た症例以外に，臨床所見から診断された症例も含んでいるため，施設間で診断方法が異なっている可能性が挙げられる．しかし，本調査の目的はAK発症者数の動向を探ることであり，同一施設内における診断方法が調査期間内で一定であれば，経年的な動向をみるうえで施設間の差はあまり問題にならないと思われる．また，本調査は大学附属病院を対象としたものであり，他の角膜を専門とする医師が所属する病院などは調査対象に含まれていないことも，留意しておかなくてはならない点である．AKに関する知識が眼科医の間に浸透してきている近年，AKを診断・治療できる施設は少なからず増えてきていることが予想され，それに伴い大学病院にAK症例が紹介される機会が減少している可能性も否定できない．しかし，角膜を専門とする医師を擁する基幹病院や眼科専門病院が複数存在するような都市部だけでなく，地方においても同様の減少傾向が認められていることから，そのような事象が今回の調査結果に及ぼしている影響は少ないと考えられる．

長年にわたり，多くの眼感染症やCLの専門家が警鐘を鳴らし続けてきたにもかかわらず増加の一途を辿っていたAKが，近年になりようやく減少に転じたことは非常に喜ばしいことである．しかし，依然として多数の発症者がいるのは論じるまでもなく，装用者側の補助操作に依存しているMPSという消毒システムの不確実性，現行のSCL消毒剤評価法の不完全性，増加するインターネットによるCL販売など，課題は山積みである．これらの課題と真摯に向き合い，今後もAKの発症動向や装用者のケア状況につき調査を続けつつ，さらなる発症抑止を目指し，CL診療に携わるすべての眼科医が装用者への啓発活動を徹底していく必要がある．

利益相反：利益相反公表基準に該当なし

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ORIGINAL ARTICLE

Moxifloxacin as Postoperative Prophylaxis for *Enterococcus faecalis*-Induced Endophthalmitis After Cataract Surgery in Aphakic Rabbits

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Abstract

Purpose: The development of endophthalmitis after cataract surgery often results in a significant vision loss. Inhibition of bacterial proliferation in the anterior chamber using antibiotic eye drops is important to prevent endophthalmitis after cataract surgery. We aimed to determine the sensitivity of fluoroquinolones against *Enterococcus faecalis* ocular isolates and the efficacy of fluoroquinolones to prevent *E. faecalis*-induced endophthalmitis in aphakic rabbits.
Methods: The minimum inhibitory concentrations (MICs) of moxifloxacin (MFLX) and levofloxacin (LVFX) used in ophthalmic solutions for 13 *E. faecalis* isolates obtained from the conjunctival sac or endophthalmitis cases were determined. Eye drops containing MFLX (0.5%), LVFX (0.5%), or saline were administered to aphakic rabbits with endophthalmitis induced by *E. faecalis.* The eye drops were administered immediately after lensectomy and at 3 and 6 h after cataract surgery (early instillation group) or immediately after lensectomy and at 12 and 15 h after cataract surgery (delayed instillation group). Bacterial growth, electroretinography (ERG), and slit-lamp examination (SLE) were determined throughout the course of infection.
Results: *In vitro* susceptibility testing revealed that the MICs of MFLX for *E. faecalis* isolates were lower than those of LVFX. In the early ocular instillation groups, MFLX significantly improved SLE scores, ERG, and viable bacterial counts compared with LVFX and saline (all, $P<0.05$). There were no significant differences in any parameter between MFLX and saline in the delayed ocular instillation groups.
Conclusions: Early ocular instillation of MFLX delays retinal damage compared with LVFX when used to treat *E. faecalis*-induced endophthalmitis after cataract surgery.

Introduction

Bacterial endophthalmitis is characterized by massive inflammation and tissue damage caused by bacteria and the host's immune response. Bacterial endophthalmitis usually occurs in the context of ocular surgery, trauma, microbial keratitis, or hematogenous spread of an opportunistic microorganism to the eye. The overall incidence of postoperative endophthalmitis in Japan is 0.052%,[1] suggesting that up to 500 of the 1 million patients undergoing cataract surgery each year in Japan may experience vision loss. In other countries, the incidence of postoperative endophthalmitis has tended to decrease (from 0.20% to 0.04%)[2] mainly because of the introduction of new cataract surgical techniques.[3]

Pre- and postoperative prophylaxis is widely performed to prevent endophthalmitis.[3] Antibiotics could inhibit bacterial proliferation after cataract surgery and prevent endophthalmitis. Several reports have shown that intracameral antibiotics are effective for the prevention of endophthalmitis.[4,5] Antibiotic eye drops and intracameral antibiotics are often used for the prevention of endophthalmitis. Generally, antibiotics are administered immediately after surgery and on the following day as postoperative prophylaxis for endophthalmitis. Wallin et al. reported that starting topical antibiotic administration on the day after surgery, rather than on the day of surgery, was associated with increased risk of endophthalmitis.[6] These results suggest that bacteria proliferate and express virulence factors as early as 1 day after surgery. Indeed, Wada et al. reported that immediate postoperative prophylaxis

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reduced the risk of *Enterococcus faecalis* endophthalmitis.[7] To date, however, no study has examined the effects of antibiotic administration on tissue damage associated with endophthalmitis, or the optimal timing of administration.

In our previous study, we established an aphakic rabbit model of endophthalmitis that accurately reflects postoperative endophthalmitis in clinical settings.[8] The model is established by inoculating pathogens into the lens capsule and anterior chamber after lensectomy. In this model, *E. faecalis* shows a high growth rate in the eye (1×10^9 colony forming unit [CFU]/mL within 3–7 h). The infected iris is partially covered with fibrin and/or hypopyon after 12 h, and none of the pupil is visible after 24 h because of inflammation. Microscopic analysis confirmed the presence of bacteria on the lens and bacterial migration to the vitreous, while electroretinographic (ERG) analysis confirmed the development of severe functional damage to the eye at 48 h because of the bacterial infection. Thus, this model is consistent with clinical postoperative endophthalmitis, and allows us to evaluate the efficacy of various treatments/procedures.

In this context, the first aim of this study was to compare the antimicrobial activities of 2 antibiotic fluoroquinolones [moxifloxacin (MLFX) and levofloxacin (LVFX)] against *E. faecalis* isolates. Our second aim was to examine, *in vivo*, the efficacy of MLFX and LVFX against *E. faecalis* in terms of visual outcomes. Our third aim was to compare the effects of different times of starting antibiotic administration on retinal damage.

Methods

Bacteria

We obtained a laboratory strain of *E. faecalis* OG1S, which produces a secretory protease, and 13 clinical isolates (10 isolates from conjunctivitis cases and 3 isolates from endophthalmitis cases from Ehime University Hospital). The bacteria were grown in a brain–heart infusion broth (Difco Laboratories, Detroit, MI) for 18 h at 37°C, and then washed twice with and resuspended in sterile physiological saline. The concentration of bacteria in the suspension was determined spectrophotometrically and adjusted to $\sim2\times10^5$ CFU/mL with sterile physiological saline.

Antibiotics

MFLX 0.5% ophthalmic solution (Vigamox®; Alcon Japan Ltd., Tokyo, Japan) and LVFX 0.5% ophthalmic solution (Cravit®; Santen Pharmaceutical Co. Ltd., Osaka, Japan) were purchased from their respective manufacturers.

Susceptibility tests

In vitro susceptibility tests were conducted by a commercial laboratory (Mitsubishi Chemical Medience Corp., Tokyo, Japan) to determine the minimum inhibitory concentration (MIC) of each antibiotic in accordance with the Clinical and Laboratory Standards Institute (CLSI) recommendations for antimicrobial susceptibility testing.[9] All strains were tested for antimicrobial susceptibility by the broth microdilution method using microdilution panels and broth reagents from Eiken Chemical Co. Ltd. (Tokyo, Japan). Cation-adjusted Mueller Hinton broth was used in these experiments. The MICs were recorded as the lowest concentration that inhibited visible growth. The range of MICs and the MICs at which 50% or 90% of the isolates are inhibited (MIC50 and MIC90, respectively) are shown.

Animal model and drug administration

Experimental endophthalmitis was induced in Japanese albino rabbits (1.6–2.4 kg; Kitayama Labs Co. Ltd., Ina, Japan) by inoculating *E. faecalis*, as previously described.[8] Briefly, lensectomy was performed on the rabbit's left eye by phacoemulsification (Black Max, Advanced Medical Optics, Santa Ana, CA). After lensectomy, the corneal incision was closed with 10-0 nylon sutures to prevent any leakage. Then, using a 27-gauge blunt needle, the eyes were inoculated from a capsular bag containing 0.1 mL of the *E. faecalis* OG1S strain (5.21 ± 0.29 $\log_{10}$ CFU/mL) or sterile physiological saline as a control. The rabbits ($n=50$) were then divided into 5 groups and treated with MFLX, LVFX, or saline, as described in Table 1. To compare *in vivo* antimicrobial activities between fluoroquinolones, we used commercially available eye drops containing the same concentrations of active drug (i.e., 0.5% MFLX and 0.5% LVFX). To limit the number of animals used and considering the limited efficacy of LVFX, we did not establish a delayed LVFX group. Animals in which the study drug was not administered correctly were excluded from the analysis.

The study protocols conformed to the Institutional Animal Care and Use Committee guidelines and the Association for Research in Vision and Ophthalmology Statement for the Use of Laboratory Animals in Ophthalmic and Vision Research. All procedures involving rabbits were approved by the Committee of Animal Experimentation, Ehime University School of Medicine.

Intraocular inflammation score

Inoculated eyes were evaluated at 6, 12, 24, and 48 h after surgery by slit-lamp biomicroscopy (Topcon SL-14; Kogaku Kikai, Tokyo, Japan). The investigators, who were blinded to the treatment, graded intraocular inflammation using the following scores: 0, normal; 1, a small amount of fibrin on the pupil; 2, the iris is partially covered with fibrin and/or hypopyon; 3, the iris is covered with fibrin and/or hypopyon; 4, the pupil is not visible.

Bacterial counts

The eyes were removed at 48 h after inoculation, and the *E. faecalis* bacterial counts in ocular tissues were determined. The surface of the enucleated globe was cleaned with ethanol

Table 1. Treatment Regimens

Group	*Drug*	*Time of first drop (h)*	*Time of second drop (h)*	*Time of third drop (h)*
Early				
Control	Saline	0	3	6
MFLX	0.5% MFLX	0	3	6
LVFX	0.5% LVFX	0	3	6
Delayed				
Control	Saline	0	12	15
MFLX	0.5% MFLX	0	12	15

MFLX, moxifloxacin; LVFX, levofloxacin.

using a cotton swab and then rinsed with sterile physiological saline. The aqueous humor was collected with a 23-gauge needle. The cornea was removed aseptically, and the contents of the anterior chamber (fibrin and hypopyon) were collected and mixed with the aqueous humor. The iris and lens were dissected away, and the surface of the vitreous body was rinsed with sterile physiological saline. The vitreous was then collected. Bacterial counts in the anterior chamber or the vitreous were determined by plating serial dilutions on a brain–heart infusion agar.[8]

Retinal responsiveness

The degree of postoperative retinal function was evaluated by recording the scotopic b-wave amplitude for each eye by flash ERG (LE-3000; Tomey Corp., Nagoya, Japan) at 12, 24, and 48 h postinoculation.[8] Acute and permanent damage was assessed at each of these times. Retinal function was determined as the b-wave amplitude for the experimental eye, expressed as a percentage of that in the contralateral control eye.

Histology

Both eyes of each rabbit were removed at 48 h. Globes were fixed in phosphate-buffered 10% formalin solution for 24 h. Eyes ($n=4$ per group) were sectioned and stained with hematoxylin and eosin.[8] Retinal damage was examined by light microscopy (BX50; Olympus, Tokyo, Japan). Each eye was checked to confirm whether the 5 retinal layers were intact.

Statistical analysis

Mann–Whitney tests were used to compare intraocular inflammation scores and changes in retinal responsiveness between the MFLX and control groups in both regimens. Tukey–Kramer tests were used to compare outcomes between the 2 ocular formulations and the control group. Values of $P<0.05$ were considered statistically significant. Values are shown as means ± standard deviation of 6–10 eyes per group.

Results

In vitro *antibacterial activity*

The 50% and 90% MICs of MFLX and LVFX against *E. faecalis* isolates are shown in Table 2. Because the MICs of MFLX and LVFX against some strains were high, the ranges of MIC values were wide. However, MFLX showed excellent activity against most of the strains, except for the highly resistant strains. Based on the 50% MICs, MFLX was the most active agent against *E. faecalis* isolates, being 8-fold more active than LVFX. The MICs of MFLX and LVFX against OG1S were 0.5 and 2.0 μg/mL, respectively.

Intraocular inflammation score

Using our established endophthalmitis rabbit model, we infected the lenses of the rabbits with *E. faecalis* and then treated them with 1 of 5 eye drop regimens: early MFLX, delayed MFLX, early LVFX, early saline (control), or delayed saline (control) (Table 1). The eyes were then examined at the designated times to assess intraocular inflammation scores (Fig. 1 and Table 3). At endpoint (i.e., 48 h), the mean score in the early MFLX group (2.3 ± 0.95) was significantly lower than those in the control groups (early control: 3.6 ± 0.52, $P<0.01$; delayed control: 3.4 ± 0.97, $P<0.01$) and the delayed MFLX group (3.5 ± 0.76; $P<0.05$). The intraocular inflammation score in the early MFLX group was also significantly lower than that in the LVFX group (3.3 ± 0.95; $P<0.05$). There were no differences between the LVFX and control groups.

Table 2. Minimum Inhibitory Concentrations of Fluoroquinolones Against 13 Clinical Isolates of *Enterococcus faecalis*

Source of E. faecalis *(no. of isolates)*	*MIC (μg/mL)*[a] *MFLX*	*MIC (μg/mL)*[a] *LVFX*
Isolates from conjunctivitis (10)		
1	0.25	1.0
2	0.25	1.0
3	0.5	2.0
4	0.5	2.0
5	0.5	2.0
6	0.5	2.0
7	8.0	32
8	16	32
9	32	64
10	32	64
Isolates from endophthalmitis (3)		
11	0.5	2.0
12	16	64
13	16	64

	MFLX 50%	MFLX 90%	MFLX Range (μg/mL)	LVFX 50%	LVFX 90%	LVFX Range (μg/mL)
	0.25	32	0.25–32	2.0	64	1.0–64

[a]50% and 90%, MICs for 50% and 90% of the isolates tested, respectively.
MICs, minimum inhibitory concentrations.

Bacterial counts

Inhibition of bacterial growth in the anterior chamber was assessed. Inhibition of bacterial growth was not significantly

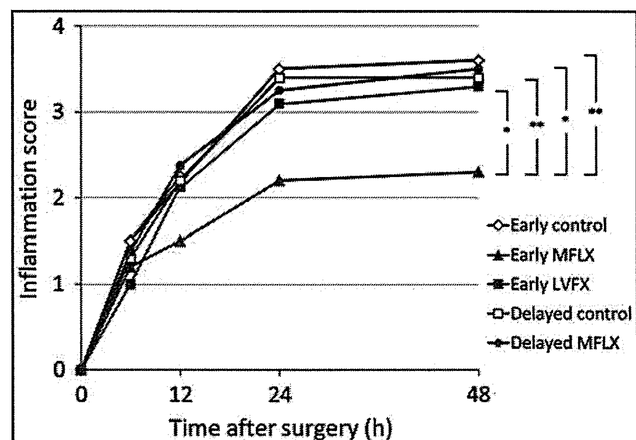

FIG. 1. Intraocular inflammation scores at the specified times after surgery and inducing *Enterococcus faecalis* endophthalmitis. Data are means ($n=10$). MFLX, moxifloxacin; LVFX, levofloxacin; $^{*}P<0.05$; $^{**}P<0.01$.

TABLE 3. INTRAOCULAR INFLAMMATION SCORES

	Time (h)			
Group	6	12	24	48
Early MFLX	1.2±0.42	1.5±0.55	2.2±0.79	2.3±0.95
Early LVFX	1±0	2.1±0.35	3.1±0.74	3.3±0.95
Early saline	1.5±0.53	2.25±0.46	3.5±0.53	3.6±0.52
Delayed MFLX	1.4±0.52	2.4±0.52	3.3±0.71	3.5±0.76
Delayed saline	1.3±0.48	2.2±0.42	3.4±0.70	3.4±0.97

different between the early MFLX group (3.4±2.2 $\log_{10}$ CFU/mL; $n=6$) and the control (5.3±1.2 $\log_{10}$ CFU/mL; $n=6$; $P=0.17$) or early LVFX (4.9±0.6 $\log_{10}$ CFU/mL; $n=6$; $P=0.23$) groups (Fig. 2). The bacterial count in the delayed MFLX group (3.2±2.2 $\log_{10}$ CFU/mL; $n=8$; $P=1.0$) was similar to that in the early MFLX group.

In the vitreous, bacterial growth in the early MFLX group (5.3±2.0 $\log_{10}$ CFU/mL; $n=6$) was significantly reduced compared with that in the control group (7.3±0.8 $\log_{10}$ CFU/mL; $n=6$; $P<0.05$). Bacterial counts were also lower in the early MFLX group compared with those in the delayed MFLX (6.4±1.5 $\log_{10}$ CFU/mL; $n=8$; $P=0.40$) and early LVFX (7.1±0.7 $\log_{10}$ CFU/mL; $n=6$; $P=0.09$) groups, although these differences were not statistically significant.

Retinal responsiveness

The retinal responsiveness of eyes infected with *E. faecalis* was determined by ERG analysis (Fig. 3). Retinal function at 48 h was significantly greater in the early MFLX group (responsiveness level 89.3%±40.3%) compared with the early control (16.7%±8.6%; $P<0.001$), delayed MFLX (52.2%±42.8%; $P<0.05$), and early LVFX (41.1%±41.7%; $P<0.05$) groups.

Histological examination

Stained retinas from the eyes of rabbits infected with *E. faecalis* were examined by light microscopy. Samples from the control group showed disruption of the retinal layers with abundant inflammatory cells, and that the photoreceptors could not be observed (Fig. 4A). The retinal layers in the early MFLX groups were intact and had a normal appearance (Fig. 4B). Although the retinal layers in the early LVFX group were almost intact, the photoreceptors were destroyed, and there were fewer cells in the outer nuclear layer (Fig. 4C). Similar observations were found in all of the samples from each group.

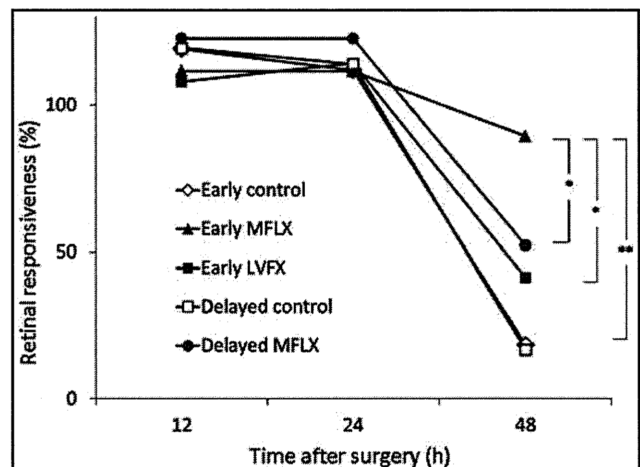

FIG. 3. Effects of topical MFLX and LVFX on electroretinographic measurements of retinal responsiveness at the specified times after surgery and inducing *E. faecalis* endophthalmitis. Data are means ($n=10$). *$P<0.05$; **$P<0.001$.

Discussion

The causative agents of endophthalmitis and their pathogenic potentials need to be identified to determine visual prognosis, and to guide treatment decisions. The Endophthalmitis Vitrectomy Study Group identified several causative agents, the most common being gram-positive, coagulase-negative staphylococci (CNS) (70.0%), *Staphylococcus aureus* (9.9%), *Streptococcus* species (9.0%), and *Enterococcus* species (2.2%), as well as other gram-negative (5.9%) and gram-positive (3.1%) species.[10,11]

Although CNS are among the most common causative bacteria, they do not produce strong toxins or proteases that damage ocular tissue, offering good visual prognosis. These properties of CNS may explain the significantly better visual outcomes in patients with CNS infection than in patients infected with other organisms.[10,12]

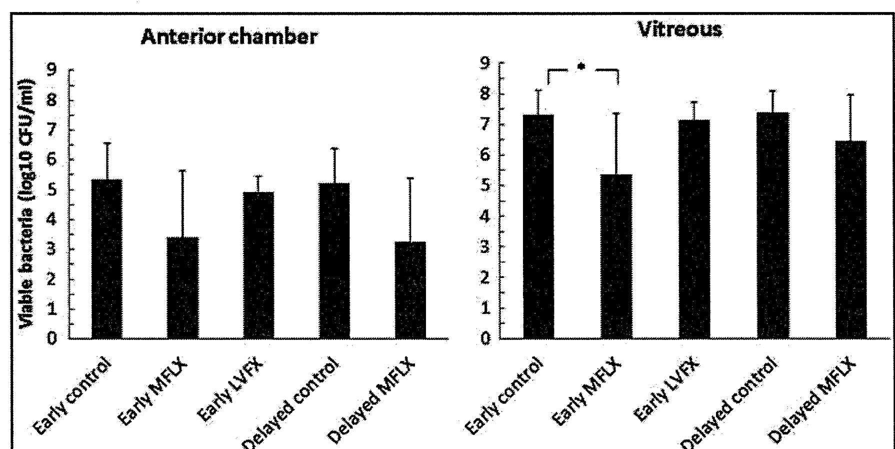

FIG. 2. Effects of topical MFLX and LVFX on the bacterial count in the anterior chamber (left) and vitreous (right). Data are means±standard deviation ($n=6$–8). *$P<0.05$.

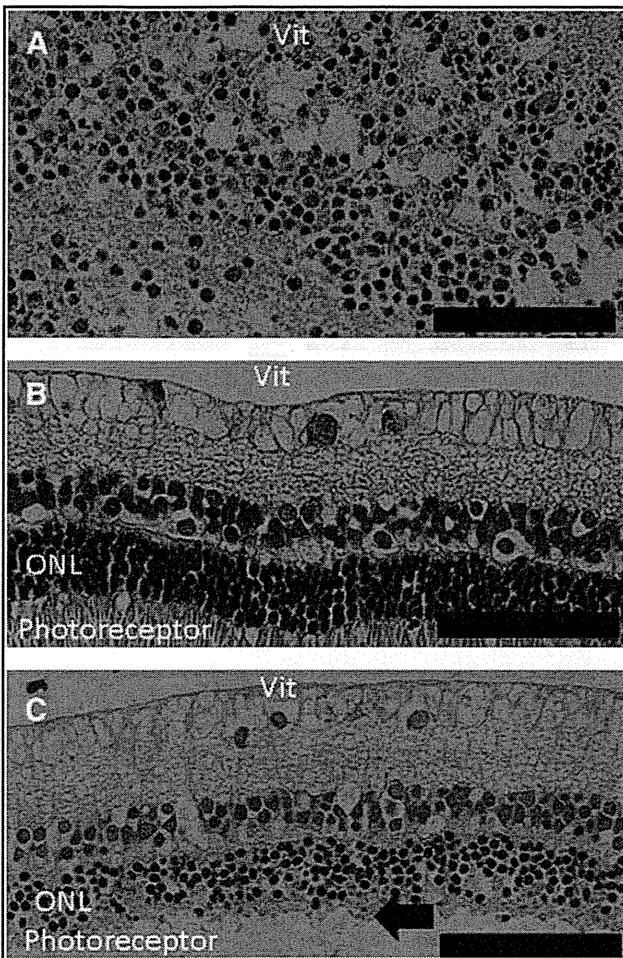

FIG. 4. Representative hematoxylin-and-eosin-stained retina tissue sections in the early saline **(A)**, early MFLX **(B)**, and early LVFX **(C)** groups at 48 h after inducing *E. faecalis* endophthalmitis. Vit, vitreous cavity; ONL, outer nuclear layer. Black arrow indicates destruction of photoreceptors. Scale bar = 50 μm.

E. faecalis is a commensal bacterium present in the gastrointestinal flora and causes many infectious diseases, including endocarditis.[13] It can rapidly induce postoperative endophthalmitis, often within 2–4 days,[14] and can cause substantial vision loss upon infection.[13,15,16] Notably, patients with *E. faecalis*-related endophthalmitis had the worst visual outcome in the Endophthalmitis Vitrectomy Study.[10]

Several studies have since examined the contribution of enterococcal virulence factors to the pathogenesis of *E. faecalis* endophthalmitis.[17–19] We recently demonstrated that proteases secreted from *E. faecalis*, such as gelatinase and serine protease, play important roles in the development of endophthalmitis in aphakic eyes after cataract surgery.[8] These proteases seem to promote the spread of organisms by penetrating the posterior lens capsule, allowing the bacteria to migrate from the anterior chamber to the posterior chamber. This migration is a key event in the progression of postoperative endophthalmitis and the consequent severe retinal damage. In Japan, *E. faecalis* is the causative agent in 12.2% of all ocular infections,[20] which is more than 6-fold higher than the rate reported in the United States.[11] *E. faecalis* also has a high growth rate *in vitro* (1×10^9 CFU/mL within 3–7 h).[14]

Considering these properties of *E. faecalis*, particularly its potent enzymatic activity, infection with this pathogen could rapidly progress to severe visual complications if the infection is not treated promptly and efficiently. Administration of antibiotic eye drops early in the infective course, or even prophylactically before infection develops, may help prevent *E. faecalis* infection and its complications, thereby reducing the risk of postoperative vision loss.

In this study, early postoperative MFLX administration (i.e., at 0, 3, and 6 h after surgery) achieved greater reductions in the inflammation score and bacterial count in the vitreous compared with delayed administration (i.e., at 0, 12, and 15 h after surgery). We also found that postoperative MFLX at the standard topical dose used in humans significantly reduced eye inflammation and maintained retinal function in our rabbit model as compared with LVFX. The standard errors for intraocular inflammation scores were quite large in many of the groups. However, this is unsurprising, as the extent of intraocular inflammation, including the abundance of fibrin and hypopyon, is highly variable in this model. This was to be expected based on the results of our earlier study.[8] In other studies using staphylococci, 0.5% MFLX provided effective prophylaxis to prevent endophthalmitis.[21,22] Interestingly, in those studies, MFLX was more effective than LVFX in preventing experimental fluoroquinolone-resistant methicillin-resistant Staphylococcus aureus in an endophthalmitis model, consistent with our findings.

The timing of antibiotic administration is important to reduce the risk of vision loss caused by postoperative *E. faecalis* endophthalmitis. *E. faecalis* has a high bacterial activity that may result in poor visual outcomes after cataract surgery. Therefore, immediate postoperative antibiotic administration is essential to improve bacterial killing and prevent infection by blocking the rapid penetration of *E. faecalis* to the anterior chamber. Antibiotic eye drops are generally administered immediately after cataract surgery, and the eye is patched. Additional antibiotic eye drops are then administered 1 day after surgery. However, one study found a significantly higher risk of endophthalmitis when antibiotics were administered 1 day after surgery than when administered on the day of surgery.[6] Their findings suggest that antibiotic eye drops should be administered starting on the day of surgery, consistent with our findings.

There are several possible reasons to explain why MFLX showed superior effects to LVFX in this study. First, MFLX was reported to achieve higher concentrations in ocular tissue.[23,24] For example, Fukuda and Sasaki reported that the maximum concentrations of MFLX in all rabbit eye tissues tested (cornea, aqueous humor, iris/ciliary body, and vitreous body) were highest of 5 ophthalmic fluoroquinolone solutions administered.[23] Moreover, topical preoperative administration of 0.5% MFLX achieved greater corneal and aqueous penetration in humans than did 0.3% gatifloxacin.[24] Second, the available formulations of MFLX can exceed the MIC of many pathogens in ocular fluids and tissues in animal models[25,26] and in humans,[27] which is particularly important for effective postoperative endophthalmitis prophylaxis. In this study, we determined the MICs of MFLX and LVFX against 13 *E. faecalis* isolates using the methods recommended by the CLSI.[9] *In vitro* susceptibility testing revealed that MFLX (50% MIC:

0.25 μg/mL) was more potent than LVFX (2 μg/mL). MFLX was reported to achieve anterior chamber concentrations of 2 μg/mL, and by achieving concentrations in excess of the 50% MIC, MFLX could prevent the development of less-susceptible pathogens.[28,29] Both the low MIC and good ocular penetration of MFLX are characteristics essential for effective prophylactic therapy.[21,22] However, several strains, including some that cause endophthalmitis, showed resistance to MFLX and LVFX. Thus, we need other antibiotics to target fluoroquinolone-resistant *E. faecalis*. Third, MFLX, a fourth-generation 8-methoxyfluoroquinolone broad-spectrum antibiotic, shows very broad antibacterial activity.[28,30,31] MFLX also shows superior antibiotic activity and sensitivity for gram-positive and gram-negative ocular pathogens compared with earlier-generation fluoroquinolones.[30,32,33]

The results of our study should be interpreted with care, considering the limitations. First, this study was conducted in a rabbit model with experimentally induced *E. faecalis* endophthalmitis. Although this closely resembles the clinical situation, it is not identical to it. For example, endophthalmitis can be caused by other bacteria, although *E. faecalis* is potentially the most severe causative bacteria in terms of visual outcomes. Second, the formulations of MFLX and LVFX used *in vivo* were the same as those used in clinical practice. Therefore, the effective intraocular concentration in this animal model may exceed that achieved in humans, considering the differences in the eye size.

In conclusion, the results of this study indicate that MFLX delays retinal damage compared with LVFX when used to treat endophthalmitis induced by *E. faecalis* after cataract surgery. Prospective clinical studies are needed to confirm the potency, efficacy, and safety of early postoperative MFLX prophylaxis for the prevention and treatment of bacterial endophthalmitis.

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Author Disclosure Statement

The authors have no commercial or financial interests associated with this article.

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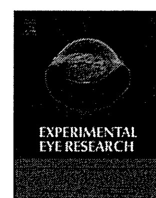

Involvement of P38MAPK in human corneal endothelial cell migration induced by TGF-β_2

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ABSTRACT

Because human corneal endothelial cells do not proliferate once the endothelial monolayer is formed, corneal wound healing is thought to be mediated by cell enlargement or migration rather than proliferation. However, the cellular mechanisms involved in corneal wound healing have not been fully determined. Because transforming growth factor-β_2 (TGF-β_2) isoform is present in high concentrations in normal human aqueous humor, it may play a role in human corneal endothelial cell wound healing. The purpose of this study was to determine the effect of TGF-β_2 on the proliferation and migration of cultured human corneal endothelial cells (HCECs). To achieve this, we first examined the effect of TGF-β_2 on the wound closure rate in an in vitro HCEC wound healing model. However, unexpectedly TGF-β_2 had no effect on the wound closure rate in this model. Therefore, a real-time cell electronic sensing (RT-CES) system and the BrdU incorporation assay were used to determine the effect of TGF-β_2 (0.1–10 ng/ml) on cultured HCEC proliferation during in vitro wound healing. The specificity of this effect was confirmed by adding the TGF-β receptor I kinase inhibitor. TGF-β_2 inhibited the proliferation of HCECs in a dose dependent way and was blocked by TGF-β receptor I kinase inhibitor. Next, the Boyden chamber assay was used to determine how TGF-β_2 (10 ng/ml) affect HCEC migration. Exposure to TGF-β_2 increased cell migration, and a synergistic effect was observed when FGF-2 was added. To determine whether the mitogen-activated protein kinase (MAPK) signaling pathway is involved in the migration of HCECs, western blot analysis and Bio-Plex™ suspension array were used to detect phosphorylation of Erk1/2, p38, and JNK in HCECs stimulated by TGF-β_2 and/or FGF-2. The effect of the p38 MAPK inhibitor, SB239063 (10 μM), on TGF-β_2 and/or FGF-2-induced cellular migration was determined by the Boyden chamber assay. Both TGF-β_2 and FGF-2-induced p38 phosphorylation, and a synergistic effect was observed with exposure to both growth factors. SB 239063 inhibited TGF-β_2 and FGF-2-induced migration of HCECs. These results indicate that TGF-β_2 reduces proliferation but stimulates migration of cultured HCECs. In addition, TGF-β_2 and FGF-2 may have synergistic effects on the migration of HCECs mediated by p38 MAPK phosphorylation.

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1. Introduction

The corneal endothelium is a single layer of cells lying between the corneal stroma and the anterior chamber, which helps maintain corneal transparency by regulating corneal hydration. It is widely accepted that corneal endothelial cells do not proliferate in humans once the endothelial monolayer is formed (Murphy et al., 1984). Different types of corneal injuries including surgical stress during intraocular surgery, corneal trauma, and viral infections cause a decrease in corneal endothelial cell density. The damaged corneal endothelium is believed to be repaired by enlargement and/or migration of the remaining corneal endothelial cells rather than proliferation during human corneal endothelial wound healing (Joyce, 2003; Landshman et al., 1988; Ling et al., 1988; Treffers, 1982).

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However, the precise mechanisms involved in human corneal endothelial cell wound healing have not been fully determined.

Because the aqueous humor bathes the corneal endothelium, the cytokines present in the aqueous humor may contribute to the healing of endothelial injury. Among the growth factors in the aqueous humor, transforming growth factor-β_2 (TGF-β_2) is the main isoform that is present in relatively high concentrations in humans (Cousins et al., 1991; Jampel et al., 1990). In other tissues, TGF-β is known to be involved in regulating cell differentiation, cell proliferation, cell migration, and other cellular functions (Furuyama et al., 1999; Gailit et al., 1994; Saika, 2004). In addition, TGF-β_2 has been reported to be associated with the arrest of corneal endothelial cells at G1 by blocking the G1 to S transition (Harris and Joyce, 1999; Kim et al., 2001a,b). This is supported by the fact that rat and rabbit corneal endothelial cell proliferation is suppressed in vitro by exposure to TGF-β_2 (Chen et al., 1999; Harris and Joyce, 1999; Kim et al., 2001a). However, the effect of TGF-β_2 on the proliferation of human corneal endothelial cells has not been fully determined.

Three isoforms of TGF-β (β_1, β_2, and β_3) bind to the serine/threonine protein kinases (TGF-β type I and type II receptors). Both TGF-β type I and type II receptors are necessary for TGF-β signal transduction. When the TGF-βs bind to their receptors, multiple signaling cascades are activated such as the Smad proteins and mitogen-activated protein kinases (MAPKs), which include the extracellular signal-regulated kinases (Erk1/2), c-Jun N-terminal kinases (JNK), and p38 (Byfield and Roberts, 2004; Itoh and ten Dijke, 2007; Massague and Gomis, 2006). In the corneal epithelium, epidermal growth factor (EGF)-induced Erk1/2 and p38 phosphorylation have been demonstrated to induce corneal epithelial cell migration (Wang et al., 2006). Recent studies have demonstrated that TGF-β enhances migration of corneal epithelial cells through the p38 MAPK pathway, but not the Smad pathway (Saika et al., 2004; Terai et al., 2011).

Although corneal endothelial cells have been shown to express the mRNA and protein for all three receptor types (TGF-β type I, type II, and type III receptors) (Harris and Joyce, 1999; Joyce and Zieske, 1997), it is unclear whether the intracellular signaling mechanisms of TGF-β_2 induce migration of human corneal endothelial cells.

It is possible that human corneal endothelial cells in vivo are affected not only by TGF-β_2 but also by other cytokines in the aqueous humor and corneal endothelial cells. The cytokines present in normal human aqueous humor include basic-fibroblast growth factor (FGF-2), hepatocyte growth factor, insulin-like growth factor binding protein, and vascular endothelial growth factor. Epidermal growth factor and transforming growth factor-α are not present or below the detectable levels in normal human aqueous humor (van Setten et al., 1996). FGF-2 is present at particularly high concentrations in normal human aqueous humor and corneal endothelial cells (Hoppenreijs et al., 1994; Rieck et al., 1995; Wilson and Lloyd, 1991), and it has been reported that FGF-2 can stimulate the proliferation and migration of corneal endothelial cells (Hoppenreijs et al., 1994; Rieck et al., 1995, 2001).

The purpose of this study was to determine whether TGF-β_2, the main isoform of TGF-β in the human aqueous humor, is involved in the proliferation and migration of cultured human corneal endothelial cells (HCECs). Another aim of this study was to investigate whether the MAPKs, Erk1/2, JNK, and p38, are involved in the migration of HCECs induced by TGF-β_2.

2. Materials and methods

All procedures including those involving human subjects were conducted in accordance with the principles of the Declaration of Helsinki, and this study protocol was approved by the Institutional Review Board of Ehime University.

2.1. Primary cultures of HCECs

All primary and passaged HCECs were cultured in a medium consisting of low glucose Dulbecco modified Eagle medium (DMEM) supplemented with 15% fetal calf serum (FCS), 30 mg/L of L-glutamine, 2.5 mg/L of Fungizone (GIBCO, Grand Island, NY), 2.5 mg/L of doxycycline (Sigma–Aldrich, St. Louis, MO), and 2 ng/mL of FGF-2 (Invitrogen, Carlsbad, CA) (Joko et al., 2007; Miyata et al., 2001). Cultured HCECs were maintained in a humidified incubator at 37 °C and 10% CO_2.

Primary cultures of HCECs were started from normal human corneas acquired from the American Eye Bank, and were isolated and cultured by procedures described in detail (Joko et al., 2007). Briefly, small explants from the endothelial cell layer including Descemet's membrane were removed and cultured in 35 mm culture dishes coated with mouse collagen type IV (BD Biosciences, San Jose, CA). When a sufficient density of proliferating HCECs was attained, cells were rinsed three times in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS(−)), trypsinized for 2 min at 37 °C, collected, and passaged. All subsequent passages were carried out using the same method. We used cultured HCECs of the fifth passage for all of the experiments.

3. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from the cultured HCECs and human corneal endothelium obtained from normal human corneas using TRIzol reagent according to the manufacturer's instructions (Invitrogen). Samples were further purified using the RNeasy kit (Qiagen, Valencia, CA). cDNA was prepared from 1 μg of total RNA by reverse transcription in a volume of 20 μl. cDNAs were synthesized with Superscript II reverse transcriptase according to the manufacturer's instructions (Invitrogen).

PCR amplification was performed with TaKaRa Ex Taq (TaKaRa, Kusatsu, Japan) with the following conditions: 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C (TGF-β receptor I) or 60 °C (TGF-β receptors II and III) for 30 s, and extension at 72 °C for 60 s. The primer pairs used for RT-PCR are listed in Table 1.

3.1. In vitro wound healing of cultured HCECs

Prior to investigating the effect of TGF-β_2 on HCEC wound healing, we first determined the minimum concentration of FCS and FGF-2 that led to healing of the cultured HCECs, because FCS and FGF-2 are known to be essential for HCEC proliferation. After

Table 1
Sequences of primers used in RT-PCR.

Primer		Sequence (5′–3′	Product size
GAPDH	F1	CGACCACTTTGTCAAGCTCA	228 bp
GAPDH	R1	AGGGGTCTACATGGCAACTG	
TGF-βR I	F1	CGTTACAGTGTTTCTGCCACCT	314 bp
TGF-βR I	R1	AGACGAAGCACACTGGTCCAGC	
TGF-βR II	F1	TTTTCCACCTGTGACAACCA	185 bp
TGF-βR II	R1	GGAGAAGCAGCATCTTCCAG	
TGF-βR III	F1	GCCTTGATGGAGAGCTTCAC	178 bp
TGF-βR III	R1	CAGACTTGTGGTGGATGTGG	

HCECs reached 100% confluence in 6 well plates coated with type IV collagen, they were serum-starved for 24 h. Then, a linear wound was made with a 1 ml pipette tip on the monolayer of cultured HCECs, and the injured HCECs were incubated for 96 h in DMEM with 30 mg/L of L-glutamine, 2.5 mg/L of Fungizone, 2.5 mg/L of doxycycline, 0%, 1%, or 3% FCS, and 0 ng/ml or 2 ng/ml of FGF-2. The wound did not close during the observation period when incubated in media without FGF-2, or with concentrations of FCS $\leq$1%. However, wound closure occurred within 48 h when cells were treated with 3% FCS and 2 ng/ml of FGF-2. Thus, we added 3% FCS and 2 ng/ml of FGF-2 to the medium used to test the effects of TGF-β_2 on HCEC wound healing and following proliferation assay including RT-CES system and BrdU incorporation assay.

To assess cultured HCEC proliferation during in vitro wound healing, wounds were created in cultured HCECs as follows. After HCECs reached 100% confluence in 6 well plates coated with type IV collagen, they were serum-starved for 24 h. Then, linear wounds consisting of 5 vertical and 5 horizontal lines were made with a 1 ml pipette tip on the monolayer of cultured HCECs. The plates were rinsed with PBS(−) twice to remove the unattached cells. The plates were photographed, and the remaining wounded area was measured at 0, 3, 6, 12, 24, and 48 h after the scraping using Canvas™ ver 6.0 (Deneba Software, Miami, FL).

To investigate the effect of TGF-β_2 on the HCEC wound healing, injured HCECs were cultured with serial concentrations of TGF-β_2 from 0.1 to 10.0 ng/ml.

3.2. Cell proliferation assay by RT-CES system

The rate of cellular proliferation was determined by a real-time cell electronic sensing (RT-CES) system (ACEA Bioscience, San Diego, CA) as described in detail (Joko et al., 2007). Cells were grown on the surface of microelectronic sensors which were composed of circle-on-line electrode arrays integrated into the bottom surface of the microtiter plate. Changes in the cell number were monitored and quantified by measuring the changes in the electrical impedance of the detecting sensors. Cell index (CI) values obtained by the RT-CES system have been shown to be quantitatively correlated with the cell number (Solly et al., 2004; Xing et al., 2005). For this measurement, HCECs at 80% confluence were serum-starved for 24 h to be synchronized in the G0 phase. Then the cells were trypsinized and seeded into 16-wells at a density of 2×10^4 cells/well. The cells were maintained for 72 h in DMEM containing 3% FCS, 2 ng/ml of FGF-2 and human recombinant TGF-β_2 (0.1–10 ng/ml; R & D, Minneapolis, MN). Additional samples were incubated with 5.0 μM of TGF-β receptor I kinase inhibitor (Calbiochem, San Diego, CA) and human recombinant TGF-β_2 (5.0 ng/ml or 10.0 ng/ml). The RT-CES system was placed in a 5% CO_2 incubator, and the CI value was determined every hour automatically by the RT-CES system for up to 72 h.

Under these conditions, normal HCECs grow exponentially from 16 h through 48 h. Therefore, we elected to determine the proliferation rate from 24 h to 40 h, and defined the proliferation rate as CI/hour. The formula used to calculate the proliferation rate was,

$$(\text{Cell index at 40 h}) - (\text{Cell index at 24 h})/16\text{ h}.$$

Each proliferation rate is presented relative to that of the controls.

3.3. Effect of TGF-β_2 on BrdU incorporation into HCECs in an in vitro wound healing model

The effect of human recombinant TGF-β_2 (0.1–10 ng/ml) on HCEC proliferation was examined by a BrdU incorporation assay. Injured cells were incubated in medium containing 3% FCS and 2 ng/ml of FGF-2. 0.1–10 ng/ml TGF-β_2 was added to the medium of the experimental samples. Additional samples were also cultured with TGF-β receptor I kinase inhibitor (0.1–5 μM) and 5.0 ng/ml of TGF-β_2. Forty-four hours after wounding, the cells were incubated in 10 μmol/l BrdU for 1 h. To detect BrdU-positive cells, the fixed cells were incubated with anti-BrdU antibody (Brdu Labeling and Detection Kit II, Roche) for 30 min at 37 °C followed by fluorescein conjugated anti-mouse IgG for 30 min at 37 °C. The BrdU positive cells in the wounded area were viewed by fluorescence microscopy and counted.

3.4. Effect of TGF-β_2 on cell migration assessed by Boyden chamber assay of HCECs

The migration of HCECs was examined by Boyden chamber assays as described in detail (Boyden, 1962). Briefly, TGF-β_2 (10 ng/ml) and/or FGF-2 (2 ng/ml) were added to the bottom wells of a 48-well Boyden chamber (Neuro Probe, Cabin John, MD), and a 10 μm pore-size polyvinylpyrrolidone-free polycarbonate membrane (Neuro Probe) was placed on the wells. The membrane was pre-coated with type I collagen (10 μg/ml in PBS; Nitta Gelatin, Osaka, Japan) at room temperature for 1 h and then washed extensively with PBS. After serum-starvation for 12 h, subconfluent HCECs were trypsinized and re-suspended in culture medium without FCS at 1×10^5 cells/ml. A 50-μl aliquot of the HCEC suspension (5000 cells/well) was added to the upper wells and incubated for 8 h at 37 °C. The cells that adhered to the upper surface of the filter membrane were removed by scraping with a rubber blade, and those that stayed on the lower surface of the membrane were taken to be the migrated cells. The membrane was fixed with 10% buffered formalin overnight and then stained with Gill's hematoxylin. The membrane was then mounted between two glass slides with 90% glycerol, and the total number of migrated cells/membrane was counted under a microscope.

To examine the effect of TGF-β_2 on cell migration, the cells were serum-starved for 12 h and preincubated for 15 min with 5.0 μM of TGF-β receptor I kinase inhibitor. Then 10 ng/ml of TGF-β_2 was added to the bottom wells, and the cells were incubated for 8 h at 37 °C.

To examine the effects of p38 MAPK on cell migration induced by TGF-β_2 and/or FGF-2, cells were serum-starved for 12 h and then preincubated for 60 min with 10 μM of p38 MAPK inhibitor SB 239063 (Calbiochem, San Diego, CA). They were then incubated for 8 h at 37 °C with TGF-β_2 (10 ng/ml) and/or FGF-2 (2 ng/ml) added to the bottom wells.

3.5. Bio-Plex beads assay for detection of phosphorylated MAP kinases

The MAPK phosphorylation of HCECs was determined by Bio-Plex phosphoprotein and a total target assay (Bio-Rad, Hercules, CA). The procedures were carried out according to the manufacturer's protocol. Briefly, serum-starved HCECs were exposed to 10 ng/ml of TGF-β_2 and/or 2 ng/ml of FGF-2, and cells were collected after 0 (control), 15, 30, 45, and 60 min. The cells were lysed with the Bio-Plex Cell Lysis Kit, and the protein concentration was adjusted to 600 μg/ml. Then 50 μl of the cell lysate was used for the assay. Fifty microliters of coupled beads, which recognize phosphorylated and total Erk1/2, p38, and JNK, were added to 96-well filter plates. The same volume of the cell lysate was added and incubated with the beads for 16 h. Then, 25 μl of detection antibodies (1×) were added and incubated for 30 min. Fifty microliters of streptavidin-PE (1×) were added and incubated for 10 min in the dark. The amount of phosphoprotein and

total MAPK protein was determined by a Luminex 100™ analyzer (Bio-Rad).

3.6. Western blot analysis for detection of phosphorylated MAP kinases

Western blot analyses were used to confirm the phosphorylation of MAPK. Briefly, serum-starved HCECs were exposed to 10 ng/ml of TGF-β_2 and/or 2 ng/ml of FGF-2, and the cells were collected at 15 and 60 min in Laemmli sample buffer (Bio-Rad) containing β-mercaptoethanol. Then, equivalent volumes of samples (5 μg/well) were separated on 5–20% gradient polyacrylamide gel containing sodium dodecyl sulfate (SDS-PAGE) and transferred to polyvinylidene (PVDF) membranes. After blocking with 5% nonfat dry milk and 0.1% Tween-20 in TBS, the membranes were incubated at 4 °C overnight with polyclonal anti-human Erk1/2 (p44/42 MAP Kinase and phospho-p44/42 MAP Kinase (Thr/202/Tyr204)), JNK (SAPK/JNK and phospho-SAPK/JNK(Thr/183/Tyr185)), and p38 (p38 MAP Kinase and phospho-p38 MAP Kinase8(Thr/Tyr182)) rabbit antibodies (Cell Signaling Technology, Danvers, MA). The membranes were washed and incubated with 1:2000 horseradish peroxidase-conjugated goat anti-rabbit antibodies (Vector Laboratory, Burlingame, CA) for 30 min. The immunoreactive products were made visible by an enhanced chemiluminescence (ECL plus) detection system according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ).

3.7. Statistical analyses

Values are presented as means ± standard deviations. The significance of differences between the groups was determined by paired or unpaired Student's t tests. All t tests were two-sided, and a P-value of <0.05 was taken to be statistically significant.

4. Results

4.1. TGF-β receptor mRNA in cultured human corneal endothelial cells

The expression of TGF-β receptors I, II, and III in human corneal endothelium have been demonstrated *in vivo* (Joyce and Zieske, 1997), however their expression in cultured HCECs has not been determined. Therefore, we first confirmed that the mRNAs of TGF-β receptors I, II, and III were expressed in both subconfluent and confluent HCECs as well as in human corneal endothelium *in situ* before beginning our experiments. The results showed that the mRNA for all three TGF-β receptor types was detected in all samples examined by RT-PCR (Fig. 1).

4.2. Effect of TGF-β_2 on cultured human corneal endothelial cell wound healing

To investigate the effect of TGF-β_2 on wound healing in cultured HCECs, injured HCECs were cultured with serial concentrations of TGF-β_2. No significant difference was observed between the speed of wound healing in the control and treated cells for up to 12 h with any concentration of TGF-β_2 from 0.1 to 10.0 ng/ml (Fig. 2A). The migrating cells were scattered after 24 h. Therefore, it was not possible to precisely measure the remaining wounded area. However, when photographic images of samples treated with various concentrations of TGF-β_2 (0–10.0 ng/ml) were compared, no difference was observed between the degree of wound healing in any sample (Fig. 2B).

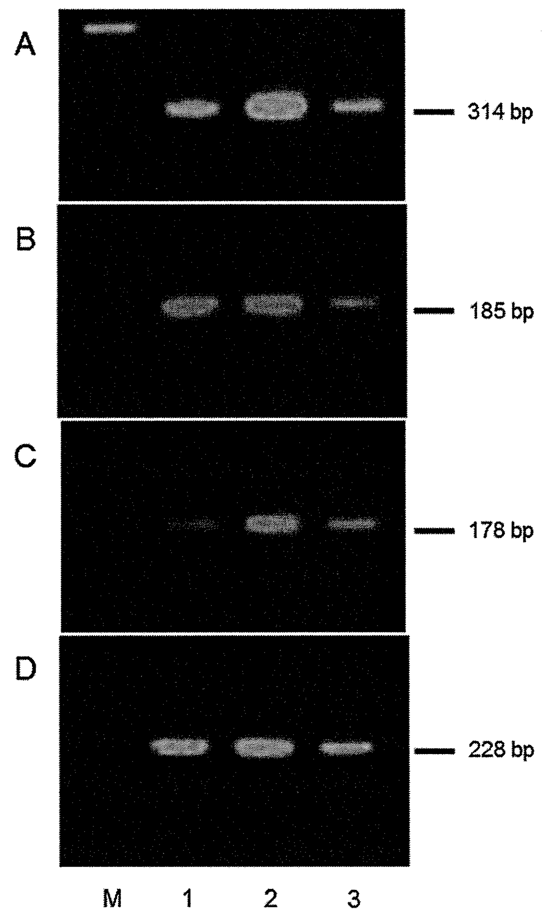

Fig. 1. Determination of the expression of the mRNA of TGF-β receptor in cultured human corneal endothelial cells (HCECs). Ethidium-bromide-stained agarose gels showing the RT-PCR products for TGF-β type I receptor (A), TGF-β type II receptor (B), TGF-β type III receptor (C) and GAPDH (D). Lane 1: Subconfluent cultured HCECs; Lane 2: 100% confluent cultured HCECs; Lane 3: in situ human corneal endothelium. M = 100 bp DNA ladder.

4.3. Effect of TGF-β_2 on cultured human corneal endothelial cell proliferation evaluated by the RT-CES system

The effect of TGF-β_2 on the proliferation of HCECs was measured with the RT-CES system. Although treatment with 0.1 ng/ml of TGF-β_2 had no effect on HCEC proliferation, exposure to TGF-β_2 at concentrations $\geq$0.5 ng/ml significantly reduced the rate of proliferation. At concentrations of TGF-β_2 $\geq$1.0 ng/ml, there was an 80–90% reduction in the proliferation rate. TGF-β receptor I kinase inhibitor (5.0 ng/ml) significantly attenuated the inhibitory effect of TGF-β_2 on HCEC proliferation (Fig. 3).

5. Effect of TGF-β_2 on BrdU incorporation in cultured human corneal endothelial cell wound healing

The effect of TGF-β_2 on the proliferation of HCECs during wound healing was investigated by culturing injured HCECs with serial concentrations of TGF-β_2 followed by BrdU. No significant difference was detected between the number of BrdU-positive cells in control samples and those exposed to 0.1 ng/ml of TGF-β_2. However, the number of BrdU-positive cells significantly decreased in a dose dependent way when cells were exposed to TGF-β_2 at concentrations between 1.0 and 10.0 ng/ml (Fig. 4A).

When injured HCECs were cultured with TGF-β receptor I kinase inhibitor, the inhibitory effect of TGF-β_2 on cell proliferation was significantly attenuated (Fig. 4B).

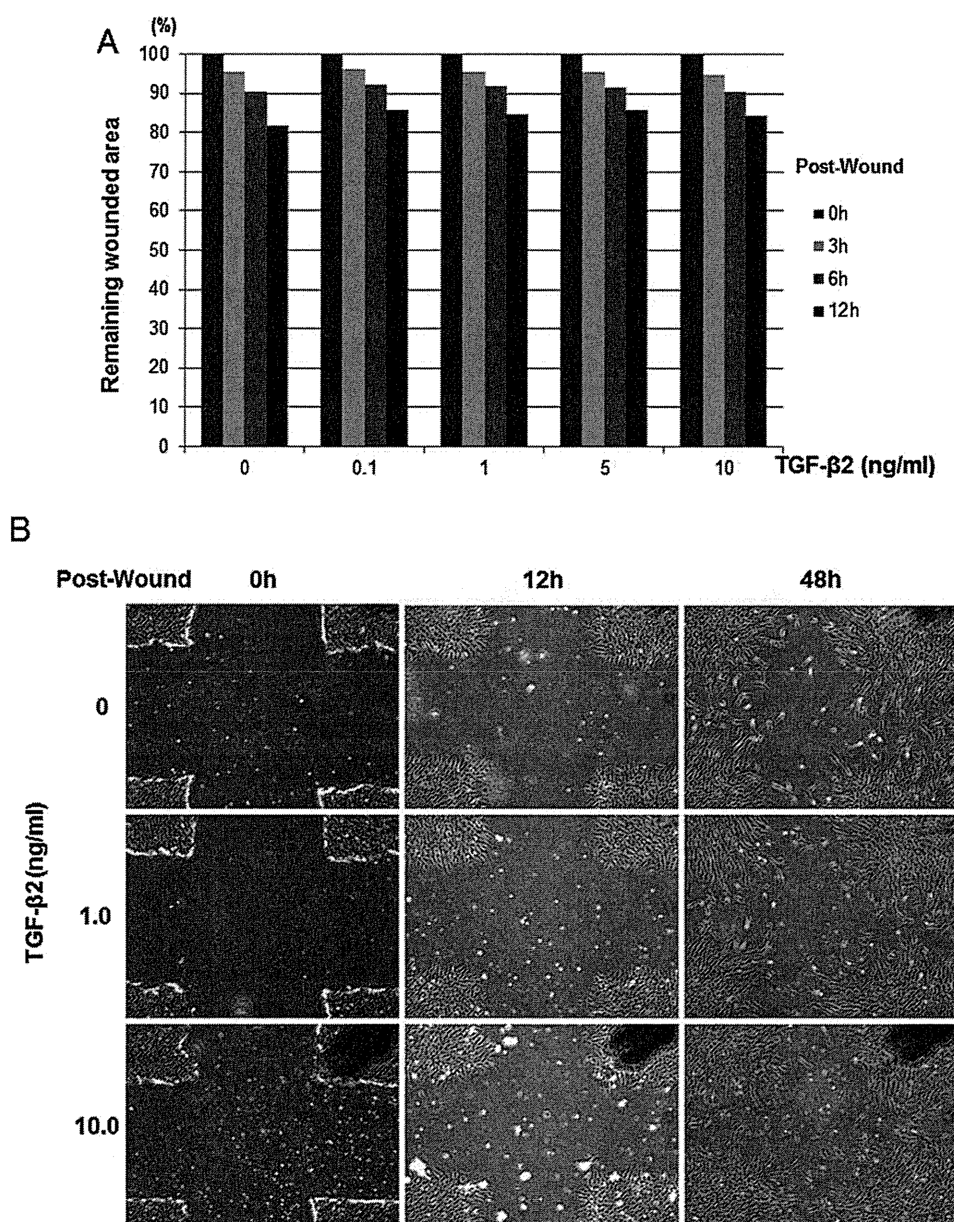

Fig. 2. Effect of TGF-β_2 on in vitro HCEC Wound Healing. Linear wounds were made with a 1 ml pipette tip and the acellular area was measured at 0, 3, 6, 12, 24, and 48 h after exposure to TGF-β_2 at concentrations of 0.1–10.0 ng/ml. A. No significant difference was observed between the speed of wound healing in the controls, and cells treated for up to 12 h with any concentration of TGF-β_2 from 0 to 10.0 ng/ml ($n = 4$ each). B. Representative micrographic images of in vitro HCEC wound healing. The migrating cells are scattered after 24 h (C, D). Therefore, it was not possible to precisely measure the remaining wounded area. However, careful examination of photographic images of samples treated with various concentrations of TGF-β_2 (0–10.0 ng/ml) showed no difference between wound healing in any of the samples.

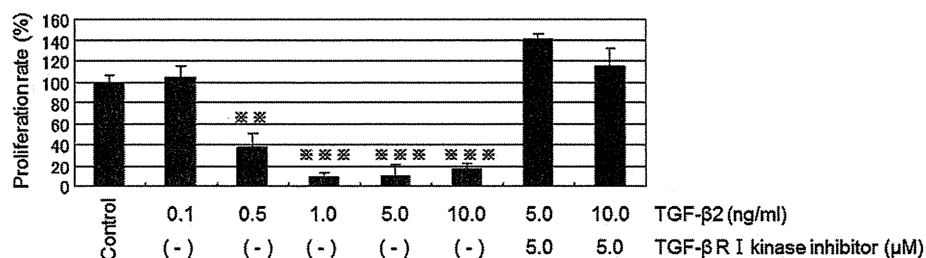

Fig. 3. Effect of TGF-β_2 on proliferation of HCECs measured by RT-CES system. Exposure to 0.5–10.0 ng/ml of TGF-β2 has a significant inhibitory effect on the proliferation of HCECs. The TGF-β receptor I kinase inhibitor (5.0 ng/ml) significantly attenuated the TGF-β_2 inhibition of HCEC proliferation. Error bars represent standard deviations. Significant difference ** ($P < 0.01$) *** ($P < 0.001$) from controls ($n = 3$ each).

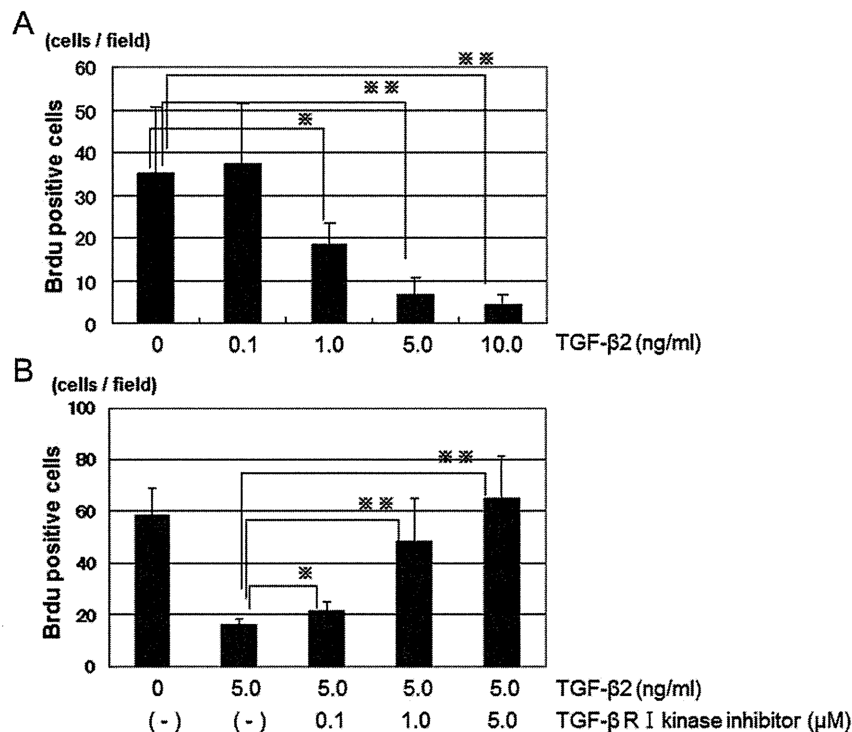

Fig. 4. Effect of TGF-β2 on HCEC proliferation during Wound Healing. The BrdU incorporation assay was carried out in an in vitro HCEC wound healing model in the presence of 0.1–10 ng/ml TGF-β2. A. The number of BrdU positive cells significantly decreased when samples were treated with 1.0–10.0 ng/ml of TGF-β2. B. Pre-incubation with TGF-β receptor I kinase inhibitor significantly inhibited the TGF-β2-induced reduction of cell proliferation. Error bars represent standard deviations. Significant difference * ($P < 0.05$), ** ($P < 0.01$) are indicated by each bar. ($n = 6$ each).

5.1. Effect of TGF-β2 and FGF-2 on migration of human corneal endothelial cells in Boyden chamber

Although treatment with TGF-β2 at concentrations from 1.0 to 10.0 ng/ml significantly reduced the rate of injured HCEC proliferation, the rate of wound healing was not significantly lower than that of the control. We hypothesized that this was due to an increase in cell migration induced by TGF-β2.

To test this, we examined the effect of TGF-β2 and FGF-2 on the migration of HCECs with the Boyden chamber assay. HCECs were incubated for 8 h with 10 ng/ml TGF-β2, with or without 2 ng/ml FGF-2, and the number of HCECs that migrated during the incubation period was counted. Compared to the control, HCEC migration significantly increased by 1.7-fold in samples incubated with either TGF-β2 or FGF-2. When TGF-β2 and FGF-2 were both added to the lower chamber, the cell migration significantly increased 2.5-fold over that of the control. This increase in migration was significantly higher than that observed with TGF-β2 or FGF-2 alone (Fig. 5A). To demonstrate the specificity of the TGF-β2-dependent cellular migration, HCECs were cultured with 5 μM of TGF-β receptor I kinase inhibitor. TGF-β receptor I kinase inhibitor significantly reduced TGF-β2-induced HCEC migration to the level of the control (Fig. 5B).

5.2. Phosphorylation of MAPKs by TGF-β2 and FGF-2

Because TGF-β2 and FGF-2 were observed to have a synergistic effect on cell migration, we investigated the mechanism of this synergy in more depth. HCECs were exposed to 10 ng/ml TGF-β2 and/or 2 ng/ml FGF-2 for 0, 15, 30, 45, and 60 min, and the state of MAPK phosphorylation was determined by the Bio-Plex Bead assay and confirmed by western blot analysis at specific times. The Bio-Plex Bead assay showed that Erk1/2 was phosphorylated as early as 15 min after exposure to FGF-2 alone or a combination of TGF-β2 and FGF-2, but not TGF-β2 alone. These results were confirmed by western blot. When p38 phosphorylation was examined by the Bio-Plex Beads assay after 45 and 60 min of incubation, phosphorylation was found to increase by about 2-fold when exposed to either TGF-β2 or FGF-2 alone, and about 4-fold when exposed to TGF-β2 combined with FGF-2 (Fig. 6).

The phosphorylation of p38 was confirmed by western blot after 60 min of incubation, and a weak increase in the phosphorylation was detected when the HCECs were exposed to either TGF-β2 or FGF-2 alone. Strong phosphorylation was detected when HCECs were exposed to TGF-β2 combined with FGF-2, consistent with the results of the Bio-Plex Bead assays (Fig. 6).

The phosphorylation of JNK was not detected by either Bio-Plex Beads assay or western blot analysis at any time point after incubation with TGF-β2 and/or FGF-2 (Fig. 6).

5.3. Effect of p38MAPK inhibitor on human corneal endothelial cell migration

To determine whether TGF-β2 and FGF-2-induced migration of HCECs is regulated by p38 MAPK activation, the migration of HCECs was quantitatively analyzed using the Boyden chamber assay in the presence of SB239063, a p38MAPK inhibitor. The results showed that SB239063 significantly reduced HCEC migration induced by TGF-β2 and/or FGF-2 to the level of the control (Fig. 7).

6. Discussion

As best we know, our experiments are the first to examine the effect of TGF-β2 on cultured HCECs. In the initial experiments, we examined the effect of TGF-β2 in an *in vitro* HCEC wound healing

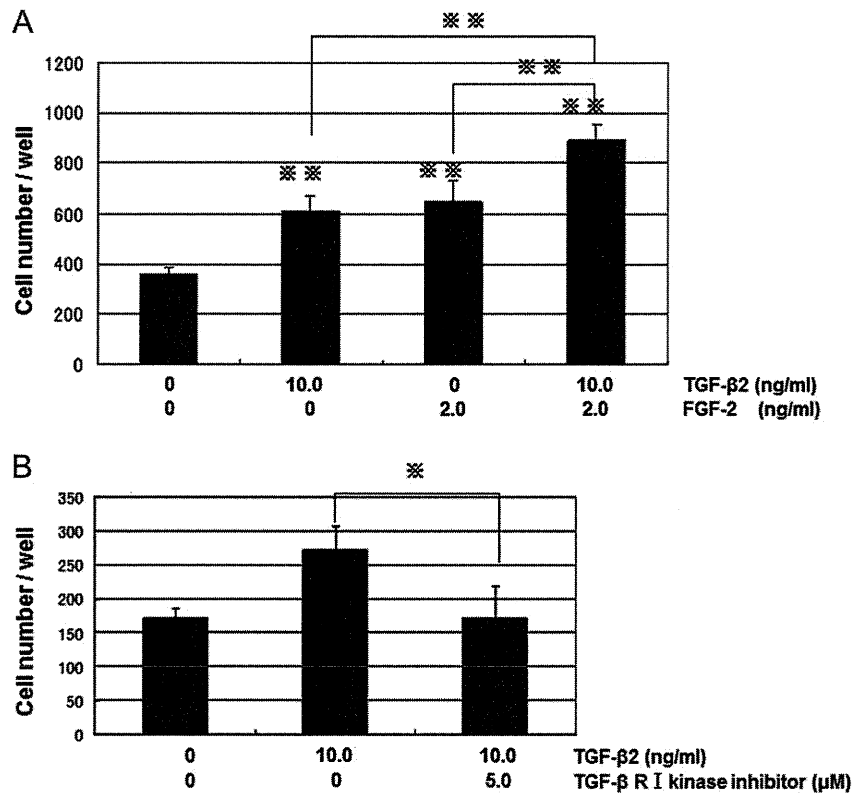

Fig. 5. Effect of TGF-β2 on HCEC migration. A. The effect of TGF-β2 and FGF-2 on the degree of HCEC migration was quantitatively determined by Boyden chamber assays. HCEC migration increased by 1.7-fold in the TGF-β2 sample, and 1.7-fold in the FGF-2 sample compared with the controls. When TGF-β2 was combined with FGF-2, cell migration increased by 2.5-fold compared with the controls, and the cell migration was significantly higher than that with TGF-β2 or FGF-2 alone. B. To confirm that cellular migration was mediated by TGF-β2, cultured HCECs were cultured with 5 μM of TGF-β receptor I kinase inhibitor. TGF-β receptor I kinase inhibitor significantly reduced the TGF-β2-induced migration of HCECs to the level of the control. Error bars represent standard deviations. Significant differences * ($P < 0.05$), ** ($P < 0.01$) between control and experimental samples are indicated by each bar ($n = 6$ each).

model and found somewhat unexpectedly that TGF-β2 had no effect on the speed of wound healing of injured cultured HCECs.

Therefore, we further investigated the effect of TGF-β2 on injured HCEC proliferation using the BrdU incorporation assay, and cell migration with the Boyden chamber assay. The number of BrdU-positive cells was reduced in a dose dependent way by exposure to TGF-β2 at concentrations of 1.0–10 ng/ml. The effect of TGF-β2 was blocked by TGF-β receptor I kinase inhibitor, confirming the results of the RT-CES assay. These findings are in good agreement with earlier results using bovine corneal endothelial cells (Thalmann-Goetsch et al., 1997). Interestingly, BrdU-positive cells were detected only around the injured area and not around non-injured areas, and these findings were also observed by ex vitro experiments carried out by Whikehart et al. (2005). Because it has been reported that contact inhibition is one of the mechanisms that leads to a reduction in the proliferation of corneal endothelial cells in vivo (Kim et al., 2001b), we believe that contact inhibition may be one of the factors that lead to the inhibition of proliferation of HCECs in the non-injured areas in our study.

The concentration of TGF-β2 in normal human aqueous humor ranges from 0.27 to 2.24 ng/ml (Picht et al., 2001; Tripathi et al., 1994). The in vivo concentration of the active form of TGF-β2 in the normal human aqueous humor is 20–830 pg/ml (Picht et al., 2001; Tripathi et al., 1994). Our RT-CES and BrdU incorporation assays showed that the concentrations of TGF-β2 $\geq$0.5 ng/ml inhibited the growth of HCECs. This indicated that the TGF-β2 level in normal human aqueous humor may be high enough to reduce the proliferation of corneal endothelial cells in vivo, even when the HCECs are released from contact inhibition due to an injury.

In contrast to cell proliferation, our results showed that TGF-β2 clearly increased the degree of migration of HCECs. This confirmed the findings of Grant et al. who tested the effect of TGF-β on cultured HCEC migration at concentrations of 0–2 pg/ml, and reported that TGF-β increased the migration with a maximum response at 1 pg/ml (Grant et al., 1992). They also tested migration at concentrations of FGF-2 ranging from 0 to 200 ng/ml and found a dose dependent increase in the migration of HCECs (Grant et al., 1992).

TGF-β2 and FGF-2 were observed to have a synergistic effects on HCECs migration. The total concentration of FGF-2 in normal human aqueous humor ranges from 0.48 to 1.44 ng/ml (Tripathi et al., 1994). We found that cell proliferation was inhibited with 1.0 ng/ml of TGF-β2 if combined with 2.0 ng/ml of FGF-2. Thus, the presence of TGF-β2 and FGF-2 in the human aqueous humor may keep the corneal endothelial cells in a non-proliferative state. On the other hand, these two cytokines may promote the migration of corneal endothelial cells in the wound healing process.

The MAPKs have been shown to play key roles in cell proliferation, cell migration, cell differentiation, inflammation, stress responses, and oncogenesis. Recently, the results of several studies have indicated that the p38 pathway is involved in the migration of different cell types (Huang et al., 2004; Saika et al., 2004; Sharma et al., 2003; Wang et al., 2006). The TGF-β2 mediated phosphorylation of p38 through the MEKK1 and MKK 3/6 pathways has been discussed (Cuadrado and Nebreda, 2010), and it has been demonstrated that SB203580, a specific inhibitor of p38 MAPK, reduced the degree of migration of corneal epithelial cells induced by hepatocyte growth factor and TGF-β (Saika et al., 2004; Sharma

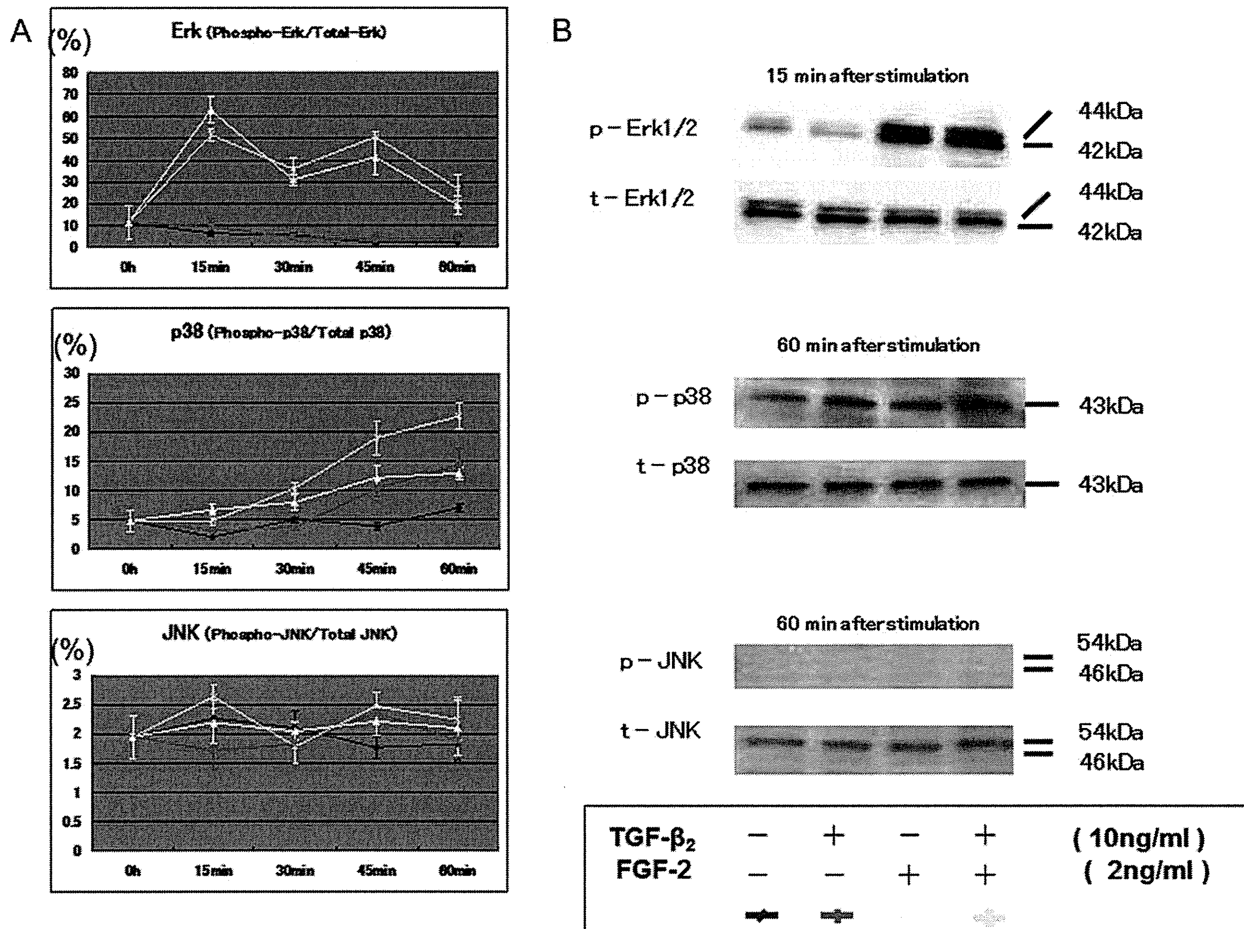

Fig. 6. Activation of MAPK by TGF-β2 and/or FGF-2 in HCECs. The effect of 10 ng/ml TGF-β_2 and/or 2 ng/ml FGF-2 on the phosphorylation of Erk, p38 and JNK was determined by Bio-Plex™ Suspension array system ($n = 4$) (A) and Western blot analysis (B). A. Erk1/2 was phosphorylated as early as 15 min after treatment with FGF-2 alone, or TGF-β_2 and FGF-2 together, but not with TGF-β_2 alone. P38 phosphorylation at 45 and 60 min increased by about 2-fold when samples were exposed to TGF-β_2 or FGF-2 alone, and about 4-fold when samples were exposed to TGF-β_2 combined with FGF-2. JNK phosphorylation was not detected by Bio-Plex Beads assay at any time after exposure to TGF-β_2 or FGF-2. B. Erk1/2 phosphorylation was confirmed by western blot at 15 min. Phosphorylation occurred after exposure to FGF-2 alone, or TGF-β_2 combined with FGF-2, but not after TGF-β_2 exposure alone. p38 phosphorylation was confirmed by western blot at 60 min. Weak phosphorylation was detected when samples were exposed to TGF-β_2 or FGF-2 alone, while stronger phosphorylation was detected after exposure to TGF-β_2 combined with FGF-2, consistent with the results of the Bio-Plex Beads assay. JNK phosphorylation was not detected by western blot analysis at any time after exposure to TGF-β_2 or FGF-2.

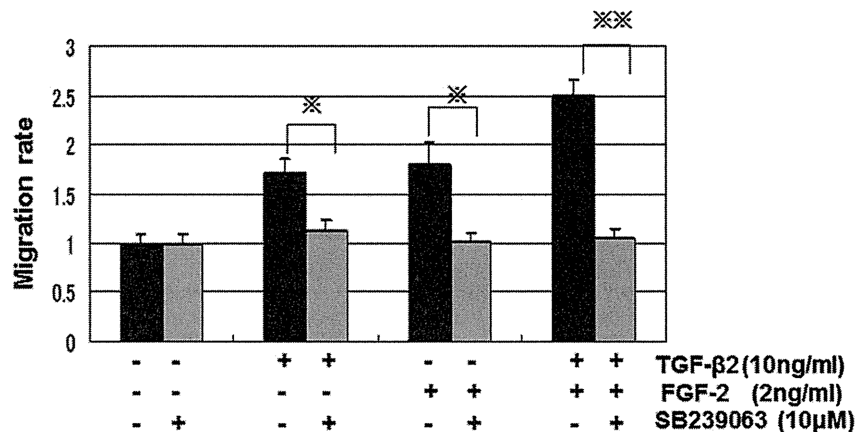

Fig. 7. Effect of p38 MAPK inhibitor on HCEC Migration to determine whether the TGF-β_2- and/or FGF-2-induced migration of HCECs was regulated by p38 MAPK activation, the migration of HCECs was determined quantitatively by Boyden chamber assays in the presence of a p38MAPK inhibitor, SB239063. SB239063 significantly reduced TGF-β_2 and/or FGF-2- induced HCEC migration to the level of the control. The data are shown as number of migrating cells relative to that of the controls. Error bars represent standard deviations. *Significant difference ($P < 0.05$), ** ($P < 0.01$) ($n = 3$ each).

et al., 2003). We found that TGF-β_2 increased the phosphorylation of p38 by 2-fold over that of the controls, but did not affect the phosphorylation of Erk1/2 or JNK. In addition to TGF-β_2, the FGF-mediated phosphorylation of p38 through the MKK 3/6 pathway has also been demonstrated, Thus, combined exposure to TGF-β_2 and FGF-2 exerted a synergistic effect on p38 phosphorylation, and the p38 phosphorylation rates in cultured HCECs exposed to both TGF- β_2 and FGF-2 were consistent with the results of the migration assays. We also demonstrated that SB239063 significantly reduced the TGF-β_2- and/or FGF-2-induced migration of HCECs to the level of the control. These findings for the first time indicated that the phosphorylation of p38 MAPK plays an important role in TGF-β_2- and/or FGF-2-induced HCEC migration. Thus, FGF-2 and TGF-β_2 may induce wound healing by stimulating cell migration through the p38 MAPK pathway rather than stimulating cell proliferation. We have not shown that FGF-2-induced proliferation of HCECs, but it has been demonstrated that FGF induced different signal pathways for proliferation and migration. Boilly et al suggested that the FGF induced cell proliferation is more specific through p38 MAPK, and cell migration is more specific through ERK1/2 in many cell types. Chen et al demonstrated an involvement of ERK1/2 activation in rabbit corneal endothelial cell wound healing (Chen et al., 2009), and more recently Lee et al have demonstrated that the FGF-2 mediated HCECs proliferation is through PI 3-kinase and its downstream ERK1/2 pathways (Lee et al., 2011). These findings are in good agreement with our results that FGF-2 increased the phosphorylation of both of p38 and Erk1/2.

Schematic pathways of HCECs proliferation and migration regulated by TGF-β_2 and FGF-2 by our results and the literature are shown in Fig. 8. TGF-β is also known to activate the SMAD pathways which are different from the MAPK pathways, however how these pathways regulate corneal endothelial cells have not been examined extensively. Funaki et al. reported that smad7 suppresses the inhibitory effect of TGF-β_2 on the proliferation of rabbit corneal endothelial cells, but not human cells. It will be necessary to determine more precisely the mechanism of regulating human CECs for future clinical application.

In conclusion, we have shown that TGF-β_2 reduces the proliferation but also stimulates migration of cultured HCECs. In addition, TGF-β_2 and FGF-2 may have synergistic effects on the migration of HCECs through p38 MAPK phosphorylation.

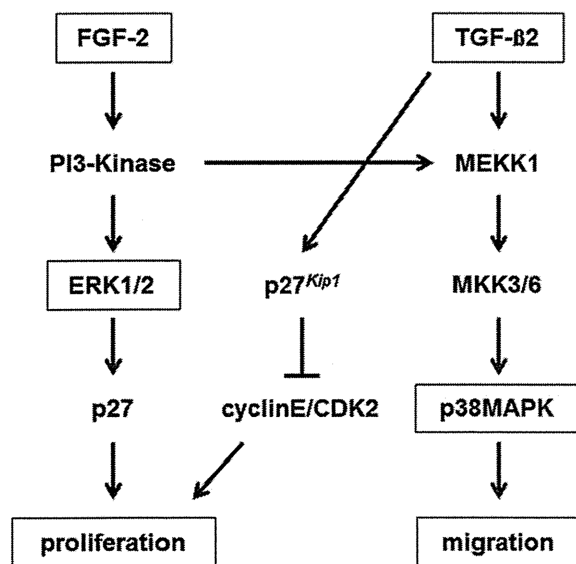

Fig. 8. Schematic pathways of HCECs proliferation and migration regulated by TGF-β_2 and FGF-2. The factors examined in this work were enclosed by lines.

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Combination Effect of Antibiotics Against Bacteria Isolated From Keratitis Using Fractional Inhibitory Concentration Index

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Purpose: To study the in vitro interaction of fluoroquinolones such as levofloxacin (LVFX), gatifloxacin (GFLX), or moxifloxacin (MFLX) in combination with tobramycin (TOB) or cefmenoxime (CMX) against clinical isolates of bacteria from keratitis.

Methods: The activity of each drug alone was determined by an agar dilution method. Checkerboard synergy testing was then performed against 47 isolates, including *Staphylococcus* species, *Streptococcus* species, and *Pseudomonas aeruginosa*. Antimicrobial combinations were classified as synergistic, additive, indifferent, or antagonistic, according to their fractional inhibitory concentration.

Results: The average fractional inhibitory concentration indexes of combined use of LVFX/CMX or GFLX/CMX in *Staphylococcus* species and *Streptococcus* species, and LVFX/CMX, GFLX/CMX, MFLX/CMX in gram-negative rods were low. The additive activity of the following drug combinations were seen in more than 70% of isolates: LVFX/CMX and GFLX/CMX against gram-positive cocci and LVFX/CMX, GFLX/CMX, MFLX/CMX against gram-negative rods. No consistent synergistic or antagonistic effect was observed with the combinations used.

Conclusion: The combination of LVFX/CMX or GFLX/CMX was predominantly additive for all tested isolates.

Key Words: antibiotics, keratitis, synergy, fluoroquinolone, minimum inhibitory concentration

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Keratitis is an infection of the cornea that occurs after injury or in association with wearing of contact lenses. Bacterial infectious keratitis is characterized by corneal inflammation and defects caused by replicating bacteria. When initial therapy against keratitis fails, these infections can progress rapidly and with devastating consequences, including corneal scarring and loss of vision. Common bacterial species causing keratitis have been described in various reports.[1–3] By comparing the findings of keratitis from patient's history and clinical examination with epidemiological data, possible causative bacteria can be inferred at the initial visit.

Because of their drug stability and broad spectra, fluoroquinolone eye drops are increasingly used for treatment of bacterial keratitis in general clinical practice. Monotherapy with fluoroquinolone eye drops has been proven effective, as described in many reports, and has high significance for treatment.[3,4] However, as increasingly more ophthalmic clinical isolates exhibit resistance to fluoroquinolones,[5–8] monotherapy may become ineffective in the future. For reducing not only the development of resistance but also the selective pressure for resistant bacteria, combination therapy with fluoroquinolone + an additional antibacterial eye drop is considered a feasible option. Besides fluoroquinolones, cephalosporin, chloramphenicol, aminoglycosides such as tobramycin and gentamicin, and macrolides such as erythromycin and azithromycin are used as eye drops. Combination of fluoroquinolone with one of these eye drops, as an empiric therapy, has significance in covering many bacterial species and broadening antibacterial spectrum. However, if possible causative bacteria could be inferred, efficacy of combined use of antibacterial eye drops is uncertain.

Recently, in in vitro studies of antibacterial combinations, fractional inhibitory concentration (FIC) index has been used to examine increased activity.[9] However, there are few reports on interactions between fluoroquinolone eye drops, which are used against isolates from keratitis, and other widely used antibacterial eye drops. In this study, we calculated FIC indexes of several combinations of fluoroquinolone eye drops + additional antibacterial drug against bacteria isolated from patients with bacterial keratitis and investigated whether antibacterial combinations may result in increased activity.

MATERIALS AND METHODS

We used 47 bacterial isolates obtained from infectious keratitis at the Department of Ophthalmology of the Ehime University Hospital between 2002 and 2008 (Table 1). Minimum inhibitory concentration (MIC) and FIC index of antibacterial drugs were determined using a checkerboard microdilution method in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI)[10,11] and the American Society for Microbiology (ASM).[12] Liquid

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TABLE 1. Bacterial Strains Used in This Study

Organism	No. Isolates
Methicillin-susceptible *Staphylococcus aureus*	8
Methicillin-resistant *Staphylococcus aureus*	1
Coagulase-negative *Staphylococcus*	20
Pseudomonas aeruginosa	10
Streptococcus pneumoniae	3
Streptococcus species other than *S. pneumoniae*	3
Klebsiella oxytoca	1
Serratia marcescens	1
Total	47

media containing 2 drugs at various concentrations [cation-adjusted Mueller-Hinton broth (CAMHB); for *Streptococcus* species, CAMHB + 2.5% horse hemolysate was used] were inoculated with bacterial suspension and cultured under aerobic conditions at 35°C for 16 to 24 hours. MIC was then determined from minimum drug concentration of wells without bacterial growth, and FIC index was calculated according to the following equation: FIC index = [MIC of drug (a) in combination/MIC of drug (a) alone] + [MIC of drug (b) in combination/MIC of drug (b) alone]. FIC index was rounded to 3 decimal places. Based on the obtained FIC index, interactions were classified as follows: FIC index ≤ 0.5 as synergistic; $0.5 <$ FIC index ≤ 1 as additive; $1 <$ FIC index ≤ 2 as indifferent; and FIC index > 2 as antagonistic.

In experiment 1, levofloxacin (LVFX), a new quinolone antimicrobial, was combined with cefmenoxime (CMX), tobramycin (TOB), erythromycin (EM), or chloramphenicol (CP) and tested against a total of 34 isolates of common bacteria causing keratitis: *Staphylococcus aureus* (6 isolates), coagulase-negative staphylococci (16 isolates), *Streptococcus* species (5 isolates), and gram-negative rods (7 isolates, comprising 5 of *Pseudomonas aeruginosa*, 1 of *Klebsiella oxytoca*, and 1 of *Serratia marcescens*).

In experiment 2, levofloxacin (LVFX), moxifloxacin (MFLX), or gatifloxacin (GFLX) was combined with cefmenoxime (CMX) or tobramycin (TOB) and tested against all the 47 clinical isolates.

RESULTS

Experiment 1. Combined Activity of LVFX + Additional Antibacterial Drug

Mean ± SD and classification according to the criteria of FIC index are shown in Table 2. Against staphylococci (22 isolates), the lowest, and therefore most favorable, FIC index was obtained with LVFX + CMX (1.05 ± 0.48), followed by LVFX + TOB (1.38 ± 0.59). Additive activities of LVFX + CMX and LVFX + TOB were observed in 82% and 55% of isolates, respectively, whereas additive activities of LVFX + EM and LVFX + CP were limited (9% and 5%, respectively). Against *Streptococcus* species (5 isolates), just as against staphylococci, LVFX + CMX had the lowest FIC index (1.05 ± 0.33). LVFX + EM and LVFX + CP also showed favorable FIC indexes. Additive activities of LVFX + CMX and LVFX + CP were observed in 80% of isolates. Against gram-negative rods (7 isolates), FIC indexes were low for all combinations, and especially favorable for LVFX + CMX (1.04 ± 0.44) and LVFX + TOB (1.04 ± 0.46). Additive activities against isolates were observed at high rates. In this experiment, synergistic or antagonistic drug combinations were not found in any isolates.

Experiment 2. Combined Activity of Quinolone + CMX or TOB

LVFX, GFLX, or MFLX was combined with CMX or TOB that showed beneficial interaction with LVFX in experiment 1, and FIC indexes of the combinations were calculated against 35 isolates of gram-positive cocci (29 isolates of *Staphylococcus* species and 6 isolates of *Streptococcus* species) (Table 3). FIC indexes of quinolone + CMX were lower than those of quinolone + TOB. Among quinolone + TOB combinations, LVFX + TOB showed the lowest FIC index. Among quinolone + CMX combinations, GFLX + CMX and LVFX + CMX had similarly low FIC indexes and high rates of additive activity against isolates. FIC indexes were also calculated against 12 isolates of gram-negative rods (Table 4). FIC indexes of quinolone + CMX were lower than those of quinolone + TOB. Among quinolone + TOB combinations, LVFX + TOB showed the lowest FIC index. Among quinolone + CMX combinations, GFLX + CMX and MFLX + CMX had similarly low FIC indexes. In addition, values of MIC90, that is, the minimum concentration required to inhibit the growth of 90% of bacterial isolates, are shown for quinolones alone or in combination with CMX or TOB (Table 5). Against gram-positive cocci, MICs of LVFX combined with TOB and CMX combined with LVFX or GFLX were 1/4 of those of LVFX alone and CMX alone. The lowest MIC90 was obtained with CMX combined with LVFX or GFLX. Against gram-negative rods, MICs of GFLX + CMX and MFLX + CMX were 1/4 of those of GFLX alone and MFLX alone. The lowest MIC90 was obtained with TOB alone or combined with quinolone, LVFX combined with TOB, and LVFX or GFLX combined with CMX.

DISCUSSION

When considering the treatment strategy for bacterial keratitis, it is ideal to infer and administer a drug to which causative bacteria would exhibit the highest sensitivity. Meanwhile, early drug administration is desirable because bacterial keratitis sometimes increases in severity, and visual prognosis may be poor if corneal scarring persists. Because instillation of antimicrobials with high activity, that is, low MIC, is associated with treatment effects on keratitis,[4] it is preferable to use eye drops that have lower MIC against causative bacteria. Although empiric therapy may include monotherapy with an eye drop of third- or fourth-generation fluoroquinolone with broad spectrum and high activity, combination therapy may be a feasible option to treat and reduce infections with fluoroquinolone-resistant bacteria. The present study suggested that antibacterial activity is expected to increase in combination therapy.

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