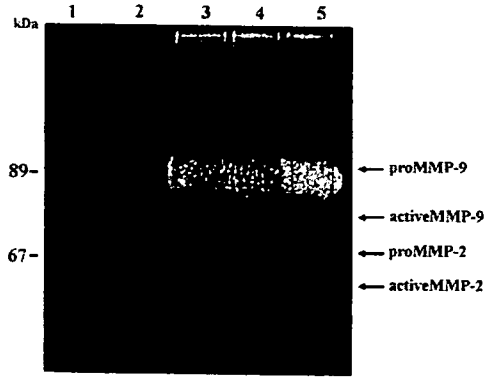

Fig. 4. Gelatin zymography. Gelatin zymography of normal corneal extracts (lane 1) or corneal extracts infected with *P. aeruginosa* obtained at 3 h (lane 2), 12 h (lane 3), 24 h (lane 4), and 72 h (lane 5). Proteins possessing gelatinolytic activity are indicated as clear lytic bands against the blue background. Note that larger amounts of gelatinolytic activity including MMP-2, MMP-9, and activated form of MMP-9 are detected in the 12-, 24-, and 72-h samples.

demonstrated at 12 h p.i., and was strongly enhanced at 24 h p.i. (Fig. 6). MMP-9 mRNA expression was then slightly reduced at 72 h p.i. Additionally, its relative concentration was also increased at 24 h p.i., and was slightly reduced at 72 h p.i. (Fig. 6). Similarly, TIMP-1 mRNA was faintly expressed at 3 and 12 h p.i., and its expression and its relative concentration significantly increased at 24 h p.i. (Fig. 6). However, its expression was somewhat reduced at 72 h p.i. Expression of TIMP-2 mRNA was detected even in control samples. Although its expression was moderate at 3–12 h p.i., it was slightly increased at 24–72 h p.i. (Fig. 6). Relative TIMP-2 mRNA concentration was increased at 72 h p.i.

4. Discussion

Our present study demonstrated that typical corneal ulcers with ring abscesses observed in rabbits by 12–24 h post-infection with a clinical isolate of *P. aeruginosa* (Fig. 1) were morphologically characterized by liquefactive necrosis, and accumulation of massive inflammatory cells, mainly PMNs (Fig. 2B). We investigated mechanisms of corneal ulceration in experimental pseudomonal keratitis in rabbits focusing on MMPs, especially MMP-2 and MMP-9, because these MMPs have been shown to be important in tissue repair, wound healing, and stromal ulceration (Fini et al., 1998).

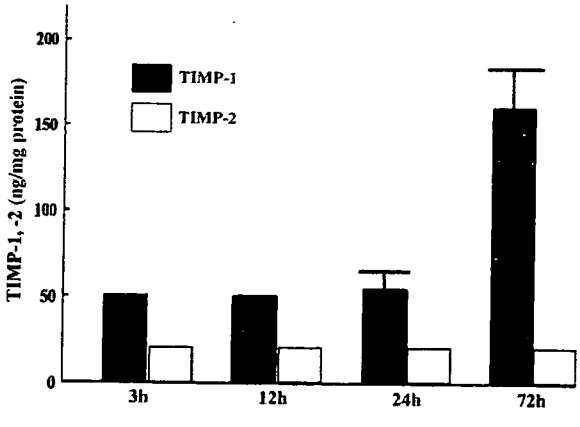

Fig. 5. Quantification of TIMP-1 and TIMP-2 concentrations using ELISA. Expressions of TIMP-1 and TIMP-2 in infected rabbit corneas were quantified using an ELISA detection kit. Results are presented as means (±standard errors) in ng/ml. Note the significant expression of TIMP-1 in the 72-h sample. On the other hand, expression of TIMP-2 was below detection limits during the experiments.

Our gelatin zymography (Fig. 4) results indicated that MMP-2 was constitutively expressed, while MMP-9 was inducible, and its expression was enhanced by pseudomonal infection. Fini et al. (1992a,b) also revealed that MMP-2 was constitutively expressed in healthy corneal cells under physiological conditions, but expression of MMP-9 was enhanced when the cornea was damaged. Moreover, Sakimoto et al. (2003) showed that even in pseudomonal keratitis with severe ulcers, active MMP-2 was not detected in the tear fluid. Matsubara et al. (1991a) suggested that MMP-2 and MMP-9 played different roles following corneal injury; namely, MMP-9 was synthesized and secreted by corneal cells with time, corresponding to a role in basement membrane degradation, whereas MMP-2 appeared during healing of corneal ulcers. Matsubara et al. (1991b) also suggested that MMP-2 performed a surveillance function in the normal cornea, catalyzing degradation of collagen molecules when the cornea was damaged. Pflugfelder et al. (2005) reported that increased MMP-9 activity on the ocular surface in response to dryness disrupted the corneal epithelial barrier function perhaps by cleaving occludins in tight junctions. Fini et al. (1991) showed that efficacy of tumor cell collagenase inhibitor in blocking progression to ulceration might be attributable to its action against MMP-9. Taken together, MMP-9 is more important than MMP-2 for tissue destruction.

Most MMPs are secreted as inactive proenzymes, and have to be cleaved and activated before performing their functions. A number of studies suggested that various factors activated MMPs. Matsumoto et al. (1992) and Matsumoto (2000) showed that secreted inactive corneal MMP-2 was activated by pseudomonal elastase. Nagano et al. (2001) suggested that pseudomonal elastase induced conversion of inactive precursors of MMP-1, -2, -3, and -9 produced by keratocytes to active forms of the enzymes. Twining et al. (1993) also demonstrated similar results. Okamoto et al. (1997) showed that purified MMP-1 (derived from human fibrosarcoma cells), MMP-8, and -9 (derived from human PMNs) were activated via limited proteolysis by bacterial proteases such as pseudomonal elastase and thermolysin. These results suggested that activated form of MMP-9 observed in our gelatin zymography was brought about via proteolytic activation by pseudomonal elastase.

Next, we investigated localizations of MMPs and TIMPs during pseudomonal infection using immunohistochemistry. Our immunostaining data for MMPs indicated that MMP-2 labeling was dependent on keratocytes while that of MMP-9 was dependent on PMN (Fig. 3A). Namely, in corneas with liquefactive necrosis, MMP-2 was detected in the limbal area rather than in the central cornea, where most keratocytes were

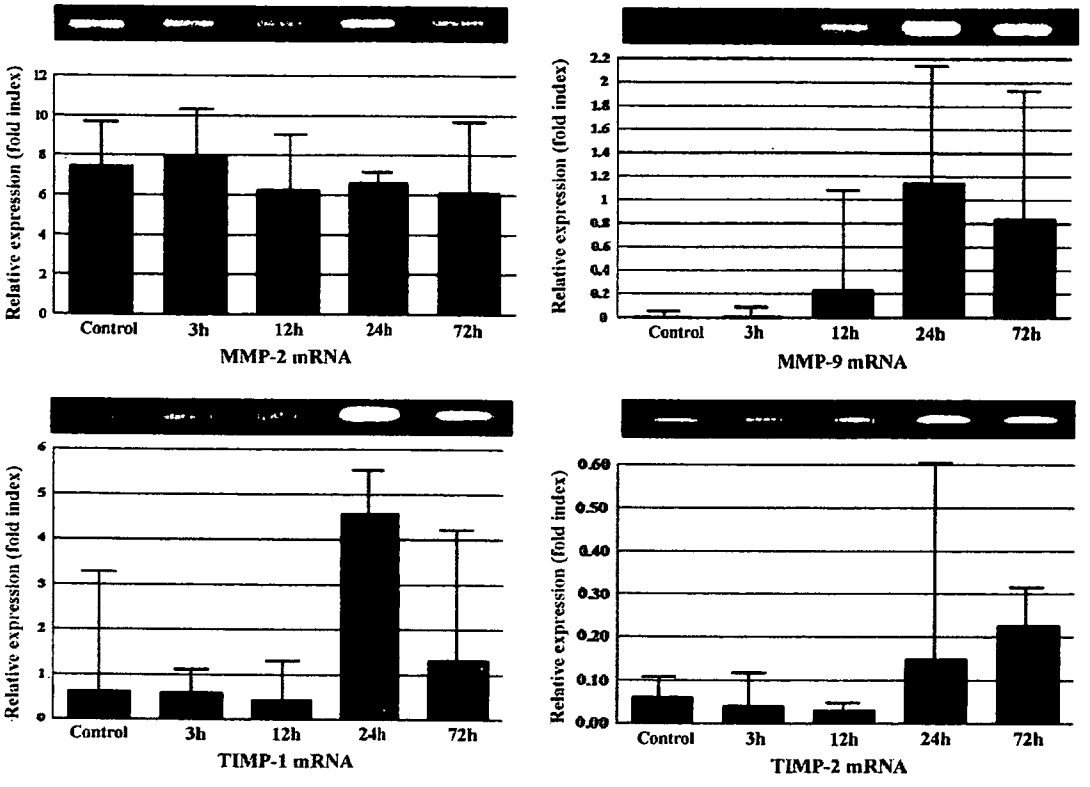

Fig. 6. Analysis of mRNAs of MMP-2, MMP-9, TIMP-1, and TIMP-2 using RT-PCR. The upper figure shows representative results of 4 independent experiments, and the lower figure shows quantitative analysis using real time PCR.

destroyed (data not shown). MMP-9 immunostaining was present in PMNs rather than in keratocytes and epithelial cells from the early stages of infection, and staining intensity gradually increased with time (Fig. 3B). However, in the later stages of infection, MMP-9 labeling was more intense in the limbal area than in the central cornea, where liquefactive necrosis was observed. We speculate that active or live PMNs exist in the limbal area, whereas dead or disrupted PMNs are present in the liquefactive necrosis area. Yang et al., 2003 showed that MMP-2 and -9 stainings were particularly intense in the proximity of ulcers, and in areas of PMN infiltration in herpetic rat keratitis models. Our findings were consistent with these notions.

Activity of MMPs is generally regulated by TIMPs. So far, 4 distinct TIMPs have been shown to exist in mammals. Balance between MMPs and TIMPs may determine net enzymatic activity in the cornea. Previous studies also demonstrated relationships between enzymes and their inhibitors (Ottinno et al., 2002). TIMP-1 is known to bind to the latent form of MMP-9. Kernacki et al. reported that TIMP-1 neutralization resulted in an overall increase in corneal MMP-9 production (Kernacki et al., 2004), and adequate endogenous expression of TIMP-1 in the cornea protected against extensive corneal tissue destruction after *P. aeruginosa* infection (Kernacki et al., 1999). Riley et al. (1995) also suggested that reduced levels of TIMP-1 expression were consistent with increased collagenase activity and tissue destruction. TIMP-1 may influence recruitment of PMNs into the infected cornea (Kernacki et al., 1999). With regards to TIMP-2, it not only acts as an inhibitor of MMP-2 by binding to MMP-2, but can also function as a co-activator of proMMP-2 (Wang et al., 2000).

Therefore, we investigated expressions and localizations of TIMP-1 and TIMP-2 in corneas after pseudomonal infection. Our immunohistochemical results demonstrated that TIMP-1 and TIMP-2 were both present in keratocytes and inflammatory cells in the area close to the limbus 12–72 h p.i. (Figs. 3C and D). However, in the central cornea where marked liquefactive necrosis was observed, intensity of TIMP-1 immunostaining was very faint during the experiments, whereas that of TIMP-2 increased with time. Immunostaining patterns for TIMP-1 and -2 agreed with previous reports (Kenney et al., 1998; Yang et al., 2003). We then quantified amounts of TIMP-1 and TIMP-2 using an ELISA detection kit. As expected, TIMP-1 concentration was enhanced at 24 and 72 h p.i., whereas that of TIMP-2 was lower than detection limits during the experiments.

In order to confirm de novo synthesis of MMPs and TIMPs, we further investigated expressions of mRNAs of MMPs and TIMPs using RT-PCR. As expected, MMP-2 mRNA was detected in control and infected corneas (Fig. 6). MMP-2 mRNA level was almost constant throughout the experiments, indicating that MMP-2 was constitutive produced (Fig. 6). On the other hand, expression of MMP-9 mRNA was significantly enhanced following pseudomonal infection (Fig. 6). These

results indicated intracorneal synthesis of MMP-9 after pseudomonal infection. From immunostaining results, it was obvious that enhanced expression of MMP-9 mRNA predominantly derived from PMNs. However, further studies are required to clarify the source of MMP-9 mRNA using in situ hybridization. With regards to TIMP-1 mRNA, its amount increased by 24 h p.i. and then decreased somewhat at 72 h p.i. (Fig. 6). On the other hand, expression of TIMP-2 mRNA was enhanced at 24 h p.i. (Fig. 6). Kernacki et al. (1998) reported that TIMP-1 mRNA was undetectable in either wounded or unwounded, non-infected corneal tissues, but increased in a time-dependent manner after corneal wounding and bacterial infection. Moreover, TIMP-2 mRNA was constitutively detected with low levels in both groups, and amounts were unchanged after corneal abrasion and bacterial inoculation. Taken together, these results indicated that TIMP-1 was inducible, and was the most important inhibitor of MMPs, especially MMP-9, in pseudomonal keratitis.

Madlener (1998) showed that both MMP-9 and TIMP-1 were strongly induced within 24 h in murine wound models. Kernacki et al. (2004) showed that adequate endogenous expression of TIMP-1 in the cornea protected the basement membrane and from stromal degradation by extensive tissue destruction via multiple processes. On the other hand, in ulcerative corneal diseases, there are lines of evidence suggesting that alterations in the ratio of MMPs vs TIMPs play a role in progressive stromal degradation. We believe that enhancement and activation of MMP-9 are much faster and stronger than those of TIMP-1, thereby facilitating tissue destruction in the cornea after pseudomonal infection. In this regard, topical application of either recombinant TIMPs or systemic MMP inhibitors would prevent or delay corneal ulceration in various disease models including pseudomonal keratitis. Furthermore, it is known that expression of MMPs is regulated by inflammatory cytokines such as IL-1β, TNF-α, and chemokines (Asano et al., 2004; Chen et al., 2004; Fini et al., 1995; Ishikawa et al., 2005; Kim et al., 2005; Xue et al., 2003). McClellan et al. (2005) reported that MMP-9 regulated immune functions in the cornea by proteolysis, potentiating *P. aeruginosa* keratitis by degrading collagen IV, and upregulating chemotactic cytokines/chemokines IL-1β and MIP-2. We are currently investigating expressions and roles of inflammatory cytokines and chemokines in pseudomonal keratitis in rabbits.

In conclusion, our present study demonstrated that MMP-9 (active form) derived mainly from PMNs was an important pathogenic factor contributing to corneal ulceration in pseudomonal keratitis.

Acknowledgments

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CASE REPORT

Loss of vision due to a physiologic pituitary enlargement during normal pregnancy

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Abstract
Background Physiologic pituitary enlargement during normal pregnancy is well known, but we are unaware of previous reports on a natural course of visual loss due to this disease.
Methods A 30-year-old woman presented blurred vision in the left eye from the 30th week of pregnancy. At 38 weeks visual acuity was 0.9 in the left eye. Automated perimetry revealed a mild central visual defect in the left eye. A magnetic resonance imaging (MRI) scan revealed pituitary enlargement with compression of the anterior optic chiasm. We observed the natural course of this case.
Results At 16 weeks after delivery, visual acuity was 1.5 in both eyes with normal visual field, and an MRI scan revealed a normal-sized pituitary without compression of the optic chiasm.
Conclusions Ophthalmologists should be aware of visual loss by physiologic pituitary enlargement to avoid unreasonable neurosurgical procedures.

Keywords Pituitary · Enlargement · Pregnancy · Vision

Introduction

Physiologic pituitary enlargement during normal pregnancy is well known. Magnetic resonance imaging (MRI) studies have revealed that the size of the pituitary gland increases an average of 120% during a normal pregnancy [2]. However, we are unaware of previous reports on a natural course of visual loss due to physiologic pituitary enlargement.

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Materials and methods

A 30-year-old woman presented blurred vision in the left eye from the 30th week of pregnancy. At 38 weeks she first visited our department; visual acuity was found to be 1.5 in the right eye and 0.9 in the left eye, with normal light reaction in both eyes. Automated perimetry revealed a mild central visual defect in the left eye (Fig. 1a). Slit lamp examination, intraocular pressure measurements, and funduscopy examination were normal in both eyes. An MRI scan revealed pituitary enlargement, which was 12×10×11 mm, homogeneous, and isointense, with compression of the anterior optic chiasm (Fig. 1b). The size was increased by 71.4% compared to normal pituitary size [2]. The patient did not consent to the administration of gadolinium diethylenetriaminepentaacetic (DTPA) during pregnancy. Endocrinologic studies revealed a prolactin level of 331.8 ng/ml with levels of the other pituitary hormones appropriate for gestational age. Routine chemistries were normal. Past medical history included sarcoidosis, which was stable with no medication, and one previous pregnancy, which was uncomplicated. Her family history was unremarkable. We observed the natural course of this case.

Results

At 2 weeks after delivery, an MRI scan revealed the same size of the pituitary and homogeneous enhancement of the

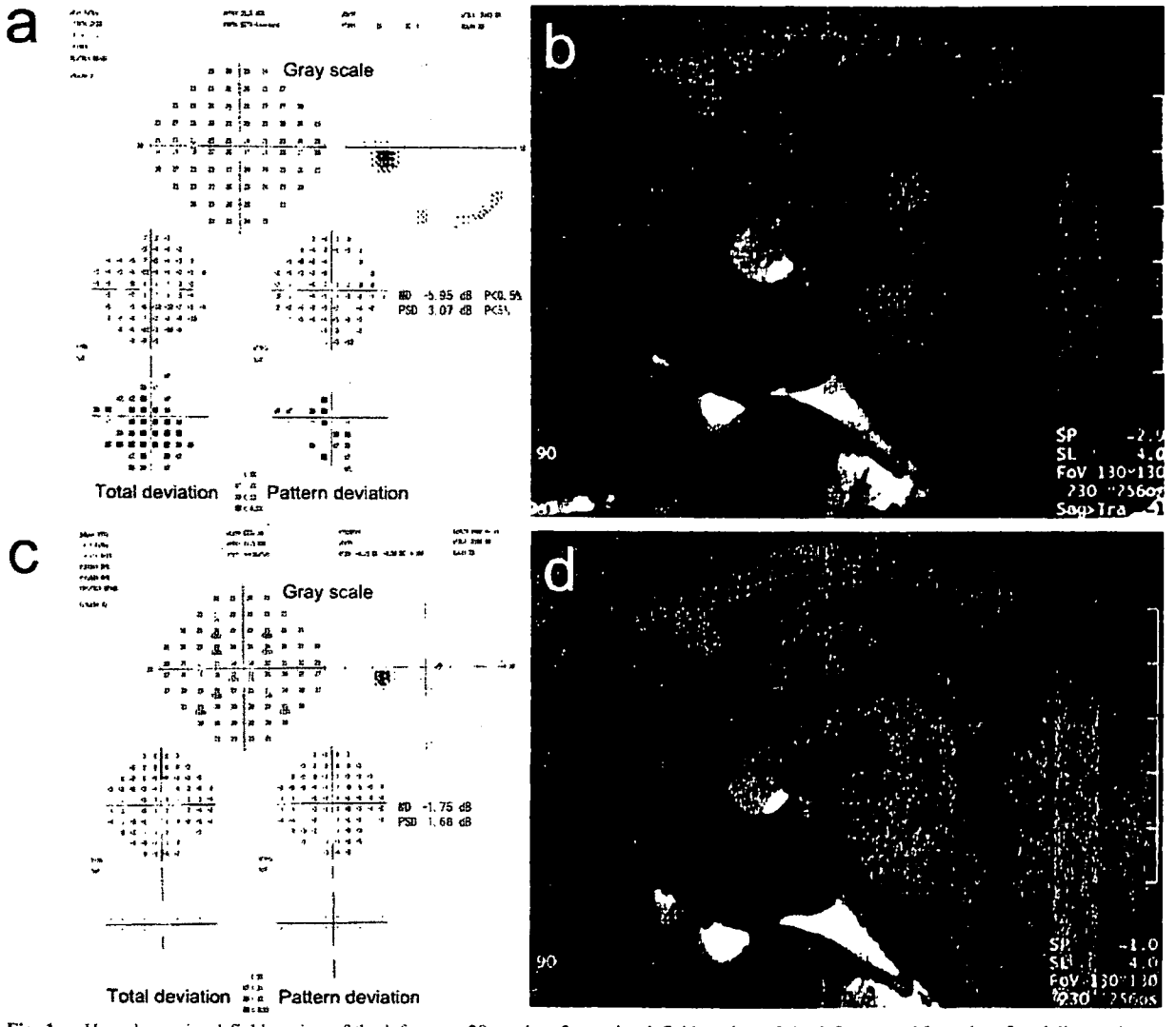

Fig. 1 **a** Humphrey visual field testing of the left eye at 38 weeks of pregnancy shows a subtle reduction in central sensitivity. **b** Sagittal view of an MRI scan of the head at 38 weeks of pregnancy, showing pituitary enlargement, which is 12×10×11 mm, homogeneous, and isointense, with compression of the anterior optic chiasm. **c** Humphrey visual field testing of the left eye at 16 weeks after delivery shows normal sensitivity. **d** Sagittal view of an MRI scan at 16 weeks after delivery, showing a normal-sized pituitary without compression of the optic chiasm

lesion after administration of gadolinium DTPA. Endocrinologic studies revealed a prolactin level of 23.1 ng/ml. At 16 weeks after delivery, visual acuity was 1.5 in both eyes with normal visual field (Fig. 1c), and an MRI scan revealed a normal-sized pituitary without compression of the optic chiasm (Fig. 1d).

Discussion

The differential diagnosis of pituitary enlargement during pregnancy is difficult because MRI is not sufficiently specific to differentiate each condition. Although prolactinoma is the most common cause of pituitary enlargement during pregnancy [3], adenoma can be ruled out in this case because of the smooth recovery after delivery. Moreover, an elevated serum prolactin level is a normal occurrence during pregnancy [4]. Considering her past medical history, granulomatous hypophysitis due to sarcoidosis was also suggested. Since this patient did not exhibit any sign of systemic sarcoid activity or show predilections for sarcoidosis, such as a lesion in the posterior pituitary gland or hypopituitarism, this seemed unlikely. Lymphocytic hypophysitis, although rare, should be suspected. Since this

condition is usually associated with autoimmune diseases [1], this was not initially considered. However, a diagnostic trial of corticosteroids should be initiated in cases with progressive visual loss after delivery, because most patients with lymphocytic hypophysitis respond well to steroids. After excluding these diseases, a clinical diagnosis of physiologic pituitary enlargement was made.

Histological examinations by a neurosurgical procedure would be needed to make a definitive diagnosis, which was not reasonable in this case because there was only mild visual loss. Although the diagnosis of this lesion is difficult, not only neurosurgeons but also ophthalmologists should be aware that physiological pituitary enlargement can cause visual loss and so should avoid unreasonable neurosurgical procedures.

Competing interests None of the authors has a proprietary interest in any products mentioned in this study.

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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

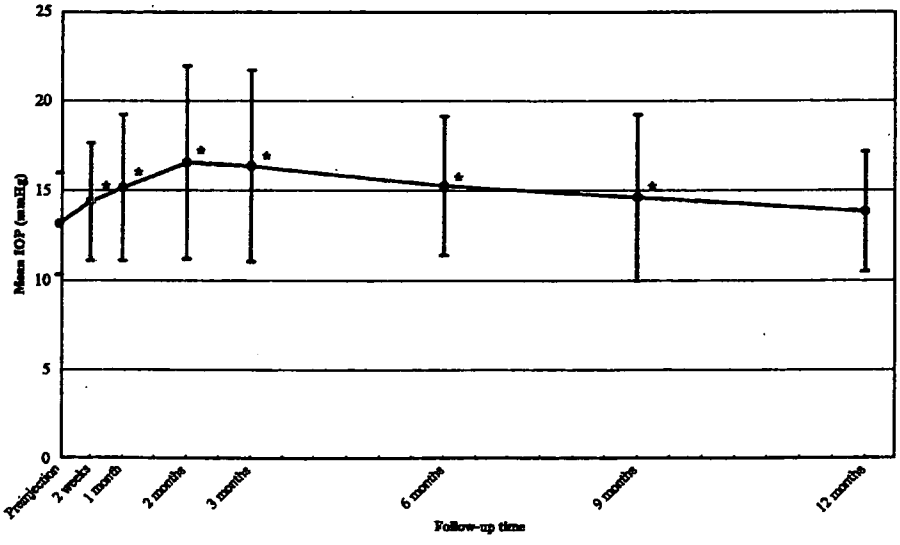

FIGURE 1. The mean IOP at each time point after PST injection of TA. The IOPs at 2 weeks, and 1, 2, 3, 6, and 9 months, were significantly higher than those before the injection. *P<0.05; Mann-Whitney U test.

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

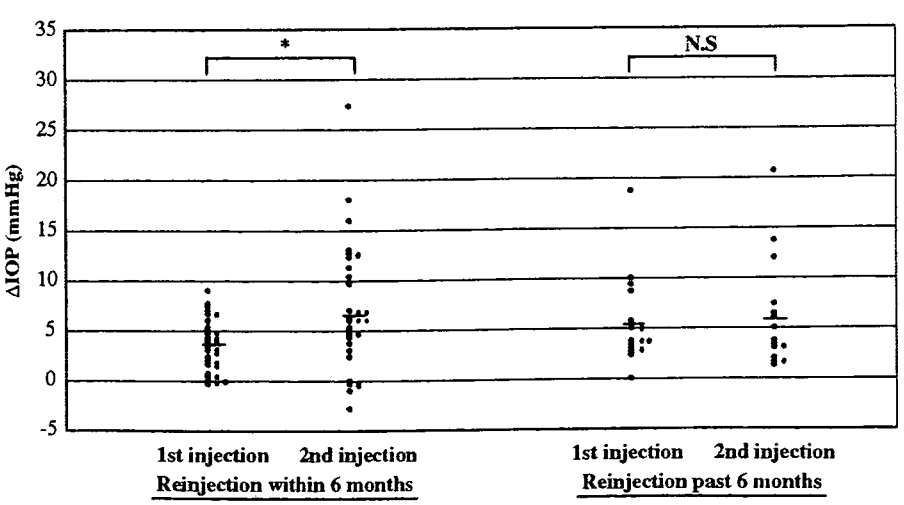

FIGURE 2. IOP elevation augmented by an additional injection of TA after a short time interval. The ΔIOP was calculated for the first and second injections, respectively. When additional injections were performed within 6 months of the first, the ΔIOPs for the second injection were significantly higher 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.

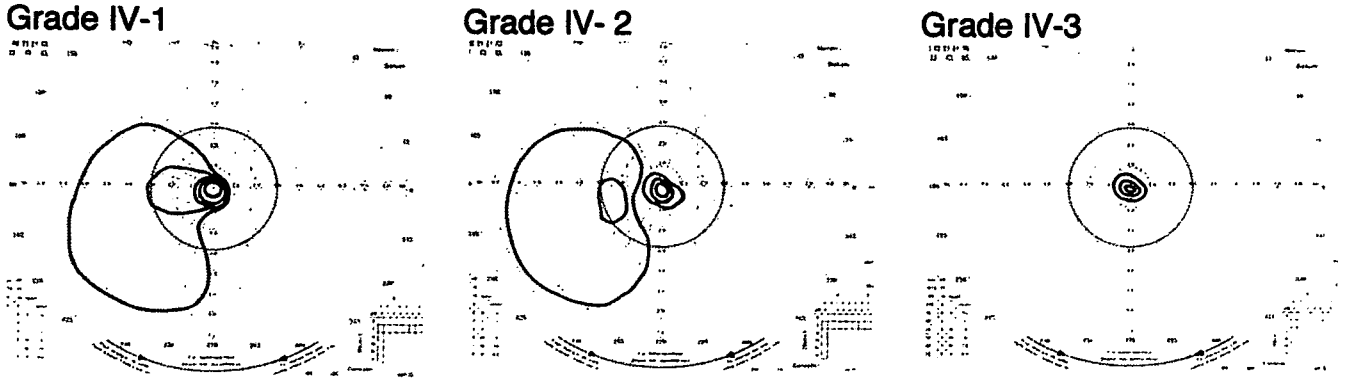

Figure 1. Visual field defects of the 49 eyes in this study as classified by Goldmann perimetry. There were 23 eyes classified as Grade IV-1, 8 eyes as Grade IV-2, and 18 eyes as Grade IV-3.

Jpn J Ophthalmol
Vol 51: 116–120, 2007

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 ≤ 4mmHg) 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 12mmHg) to determine cumulative success probabilities. Postoperative visual acuity at the long-term follow-up was examined on the final visit.

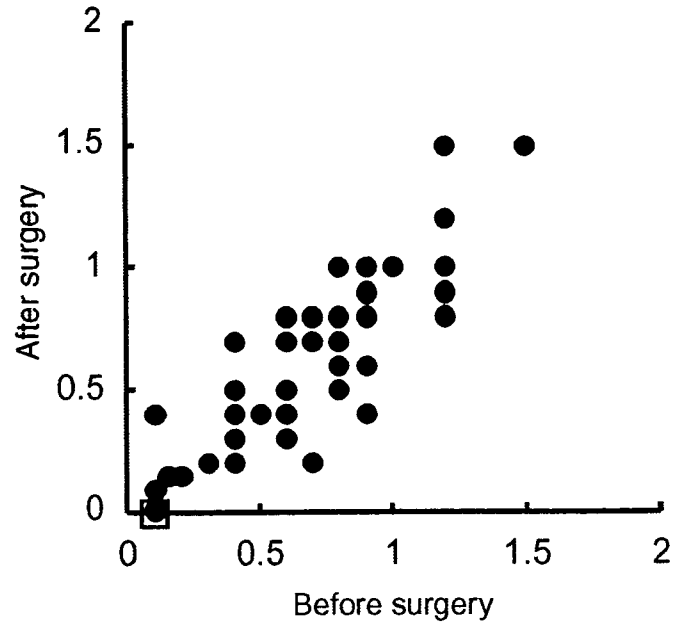

Figure 2. Corrected visual acuity before and 2 months after surgery in the 49 eyes. One outlier (▣) shows an eye with severe hyphema. In this case, a vitreous hemorrhage developed from hyphema as the eye was aphakic. Visual acuity was recovered to presurgery levels when the vitreous hemorrhage disappeared.

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.

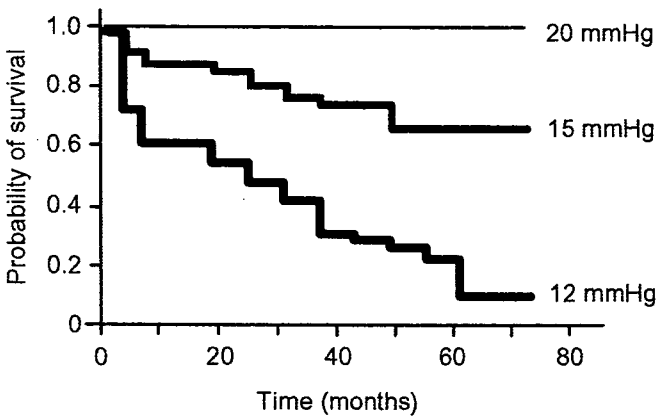

Figure 3. Kaplan-Meier survival curve of intraocular pressure control. Data are for patients both with and without antiglaucoma medication use.

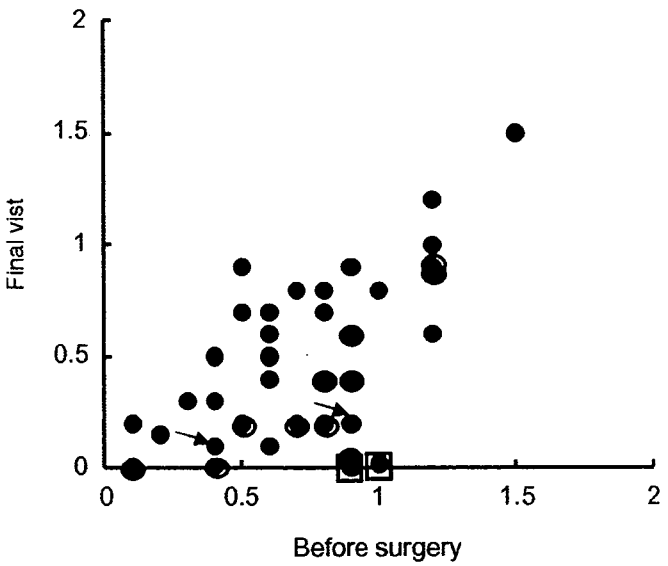

Figure 4. Corrected visual acuity before surgery and at the final visit in the 49 eyes. Eleven outliers (◉) show eyes with a progression of cataracts during the follow-up period. Two outliers (▣) indicate eyes with central visual field loss during the follow-up period. Two outliers (*arrows*) indicate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

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.

Acknowledgments. Grants from the Ministry of Education, Science, Sports, Culture and Technology, Japan; the Ministry of Health, Labour and Welfare, Japan; the Japan National Society for the Prevention of Blindness; and the Imai Memorial Glaucoma Research Foundation supported this work.

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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

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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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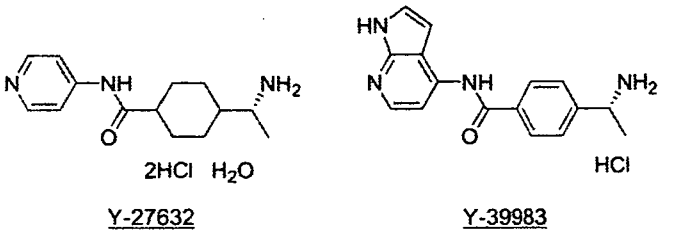

FIGURE 1. Molecular structures of Y-27632 and Y-39983.

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