Ⅰ．臨床所見
　　① 小円形に配列する白色の角膜後面沈着物（コインリージョン）
　　② ①以外の角膜後面沈着物を伴う角膜浮腫
　　③ 角膜内皮細胞密度の減少
　　④ 再発性・慢性虹彩毛様体炎
　　⑤ 眼圧上昇もしくはその既往
Ⅱ．前房水 PCR 検査所見
　　① Cytomegalovirus DNA が陽性
　　② Herpes simplex virus DNA および varicella-zoster virus DNA が陰性

＜診断基準＞
　確定例　　　　　　Ⅰ-①および，Ⅱ-①，②に該当するもの．
　臨床的疑い例　　　ⅠのうちⅠ-②を含む 3 項目以上，および
　　　　　　　　　　Ⅱ-①，②に該当するもの．

＜注釈＞
　1．角膜移植術後の場合は次のような点から拒絶反応が否定的であること．
　　　① 臨床所見で host 側に角膜浮腫がある，あるいは graft 側にのみ角膜浮腫があるが，角膜浮腫と透明角膜の境界に host-graft junction に一致した部分がない．
　　　② 副腎皮質ステロイド薬あるいは免疫抑制薬による治療効果が乏しい．
　2．治療に対する反応も参考所見となる．
　　　① ガンシクロビルあるいはバルガンシクロビルにより臨床所見の改善が認められる．
　　　② アシクロビル・バラシクロビルにより臨床所見の改善が認められない．

（10 mg/kg/日，2 回に分けて点滴）を 10〜14 日間行い，維持治療として 0.5％ガンシクロビル点眼（自家調整薬，1 日 4〜6 回）を行う．低濃度ステロイド点眼薬（0.1％フルオロメトロンなどを 1 日 4 回）を併用する．内服投与が可能なバルガンシクロビルも用いられる．本疾患に対するガンシクロビル，バルガンシクロビルの使用は保険適用外となるため，大学倫理委員会などで承認されたプロトコールに従って，患者の同意を得て行う必要がある．筆者らの症例では，発症後早期にガンシクロビル全身投与を行った場合には速やかに角膜浮腫や KPs が改善され，角膜内皮機能を維持することが可能であった．しかし，発症から確定診断までに長期間が経過した症例では，診断時にすでに著明な角膜内皮密度の低下を生じており水疱性角膜症となる場合があった．筆者らは，全身投与で角膜内皮炎が軽快した症例においても，再発を予防する目的でガンシクロビル点眼を使用することが望ましいと考えているが，いつまで継続する必要があるのかについては今後の検討が必要である．

【CMV は他の前眼部炎症性疾患の原因としても注目されている】

　再発性の虹彩毛様体炎，Posner-Schlossman 症候群や Fuchs 虹彩異色性虹彩毛様体炎においても CMV などのウイルスの関与が報告されており，CMV 虹彩毛様体炎の症例のなかに角膜内皮障害を合併するものがあることが報告されている．これらの前眼部炎症と角膜内皮炎は一連の疾患の異なる病期をとらえている可能性がある．Suzuki ら[4]は角膜内皮炎を含むウイルスが関与する前眼部炎症性疾患を anterior chamber-associated immune deviation（ACAID）-related syndrome として包括的にとらえる新しい概念を提唱しており，興味深い．

【CMV 角膜内皮炎の病態は不明である】

　日本の成人では CMV に既感染である場合が多く，潜伏感染したウイルスが角膜内皮細胞，あるいは隅角組織など角膜内皮近傍の組織において再活性化されて角膜内皮細胞に感染し，炎症を惹起するものと推測されるが，病態は明らかにはされていない．HSV による角膜内皮炎の発症には，ACAID（前房関連免疫偏位：anterior chamber-associated immune deviation）とよばれる前房の特殊な免疫状態が関連していることが Ohashi らによって報告されており，CMV 角膜内皮炎の発症にも ACAID が関与している可能性が高い．しかし骨髄前駆細胞やマクロファージなどに潜伏感染していると考えられている CMV がどのようなルートで角膜内皮細胞に感染を生じるのかについては不明で，今後の課題である．

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Cytomegalovirus Retinitis after Multiple Ocular Surgeries in an Immunocompetent Patient

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Key Words

Cytomegalovirus retinitis · Immunocompetent patient · Multiple ocular surgeries

Abstract

Purpose: To describe the case of a 74-year-old man who developed cytomegalovirus (CMV) retinitis after multiple ocular surgeries.
Methods: Observational case report.
Results: A 74-year-old man who had a history of multiple ocular surgeries developed unilateral retinitis with whitening of the entire peripheral retina. A presumptive diagnosis of viral retinitis was considered, and polymerase chain reaction of the aqueous fluid was positive for CMV DNA. Laboratory examination revealed that the patient was completely immunocompetent. Moreover, the patient did not have any subtenon or intravitreal injection of triamcinolone acetonide (TA). The patient responded well to intravenous ganciclovir and oral valganciclovir.
Conclusion: CMV retinitis can occur to immunocompetent patients without local immunosuppression with TA injection.

Introduction

Cytomegalovirus (CMV) retinitis is a viral inflammation of the retina in immunosuppressed patients, which sometimes results in severe visual loss. We report an immunocompetent patient who developed unilateral CMV retinitis after multiple ocular surgeries.

Case Report

A 74-year-old man who had a cataract surgery, 8 glaucoma surgeries for secondary glaucoma, a vitrectomy for retinal detachment, and a deep lamellar endothelial keratoplasty and 2 penetrating

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keratoplasties for bullous keratopathy for his right eye visited our clinic for a routine examination. He had used 0.05% cyclosporine eye drops once a day for over ten years after the keratoplasties. Funduscopic examination revealed areas of confluent exudate in the entire peripheral retina in his right eye (fig. 1). However, slit-lamp examination revealed no inflammatory cells in the anterior chamber or the anterior vitreous. Although the retinitis did not reach the macular area, the patient's best corrected visual acuity (OD) was hand motion because of optic nerve atrophy due to glaucoma. Fluorescein angiography demonstrated dye leakage in the corresponding peripheral retina, and severely reduced retinal blood flow due to retinal atrophy caused by multiple ocular surgeries (fig. 2). A presumptive diagnosis of viral retinitis was considered, with possible causative organisms including herpes simplex virus (HSV), varicella-zoster virus (VZV), or cytomegalovirus (CMV). Aqueous fluid was aspirated for use in real-time polymerase chain reaction (PCR) analysis, and prior to getting results, 1,500 mg acyclovir was administered intravenously. Real-time PCR of the aqueous fluid was positive for CMV DNA (3.89×10^6 copies/ml) and negative for HSV DNA or VZV DNA. Laboratory examination revealed that the patient was negative for human immunodeficiency virus and had a normal CD4 cell count (611/µl). Immunoglobulin G and A were within the normal range. CMV antigenemia was negative. There were no signs of systemic CMV infection or systemic disease such as diabetes mellitus except for a history of acute myocardial infarction. Since the laboratory analyses indicated CMV retinitis, the cyclosporine eye drops were stopped and the patient was treated with 300 mg intravenous ganciclovir twice daily for 14 days, and two intravitreal ganciclovir injections (0.4 mg). The retinitis lesion slowly decreased in size, but real-time PCR of the aqueous fluid after intravenous ganciclovir administration revealed 9.37×10^5 copies/ml of CMV DNA. Thus, the patient was treated with oral valganciclovir (900 mg/day) for 8 weeks. After disappearance of the retinitis, real-time PCR of aqueous specimens did not detect CMV DNA. However, a recurrence of CMV retinitis was observed two months after finishing oral ganciclovir. After two additional weeks of oral valganciclovir, the retinitis resolved (fig. 3). The left eye showed no CMV retinitis at any time during the follow-up period.

Discussion

CMV retinitis usually affects severely immunosuppressed individuals. However, previous studies have also reported CMV retinitis in immunocompetent patients after intravitreal or subtenon injection of triamcinolone acetonide (TA) [1–3]. Interestingly, most patients who have developed CMV retinitis after using steroids had diabetes mellitus [1–3] and it has been suggested that CMV retinitis and diabetes mellitus may be related [3, 4]. Our patient was immunocompetent based on the results of laboratory examinations and did not have systemic disorders such as diabetes mellitus, nor any history of TA injection. CMV retinitis in immunocompetent patients typically manifests with anterior and vitreous inflammation [1–3], but no inflammatory reaction was observed in our case, similar to the typical manifestation in an immunocompromised patient. Thus, it seems likely that the immune reaction in our patient's right eye was severely suppressed. Although topical betamethasone was administered after the second keratoplasty, it was stopped about six months before the onset of the CMV retinitis. Since the patient had received cyclosporine eye drops for over ten years after the first keratoplasty, one possibility is that long-term use of cyclosporine eye drops induced local immunosuppression, leading to CMV retinitis. However, previous work shows that penetration of cyclosporine eye drops into intraocular tissues is poor [4]. Although our patient had penetrating keratoplasty for the right eye, there is few epithelial damage of the cornea in his right eye, thus it is supposed that his right eye had almost normal corneal barrier function. Moreover, since this patient exhibited recurrences of CMV retinitis even after discontinuing cyclosporine eye drops, other possibilities should be considered. Retinal vasculopathy, such as damaged retinal vascular endothelium and reduced blood flow, is thought to promote leukocyte

entrapment in the retina and reactivation of latently infecting CMV [5, 6]. In our patient, fluorescein angiography demonstrated that retinal blood flow in his right eye was extremely decreased due to multiple ocular surgeries, and this may have some relevance to local immunosuppression.
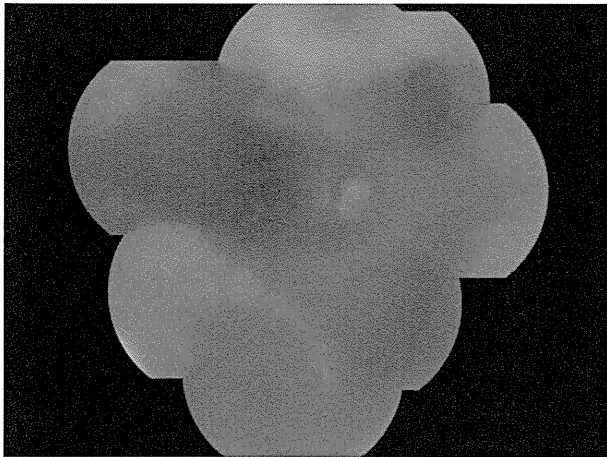

Fig. 1. Fundus photograph of the right eye. Confluent retinitis with whitening of the entire peripheral retina was observed. The optic nerve was atrophic due to glaucoma.
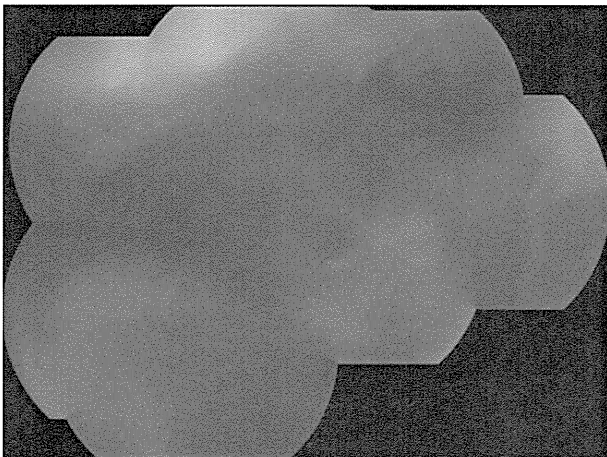

Fig. 2. Fluorescein angiography of the right eye. Dye leakage was observed corresponding with retinal white lesion, and retinal blood flow was severely reduced due to retinal atrophy caused by multiple ocular surgeries.
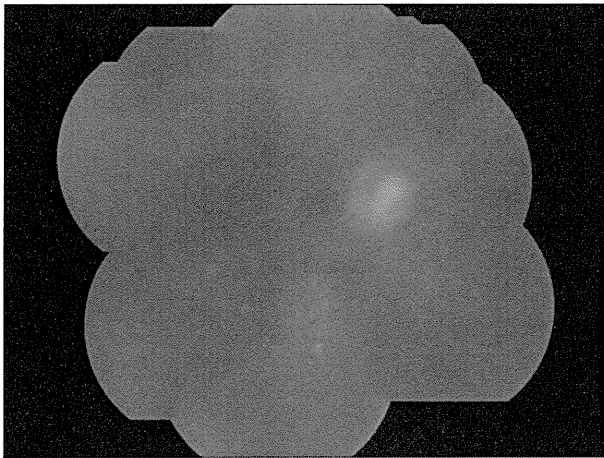
Fig. 3. Fundus photograph after treatment. The whitened retinal area had disappeared. No inflammation was observed during the follow-up period.

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BMC
Ophthalmology

In vivo confocal microscopic and histological findings of unknown bullous keratopathy probably associated with pseudoexfoliation syndrome

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Abstract

Background: Bullous keratopathy (BK), a severe sight-threatening disorder can have a variety of etiologies such as prophylactic laser iridotomy, intraocular surgery, trauma, and other ocular disorders. However, there are cases of unknown origins, among which a unique clinical entity namely pseudoexfoliation syndrome (PEX) is having increased importance.

Case presentation: In this case note, we report the clinical features and in vivo confocal microscopic and pathological findings of two BK cases of unknown cause.

Conclusions: Our findings suggest that the BK was caused by the corneal endotheliopathy of PEX, a common disease that could affect up to 30% of people over 60 years old and is more prevalent than we have believed.

Keywords: Cornea, Bullous keratopathy, Pseudoexfoliation syndrome, Electron microscopy, In vivo confocal microscopy

Background

Bullous keratopathy (BK), a sight-threatening disorder caused by endothelial cell dysfunction, is one of the leading causes for corneal transplantation in many countries. A recent national survey in Japan showed the most common cause of BK was cataract surgery, followed by prophylactic laser iridotomy, glaucoma surgery, trauma, and other ocular diseases [1]. However, there are cases of which the etiology is unknown. In this note, we report the clinical features and in vivo confocal microscopic (IVCM) and pathological findings of two BK patients diagnosed with an unknown cause. Our findings suggest that the BK was probably caused by the corneal endotheliopathy of pseudoexfoliation syndrome (PEX), a common disease that could affect up to 30% of people over 60 years old [2].

Case presentation

Case 1

An 82-year-old man complained of blurred vision in his right eye of one year duration visited our hospital. He had no history of intraocular surgery and trauma, and his medical records showed that he was not taking any medication, such as major tranquilizers, that could cause corneal endotheliopathy.

On our examination, his best-corrected visual acuity (BCVA) was 0.04 (Snellen: 20/500) OD and 0.8 (20/25) OS. The intraocular pressure was 12 mmHg OD and 10 mmHg OS. The right cornea was diffusely edematous and the details of the iris and lens were barely visible (Figure 1A). No inflammatory signs were found in the anterior chamber and vitreous, and the retinal reflex was normal. IVCM showed characteristic changes of the endothelial cells with increased polymorphism and pleomegatism. Abundant PEX materials were found deposited on the corneal endothelial cells (Figure 1B). It was noteworthy that PEX materials were also found in the subbasal layer and stroma of the cornea (Figure 1C).

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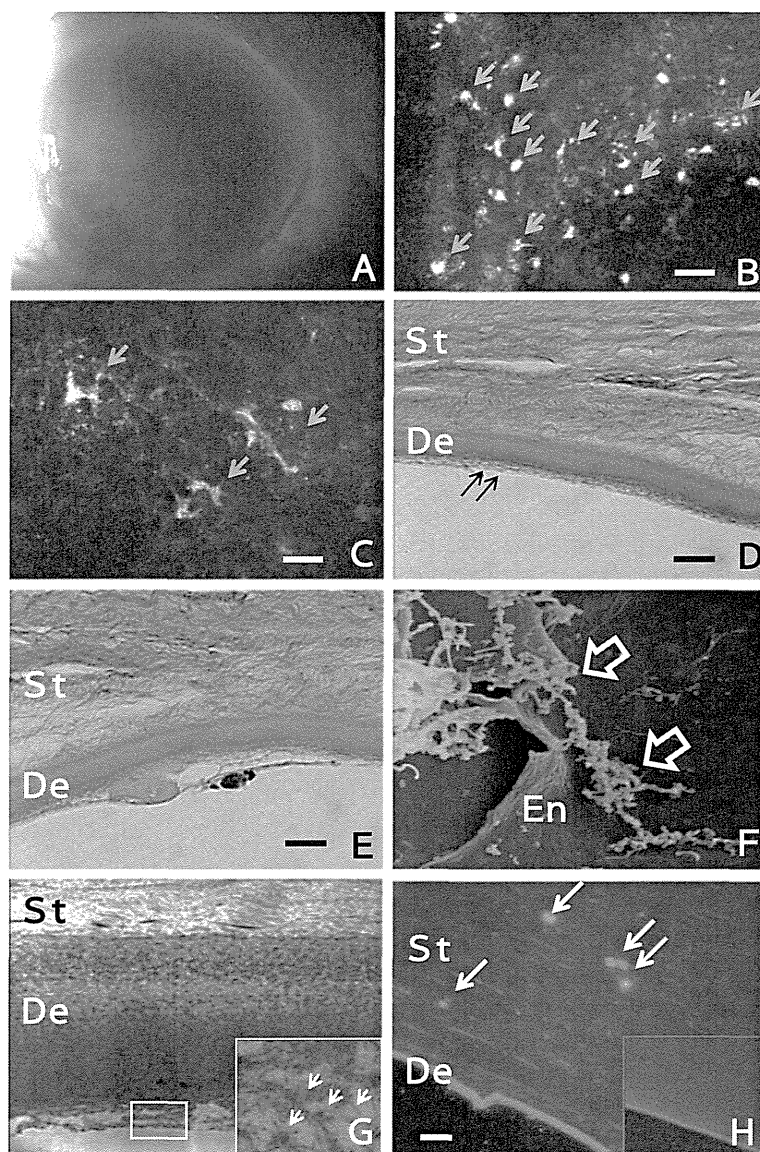Full list of author information is available at the end of the article

Figure 1 Findings in a patient with unknown bullous keratopathy (Case 1). A. Slit-lamp photograph showing diffused corneal edema. B and C. In vivo confocal microscopy of the endothelial layer showing abundant irregular hyperreflective materials most likely PEX materials in the endothelium (B, arrows; Bar = 50 µm) and stroma of the cornea (C, arrows; Bar = 50 µm). D and E. Hematoxylin-eosin staining of light microscopy showing apparent loss of endothelium and formation of fibrillar layer (D, double arrows) and the fibroblast-like change of an endothelium (E). St: stroma, De: Descemet membrane, Bar = 10 µm. F and G. Scanning electronic microscopy showing PEX fibers (open arrows) adjacent to destroyed endothelium (F; En: endothelium) and transmission electron microscopic identification of PEX fibers (arrows in enlarge window) in the thickened Descemet membrane (G; St: stroma, De: Descemet membrane, original magnification: x5000). H. Anti-LOXL1 immunohistochemical staining showing positive staining within the stroma of the cornea (arrows). St: stroma, De: Descemet membrane, Bar = 10 µm. Window image shows negative control staining without LOXL1 antibody.

With an informed consent, this patient underwent penetrating keratoplasty in combination with cataract extraction and intraocular lens insertion. He recovered well with a clear corneal graft and his final BCVA was 0.8 (Snellen 20/25) at his final visit to our department at 3 years after surgery.

The removed cornea was processed for pathology. Histological study showed that there was a marked loss of endothelial cells, formation of fibrillar layer (Figure 1D), and fibroblast-like changes of the remaining cells (Figure 1E). Electron microscopy identified abundant PEX materials deposited on the corneal endothelium and within the thickened Descemet layer (Figures 1F and 1G). Immunohistochemical studies using anti-LOXL1 antibody confirmed positive staining correlating with the PEX materials deposited in the stroma (Figure 1H).

Case 2

A 76-year-old woman had decreased vision in her left eye and was diagnosed with BK of unknown cause. She also had no history of intraocular surgery, laser therapy, and trauma before the onset of BK. On her visit to our hospital, her BCVA was 0.1 (Snellen 20/200) in the left eye, and the intraocular pressure was 13 mmHg. Diffused corneal edema was present and there were no signs of inflammation in this eye (Figure 2A). Both of her eyes had PEX materials in the pupillary area and right lens capsule (Figure 2B). IVCM showed typical PEX materials deposits on the endothelial layers and the anterior stroma of the edematous cornea (Figure 2C).

DSAEK was performed and the removed endothelial layer was examined by electron microscopy. Extensive PEX materials were found on the destroyed endothelial cell layer (Figure 2D). She recovered uneventful after the surgery and her BCVA was 0.9 (Snellen 20/22) at her latest visit 9 month after the surgery.

Discussion

PEX is a common age-related disorder of the extracellular matrix and is frequently associated with severe chronic secondary open angle glaucoma and cataracts [2]. Recently, a mutation of the *LOXL1* gene was shown to be responsible for PEX, indicating a systemic abnormality in this clinical entity [3]. Evidence has been accumulating documenting the morphological alterations in almost all cell layers of the cornea in PEX [4]. The PEX can lead to corneal endothelial cell decompensation, which can result in severe BK, a vision-threatening disorder [2,5].

In our two cases, PEX was the only recognizable factor that could be responsible for the development of BK. To date, there has been no report on simultaneous description of the IVCM and electron microscopic findings in the PEX related BK in the literature. The IVCM findings in this report were in agreement with our earlier observations that the endothelial cell density was significantly decreased and PEX materials were precipitated on the endothelial cells. More interestingly, the PEX materials were also found in the stroma as detected by our immunohistochemical study.

The origin of the PEX materials is still controversial although fibroblastic changes of endothelial cells have been suggested to be responsible for PEX material formation [2]. Our findings also showed that IVCM is a useful noninvasive and rapid method for diagnosing PEX endotheliopathy. Early diagnosis of these patients before intraocular surgery or laser iridotomy is critical for better preservation of the endothelial function. Our recent survey showed that the PEX endotheliopathy comprises up to 50% of the unknown BK cases in Japan (Japanese Ministry of Health PEX Endotheliopathy Study Group). Alert should be raised to this unique clinical entity that relates to aging process, bilaterally involved and is probably more prevalent than we have believed.
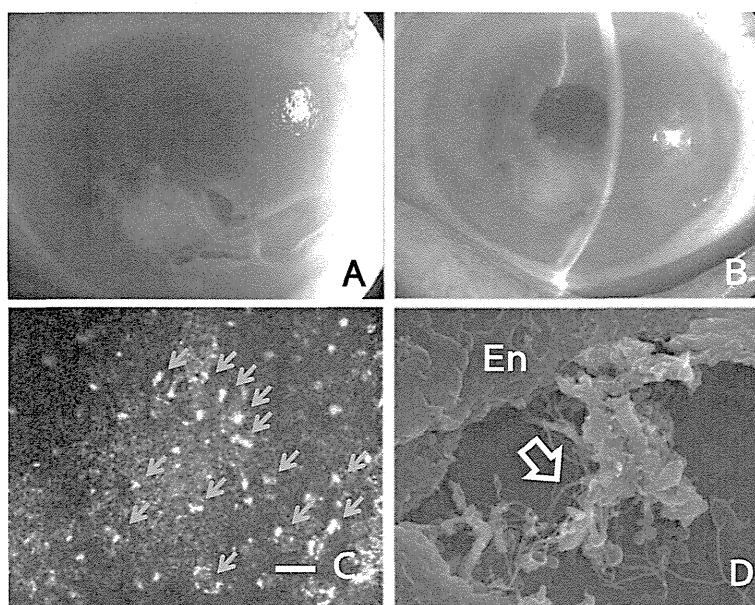

Figure 2 Findings in another patient with unknown bullous keratopathy (Case 2). A. Slit-lamp photograph showing diffused corneal edema and giant epithelial bullae. B. Slit-lamp photograph showing PEX materials on the pupillary iris. C. In vivo confocal microscopy showing PEX materials on the endothelial cells (arrows; Bar = 50 µm). D. Scanning electronic microscopy showing fabric PEX materials (open arrow) adjacent to a destroyed endothelium. En: endothelium, original magnification: x5000.

Conclusions

Our in vivo confocal microscopic and histopathological studies showed the endothelial dysfunction of the two cases are probably associated with pseudoexfoliation syndrome. PEX related corneal endotheliopathy should be taken into consideration as one possible etiology for bullous keratopathy of unknown origin. In vivo confocal microscopy is a useful tool for assistance of the diagnosis.

Consent

Written informed consent was obtained from the two patients for publication of this report and any accompanying images.

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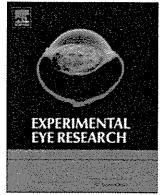# Involvement of P38MAPK in human corneal endothelial cell migration induced by TGF-β2

Takeshi Joko [a], Atsushi Shiraishi [a,b,*], Yoko Akune [a], Sho Tokumaru [c], Takeshi Kobayashi [b,d], Kazunori Miyata [f], Yuichi Ohashi [a,e]

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ARTICLE INFO

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-β2 (TGF-β2) 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-β2 on the proliferation and migration of cultured human corneal endothelial cells (HCECs). To achieve this, we first examined the effect of TGF-β2 on the wound closure rate in an in vitro HCEC wound healing model. However, unexpectedly TGF-β2 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-β2 (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-β2 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-β2 (10 ng/ml) affect HCEC migration. Exposure to TGF-β2 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-β2 and/or FGF-2. The effect of the p38 MAPK inhibitor, SB239063 (10 μM), on TGF-β2 and/or FGF-2-induced cellular migration was determined by the Boyden chamber assay. Both TGF-β2 and FGF-2-induced p38 phosphorylation, and a synergistic effect was observed with exposure to both growth factors. SB 239063 inhibited TGF-β2 and FGF-2-induced migration of HCECs. These results indicate that TGF-β2 reduces proliferation but stimulates migration of cultured HCECs. In addition, TGF-β2 and FGF-2 may have synergistic effects on the migration of HCECs mediated by p38 MAPK phosphorylation.

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 Can-vas™ 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).
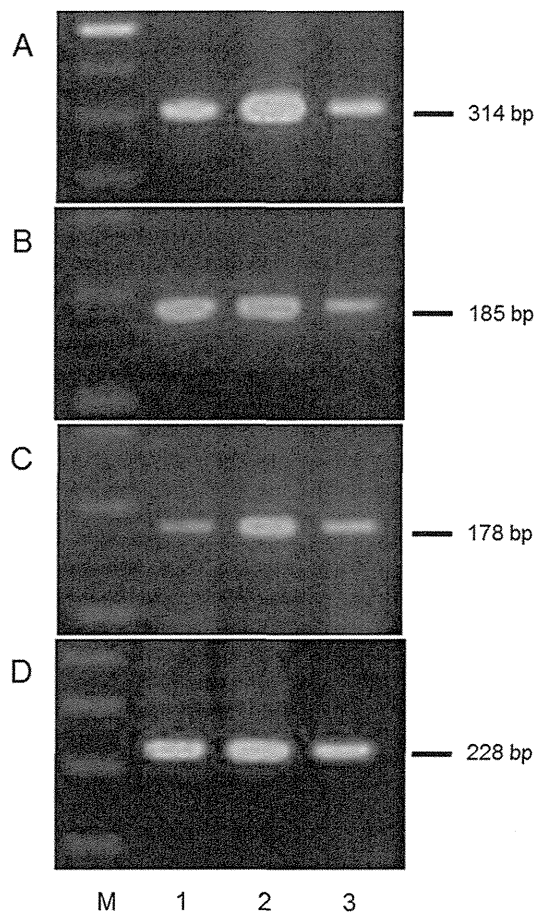

Fig. 1. Determination of the expression of the mRNA of TGF-β receptor in cultured human corneal endothelial cells (HCECs). Ethidium-bromide-stained agarose gels showing the RT-PCR products for TGF-β type I receptor (A), TGF-β type II receptor (B), TGF-β type III receptor (C) and GAPDH (D). Lane 1: Subconfluent 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).
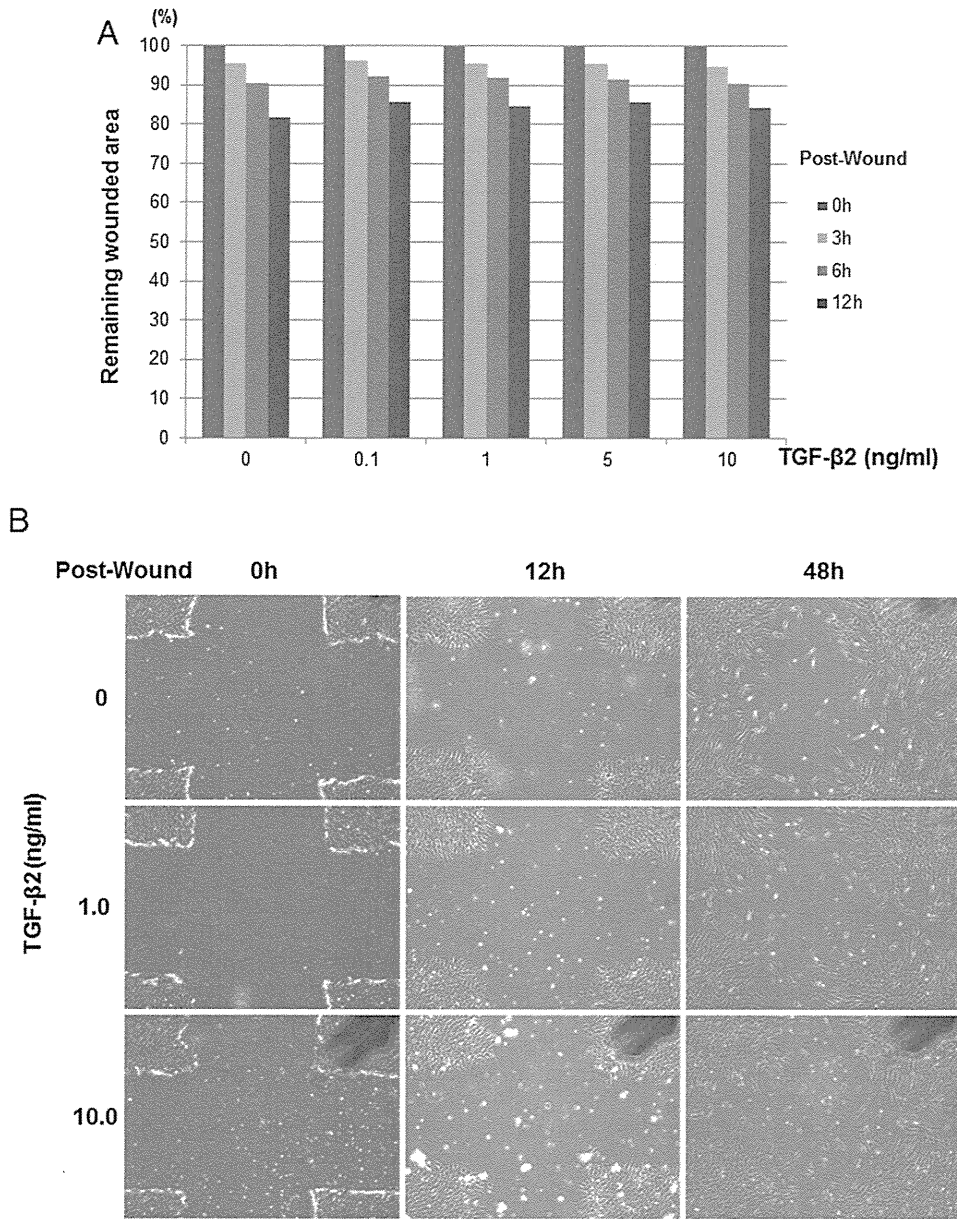
Fig. 2. Effect of TGF-β_2 on in vitro HCEC Wound Healing. Linear wounds were made with a 1 ml pipette tip and the acellular area was measured at 0, 3, 6, 12, 24, and 48 h after exposure to TGF-β_2 at concentrations of 0.1–10.0 ng/ml. A. No significant difference was observed between the speed of wound 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.
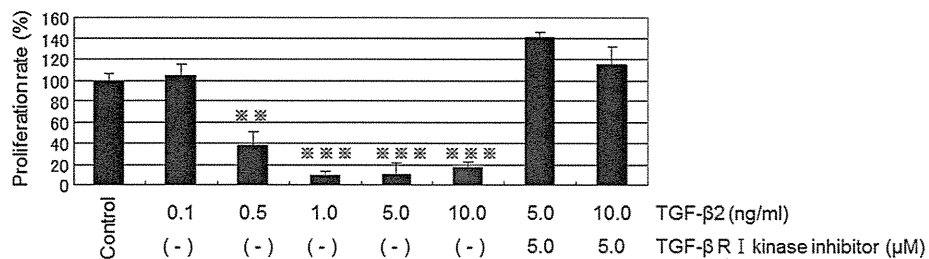
Fig. 3. Effect of TGF-β_2 on proliferation of HCECs measured by RT-CES system. Exposure to 0.5–10.0 ng/ml of TGF-β2 has a significant inhibitory effect on the proliferation of HCECs. The TGF-β receptor I kinase inhibitor (5.0 ng/ml) significantly attenuated the TGF-β_2 inhibition of HCEC proliferation. Error bars represent standard deviations. Significant difference ** ($P < 0.01$) *** ($P < 0.001$) from controls ($n = 3$ each).
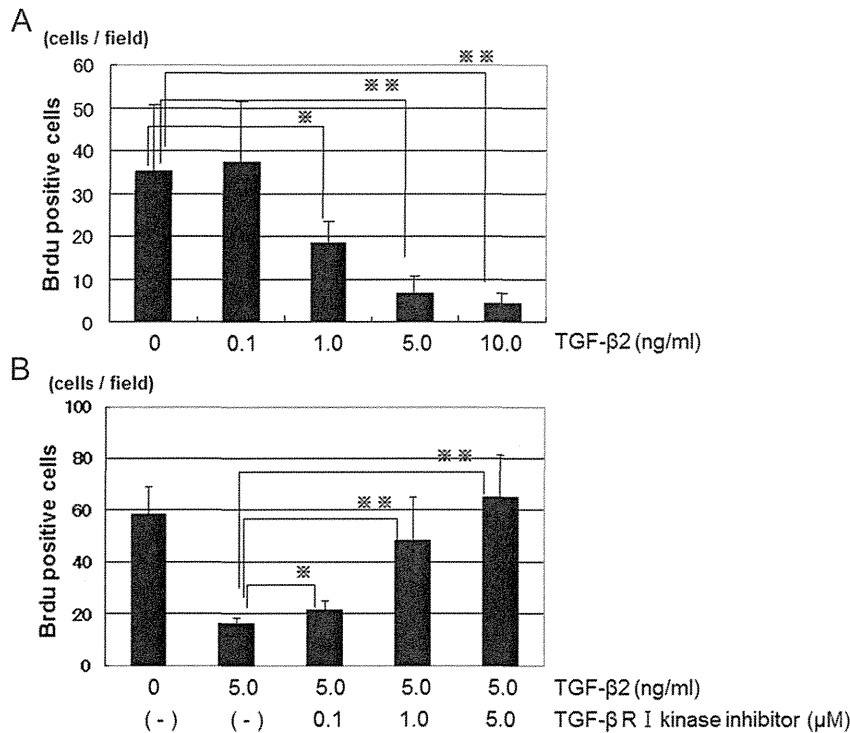
Fig. 4. Effect of TGF-β_2 on HCEC proliferation during Wound Healing. The BrdU incorporation assay was carried out in an in vitro HCEC wound healing model in the presence of 0.1–10 ng/ml TGF-β_2. A. The number of BrdU positive cells significantly decreased when samples were treated with 1.0–10.0 ng/ml of TGF-β_2. B. Pre-incubation with TGF-β receptor I kinase inhibitor significantly inhibited the TGF-β_2-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-β_2 and FGF-2 on migration of human corneal endothelial cells in Boyden chamber

Although treatment with TGF-β_2 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-β_2.

To test this, we examined the effect of TGF-β_2 and FGF-2 on the migration of HCECs with the Boyden chamber assay. HCECs were incubated for 8 h with 10 ng/ml TGF-β_2, 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-β_2 or FGF-2. When TGF-β_2 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-β_2 or FGF-2 alone (Fig. 5A). To demonstrate the specificity of the TGF-β_2-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-β_2-induced HCEC migration to the level of the control (Fig. 5B).

5.2. Phosphorylation of MAPKs by TGF-β_2 and FGF-2

Because TGF-β_2 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-β_2 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-β_2 and FGF-2, but not TGF-β_2 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-β_2 or FGF-2 alone, and about 4-fold when exposed to TGF-β_2 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-β_2 or FGF-2 alone. Strong phosphorylation was detected when HCECs were exposed to TGF-β_2 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-β_2 and/or FGF-2 (Fig. 6).

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

To determine whether TGF-β_2 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-β_2 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-β_2 on cultured HCECs. In the initial experiments, we examined the effect of TGF-β_2 in an *in vitro* HCEC wound healing
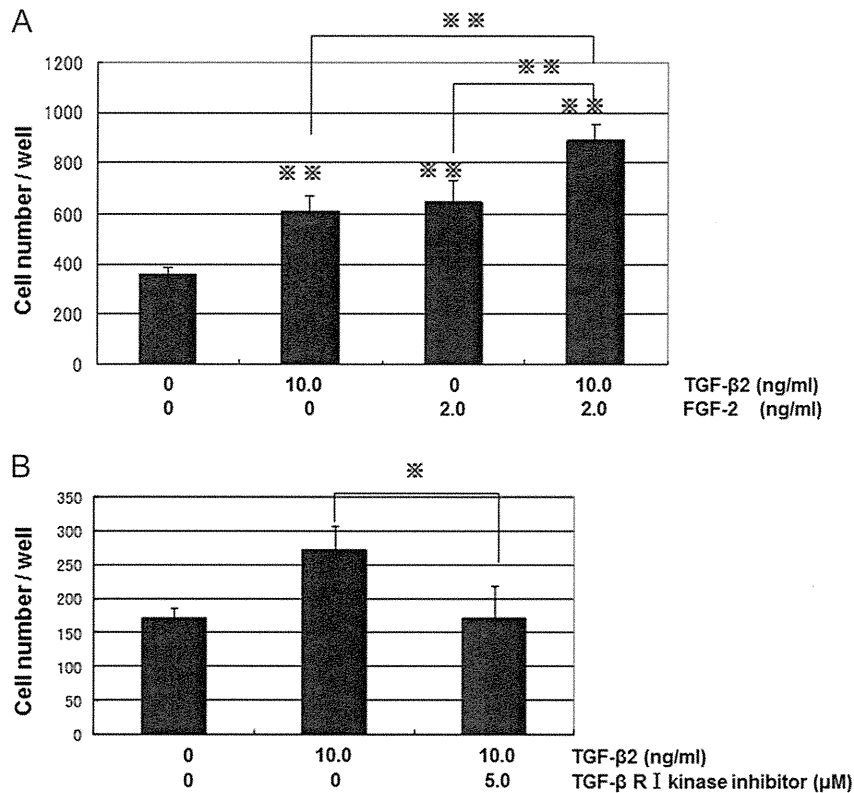
Fig. 5. Effect of TGF-β_2 on HCEC migration. A. The effect of TGF-β_2 and FGF-2 on the degree of HCEC migration was quantitatively determined by Boyden chamber assays. HCEC migration increased by 1.7-fold in the TGF-β_2 sample, and 1.7-fold in the FGF-2 sample compared with the controls. When TGF-β_2 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-β_2 or FGF-2 alone. B. To confirm that cellular migration was mediated by TGF-β_2, cultured HCECs were cultured with 5 μM of TGF-β receptor I kinase inhibitor. TGF-β receptor I kinase inhibitor significantly reduced the TGF-β_2-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-β_2 had no effect on the speed of wound healing of injured cultured HCECs.

Therefore, we further investigated the effect of TGF-β_2 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-β_2 at concentrations of 1.0–10 ng/ml. The effect of TGF-β_2 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-β_2 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-β_2 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-β_2 $\geq$ 0.5 ng/ml inhibited the growth of HCECs. This indicated that the TGF-β_2 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-β_2 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-β_2 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-β_2 if combined with 2.0 ng/ml of FGF-2. Thus, the presence of TGF-β_2 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-β_2 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
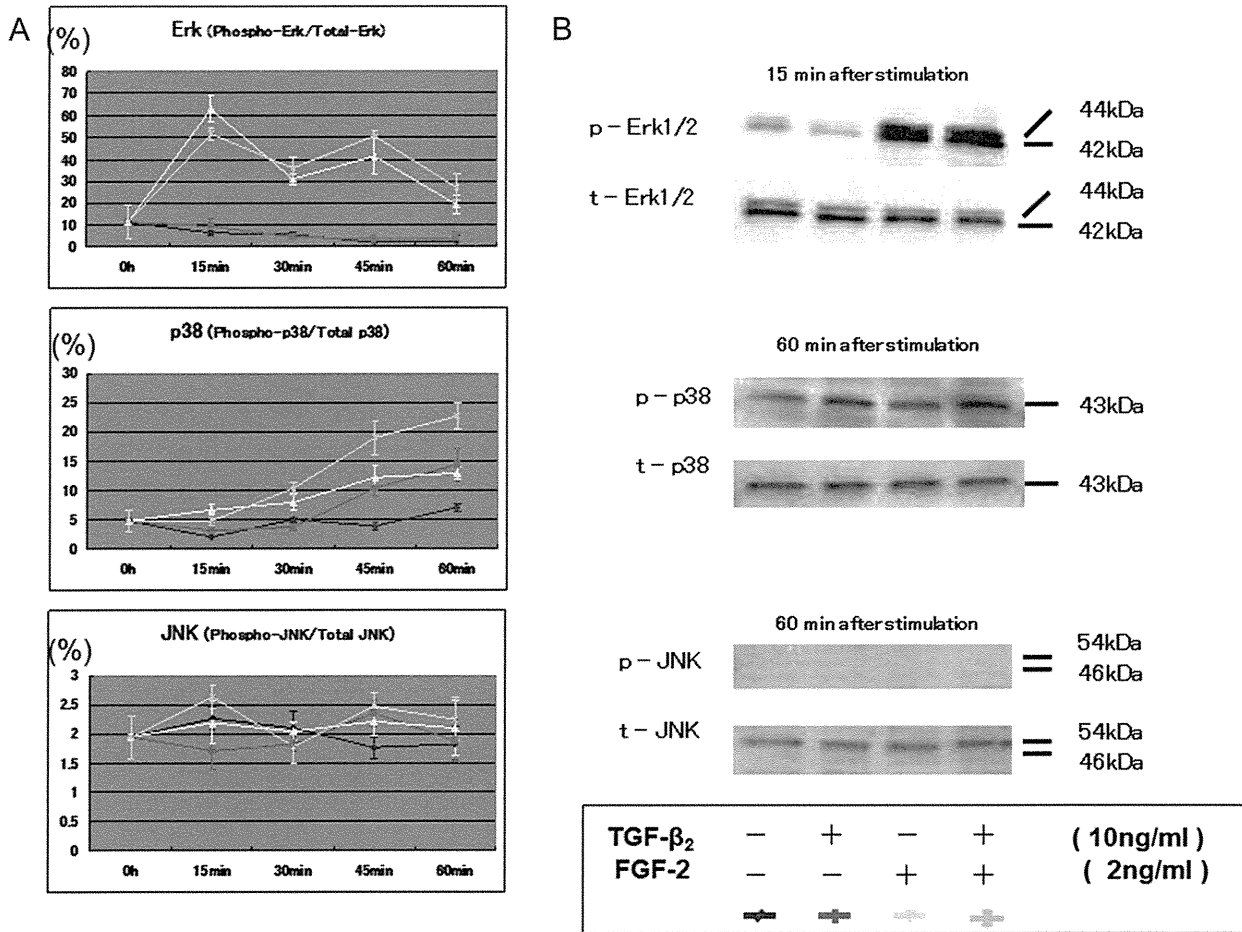
Fig. 6. Activation of MAPK by TGF-β2 and/or FGF-2 in HCECs. The effect of 10 ng/ml TGF-β_2 and/or 2 ng/ml FGF-2 on the phosphorylation of Erk, p38 and JNK was determined by Bio-Plex™ Suspension array system ($n = 4$) (A) and Western blot analysis (B). A. Erk1/2 was phosphorylated as early as 15 min after 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.
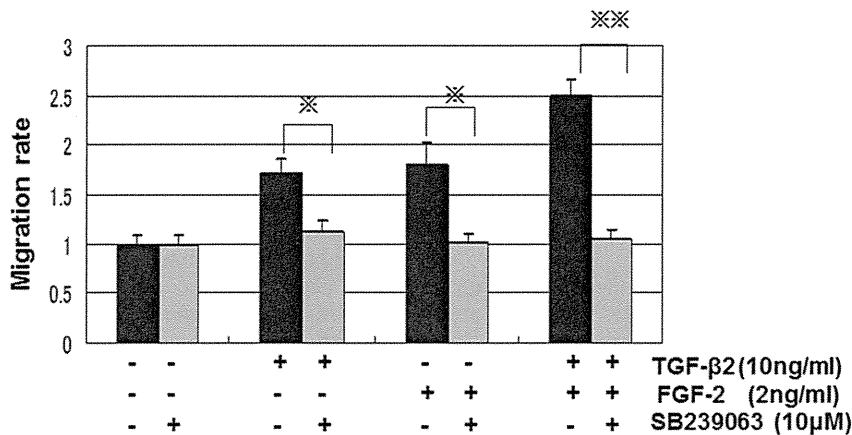

Fig. 7. Effect of p38 MAPK inhibitor on HCEC Migration to determine whether the TGF-β_2- and/or FGF-2-induced migration of HCECs was regulated by p38 MAPK activation, the migration of HCECs was determined quantitatively by Boyden chamber assays in the presence of a p38MAPK inhibitor, SB239063. SB239063 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.
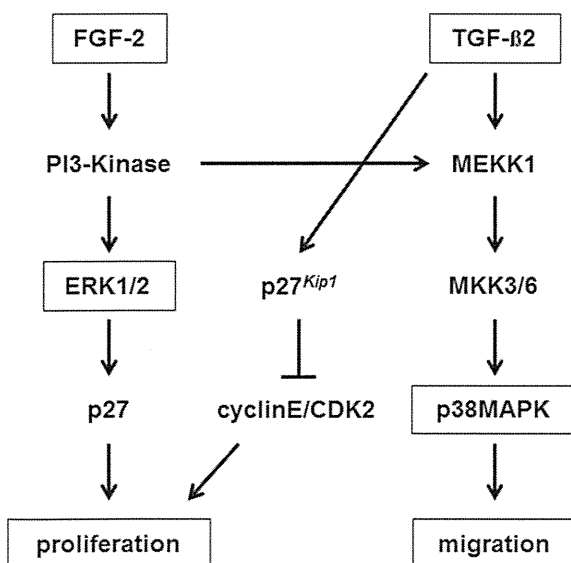

Fig. 8. Schematic pathways of HCECs proliferation and migration regulated by TGF-β_2 and FGF-2. The factors examined in this work were enclosed by lines.

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Clinical science

Multicentre clinical study of the herpes simplex virus immunochromatographic assay kit for the diagnosis of herpetic epithelial keratitis

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ABSTRACT

Background/aims The novel immunochromatographic assay (ICGA) kit was recently developed to diagnose herpes simplex virus (HSV) infection. This multicentre study aimed to evaluate the value of the ICGA kit for the diagnosis of herpetic epithelial keratitis by comparing it with immunofluorescence assay (IFA) and real-time PCR.
Methods Corneal scrapings were collected from 117 patients, including 77 with herpetic keratitis as their final clinical diagnosis as well as 40 others at 21 facilities. These samples were tested by the ICGA kit, IFA and real-time PCR.
Results The positive concordance between final clinical diagnosis and ICGA was 46.7% (35/75 cases) and the negative concordance was 100% (39/39). The positive and negative concordance between real-time PCR and ICGA were 57.4% (35/61 cases) and 100% (53/53), respectively. The positive and negative concordance between IFA and ICGA were 61.1% (22/36 cases) and 83.3% (55/66), respectively. In 92 cases where anti-HSV drugs were not prescribed prior to corneal scraping, the positive and negative concordance between final clinical diagnosis and ICGA were 55.0% (33/60 cases) and 100% (32/32), respectively.
Conclusions The ICGA kit has moderate sensitivity and high specificity, indicating clinical utility in the diagnosis of herpetic epithelial keratitis.

INTRODUCTION

Herpetic epithelial keratitis has been diagnosed mostly based on clinical manifestation as it is easily recognised as dendritic keratitis with terminal bulb in typical textbook cases. However, in actual clinical settings, there are plenty of atypical cases in which diagnosis is difficult by clinical manifestation alone, sometimes leading to misdiagnosis.

For accurate diagnosis, the detection of herpes simplex virus (HSV) is essential. The gold standard of HSV detection is culture of live HSV; however it is very low sensitivity[1–4] and time-consuming. It requires constant preparation of indicator cells and special equipment and expertise, and is therefore not suitable for clinical examination. Immunofluorescence assay (IFA)[4 5] and PCR[5–8] have been available as practical means of HSV detection in clinical settings; however both necessitate expensive equipment and special expertise and are difficult

procedures for general ophthalmologists to perform. In addition, PCR is too sensitive to detect only pathological-level HSV. HSV is constantly shedding in the ocular surface, and can be detected in normal individuals by highly sensitive PCR.[9–11]

Recently the novel immunochromatographic assay (ICGA) kit (Checkmate Herpes Eye, Wakamoto Pharmaceutical Co., Tokyo, Japan) has been developed to diagnose HSV keratitis.

This ICGA kit utilises the monoclonal antibody against HSV glycoprotein D, which is expressed in the virions, infected cells in all HSV strains; it is essential for infectivity,[12] and highly conserved between HSV-1 and HSV-2 as a type-common antigen.[13 14] This kit can be performed as an 'in-the-office' diagnostic test within 15 min, and does not require specialised equipment and expertise.

The aim of this study was to evaluate the utility of the newly developed ICGA for the diagnosis of herpetic epithelial keratitis by comparing it with IFA and real-time PCR in a multicentre study.

MATERIALS AND METHODS
Subjects

A total of 117 patients with corneal epithelial lesions necessitating differential diagnosis of herpetic keratitis at 21 ophthalmological facilities belonging to the HSV ICGA Kit Study Group participated in this study from June 2008 to August 2009. There were 66 men and 51 women, with a mean age of 60.3±18.2 years. Corneal scrapings from 48 right eyes and 69 left eyes were collected by cotton swab after topical anaesthesia was applied; these scrapings were used in ICGA (Checkmate Herpes Eye), real-time PCR and IFA.

The criteria for the clinical diagnosis of herpetic epithelial keratitis were defined as follows: typical dendritic keratitis was defined as a dendritic form epithelial defect with terminal bulb, intraepithelial infiltration on the border and normal appearance of the epithelium surrounding the area of the lesion, and diagnosed as herpetic epithelial keratitis in cases where there was a response to anti-HSV treatment. In addition, atypical dendritic form, stellate form, and geographic form epithelial lesions were diagnosed as herpetic epithelial