. Our data suggest that the modern surgical procedures of trabeculectomy with MMC, tight suture closure of scleral flaps, postoperative laser suture lysis, and ocular massages decrease the risk of fixation loss in eyes with advanced glaucoma because of the reduced occurrence of hypotony and excess IOP fluctuations after surgery.

In the present study, however, one eye developed fixation loss during the long-term follow-up period. The IOP levels of this eye were controlled between 8–11 mmHg. This suggests that the mechanism of fixation loss during the chronic posttrabeculectomy phase differs from that in the early postsurgery phase. Recently, it has been suggested

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that several IOP-independent factors such as circulatory disorders, autoimmunity, and glutamate toxicity are associated with the progression of glaucomatous optic neuropathy.[18–20] Further studies will be required to elucidate the risk factors related to fixation loss during the chronic postsurgery phase in eyes with advanced glaucoma.

In conclusion, trabeculectomy with MMC and tight sutures for the closure of scleral flaps, followed by postoperative laser suture lysis, results in postoperative hypotony and fixation loss being rare. We suggest that these procedures successfully lower the IOP of advanced glaucoma patients.

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Effects of Topical Administration of Y-39983, a Selective Rho-Associated Protein Kinase Inhibitor, on Ocular Tissues in Rabbits and Monkeys

Hideki Tokushige,[1] *Masaru Inatani,*[2] *Shingo Nemoto,*[1] *Hideyuki Sakaki,*[1] *Koushirou Katayama,*[3] *Masayoshi Uehata,*[3] *and Hidenobu Tanihara*[2]

PURPOSE. To elucidate the intraocular pressure (IOP)-lowering effects and associated characteristics of Y-39983, a selective Rho-associated coiled coil-forming protein kinase (ROCK) inhibitor derived from Y-27632, in animal eyes.

METHODS. Y-39983 was compared with Y-27632 for selectivity of ROCK inhibition by biochemical assay. The IOP was monitored by pneumatonometer in albino rabbits and cynomolgus monkeys that were given topically administered Y-39983. The total outflow facility and uveoscleral outflow were measured by two-level constant-pressure perfusion and perfusion technique using fluorescein isothiocyanate-dextran, respectively, at 2 hours after topical administration of Y-39983 in albino rabbits. The ocular toxicologic effects of topical administration of Y-39983 were observed in albino rabbits and cynomolgus monkeys.

RESULTS. A biochemical assay showed that Y-39983 inhibited ROCK more potently than Y-27632. In rabbits, topical administration of Y-39983 significantly increased conventional outflow by 65.5%, followed by significant, dose-dependent reduction in IOP. Maximum IOP reduction was 13.2 ± 0.6 mm Hg (mean ± SE) at 0.1% Y-39983 in rabbits. In monkeys, at 3 hours after topical administration of 0.05% Y-39983, maximum reduction of IOP was 2.5 ± 0.8 mm Hg. No serious side effects were observed in ocular tissues except sporadic punctate subconjunctival hemorrhage during long-term topical administration of Y-39983 four times a day (at 2-hour intervals) in rabbits or monkeys. However, punctate subconjunctival hemorrhage was not observed with administration twice daily (at a 6-hour interval) or three times a day (at 5-hour intervals).

CONCLUSIONS. Y-39983 causes increased outflow facility followed by IOP reduction. Y-39983 ophthalmic solution may be a candidate drug for lowering of IOP, since it increases conventional outflow and produces relatively few side effects. (*Invest Ophthalmol Vis Sci.* 2007;48:3216-3222) DOI: 10.1167/iovs.05-1617

Because the small GTPase Rho plays critical roles in signaling pathways that lead to formation of actin stress fibers and focal adhesions,[1-4] it regulates various cellular behaviors including cytoskeletal rearrangement,[5,6] cell morphology,[7] cell motility,[8] cytokinesis,[9] and smooth muscle contraction.[10,11] These effects of Rho are mediated by downstream Rho effectors such as Rho-associated coiled coil-forming protein kinase (ROCK) and mDia. The GTP-bound forms of Rho activate these Rho effectors that control the actin cytoskeleton, resulting in changes in morphology and adhesion of fibroblasts and epithelial cells.[12-16] Inhibitors of ROCK have been developed because of their potential for use in treating metastasis and axon injury. Among these inhibitors, Y-27632 is the first identified specific inhibitor of the ROCK family of protein kinases.[17] In our previous studies, Y-27632 was found to lower intraocular pressure (IOP) in rabbit eyes.[18,19] Our previous studies also revealed that Y-27632 altered the contractility of the trabecular meshwork (TM) cells and ciliary muscle (CM). Recently, alteration in contractility, focal adhesion, and stress fiber formation in Schlemm's canal (SC) cells, TM cells, and CM have been proposed to lower IOP.[18-30] ROCK inhibitors are thus considered candidates for novel IOP-lowering antiglaucoma drugs.[18,19,23,25,26]

In addition, it has been reported that other protein kinase inhibitors such as H-7 and HA-1077 have ROCK inhibitory activity, though their specificity for ROCK is less than that of Y-27632.[17] H-7 and HA-1077 also reduce IOP by increasing conventional outflow by altering the contractility of TM and the cellular behavior of TM cells.[27-30] These inhibitors may also have potential for development as antiglaucoma drugs to lower IOP.

Thus, inhibition of the Rho-ROCK signaling pathway is a new target for glaucoma treatment. In the present study, a novel selective ROCK inhibitor, Y-39983, inhibited Rho-ROCK signaling more potently than Y-27632, and topical administration of it facilitated aqueous conventional outflow, resulting in a lowering of IOP. We also examined the toxicologic effects of topical administration to evaluate the possibility of clinical use of Y-39983.

MATERIALS AND METHODS

Animals

In pharmacological studies (measurements of IOP and aqueous outflow), adult male Japanese white (albino) rabbits weighing 2.0 to 2.8 kg and adult male cynomolgus monkeys (*Macaca fascicularis*) weighing 6.0 to 8.9 kg were used. In this experiment, adult cynomolgus monkeys were trained for measurement of IOP in conscious condition (without systemic anesthesia). In toxicologic studies, adult male Japanese white rabbits weighing 1.8 to 2.7 kg and adult male and female cynomolgus monkeys weighing 2.2 to 3.5 kg were used. All studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

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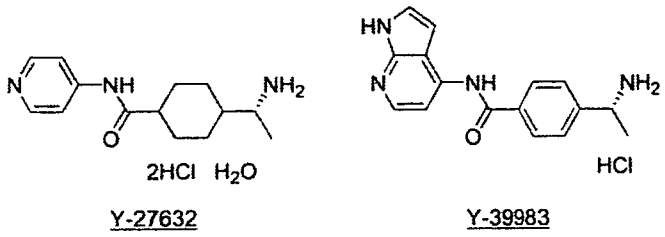

FIGURE 1. Molecular structures of Y-27632 and Y-39983.

For IOP measurements in rabbits and monkeys, the eyes were anesthetized by topical instillation of 0.04% and 0.4% oxybuprocaine hydrochloride, respectively.

Chemicals and Drug Preparation

Y-27632 (molecular weight [MW] 338.3) and Y-39983 (MW 316.8) were synthesized by Mitsubishi Pharma Corp. (Osaka, Japan). The structures of Y-27632 and Y-39983 are shown in Figure 1. Staurosporine, a nonspecific protein kinase inhibitor, was purchased from Wako Pure Chemical (Osaka, Japan). In the topical administration experiments, Y-39983 was used as an ophthalmic solution containing preservative for clinical use. In addition, 0.005% latanoprost (Xalatan; Pfizer, Tokyo, Japan) was used as a comparator in examination of IOP-lowering effects.

Measurement of Inhibition of ROCK, Protein Kinase C, and Calmodulin-Dependent Protein Kinase II

Recombinant ROCK (ROK α/ROCK II) and purified protein kinase C (PKC: mixture of α, β, γ isoforms) were purchased from Upstate Biotechnology (Lake Placid, NY). Recombinant calmodulin-dependent protein kinase II (CaMK II) was purchased from Daiichi Pure Chemical (Tokyo, Japan). ROCK (0.2 U/mL) was incubated with 1 μM [γ-^{32}P] ATP and 10 μg/mL histone as substrates in the absence or presence of various concentrations of Y-27632, Y-39983, or staurosporine at room temperature for 20 minutes in 20 mM MOPS (3-(*N*-morpholino)propanesulfonic acid) buffer (pH 7.2) containing 0.1 mg/mL bovine serum albumin (BSA), 5 mM dithiothreitol [DTT], 10 mM β-glycerophosphate, 50 μM Na_3VO_4, and 10 mM $MgCl_2$ in a total volume of 100 μL. PKC (10 ng/mL) was incubated with 1 μM [γ-^{32}P] ATP and 20 μM PKC substrate (Peptide Institute, Osaka, Japan) in the absence or presence of various concentrations of Y-27632, Y-39983, or staurosporine at room temperature for 30 minutes in 20 mM MOPS buffer (pH 7.5) containing 0.1 mg/mL BSA, 10 mM DTT, 10 mM β-glycerophosphate, 50 μM Na_3VO_4, 2 mM $CaCl_2$, 20 μg/mL phosphatidyl-L-serine, and 10 mM $MgCl_2$ in a total volume of 100 μL. CaMK II (125 U/mL) was incubated with 1 μM [γ-^{32}P] ATP, 10 μM calmodulin, and 20 μM CaMK II substrate (Daiichi Pure Chemical), in the absence or presence of various concentrations of Y-27632, Y-39983, or staurosporine at room temperature for 30 minutes in 20 mM MOPS buffer (pH 7.5) containing 0.2 mg/mL BSA, 0.5 mM DTT, 0.1 mM β-glycerophosphate, 50 μM Na_3VO_4, 1 mM $CaCl_2$, and 5 mM $MgCl_2$ in a total volume of 100 μL. Incubation was terminated by the addition of 100 μL of 0.7% phosphoric acid. A 160 μL portion of the mixture was transferred to Multiscreen-PH plate (Millipore, MA). A positively charged phosphocellulose filter absorbed the substrate that bound ^{32}P (Multiscreen-Vacuum manifold; Millipore). The filter was washed with 300 μL of 0.5% phosphoric acid and then twice with purified water and then dried. The radioactivity of the dried filter was measured with a liquid scintillation counter (LS6500; Beckman Instruments, Fullerton, CA). Results are presented as 50% inhibitory concentrations and 95% confidence intervals (CIs).

IOP Measurements

Pneumotonometers (Alcon, Fort Worth, TX, or Medtronic Solan, Jacksonville, FL) were used to monitor IOP. In the experiments involving single topical administration in rabbits and monkeys, 50 μL of Y-39983 at concentrations of 0.003% to 0.1% (0.1% in rabbits only) was topically administered to one eye. In addition, 0.005% latanoprost was topically administered as a comparator to one eye in monkeys. Saline was topically administered to the contralateral eyes in both species. IOPs were measured before topical administration and at 1, 2, 3, 5, 7, 9, and 12 hours (12 hours in monkeys only) after administration. In the experiments on repeated topical administration using rabbits, 50 μL of 0.03% Y-39983 was topically administered to one eye four times a day (QID; 10:00, 13:00, 16:00, and 19:00, at 3-hour intervals) for 28 days. The contralateral eyes were not treated. IOPs were measured at maximum reduction (2 hours after topical administration in the morning) at 7, 14, 21, and 28 days after administration. The vehicle of Y-39983 was used as the control. IOPs were calculated from the difference between results for Y-39983 or its vehicle-treated eyes and the contralateral saline-treated or nontreated eyes at each time point.

Measurement of Total Outflow Facility and Uveoscleral Outflow

Total outflow facility was determined by two-level constant pressure perfusion (25 and 35 mm Hg) at 2 hours after topical administration of 50 μL of 0.05% Y-39983 in one eye and its vehicle in the contralateral eye, according to the method of Bárány.[31] Briefly, the anterior chambers of rabbits anesthetized with 40% urethane were perfused with mock aqueous humor (Opeguard MA; Senju Pharmaceutical, Osaka, Japan) with a constant pressure of either 25 or 35 mm Hg, which was alternately applied for 10-minute intervals. During each 10-minute period, fluid flow was measured for 8 minutes, beginning 2 minutes after pressure change.

Uveoscleral outflow was determined with a perfusion technique using fluorescein isothiocyanate-dextran (FITC-dextran, mean MW, 71,200; Sigma-Aldrich, St. Louis, MO)[32,33] at 2 hours after topical administration of 50 μL of 0.05% Y-39983 in one eye and its vehicle in the contralateral eye. Rabbits were anesthetized with 40% urethane, and two 23-gauge needles connected to a pair of syringes were inserted into the anterior chamber of each eye of each rabbit. The pair of syringes was controlled by an infusion–withdrawal pump (Model 55-1382; Harvard Apparatus, S. Natick, MA), and the infusion syringe was filled with 10^{-4} M FITC-dextran. One milliliter of the FITC-dextran solution was washed through the anterior chamber from the syringes at a rate of 0.5 mL/min. The IOP level was then set to 20 mm Hg. The FITC-dextran solution was perfused continuously through the anterior chamber at a rate of 10 μL/min for 30 minutes. The anterior chamber was washed with 2 mL of PBS at a rate of 0.5 mL/min. Each eye was then enucleated and dissected into the following sample groups: anterior uvea, anterior sclera, posterior sclera plus posterior uvea, and posterior segment fluid plus vitreous. All samples were homogenized and centrifuged, and then each volume was measured. The supernatant was measured to determine FITC-dextran concentration using a fluorophotometer. Uveoscleral outflow (Fu) was calculated as follows

$$\text{Fu}\ (\mu\text{L/min}) = \frac{\sum (a \times b)(\text{ng})}{C(\text{ng}/\mu\text{L}) \times T(\text{min})} \quad (1)$$

where a is the volume of each sample, b is the concentration of FITC-dextran in each sample, C is the concentration of FITC-dextran in the perfusion fluid (10^{-4} M = 7120 ng/mL); and T is the time of perfusion (30 minutes).

Ocular Toxicology

Ocular toxicologic properties of Y-39983 were evaluated in rabbits and monkeys. In the QID study, performed to test severe conditions, 100 μL of Y-39983 (0.003%–0.03%) or saline as a control was topically administered to both eyes of the rabbits at 2-hour intervals for 4 weeks (n = 5). In addition, 50 μL of Y-39983 (0.003%–0.05%) or its vehicle was topically administered four times a day at 2-hour intervals for 26

TABLE 1. Selective Inhibitory Effect of Y-39983 on ROCK

Compounds	IC_{50} (μM) ROCK	PKC	CaMKII
Y-39983	0.0036 (0.0025-0.0051)	0.42 (0.36-0.49) [117 times]	0.81 (0.67-0.97)] [225 times]
Y-27632	0.11 (0.074-0.17)	9.0 (7.1-11) [82 times]	26 (21-32) [236 times]
Staurosporine	0.0011 (0.00078-0.0015)	0.00026 (0.00024-0.00030) [0.24 times]	0.00036 (0.00033-0.00040) [0.33 times]

In parentheses, 95% confidence interval; in brackets, comparison with the IC_{50} of ROCK.

weeks ($n = 8$). In slit lamp examinations, the cornea (epithelial defects revealed by fluorescein biostaining, opacity, and neovascularization), conjunctiva (hyperemia, swelling), anterior chamber (flare), and iris (hyperemia, swelling) were observed. The lens, vitreous, and retina were observed in eyes under mydriasis, with a slit lamp and binocular indirect ophthalmoscope. Tear quantity was measured by phenol red thread (Zonequick; Menicon, Nagoya, Japan). The electroretinogram (ERG) was measured to evaluate retinal safety. In darkness and under mydriasis with systemic anesthetization, a contact lens-type electrode was fitted to the eye. Results of light stimulation and the ERG were recorded using a veterinary ERG system. Amplitudes of the a- and b-waves and the peak latency were determined. In addition, histologic examination was performed by the usual method after the last observation. The tissues observed were the eye including the palpebral and bulbar conjunctiva, and optic nerve, lacrimal glands, internal organs including the liver, gallbladder, kidneys, spleen, heart, aorta, gullet, stomach, intestines, lungs, and bronchial tube, sex organs, brain, bone, muscle, skin, and other tissues.

In addition, to investigate the safety of Y-39983 at various frequencies of administration, the drug was administered two and three times a day (BID and TID) to rabbits and monkeys. In rabbits in the BID study, 0.05% or 0.1% Y-39983 or saline was topically administered to both eyes twice daily (with a 6-hour interval) for 4 weeks ($n = 3$). In rabbits in the TID study, 0.1% Y-39983 or saline was topically administered three times a day at 5-hour intervals for 2 weeks ($n = 5$). In monkeys in the BID study, 0.05%, 0.1%, or 0.2% Y-39983 or saline was topically administered twice daily at a 6-hour interval for 4 weeks ($n = 3$). Ocular tissues were observed in the same fashion as for administration four times a day.

Effects on Cultured Human Umbilical Venous Endothelial Cells

Human umbilical venous endothelial cells (HUVECs) were purchased from Dainippon Pharmaceutical (Osaka, Japan). HUVECs were cultured in CS-C medium (Dainippon Pharmaceutical) and maintained in a 95% air-5% CO_2 atmosphere at 37°C and passaged using the trypsin-EDTA method. HUVECs were seeded into 24-well plates. After seeding, HUVECs were incubated in medium containing 1 μM Y-39983 for 15 or 30 minutes and observed by phase-contrast microscopy. Medium was then removed, and HUVECs were incubated in medium without Y-39983 for 1 hour to evaluate recovery from the morphologic changes induced by Y-39983.

RESULTS

Selective Inhibitory Effect of Y-39983 on ROCK Activity

Results are summarized in Table 1. The 50% inhibitory concentration (IC_{50}) of Y-27632 for ROCK (0.11 μM; 95% CI, 0.074-0.17 μM), was 30.6 times that of Y-39983 (0.0036 μM; 95% CI, 0.0025-0.0051 μM). In contrast, in the examination of inhibition of PKC and CaMKII, the IC_{50}s of Y-27632 and Y-39983 for PKC were 9.0 μM (95% CI, 7.1-11 μM) and 0.42 μM (95% CI, 0.36-0.49 μM), respectively, whereas the IC_{50}s of Y-27632 and Y-39983 for CaMKII were 26 μM (95% CI, 21-32 μM) and 0.81 μM (95% CI, 0.67-0.97 μM), respectively. The IC_{50}s of Y-27632 and Y-39983 for PKC were 82 and 117 times those for ROCK, respectively, whereas the IC_{50}s of Y-27632 and Y-39983 for CaMKII were 236 and 225 times those for ROCK, respectively. In addition, the same experiments were performed as controls using staurosporine, a nonspecific protein kinase inhibitor. Staurosporine exhibited the same inhibitory effects on all three kinases—ROCK, PKC, and CaMKII—as shown in Table 1. These findings indicate that Y-39983 more potently inhibits ROCK than Y-27632 and has the same selectivity for ROCK as Y-27632. In addition, Y-39983 was more selective for ROCK than staurosporine.

IOP Measurements in Rabbit Eyes

In rabbits, Y-39983 lowered IOP in a dose-dependent fashion, as shown in Figure 2A. Statistically significant IOP-lowering effects were found at concentrations of Y-39983 equal to or higher than 0.01% at 2 hours after topical administration. IOP reduction was at maximum between 2 and 3 hours after administration of 0.01% to 0.1% Y-39983. Maximum IOP reduction (mean ± SE) was 2.5 ± 0.8 ($P = 0.28$ vs. vehicle-treated eyes, Williams' test, one-sided), 7.0 ± 1.6 ($P = 0.0009$), 11.0 ± 1.0 ($P < 0.0001$), 12.1 ± 1.5 ($P < 0.0001$), and 13.2 ± 0.6 ($P < 0.0001$) at 0.003%, 0.01%, 0.03%, 0.05%, and 0.1% Y-39983, respectively (Fig. 2B). This result demonstrated the potent IOP-lowering effects of Y-39983 in rabbit eyes.

In addition, repeated topical administration of 0.03% Y-39983 (four times a day) was performed in rabbit eyes. Figure 3 shows the time course of changes in peak IOP reduction over 28 days. Mean reduction of IOP was between 7.0 and 9.6 mm Hg during the 28-day period, demonstrating that maintenance of IOP reduction is obtained with repeated administration.

IOP Measurements in Monkey Eyes

In monkeys, Y-39983 dose dependently lowered IOP, as shown in Figure 4A. Compared with vehicle-treated eyes, 0.05% Y-39983-treated eyes in particular exhibited significant reduction of IOP between 2 and 7 hours after topical administration ($P < 0.05$, the Dunnett's test, one-sided). The reduction of IOP was at maximum 3 hours after administration of 0.05% Y-39983. The reductions of IOP (mean ± SE) at 2, 3, 5, and 7 hours after administration of 0.05% Y-39983 were 1.9 ± 0.3, 2.5 ± 0.4, 1.7 ± 0.3, and 0.8 ± 0.2 mm Hg, respectively. Maximum IOP reduction (mean ± SE) was 0.4 ± 0.1, 0.4 ± 0.2, 1.4 ± 0.3 ($P < 0.05$ vs. vehicle-treated eyes, Williams' test, one-sided),

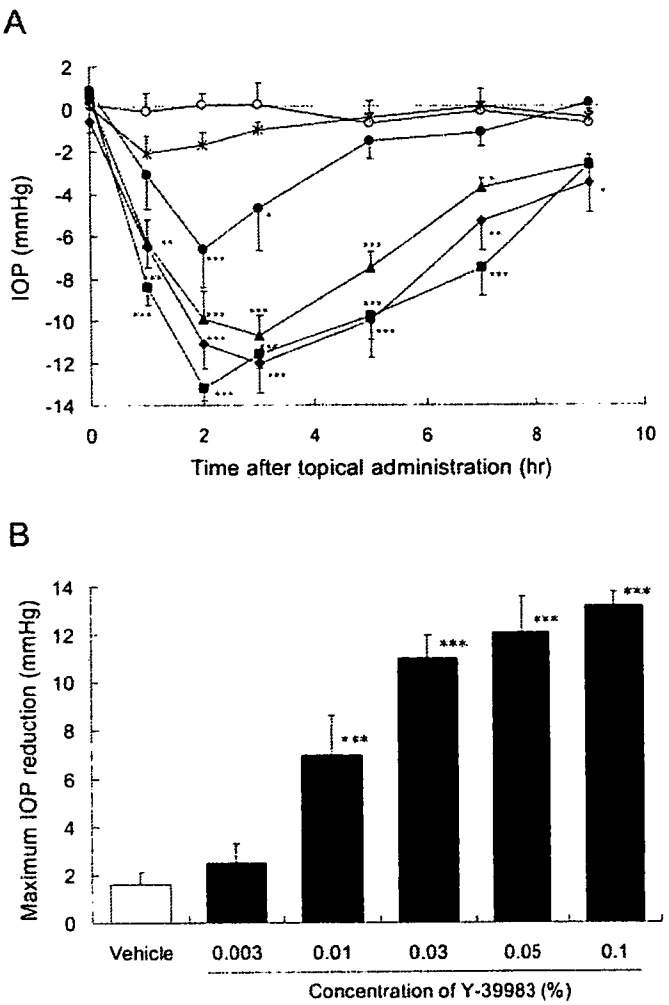

FIGURE 2. Effect of topical administration of Y-39983 on IOP in rabbit eyes. Y-39983 or its vehicle was topically administered to one eye in rabbits. The contralateral eyes were treated with the same volume of saline (n = 5). (A) Time course of changes in IOP. (○) Vehicle; (×) 0.003%; (●) 0.01%; (▲) 0.03%; (♦) 0.05%; and (■) 0.1% Y-39983. IOPs were calculated as the difference between the value in the Y-39983- or the vehicle-treated eye and the contralateral saline-treated eyes at each time point. Data are the mean mm Hg ± SE. The significance of findings was evaluated by the Dunnett's test (one-sided); $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with the vehicle group at each time point. (B) Maximum IOP reduction. Data are the mean mm Hg ± SE. The significance of findings was evaluated by the Williams' test (one-sided); $^{***}P < 0.001$, compared with the vehicle group. Baseline IOPs were 20.7 ± 0.9, 20.5 ± 1.2, 22.9 ± 0.6, 19.6 ± 0.5, 22.0 ± 0.6, and 21.5 ± 0.7 mm Hg (mean ± SE) with vehicle, 0.003%, 0.01%, 0.03%, 0.05%, and 0.1% Y-39983, respectively.

and 2.5 ± 0.8 mm Hg ($P < 0.05$) at 0.003%, 0.01%, 0.03%, and 0.05% Y-39983, respectively (Fig. 4B). Statistically significant reduction of IOP was obtained at concentrations of Y-39983 equal to or greater than 0.03%. Administration of 0.005% latanoprost lowered IOP by 2.5 ± 0.2 mm Hg ($P < 0.001$ vs. vehicle-treated eyes, t-test, one-sided), demonstrating that the IOP-lowering effect of 0.05% Y-39983 was similar to that of 0.005% latanoprost.

Measurements of Total Outflow Facility and Uveoscleral Outflow

Outflow facility was measured 2 hours after topical administration of 0.05% Y-39983, when maximum IOP reduction was observed. As summarized in Table 2, outflow facility (mean ± SE) in eyes treated with Y-39983 (0.168 ± 0.018 μL/min/mm Hg) was approximately 1.7 times (+65.5%) that in the contralateral, vehicle-treated eyes (0.111 ± 0.014 μL/min per mm Hg. This difference was significant ($P < 0.001$, paired t-test, one-sided). In contrast, there were no significant differences in uveoscleral outflow between eyes treated with Y-39983 and those treated with vehicle.

Ocular Toxicologic Effects of Topical Administration of Y-39983

Ocular toxicologic properties were evaluated for long-term topical administration of Y-39983. In the QID study, rabbit eyes were treated with 0.003% to 0.03% Y-39983 four times a day (at 2-hour intervals) for 4 weeks, and monkey eyes with 0.003% to 0.05% Y-39983 at the same dosage for 26 weeks. In neither species were significant abnormalities of the corneal surface, anterior chamber, lens, vitreous, or retina observed on slit lamp examination, nor were significant findings of toxicity detected on histologic examination. ERG analysis revealed no abnormalities in eyes treated with Y-39983 of either species. At week 4, thread-wetting values (mean millimeters ± SE) for rabbits determined by the phenol red thread method were 29.4 ± 1.7, 29.4 ± 0.9, 28.8 ± 1.0, and 29.0 ± 1.1 mm with saline, 0.003%, 0.01%, and 0.03% Y-39983, respectively. At week 25, the thread-wetting values in monkeys were 30.0 ± 1.0, 28.5 ± 1.0, 31.0 ± 0.7, 28.9 ± 1.1, and 29.1 ± 0.8 mm with vehicle, 0.003%, 0.01%, 0.03%, and 0.05% Y-39983, respectively. There were no differences in thread-wetting values between the groups of rabbits and monkeys. However, conjunctival hyperemia and punctate subconjunctival hemorrhage were observed in eyes with topical administration of Y-39983 in rabbits (Fig. 5A) and monkeys (Fig. 5B), and punctate subconjunctival hemorrhage was sporadic during the administration period in both species. In the QID study, punctate subconjunctival hemorrhages were observed in four of five rabbits receiving 0.03% Y-39983 and in two of eight monkeys receiving 0.05% Y-39983. The hemorrhages resolved during the administration period. However, in the BID and TID studies,

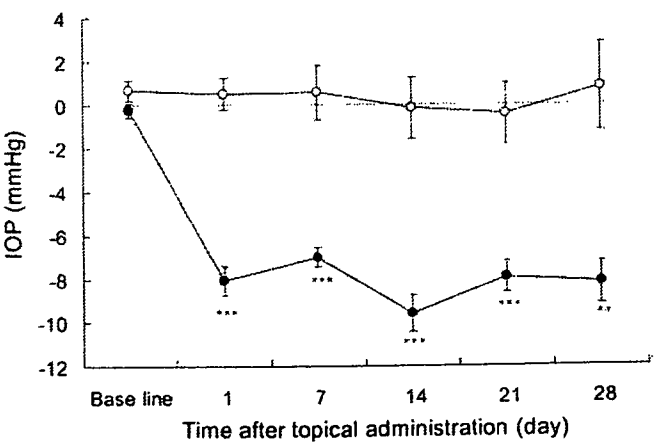

FIGURE 3. Effects of repeated topical administration of Y-39983 on IOP in rabbit eyes. Y-39983 0.03% or its vehicle was topically administered to one eye in rabbits four times a day for 28 days (n = 6). The contralateral eyes were not treated. IOPs were measured 2 hours after administration in the morning. (○) vehicle; (●) 0.03% Y-39983. IOPs were calculated as the difference between the value in the Y-39983 or vehicle-treated eye and the contralateral nontreated eye at each time point. Data are the mean mm Hg ± SE. The significance of findings was evaluated by t-test (one-sided); $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with the vehicle group at each time point. Baseline IOPs were 20.9 ± 0.5 and 21.0 ± 0.5 mm Hg (mean ± SE) with vehicle and 0.03% Y-39983, respectively.

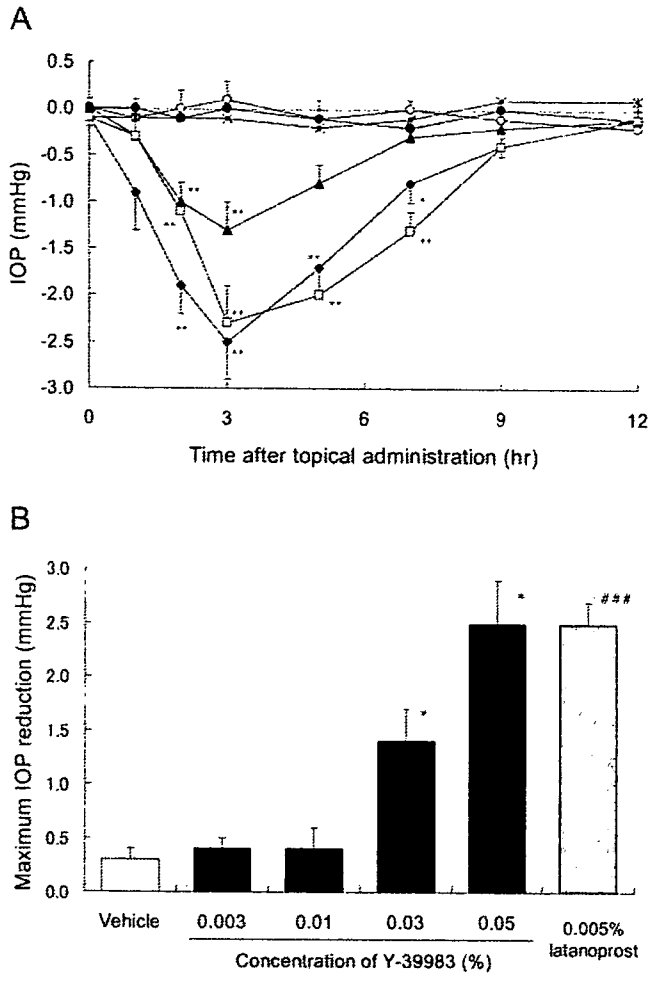

FIGURE 4. Effects of topical administration of Y-39983 on IOP in monkey eyes. Y-39983 or its vehicle was topically administered to one eye in monkeys. The contralateral eyes were treated with the same volume of saline (n = 5). (A) Time course of changes in IOP. (○) vehicle; (×) 0.003%; (●) 0.01%; (▲) 0.03%; and (◆) 0.05% of Y-39983; (□) 0.005% latanoprost. IOPs were calculated as the difference between the value in Y-39983 or vehicle-treated eyes and contralateral saline-treated eyes at each time point. Data are the mean mm Hg ± SE. The significance of findings was evaluated by the Dunnett's test (one-sided); $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the vehicle group at each time point. (B) Maximum IOP reduction. Data are the mean mm Hg ± SE. The significance of findings was evaluated by the Williams' test (one-sided) $^{*}P < 0.05$; and by t-test (one-sided) $^{###}P < 0.001$, compared with the vehicle group. Baseline IOPs were 17.8 ± 0.2, 17.9 ± 0.2, 17.8 ± 0.4, 17.3 ± 0.2, and 17.5 ± 0.2 mm Hg (mean ± SE) with vehicle, 0.003%, 0.01%, 0.03%, and 0.05% Y-39983, respectively.

punctate subconjunctival hemorrhages were not observed in eyes treated with Y-39983, although conjunctival hyperemia was observed in eyes treated with Y-39983 in both species. To elucidate the mechanisms responsible for the punctate subconjunctival hemorrhages, we examined morphologic changes in cultured HUVECs after the addition of Y-39983. In medium containing 1 μM Y-39983, HUVECs exhibited contraction (Figs. 6A–C). The morphologic changes in HUVECs were reversible and had nearly recovered by 1 hour after removal of Y-39983 (Fig. 6D).

DISCUSSION

In the present study, topical administration of a selective inhibitor of the ROCK/ROK family of protein kinases, Y-39983, significantly reduced IOP in rabbit and monkey eyes. Several clinical investigations have revealed that elevated IOP is a major factor that causes glaucomatous optic neuropathy.[34–36] Because they potently lower IOP in mammalian eyes, ROCK inhibitors have been considered potential drugs for the treatment of glaucoma.[18,19] In this study, we examined the efficacy and safety of Y-39983 for potential clinical application.

Although our previous study revealed significant IOP-lowering effects of Y-27632 in animal eyes,[18,19] for potential clinical use, this compound has the disadvantage of poor stability in solution (data not shown). A series of modifications of molecular structure was therefore conducted to develop a more suitable form and more potent ROCK inhibitory activity for clinical use. Among the forms obtained, Y-39983 exhibits potent inhibition of ROCK activity and has acceptable stability, even in solution. Furthermore, we found that the ratio of IC_{50} for inhibition of ROCK/PKC for Y-39983 was 117 while that for Y-27632 was 82, suggesting that Y-39983 has the same specificity for ROCK as Y-27632. Also, the inhibition of ROCK by Y-39983 was 30 times that by Y-27632. These in vitro findings suggest that Y-39983 is a more useful candidate for an antiglaucoma drug than Y-27632. In our previous study,[18] Y-27632 at concentrations of 0.34% to 3.4% reduced IOP by 7 to 12 mm Hg in rabbit eyes under the same conditions of administration as in this study. Reduction of IOP (ΔIOP ≥ 10 mm Hg) was observed with a lower dose of Y-39983 (0.03%–0.1%). In addition, the degree of reduction of IOP (maximum ΔIOP = 5.3 mm Hg) obtained with 0.1% Y-27632, as determined in a previous study,[19] was observed with 0.01% Y-39983 (peak ΔIOP = 6.6 mm Hg). These findings together suggest that the reduction of IOP by Y-39983 is approximately 10-fold higher than that by Y-27632. These findings appear to agree with our in vitro result that the ROCK inhibitory activity of Y-39983 is 30 times that of Y-27632.

In considering clinical application of Y-39983, the next important question is whether Y-39983 is also effective in lowering IOP in primate eyes, since primate eyes have a modality of aqueous outflow different from that of lower mammalian eyes. The present study revealed that, even in monkey eyes, 0.05% Y-39983 induces significant IOP reduction almost equal to that obtained with 0.005% latanoprost. The IOP-lowering effect of Y-39983 in monkey eyes suggests the possibility of clinical use of this compound.

In this study, we examined the IOP-lowering effects of Y-39983 in rabbits and monkeys. With 0.05% Y-39983, maximum reductions of IOP were 12.1 ± 1.5 (mean ± SE) and 2.5 ± 0.2 mm Hg in rabbits and monkeys, respectively, showing that the magnitude of effect of Y-39983 in monkeys was much less than that in rabbits. This difference between species may be explained by the difference in baseline IOPs, which were 22.0 ± 0.6 and 17.5 ± 0.2 mm Hg in rabbits and monkeys, respectively. In fact, it has been reported that the IOP-lowering effects of H-7 and prostaglandin analogues in monkeys, which have low baseline IOPs, are weaker than that in rabbits, which have high baseline IOPs.[30,37] In a preliminary

TABLE 2. Effect of 0.05% of Y-39983 on Outflow Facility in Rabbit Eyes

	Outflow Facility (μL/min per mm Hg)	Uveoscleral Outflow (μL/min)
Y-39983	0.168 ± 0.018	0.470 ± 0.034
Vehicle	0.111 ± 0.014	0.478 ± 0.031
Significance	$P < 0.001$	NS
% Change	+65.5%	−0.9%

Data are the mean ± SE (n = 10). Significance was evaluated by paired t-test (one-sided).

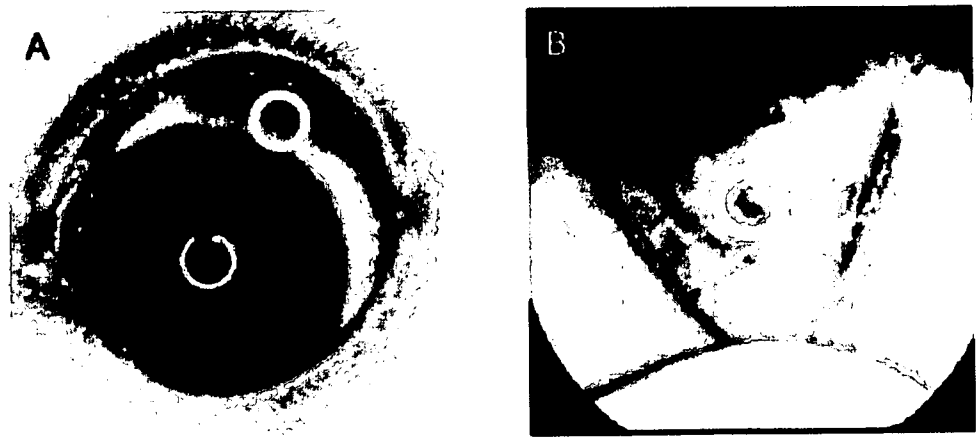

FIGURE 5. Examples of subconjunctival hemorrhage in rabbit and monkey eyes. Y-39983 was topically administered to eyes four times a day (at 2-hour intervals) for 4 and 26 weeks in rabbits and monkeys, respectively. (A) A rabbit eye treated with 0.03% Y-39983. (B) A monkey eye treated with 0.03% Y-39983.

pharmacokinetics study after topical administration of Y-39983, the disappearance of Y-39983 in tears of monkeys was faster than that in rabbits, and this difference in pharmacokinetics may be due to differences in frequency of blinking in these species. This difference in pharmacokinetics may also have resulted in the difference in IOP-lowering effects in rabbits and monkeys in this study.

There are two routes of aqueous humor outflow: that via the conventional (trabecular) and that via the unconventional (uveoscleral) pathway.[20] In primate and human eyes, conventional outflow is considered the main route and is believed to be regulated by the cellular behavior and contractility of TM cells.[38,39] Our previous study showed that Y-27632 increased conventional outflow by altering the contractility of TM cells.[18] In addition, Y-27632 has been shown to increase conventional outflow by inducing cellular relaxation and loss of cell–substratum adhesion in the TM and in SC cells.[23] Our outflow measurements suggest that Y-39983 may also affect the contractility of TM and SC cells, resulting in increased conventional outflow. Consistent with this, it has been demonstrated that TM exhibits higher levels of mRNA for ROCK and ROCK substrates than CM in human and monkey eyes, suggesting that TM is one of the major sites of regulation of IOP by ROCK.[25]

The IOP-lowering mechanism of H-7, a broad-spectrum inhibitor of serine-threonine kinases including ROCK, has been investigated.[27,28,30] H-7 also increases conventional outflow by altering the shape, actin cytoskeleton, and cell–cell adhesion of TM and SC cells, as Y-27632. Thus, alterations of TM and SC cells affect conventional outflow, and compounds causing cytoskeletal change in TM and SC cells may potentially be useful as antiglaucoma drugs.

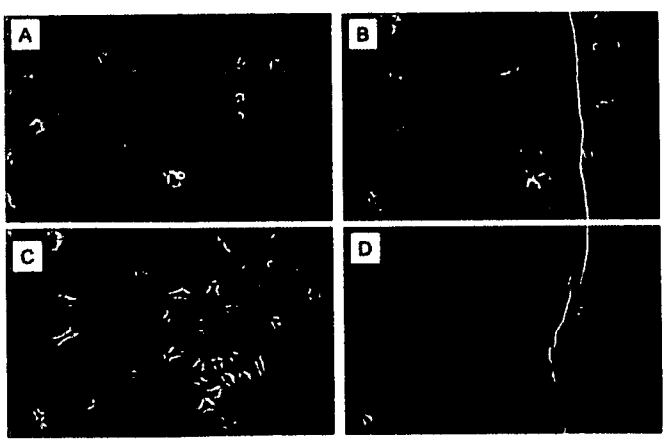

FIGURE 6. Effects of Y-39983 on morphology of HUVECs. Phase-contrast microscopic observation of HUVECs in the same region. (A) Nontreatment or (B) treatment with 1 μM Y-39983 for 15 minutes or (C) for 30 minutes. *Arrows*: contracted cells. (D) Replacement with medium without Y-39983 for 1 hour.

In our toxicologic study, no serious side effects were observed in ocular tissues of rabbits and monkeys except sporadic punctate subconjunctival hemorrhage. This type of hemorrhage was observed after frequent administration of Y-39983 (four times a day at 2-hour intervals). The side effects may be explained by impairment of barrier function or morphologic changes in vascular endothelial cells, as shown in the experiments using HUVECs. The morphologic changes in HUVECs observed after the addition of Y-39983 suggest the possibility of impairment of barrier function in vascular endothelial cells in the retina, since the Rho-ROCK signaling pathway is considered ubiquitous. However, our animal experiments did not reveal detectable hemorrhages in the iris-ciliary body or retina-choroid, suggesting that the concentration at which lowering of IOP is elicited may not be high enough to induce hemorrhage in the ocular fundus. Also, subconjunctival hemorrhage, which was encountered with frequent administration (four times a day), was not observed with administration two or three times a day. It is possible that no side effects will be induced in the conjunctiva with clinical usage of Y-39983, if excessively frequent instillation is avoided.

In summary, the present study showed that Y-39983, a selective and potent ROCK inhibitor, reduced IOP and increased aqueous outflow. Selective inhibition of the Rho/ROCK signaling pathway may be a useful new strategy for the treatment of glaucoma, and Y-39983 ophthalmic solution may be a candidate drug since it lowers IOP by increasing aqueous conventional outflow and produces fewer side effects.

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Effect of pitavastatin on experimental choroidal neovascularization in rats☆

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Abstract

The association between the use of statins and age-related macular degeneration (AMD), a leading cause of blindness, has been evaluated in many clinical studies; however, the results have been contradictory. We evaluated the effect of pitavastatin administration on laser-induced experimental choroidal neovascularization (CNV) in rats. Brown Norway rats received pitavastatin (1.0 mg/kg per day) for 1 day prior to laser-induced CNV and continued to receive the drug for 14 days. Fluorescein angiograms were graded by masked observers. CNV area and thickness were assessed by fluorescein isothiocyanate-labeled dextran angiography and histology, respectively. Vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (Ccl-2; also known as MCP-1), and intercellular adhesion molecule-1 (ICAM-1) mRNA levels were measured using reverse-transcription polymerase chain reaction (RT-PCR) and real-time quantitative RT-PCR. Pitavastatin-treated rats had significantly less fluorescence leakage compared with the vehicle-treated rats estimated by CNV score using fluorescein angiography. Both the area and the thickness of CNV in pitavastatin-treated rats were significantly reduced compared with the vehicle-treated rats. Gene expression of VEGF, Ccl-2, and ICAM-1 were significantly decreased by pitavastatin administration in experimental CNV. Thus, we demonstrated that the therapeutic dose of pitavastatin for human hypocholesterolemia effectively suppressed experimental CNV in rats. The use of pitavastatin may be helpful in preventing CNV development in AMD patients.

Keywords: choroidal neovascularization; HMG-CoA reductase inhibitor; pitavastatin; age-related macular degeneration

1. Introduction

The exudative form of age-related macular degeneration (AMD) is the major cause of visual loss in well-developed countries (Fine et al., 2000). The main pathological change of the exudative form of AMD is choroidal neovascularization (CNV). An essential element in the growth of CNV is the rupture of Bruch's membrane and the proliferation of blood vessels through breaks in the membrane. However, the pathogenesis of CNV is not completely understood (Zarbin, 2004).

In recent years, statins, the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, have been widely used in the treatment of atherosclerotic diseases and hyperlipidemia (Mahley et al., 1999; Vaughan et al., 2000). In addition to their lipid-lowering properties, statins have been thought to exert an expanded profile of non-lipid-related pleiotropic effects, including improved endothelial function and decreased low-density lipoprotein oxidation, foam cell formation, leukocyte–endothelium interactions, plaque rupture, and smooth muscle cell proliferation (Bellosta et al., 2000; Hess and Fagan, 2001; Takemoto and Liao, 2001). Furthermore, statins have been found to exert both anti-inflammatory and anti-angiogenic effects, which are relevant to vascular disease and may also be relevant in the pathogenesis of AMD (Pruefer

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