は報告が少なく，一定の見解は得られていないが，原田病のUBM所見として高度の毛様体浮腫が認められるように[10]，本症例でも毛様体浮腫を示した。HAUにおいても炎症極期においては可逆的な毛様体浮腫を引き起こしている可能性が高いのではないかと考えている。

本症例では，強力なステロイド療法により，1週間で硝子体混濁は軽快し，眼内の炎症も減少した。このことは，治療開始1週間後に施行したFA検査において，網膜血管からの造影剤漏出をほぼ認めなかったことからも推測される。

片眼性で，非常に強い硝子体混濁を呈した症例においては，急性網膜壊死，転移性眼内炎などの感染症に加え，HAUも鑑別診断の一つとして考え，詳細な問診，血清抗体価，前房水のPCR法によるウイルス検索などを考慮すべきである。

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Antibiotic Effects of WP-0405, a Thermo-Setting Ofloxacin Gel, on Methicillin-Resistant *Staphylococcus aureus* Keratitis in Rabbits

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ABSTRACT

Purpose: The chemotherapeutic effects and pharmacokinetics properties of WP-0405 (a thermo-setting *in situ* 0.3% ofloxacin-containing ophthalmic gel) and ofloxacin (a conventional 0.3% ofloxacin solution) on methicillin-resistant *Staphylococcus aureus* (MRSA) keratitis were compared in a rabbit model.

Method: The single-instillation pharmacokinetics of WP-0405 and ofloxacin in the cornea, aqueous humor, conjunctiva, and iris–ciliary body were determined in normal rabbit eyes. To compare the duration of antimicrobial action, WP-0405 or ofloxacin was instilled once-daily in an early-treatment model of keratitis, and corneas were either removed immediately or 4 or 8 h postinstillation. In another experiment, WP-0405 was instilled two or three times daily to compare its antibiotic efficacy with three-times daily instillation of ofloxacin in the same early-treatment model of keratitis; corneas were then removed after determining the extent of the abscess area. In another experiment, WP-0405 was instilled four or eight times daily to compare its effects with eight-times daily instillation of ofloxacin in a late-treatment model of keratitis, and corneas were removed. The number of viable bacteria in the corneas was determined in all experiments.

Results: C_{max} and $AUC_{0-\infty}$ in tissues treated with WP-0405 were 1.5–3.4-fold and 1.8–2.9-fold greater than those treated with ofloxacin, respectively. WP-0405 significantly reduced the number of viable bacteria for up to 8 h after a single instillation. WP-0405 not only significantly reduced the number of viable bacteria, but also the size of the abscess area at the same frequency of instillation. When compared to ofloxacin, WP-0405 exhibited an approximately equivalent antibiotic effect, with fewer administrations.

Conclusions: As a result of its pharmacokinetics, WP-0405 had a more potent, longer-acting antibiotic effect than did ofloxacin. Furthermore, because of its lower required instillation frequency, which would improve patient compliance, WP-0405 has great potential therapeutic benefits.

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INTRODUCTION

WP-0405 IS A REVERSIBLE, THERMO-SETTING, *in situ* gel-forming, 0.3% ofloxacin-containing ophthalmic solution incorporated in a base that transforms into the gel-phase when contacting the warmer ocular surface and transforms back into the sol-phase at cooler temperatures.[1–3]

In situ gelling technology is utilized to deliver ophthalmic solution. Gel formation can be triggered by changes in pH, temperature, and ionic changes in the transition environment. Gel systems are widely applicable for use with various drugs for the purpose of enhancing the drug's bioavailability by triggering the drug's accessibility and/or reducing systemic side-effects.[4–11]

Jackson and colleagues[12] reported that patients in a clinical study of twice-daily topical application of 1% fusidic acid viscous drops demonstrated significantly better compliance ($P < 0.001$) than with 0.3% tobramycin eye drops, and a significantly high number of patients reported that the formulation was convenient to use ($P < 0.001$). There were no significant differences in clinical or bacteriologic efficacy when compared with four- to six-times daily application of 0.3% tobramycin eye drops. These results suggest that a lower frequency of instillation of an antimicrobial improves patient compliance, although drug differences must be taken into account. Furthermore, high viscosity leads to prolonged contact of the antimicrobial agent with the surface of the eye, thus enhancing therapeutic efficacy.

We developed WP-0405 as an antimicrobial to (1) enhance patient compliance and ease of use; (2) reduce systemic absorption by reducing the frequency of instillation (without reducing antibiotic effectiveness in self-limiting diseases, such as conjunctivitis)[13]; and (3) increase bacterial eradication efficacy of ofloxacin for use at the same drug concentration as is now prescribed and with the same frequency of instillation for use in cases of severe infectious ocular disease, such as corneal ulcers, in which frequent eyedrop application is required.

We wanted to compare the chemotherapeutic effects and pharmacokinetic properties of WP-0405 (a thermo-setting, *in situ* 0.3% ofloxacin-containing, ophthalmic gel) and Tarivid (Santen Pharmaceuticals; Osaka, Japan) (a conventional 0.3% ofloxacin solution) on methicillin-resistant *Staphylococcus aureus* (MRSA) keratitis.

In this study, we compared duration and level of therapeutic efficacy of the two formulations using two models of severity of *S. aureus* keratitis in rabbits.

METHODS

Antibiotic

WP-0405 contains the active ingredient ofloxacin 0.3% in a gel base composed of methylcellulose (3.6% w/v), polyethyleneglycol-4000 (4.0% w/v), and sodium citrate (3.0% w/v). Tarivid is a commercially available conventional ofloxacin 0.3% ophthalmic solution. Fifty (50) μL aliquots of both drugs were applied to the surface of the eye.

Bacteria

Clinically isolated MRSA (strain 2) was a gift from Tokai University (Kanagawa, Japan). Minimal inhibitory concentrations (MIC) of ofloxacin against this organism were determined according to the broth micro dilution method.[14,15] The MIC of ofloxacin against this organism was 2 μg/mL.

Animals

Japanese white male rabbits, weighing 1.8–2.8 kg, were treated and maintained in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Resolution on the Use of Animals in Ophthalmic and Visual Research. Animals were housed at temperatures of 20–26°C, humidity of 30%–70%, and a 12-h light/dark cycle.

Pharmacokinetics

One hundred-twenty (120) eyes of 60 animals were used. WP-0405 was randomly instilled into 1 eye and Tarivid instilled into the contralateral eye of 6 rabbits from each group. Corneas, aqueous humor, inferior palpebral conjunctiva, and iris–ciliary body were removed at 5, 15, and 30 min and 1, 2, 3, 4, 6, 8, and 12 h after instillation and stored at −20°C for later assay. Ofloxacin concentrations in the ocular tissues were determined by high-performance liquid chromatography (HPLC). Ofloxacin was extracted from the cornea, conjunctiva, and iris–ciliary body with chloroform. Aliquots of appropriate concentrations extracted from tissues were loaded onto a

reversed-phase column (Mightysil RP-18 GP, 4.6 mm i.d. 150 mm; Kanto Kagaku Co., Tokyo, Japan) in an LC-10A apparatus (Shimazu Co.; Kyoto, Japan) with an aqueous mobile phase containing 50% methanol, 5% acetic acid, and 3 mM sodium octanesulfonate, at a flow rate of 0.7 mL/min at room temperature, and monitored at 290 nm (excitation wavelength) and 490 nm (emission wavelength) with an RF-10AXL fluorescence detector (Shimazu Co.). Aqueous humor was diluted, filtered, and loaded onto a column under the conditions described above. The peak concentration of ofloxacin (C_{max}) and the time to peak concentration (T_{max}) were estimated by visual inspection of concentration-versus-time data. The areas under the concentration-time curve ($AUC_{0-\infty}$) in the ocular tissues were estimated by the trapezoidal method and extrapolated to infinity as the quotient of the last quantifiable concentration and the terminal elimination rate constant, k. The rate constant, k, was estimated by log-linear, ordinary least-squares regression of three-to-six terminal concentration-time data points. The terminal half-life ($t_{1/2}$) was calculated as $\ln 2/k$. The intra- and interday coefficients of variances (%) were 0.7–1.9 and 0.8–6.9 (cornea), 0.2–1.2 and 0.7–4.8 (aqueous humor), 1.0–4.7 and 0.6–5.6 (conjunctiva), and 0.5–1.8 and 1.0–7.5 (iris–ciliary body). The limits of quantification were 2.5 ng/cornea, 20 ng/mL of aqueous humor, 1 ng/conjunctiva, and 0.5 ng/iris–ciliary body.

Keratitis model

The bacterial suspension of *S. aureus* to be used for inoculation was cultured overnight, and the appropriate concentration was diluted in Mueller-Hinton broth (MHB; Becton Dickinson, Sparks, MD) just before inoculation. The MRSA keratitis model was based on a modification of the method of Callegan and colleagues.[16] General anesthesia was induced in rabbits by intravenous injection of 30 mg/kg pentobarbital sodium. Oxybuprocaine hydrochloride (0.4% Benoxil; Santen) was applied topically to each eye. Approximately 10^4 (Experiments 1 and 2) or 10^3 (Experiment 3) colony-forming units (CFUs) of MRSA in 10 μL MHB was injected intrastromally using a 30-gauge needle attached to a 50-μL syringe (Hamilton Co.; Reno, NV) into the corneas of anesthetized rabbits. The CFU of the cooled bacterial suspension was determined just after inoculation.

Experiment 1. Determination of pharmacokinetics following single-drug instillation in the keratitis model. Eighty-four (84) eyes of 60 animals were randomly assigned to seven groups (n = 12/group). In six groups, WP-0405, Tarivid, or saline was instilled only once at 4 h postinoculation. Corneas were extracted at 8 (three groups) or 12 h (another three groups) postinoculation. To measure the number of viable bacteria at the time of drug instillation (initial CFU), corneas were removed 4 h after inoculation without instillation of any drugs in a separate group.

Experiment 2. Comparison of drug antibacterial activity during early treatment. Forty-one (41) eyes of 24 animals were randomly assigned to four groups (n = 10–11/group). Drug treatment was initiated 4 h postinoculation and continued for 3 d. The following regimen was designated as early treatment: On day 1, successive instillations were performed every 4 h for the first 8 h. Then, 16 h later (day 2), the same regimen was repeated (i.e., each successive instillation was performed every 4 h for the first 8 h). After waiting 16 h, day 3 commenced, and the same regimen was repeated. Sixteen (16) h after the final instillation on day 3, corneal abscesses were photographed and the eyes enucleated.

Experiment 3. Comparison of drug antibacterial activity during late treatment. Forty-four (44) eyes of 22 animals were randomly assigned to four groups (n = 11/group). Drug treatment was initiated 8 h post-inoculation and continued for 1 d. The following regimen was designated as late treatment: WP-0405 was instilled four or eight times every 180 or 90 min, respectively. Tarivid or saline was instilled eight times every 90 min. Each cornea was removed 32 h after inoculation.

Quantification of CFU/cornea

Corneas were removed, after which they were each placed into a sterile tube, dissected, and homogenized with aliquots of MHB. Homogenized suspensions were serially diluted by 2×10^{-1}–2×10^6-fold in MHB, plated onto MHB agar, and incubated at 37°C for 24 h. Colonies were counted to determine the number of CFUs per cornea.

Analysis of abscess

Photographs of corneas were scanned with a film-scanner (Coolscan III; Nikon Corp., Tokyo,

Japan), and areas of corneal abcesses were analyzed using the public domain National Institutes of Health (NIH; Bethesda, MD)-image software (version 1.6) program and Adobe Photoshop (version. 5.5) software (Adobe Systems, Tokyo, Japan).

Statistics

The number of viable bacteria in corneas was expressed as $\log_{10}$ CFU. The ofloxacin concentration in each tissue was expressed as the mean ± standard deviation, whereas the mean $\log_{10}$ CFU and abscess area were expressed as the mean ± standard error. Statistical analyses were performed using the StatLight program (Yukms; Tokyo, Japan). A P value of < 0.05 was considered statistically significant. For pharmacokinetics, statistical analyses were as follows: (1) Values were evaluated for significance using the Grubbs-Smirnov test. Those falling outside of the mean ± 1.96 standard deviation in each group were regarded as outliers and rejected; and (2) Nonpaired Student t test or Aspin-Welch test was performed for WP-0405- and Tarivid-treated groups.

In Experiment 1, the one-way analysis of variance (ANOVA) with Dunnett multiple comparison test was used to evaluate the statistical difference for the effect of each drug on the initial number of CFUs and on those where the corneas were removed 8 or 12 h after inoculation for determining the duration of the effect of WP-0405 or Tarivid on CFUs.

In Experiments 2 and 3, statistical analyses were as follows: (1) To determine the efficacy of Tarivid, the differences between the saline-treated group and the Tarivid-treated group were evaluated using Aspin-Welch test; and (2) To compare the efficacies of WP-0405 and Tarivid, one-way ANOVA with Dunnett multiple comparison test was done for the saline- or Tarivid-treated groups and WP-0405-treated groups.

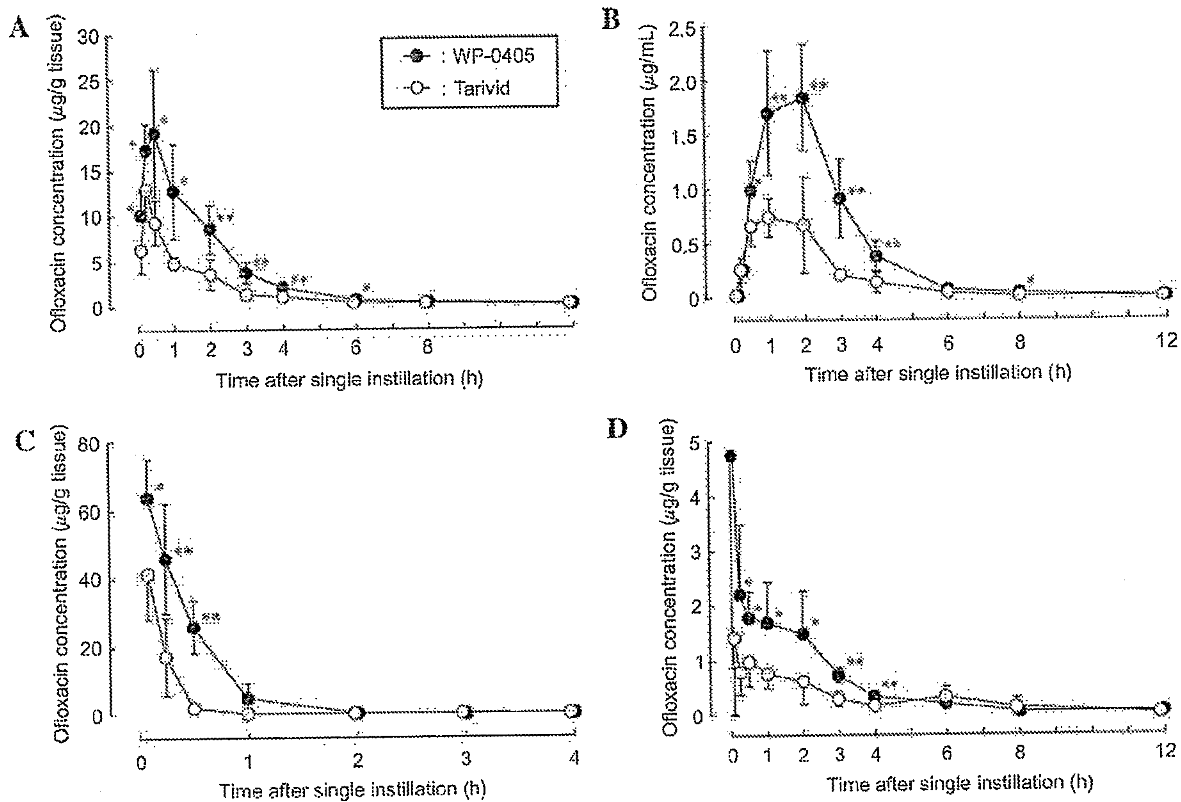

FIG. 1. Tissue levels of ofloxacin after single instillation of WP-0405 or Tarivid in normal rabbits. (A) cornea. (B) aqueous humor. (C) conjunctiva. (D) iris–ciliary body. Each tissue was extracted 5, 15, and 30 min, and 1, 2, 3, 4, 6, 8, and 12 h after instillation. Each point represents the mean ± standard deviation. (n = 5–6, each). $^*P < 0.05$; $^{**}P < 0.01$. Significant differences from Tarivid-instilled group were calculated using the Student or Aspin-Welch t test.

TABLE 1. COMPARISON OF PHARMACOKINETIC PARAMETERS OF OFLOXACIN AFTER INSTILLATION OF WP-0405 OR TARIVID IN NORMAL RABBITS

	Cornea		*Aqueous humor*		*Conjunctiva*		*Iris–ciliary body*	
	WP-0405	*Tarivid*	*WP-0405*	*Tarivid*	*WP-0405*	*Tarivid*	*WP-0405*	*Tarivid*
T_{max}	30 min	15 min	2 h	1 h	5 min	5 min	5 min	5 min
C_{max} (μg/g or mL)	19.11	12.92	1.84	0.74	63.38	41.20	4.76	1.42
WP-0405/Tarivid [ratio]	[1.5]		[2.5]		[1.5]		[3.4]	
$AUC_{0\text{-}\infty}$ (μg h/g or mL)	39.93	19.27	5.25	2.05	31.41	11.01	6.48	3.56
WP-0405/Tarivid [ratio]	[2.1]		[2.6]		[2.9]		[1.8]	
$t_{1/2}$ (h)	2.2 (6 → 12 h)	2.9 (6 → 12 h)	1.1 (3 → 8 h)	1.0 (2 → 8 h)	5.1 (3 → 12 h)	2.9 (2 → 12 h)	2.8 (4 → 12 h)	2.4 (4 → 12 h)
k (h^{-1})	0.321	0.241	0.639	0.666	0.137	0.239	0.246	0.292

Note: Figures in square brackets indicate the ratio of C_{max} or $AUC_{0\text{-}\infty}$ of ofloxacin in each tissue treated with WP-0405 compared to those treated with Tarivid. Figures in parentheses of "$t_{1/2}$ (h)" indicate the time used for calculation of $t_{1/2}$ (h).

T_{max}, time to peak concentration; C_{max}, peak concentration of ofloxacin; AUC, area under the curve; $t_{1/2}$, terminal half-life; k, terminal elimination rate constant.

RESULTS

Pharmacokinetics

Figure 1 shows that time-dependent changes in the concentration of ofloxacin in WP-0405-treated cornea (Panel A), aqueous humor (Panel B), conjunctiva (Panel C), and iris–ciliary body (Panel D) were significantly higher than in those treated with Tarivid 5 min to 6 h (cornea, 1.3–2.8-fold; $P < 0.05$ or 0.01); 30 min to 4 h (aqueous humor, 1.5–4.3-fold; $P < 0.05$ or 0.01); 5–30 min (conjunctiva, 1.5–12.6-fold; $P < 0.05$ or 0.01); and 15 min to 4 h (iris–ciliary body, 1.8–2.8-fold, $P < 0.05$ or 0.01) after instillation. Table 1 shows that the C_{max} and $AUC_{0\text{-}\infty}$ values in corneas treated with WP-0405 were 1.5- and 2.1-fold greater than in those treated with Tarivid, respectively. Similarly, the C_{max} and $AUC_{0\text{-}\infty}$ values in other tissues treated with WP-0405 were 1.5–3.4-fold and 1.8–2.9-fold greater than in those treated with Tarivid, respectively. These results suggest that ofloxacin delivery to the anterior segment is greater when treated with WP-0405 than with Tarivid.

Experiment 1. Figure 2 shows that following a single instillation of WP-0405 or Tarivid, the number of CFUs in the WP-0405-treated group decreased after 4 and 8 h, whereas the number of CFUs in the Tarivid-treated group decreased only after 4 h. The number of CFUs in corneas of the

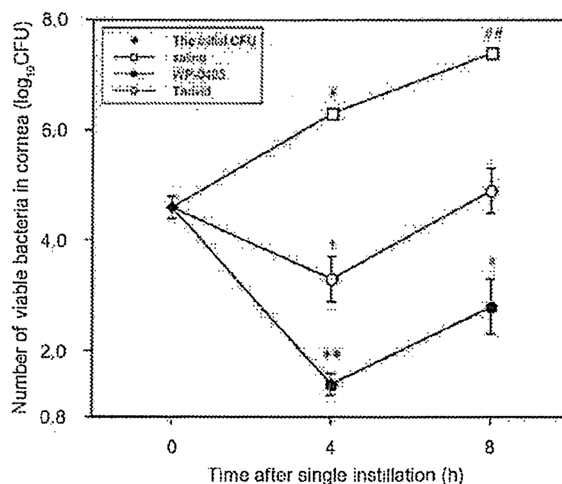

FIG. 2. Antibacterial effects of WP-0405 and Tarivid on colony-forming units (CFUs) in corneas 0, 4, and 8 h after instillation. Each drug was instilled 4 h after inoculation of MRSA, and corneas were removed 4 (at time of drug instillation, nondrug treatment: the initial CFU), 8, and 12 h after inoculation of MRSA. Each point represents the mean ± standard error. (n = 12). $^*P < 0.05$; $^{**}P < 0.01$. Significant differences from the initial CFU were calculated using one-way analysis of variance (ANOVA) with the Dunnett's multiple comparison test. $^{\dagger}P < 0.05$ Significant differences from the initial CFU were calculated using one-way ANOVA with the Dunnett multiple comparison test. $^{\#\#}P < 0.01$; $^{\#}P < 0.05$. Significant differences from the initial CFU were calculated using one-way ANOVA with the Dunnett multiple comparison test.

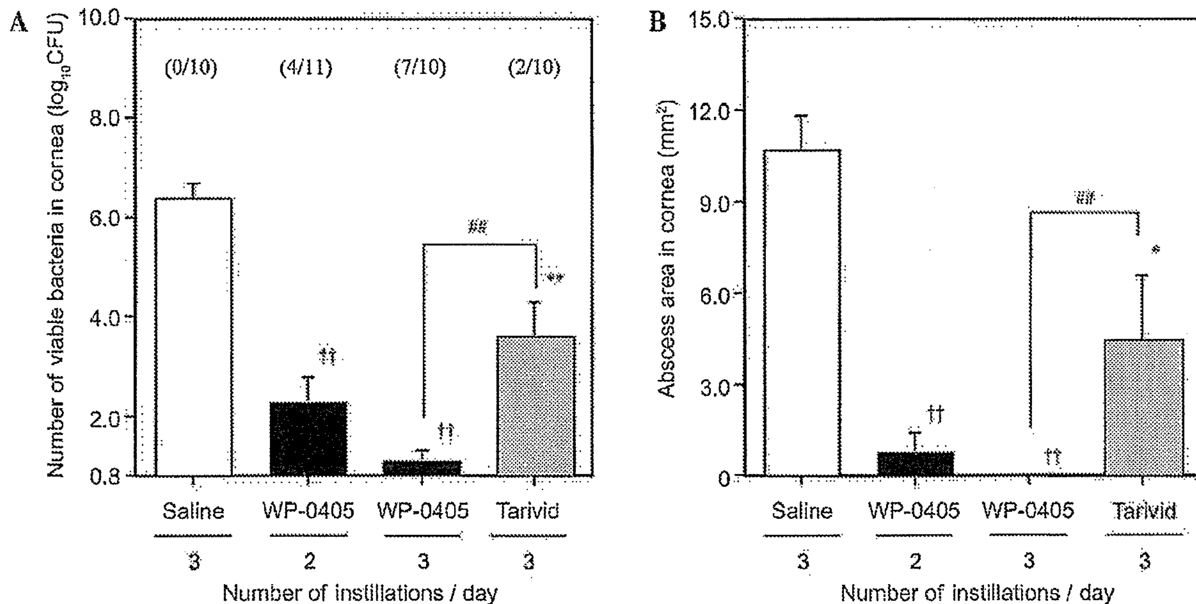

FIG. 3. Antibacterial effects of WP-0405 and Tarivid on colony-forming units (CFU) (A) and the area of abscess (B). Antibiotic treatment was initiated 4 h after inoculation. On day 1, successive instillations were performed every 4 h for the first 8 h. Then, 16 h later (day 2), the same regimen was repeated (i.e., each successive instillation was performed every 4 h for the first 8 h). After waiting 16 h, day 3 commenced, and the same regimen was repeated. Sixteen (16) h after the final instillation on day 3, corneal abscesses were photographed and the eyes enucleated. Each bar represents the mean ± standard error. (n = 10–11). $^{*}P < 0.05$; $^{**}P < 0.01$. Significant differences from the saline-instilled group were calculated using Aspin-Welch *t* test. $^{††}P < 0.01$. Significant differences from the saline-instilled group were calculated using one-way analysis of variance (ANOVA) with the Dunnett multiple comparison test. $^{\#\#}P < 0.01$. Significant difference between WP-0405- and Tarivid-instilled group were calculated using one-way ANOVA with the Dunnett multiple comparison test. The numerator of the ratios shown in the top of panel (A) for each of the four groups indicates the number of corneas in which the CFU number was below the limit of detection (<0.8 CFU).

WP-0405-instilled group removed 8 h after instillation was almost equivalent to that in corneas of the Tarivid-instilled group removed 4 h after instillation.

Experiment 2. Figure 3 shows that three consecutive instillations of WP-0405 more effectively suppressed CFU formation than did Tarivid, as evidenced by the fact that the CFUs with WP-0405 fell to a level that was only 0.3% of the value obtained with Tarivid. The greater effectiveness of WP-0405 had already been reached in corneas that only received two consecutive instillations of WP-0405. Figure 3B shows that WP-0405 had similar efficiency in reducing the size of the abscess areas in infected corneas. In fact, three instillations of WP-0405 completely eliminated abscess formation, whereas with Tarivid, the abscess area only decreased by 56%. WP-0405, therefore, is more effective than Tarivid in reducing this indicator of corneal infection.

Experiment 3. Figure 4 shows that eight consecutive instillations of WP-0405 were more effective than Tarivid in inhibiting CFU formation; with WP-0405, the remaining CFUs were only 0.3% of those obtained following Tarivid treatment. The effectiveness of only four consecutive instillations of WP-0405 was the same as that obtained with eight consecutive instillation of Tarivid. Therefore, despite a delay in the onset of treatment, WP-0405 was still quite effective in suppressing CFU formation.

DISCUSSION

It is necessary to obtain high tissue levels of antimicrobial agents to effectively treat corneal infection, owing to the fact that bacteria rapidly proliferate and can quickly develop drop resistance.[17,18] We compared the effectiveness of WP-0405 and Tarivid for treatment of corneal kerati-

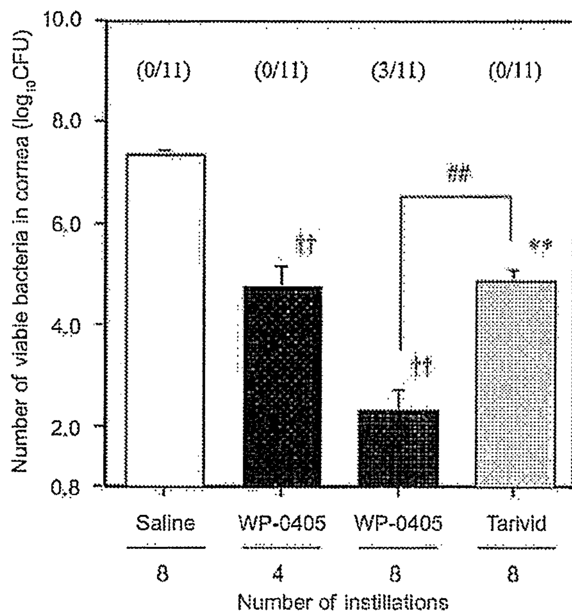

FIG. 4. Antibacterial effects of WP-0405 and Tarivid on colony-forming units (CFU) in corneas 24 h after initiation of topical application on methicillin-resistant *Staphylococcus aureus* (MRSA) keratitis in rabbits. Antibiotic treatment was initiated 8 h after inoculation. Saline and Tarivid were instilled eight times every 90 min, and WP-0405 was instilled four and eight times every 180 and 90 min, respectively. Each bar represents the mean ± (n = 11). $^{**}P < 0.01$. Significant difference from saline-instilled group was calculated using Aspin-Welch *t* test. $^{††}P < 0.01$. Significant differences from the saline-instilled group were calculated using one-way analysis of variance (ANOVA) with the Dunnett multiple comparison test. $^{\#\#}P < 0.01$. Significant differences between the WP-0405- and Tarivid-instilled groups were calculated using one-way ANOVA with the Dunnett multiple comparison test. The numerator of the ratios, shown in the top of panel for each of the four groups, indicates the number of corneas in which the CFU number was below the limit of detection (<0.8 CFU).

tis in our model system, in hopes of improving treatment of this disease by the use of a thermosetting, ofloxacin-containing gel. Our results show that WP-0405 is a more effective drug delivery system, as its pharmacokinetic parameters reflect tissue accumulation of ofloxacin. This is evident because WP-0405-delivered ofloxacin concentrations in the cornea, iris–ciliary body, and conjunctiva were 1.5–3.4-fold higher than those obtained with Tarivid. Similarly, the values for AUC in each tissue were 1.8–2.9-fold greater than those found with Tarivid.

The C_{max} value of WP-0405 exceeded reported MIC values of other tested bacterial strains by 90%; other strains included common ocular pathogens, such as *S. aureus* (0.5–1 μg/mL), *Staphylococcus epidermidis* (0.5–0.78 μg/mL), *Streptococcus pneumoniae* (1–2 μg/mL), *Pseudomonas aeruginosa* (2–12.5 or > 8 μg/mL), and *Haemophilus influenzae* (0.03–0.12 μg/mL).[19,20] In addition, the length of time that the tissue concentration exceeded MIC_{90} was longer in WP-0405-treated eyes than in Tarivid-treated eyes in most of the tissue examined. Although eye drops containing a high concentration of drug are expected to achieve high-level penetration into ocular tissues, most of the instilled solution flows into systemic circulation through the nasolacrimal duct.[21] Furthermore, the resulting higher concentration in the blood increases the probability of systemic side-effects.[22] Therefore, it is beneficial to raise the tissue concentration of drugs in the eye without increasing the quantity transferred into the systemic circulation. Such an outcome is more easily achieved with WP-0405 application.

Pharmacokinetic studies suggest that topically administered WP-0405 is likely to cure ocular infections more efficiently than Tarivid; although in either case, the concentration of ofloxacin was 0.3%. We examined whether (1) WP-0405 had the same efficacy in early treatment of infectious external ocular disease at a lower daily frequency of instillation as Tarivid and (2) WP-0405 had superior efficacy in late treatment of infectious ocular disease at the same high daily frequency of instillation as Tarivid. WP-0405 appears to be more effective as a delivery system for ofloxacin than the eye-drop instillation of Tarivid. Perhaps the greater effectiveness of WP-0405 is a result of to its prolonged contact with the ocular surface, as a consequence of the sol-to-gel transformation upon contact with the warmer ocular surface. Such a change is consistent with the higher C_{max} and AUC values obtained with WP-0405 than with Tarivid.

Craig[23] also reported that the improved efficacy of the fluoroquinolones correlates with increases in the AUC/MIC and peak/MIC ratios. Fluoroquinolones also produce postantibiotic effects, defined as the period of bacterial growth suppression after brief exposure to antimicrobials[24] against *S. aureus*.[23] Smith and colleagues,[19] however, reported that higher fluoroquinolone concentrations might be achievable in the pres-

ence of ulcerative keratitis with epithelial defects and, therefore, the actual corneal ofloxacin levels in these keratitis models might be higher than those obtained in normal rabbits. The ability of WP-0405 to produce higher ofloxacin tissue levels than Tarivid may also be advantageous for producing a better postantibiotic effect. Furthermore, it was confirmed that WP-0405 was well tolerated in the rabbit eyes by a 13-week repeated instillation toxicity study.

CONCLUSIONS

In conclusion, topical application of WP-0405 had similar effects as Tarivid, but with lower frequency of instillation and increased antimicrobial effects at the same dosages, on MRSA keratitis in rabbits. These findings suggest that WP-0405 is beneficial for patients, depending on the type and severity of external ocular infections, including conjunctivitis and keratitis. Further studies in experimental animals or clinical trials are warranted to clarify the clinical implications.

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Dendritic Keratitis Caused by an Acyclovir-Resistant Herpes Simplex Virus With Frameshift Mutation

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Purpose: To report a case of acyclovir-resistant herpes simplex virus (HSV) keratitis after long-term, inconsistent use of topical acyclovir and fluorometholone.

Methods: A 70-year-old man with dendritic keratitis caused by an acyclovir-resistant HSV strain was examined. The 50% inhibitory concentration of different antiviral agents against the isolated virus and the DNA sequence of viral thymidine kinase were determined.

Results: The 50% inhibitory concentration of acyclovir and trifluorothymidine for the isolated HSV strain was 13.75 and 0.28 μg/mL, respectively, indicating that the virus was resistant to acyclovir. DNA sequencing of the viral thymidine kinase revealed that this virus had a frameshift mutant with a G insertion in the 7Gs homopolymer. Topical trifluorothymidine was effective, and the epithelial lesion was completely resolved within 2 weeks.

Conclusion: A typical form of dendritic keratitis was caused by an acyclovir-resistant HSV with frameshift mutation in a 7Gs homopolymer region.

Key Words: acyclovir resistance, keratitis, frameshift mutation, herpes simplex virus, thymidine kinase

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Herpes simplex keratitis is still 1 of the leading causes of infectious corneal blindness in the world. Acyclovir (ACV) ointment has been extensively used as the first choice drug of herpes simplex keratitis in Japan because of its high antiviral efficacy and low cytotoxicity. This treatment has resulted in a significant reduction of patients who develop necrotizing stromal keratitis and corneal perforations.

However, the widespread use of ACV has invariably led to the emergence of herpes simplex virus (HSV) strains resistant to ACV, especially in severely immunocompromised patients. During the past several years, ACV-resistant HSV strains have been isolated from various sites including the eye. Three mechanisms may be involved in the development of ACV-resistant HSV: a loss of thymidine kinase (TK) activity (TK-deficient virus), an alteration of TK activity or substrate specificity (TK-altered virus), and an alteration of DNA polymerase activity. In 95% of the cases, ACV resistance is associated with a mutation in the *TK* gene.[1]

We report a case of dendritic keratitis that did not respond to topical ACV ointment. A novel frameshift mutation was detected in this ACV-resistant, ocular HSV isolate.

CASE REPORT

A 70-year-old, immunocompetent man was referred from a local hospital in March 2005 for recurrent dendritic keratitis that had been treated unsuccessfully with topical ACV. He complained of redness, irritation, pain, and blurred vision in his right eye. His best-corrected visual acuity was reduced to 20/25 in the affected eye. Slit-lamp examination showed dendritic keratitis with slight stromal edema but no significant signs of anterior chamber inflammation.

The epithelial lesion was found to develop at the lower portion of the cornea, a part of which was geographic in appearance (Fig. 1). Suspecting poor compliance, we gave the patient strict instructions on the use of ACV ointment 5 times a day for 1 week, but no improvement was observed. The patient had a 10-year history of recurrent dendritic and disciform keratitis and had been given topical ACV with fluorometholone for a long time, but his compliance was poor.

We considered the possibility of an ACV-resistant HSV; corneal epithelial debridement was performed under local anesthesia for virus isolation. Typical cytopathic effect was observed on the next day, and the isolated virus was identified as HSV-1 by indirect immunofluorescence. As reported previously,[2] antiviral plaque reduction assay was performed with ACV, trifluorothymidine (TFT), foscarnet (FOS), and idoxuridine (IDU) to determine the 50% viral inhibitory concentration (IC_{50}). As a result, the IC_{50} of this strain against ACV, TFT, and FOS was 13.75, 0.28, and 7.14 μg/mL, respectively. No plaque reduction occurred at any concentration of IDU. Generally, in an antiviral plaque reduction assay, HSV with IC_{50} exceeding 2 μg/mL to ACV is considered resistant.[3] The IC_{50} of this strain was 13.75 μg/mL, indicating that the dendritic keratitis was caused by an ACV-resistant HSV strain.

We therefore discontinued topical ACV and switched to 1% topical TFT, which this strain was sensitive to. The patient was given 1% topical TFT 8 times per day, and the 1% TFT solution was prepared in our facility from TFT (Sigma, St. Louis, MO). The epithelial lesion healed completely within 2 weeks.

The analysis of the *TK* gene of the isolated virus strain was performed as described[4] and compared with the *TK* gene of an ACV-sensitive clinical isolated from a patient with dendritic keratitis. A G insertion in a homopolymer harboring 7 Gs was detected in the TK

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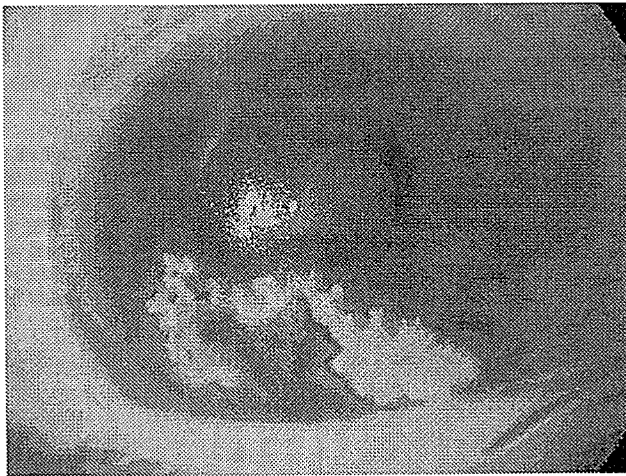

FIGURE 1. Slit-lamp photograph of right eye with fluorescein staining showing typical dendritic keratitis with a part of geographic in appearance at the lower portion of the cornea.

sequence. This insertion was located at nucleotides 429 to 436 (codon 144–146), causing a premature stop codon at 227 (Table 1).

TABLE 1. TK Gene Sequence

	Nucleotide Change	Amino Acid Change
Isolated virus	125: C→T	42: Pro→Leu
	256: G→A	89: Arg→Gln
	437: ins G	146: frameshift
		227: stop codon

DISCUSSION

Among the mutations that are associated with ACV resistance, one half are nucleotide insertions or deletions and the other half are nucleotide substitutions. Generally, such mutants have low virulence. For example, Yao et al[2] have reported 2 ACV-resistant HSV-1 mutants with a nucleotide substitution, and both had low virulence in the cornea and had a low incidence of establishing a latent infection. One had a point mutation in the ATP binding site (codon 55) of the *TK* gene, whereas the other was in codon 125.

Nucleotide insertions or deletions are usually responsible for a frameshift resulting in the synthesis of a truncated, nonfunctional TK. A mutant strain with a frameshift mutation within the 7 Gs homopolymers, however, could synthesize a part of a full-length TK because of an unusual net +1 frameshift during translation. This outcome first reported in a patient with progressive esophagitis that had developed after bone marrow transplantation. It was noted that the mutant retained some TK activity and had the ability to reactivate from latent infections in the mouse trigeminal ganglia.[5] In fact, recurrences have occurred in a bone marrow transplant patient.[6] Thus, net +1 frameshift mutants have stronger virulence and higher rate of recurrences than the more common mutants because of their specific process of translation. Our mutant also possessed strong virulence, causing typical dendritic keratitis and the ability to establish latency[5] (Zhang et al, unpublished data).

ACV-resistant mutants of HSV emerge predominantly in severely immunocompromised patients (eg, bone marrow transplants, cancer, and AIDS).[1] Although resistance to ACV rarely occurs among immunocompetent patients, there have been several reports of ACV-resistant herpes simplex keratitis in otherwise healthy individuals.[4] This finding is probably attributable to the inherent immunocompromised status of the cornea because of its avascularity. Our patient was immunocompetent according to his medical history. Therefore, we presume that the long-term, irregular concomitant use of a low dose of ACV and steroids is responsible for the emergence of this ACV-resistant mutant. Although sensory ganglia harboring latent HSV-1 are not generally reinfected with a second strain of HSV-1, superinfection has been reported to occur.[7,8] Because the strain we reported here can establish latency[5] (Zhang et al, unpublished data), a possibility of recurrence, albeit remote, should be kept in mind.

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Role of Promyelocytic Leukemia Zinc Finger Protein in Proliferation of Cultured Human Corneal Endothelial Cells

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ABSTRACT

Purpose: To review the role of promyelocytic leukemia zinc finger (PLZF), a transcriptional repressor and negative regulator of cell cycling, in the proliferation of cultured human corneal endothelial cells (HCECs).

Methods: The expression pattern of PLZF mRNA was determined by RT-PCR and real-time quantitative PCR in HCECs and normal human corneal epithelia. The effect of cell-cell contact on the expression of the PLZF gene was investigated after incubation the cultured HCECs in EDTA. The proliferation rate of cultured HCECs was assayed by a real-time electronic sensing (RT-CES) system, and DNA microarray analyses was performed to find the PLZF-regulating genes in cultured HCECs infected with LacZ- and PLZF-carrying adenoviruses (Ad-LacZ, Ad-PLZF).

Results: The mRNA of PLZF was expressed in HCECs in vivo, and in completely confluent HCECs but not in sub-confluent HCECs in vitro. Real-time PCR showed that the expression of PLZF mRNA was decreased by about 20-fold when incubated with EDTA, and returned to normal level as the cell-cell contact reformed. The cell proliferation assay by the RT-CES system showed that the infection of cultured HCECs with Ad-PLZF inhibited the proliferation.

Conclusions: These findings suggest that PLZF plays an important role in the suppression of proliferation of HCECs.

INTRODUCTION

The corneal endothelium is the single layer of cells lying between the corneal stroma and the anterior chamber, and it helps maintain corneal transparency by regulating corneal hydration. Evidence has been accumulating that once the endothelial monolayer is formed in vivo, human corneal endothelial cells (HCECs) do not proliferate.[1] Recent reports have been demonstrated that HCECs in vivo are arrested in the G1-phase of the cell cycle, and indicated that HCECs have the potential to proliferate.[2-5] Until now, there are several anti-mitotic factors that contribute to the arrest of the HCECs at G1. For example, TGF-β_2 and cAMP can inhibit the G1-to-S transition by blocking the phosphorylation of $p27^{kip1}$, which is required for the nuclear export of the inhibitory molecules for degradation.[6, 7] In addition, contact-induced inhibition is mediated, at least in part, by $p27^{kip1}$.[8, 9]

In this article, we focused on DNA binding transcriptional factors. Among the factors, the members of the BTB/POZ-zinc finger protein family are known to suppress the transcription of the genes that are positive regulators of the cell cycle.[10, 11] The BTB/POZ-zinc finger protein is a sequence-specific transcriptional repressor characterized by a BTB/POZ domain, which is responsible for transcriptional repression, and a zinc finger domain that forms the DNA binding domain.[12-14]

Expression of PLZF mRNA in Cultured HCECs and in Normal HCECs in vivo

In preliminary experiments, we investigated that the expression pattern of six human BTB/POZ-Zinc finger-containing transcription factors; promyelocytic leukemin zinc finger (PLZF), B-cell lymphoma 6 (BCL-6), Kaiso, myoneurin, KIAA0441, and zinc finger protein278 (ZNF278) in cultured HCECs and normal human corneal endothelial cells by RT-PCR. Interestingly, the expression of PLZF mRNA was detected in 100% confluent HCECs and normal human corneal endothelial cells but not in sub-confluent HCECs. While the expression of the mRNA of the other transcription factors were also detected in all samples of 100% confluent HCECs and normal HCECs in vivo but also in sub-confluent HCECs (Fig. 1).

The *PLZF* gene was first identified by its fusion to the retinoid acid receptor (RAR) α locus in a therapy-resistant form of acute promyelocytic leukemia associated with the t(11;17) translocation.[15] *PLZF* is a sequence-specific DNA binding transcriptional repressor that suppresses the transcription of genes such as cyclin A2 and c-myc.[10, 16, 17] This is relevant because both cyclin A and c-myc are involved in cell cycling. For example, NIH3T3 cells expressing PLZF stably were delayed S phase entry and inhibited the expression of endogenous cyclin A.[10]

Kinetics of PLZF mRNA in cultured HCECs

The kinetics of PLZF mRNA expression in cultured HCECs was examined by real-time PCR. PLZF mRNA was not expressed in proliferating HCECs but was first detected when the HCECs attained 100% confluency, i.e., in a non-proliferative state (Fig. 2). The relative expression level of PLZF mRNA continued to increase up to 6 days after reaching 100% confluency (Fig. 2).

Effect of Cell-Cell Contact on PLZF mRNA Expression

To determine whether cell-cell contact is involved in regulating the expression of the *PLZF* gene, cultured HCECs were incubated in 1.2 mg/ml EDTA for 120 min and returned to fresh culture medium. HCECs were collected at designated times, and the kinetics of the mRNA of PLZF were evaluated during the recovery of cell-cell contact by real-time PCR. The expression of PLZF mRNA was decreased by about 20-fold when transiently exposed to EDTA for 120 min, but returned to normal levels as the cell-cell contact reformed at 72 hr after returning the HCECs to normal media (Fig. 3). The same experiment was performed on cultured human umbilical vein endothelial cells, but the expression of PLZF mRNA was decreased by about only 2-fold when transiently incubated with EDTA (data not shown).

Effect of PLZF on Suppression of Proliferation of Cultured HCECs

To investigate the suppressive effects of PLZF on the proliferation of HCECs, adenovirus vector carrying genes encoding PLZF (Ad-PLZF) or LacZ (Ad-LacZ) were prepared using an adenovirus expression vector kit (Takara Biomedicals) and infected cultured HCECs. The rate of cellular proliferation was assayed with a real-time electronic sensing (RT-CES) system (ACEA Bioscience, San Diego, CA). The proliferation rate was continuously monitored and the infection with Ad-PLZF at a MOI 100 inhibited the proliferation of HCECs for up to 72 hours (Fig. 4A). Ad-PLZF significantly inhibited the proliferation rate by 30.3% at 24 hr and 26.5% at 48 hr compared to Ad-LacZ (Fig. 4B).

Determination of PLZF-induced Gene Expression in HCECs

The gene expression in HCECs induced by PLZF was determined by DNA microarray analysis. The Acegene Human oligo chip 30K (Hitachi Software Engineering, Japan) containing 30000 genes was used to compare gene expression in cultured HCECs infected with Ad-PLZFor Ad-LacZ. The results showed that PLZF was shown to up-regulate at least 54 genes and down-regulate at least 34 genes. Unexpectedly, the expression of *cyclin A2* and *c-myc* genes were not influenced by over-expression of the PLZF gene in HCECs (data not shown). Two growth factors including heparin-binding epidermal growth factor-like growth factor (HB-EGF) and connective tissue growth factor were most down-regulated by 0.059 fold and 0.188 fold

respectively, while of our particular interest was the finding that *transforming growth factor-* β *stimulated clone 22 (TSC-22)* gene was up-regulated by 2.32 folds (Table). The increased TSC-22 mRNA expression was also confirmed by real-time PCR (data not shown).

DISCUSSION

PLZF is a transcriptional repressor and is known to suppress the expression of several genes which regulate cell proliferation. In earlier studies, an enhanced expression of PLZF led to the suppression of proliferation in some cell lines.[10, 18] In murine 32Dcl3 cells, the cell cycling profiles of over-expressing PLZF cells were significantly altered, and up to 80% of the cells accumulated in the G0/G1 phase with a significantly smaller proportion of cells in the S phase.[18] In this article, we demonstrated that the mRNA of PLZF is expressed in normal HCECs in vivo and in 100% confluent HCECs but not in subconfluent HCECs. The kinetics of the expression of PLZF mRNA during the proliferation of HCECs clearly supported the RT-PCR results. Addition to these results, the expression of the mRNA of PLZF was decreased by about 20-fold when the cell-cell contact was reduced by EDTA, and returned to normal level as the cell-cell contact reformed in normal medium. A recent study has demonstrated that EDTA released cells from contact inhibition and promoted proliferation of corneal endothelial cells.[5] Taken together, these findings suggest that cell-cell contact may be involved in the regulation of the expression

of the PLZF gene, and/or PLZF may be involved in the contact inhibition of HCECs.

Next, we examined the effect of PLZF on the proliferation of HCECs, in which over-expression of PLZF gene in HCECs resulted in the suppression of proliferation of cultured HCECs. The results suggested that PLZF mRNA expression might be related to HCECs proliferation.

To further investigate the genes regulated by PLZF, we performed DNA microarray analyses of HCECs infected with Ad-PLZF. We found that transforming growth factor-ß stimulated clone 22 (TSC-22) mRNA was increased by over-expressing the PLZF gene, and might be considered a candidate factor for the suppression of proliferation following an over-expression of PLZF (Table1).

TSC-22, a leucine zipper transcriptional factor, was found to be an immediate-early target gene of TGF-beta 1,[19] and has the characteristics of a suppressor of cell proliferation.[20-22] TSC-22 binds to and modulates the transcriptional activity of Smad3 and Smad4, and enhances TGF-β signaling by associating with Smad4.[23]

It has been well documented that TGF-β is present in high concentrations in normal aqueous humor, and its predominant form is TGF-β_2,[24, 25] and that TGF-β_2 suppresses the proliferation of corneal endothelial cells to suppress the entry into the S-phase in vitro.[7, 26, 27] In human, we have found that 0.5 ng/ml or more concentration of TGF-β_2 suppressed the proliferation of HCECs