4）　トラブルのあった目

右目が32.0%，左目が15.5%，両目が52.6%であった（図4）。

5）　自覚症状（複数回答可）

右目ならびに左目に生じたトラブルの自覚症状を図5，6に示す。充血（右目が25.5%，左目が22.7%，以下同様），目の痛み（21.3%，19.3%）を訴えるものが多く，視力低下（4.6%，4.5%），かすみ（8.3%，8.0%），めやに（13.4%，13.1%），乾燥感（11.1%，14.8%），かゆみ（9.3%，10.2%），その他（6.5%，7.4%）であった。

6）　診断名（複数回答可）

右目ならびに左目の診断名を図7，8に示す。点状表層角膜症（13.0%，11.1%），角膜上皮びらん（角膜上皮剝離を含む）（26.1%，22.2%），角膜浸潤（4.3%，　0%），角膜潰瘍（10.9%，11.1%），角膜浮腫（2.2%，2.8%），角膜血管新生（2.2%，0%），結膜充血（19.6%，25.0%），角膜内皮障害（4.3%，2.8%）で，その他（17.4%，25.0%）であった。

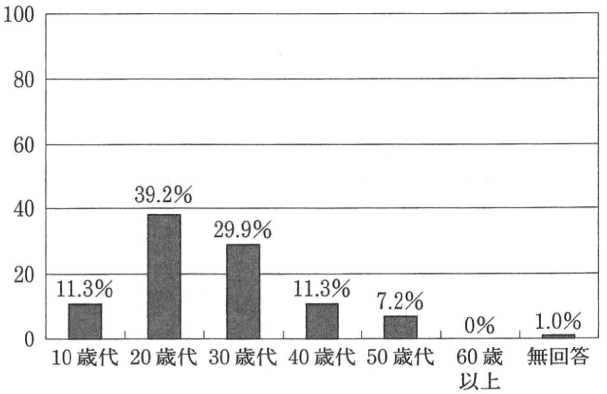

図2　年齢（年代別）

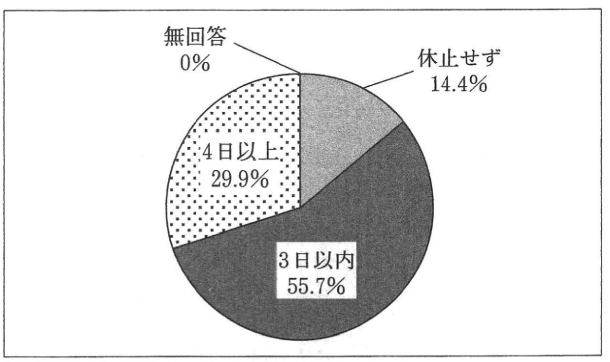

図3　休止期間

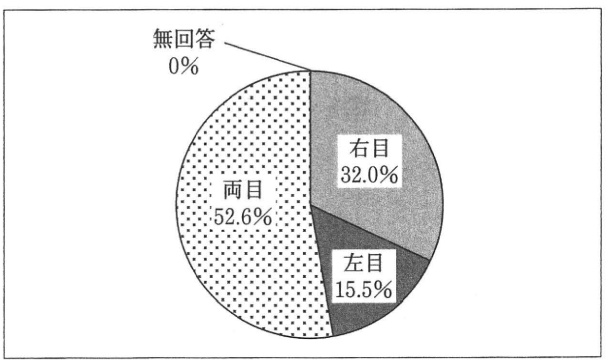

図4　トラブルのあった目

7）　CLの種類

ガス透過性レンズを含むハードコンタクトレンズ（以下HCL）が19.6%，従来型SCLが13.4%，1日使い捨てSCLが10.3%，1週間連続装用使い捨てSCLが2.1%，2週間頻回交換SCLが43.3%，1〜6ヶ月定期交換SCLが6.2%，カラーSCLが1.0%，度数のないカラーSCLが4.1%であった（図9）。

8）　CLの購入先

CL診療を主とする診療所に併設する販売店（量販店を含む）が42.3%，一般病院内または併設する販売店が3.1%，眼科医療機関に併設する販売店が27.8%，眼鏡店が10.3%，通信販売が2.1%，インターネットが7.2%，薬局が1.0%，その他が6.2%であった（図10）。

9）　CLの処方施設

大学病院が1.0%，一般病院（大学病院を除く）が4.1

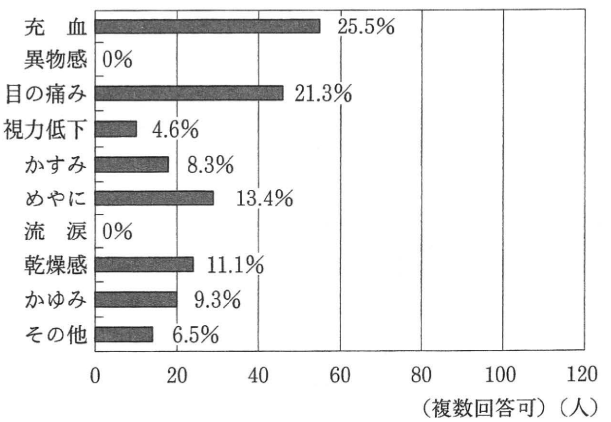

図5　自覚症状（右目）

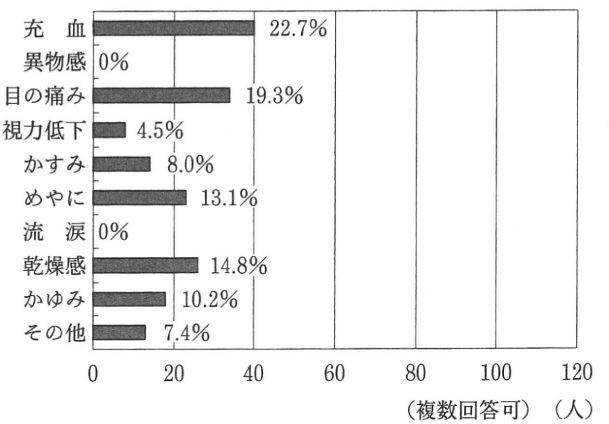

図6　自覚症状（左目）

%，一般眼科診療所が33.0%，CL診療を主とする診療所が44.3%，眼鏡店に併設する眼科診療所が4.1%，医師の処方を受けなかったが11.3%，その他が2.1%であった（図11）。

10）　装用状況

連続装用が8.2%，終日装用が91.8%であった（図12）。

11）　装用日数

毎日が67.0%，週6日が9.3%，週5日が10.3%，週3〜4日が4.1%，週1〜2日が2.1%，不規則が5.2%，その他が2.1%であった（図13）。

12）　装用時間

1日16時間以上が20.6%，8〜16時間未満が66.0%，4〜8時間未満が7.2%，4時間未満が2.1%，不規則が1.0%，その他が3.1%であった（図14）。

13）　取扱説明書の受け取り

受け取ったが86.6%，受け取らなかったが13.4%であった（図15）。

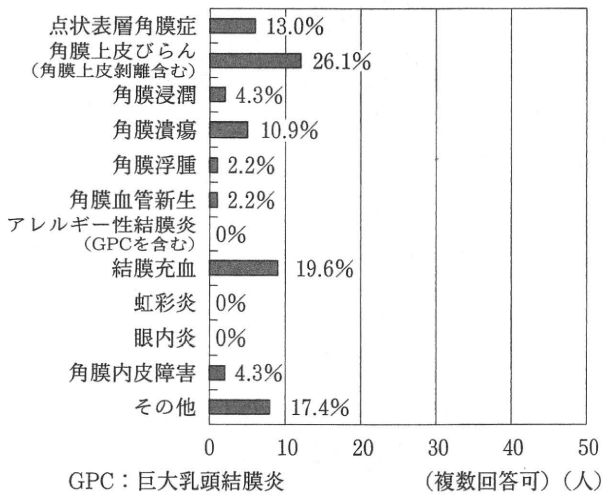

図7　診断名（右目）

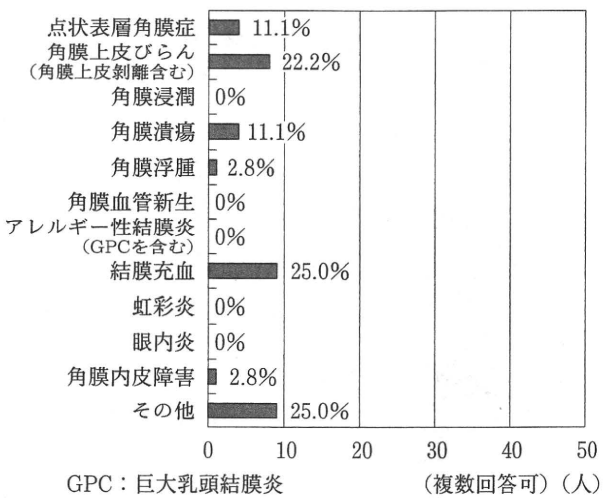

図8　診断名（左目）

14）　定期検査

1ヶ月に1回が1.0%，3ヶ月に1回が15.5%，6ヶ月に1回が18.6%，年に1回が11.3%，不定期に受けていたが20.6%，全く受けていなかったが29.9%，その他が3.1%であった（図16）。

15）　CLの洗浄

毎日洗浄していたが71.1%，週に2〜3回洗浄してい

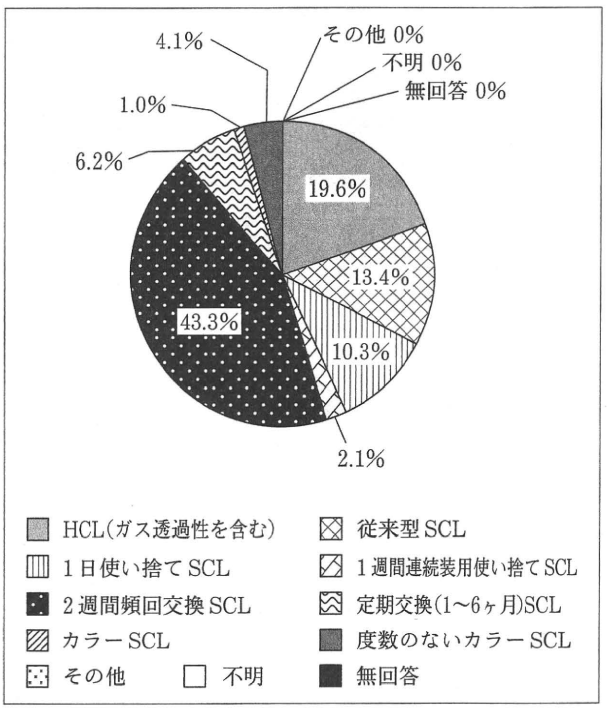

図9　CLの種類

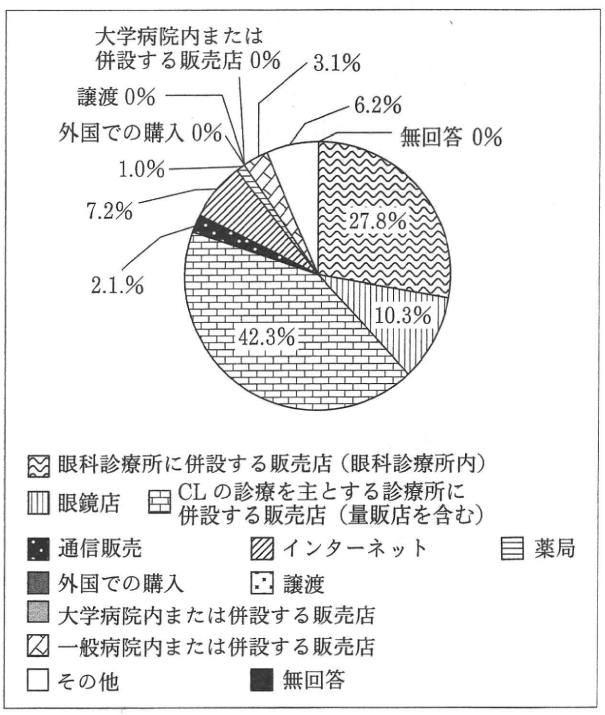

図10　購入先

たが5.2%，週に1回洗浄していたが2.1%，ほとんど洗浄していなかったが4.1%，全く洗浄していなかったが10.3%，その他が7.2%であった（図17）。

16）SCLの消毒

毎日消毒していたが42.3%，週に1回消毒していたが1.0%，週に2〜3回消毒していたが4.1%，時々消毒していたが3.1%，ほとんど消毒していなかったが3.1%，消毒はしていなかったが5.2%，その他が4.1%，無回答が37.1%であった（図18）。

17）SCLの消毒剤

マルチパーパスソリューション（MPS）が22.5%，過酸化水素消毒剤が5.6%，不明が71.8%であった（図19）。

18）装用方法の遵守（1日および1週間連続装用使い捨てSCL・2週間頻回交換SCLについて）

守っていたが46.3%，ほぼ守っていたが20.4%，時々守っていたが5.6%，守っていなかったが11.1%，無回

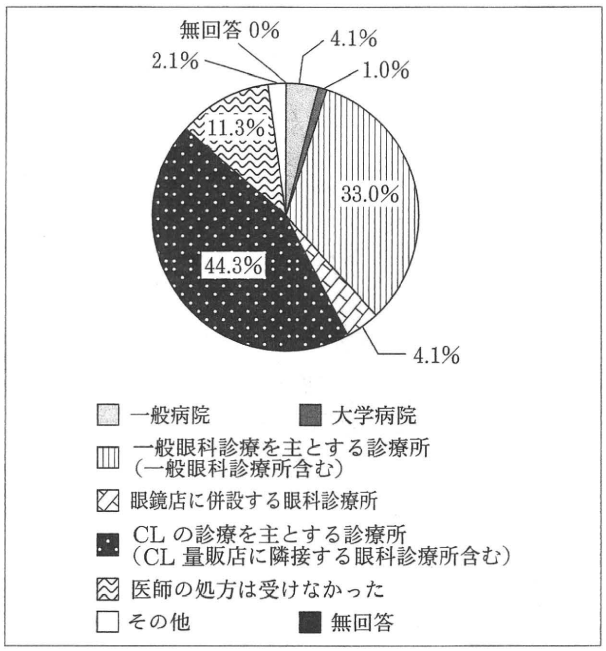

図11　処方施設

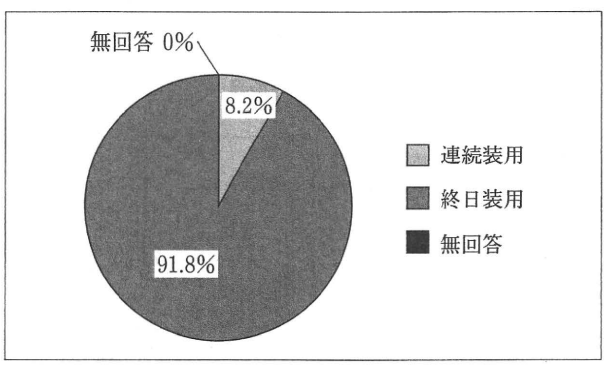

図12　装用状況

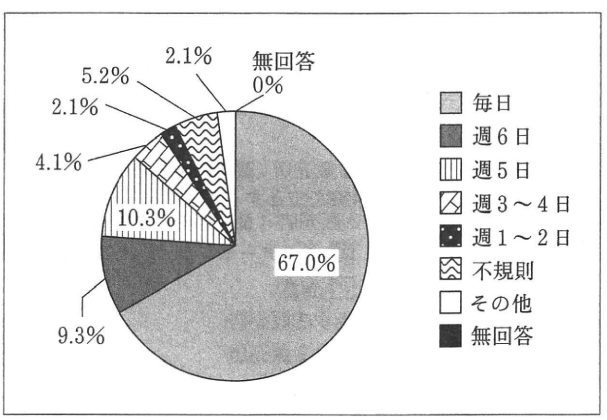

図13　装用日数

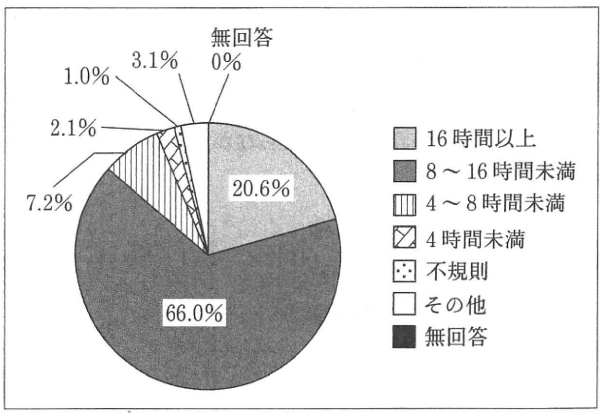

図14　装用時間

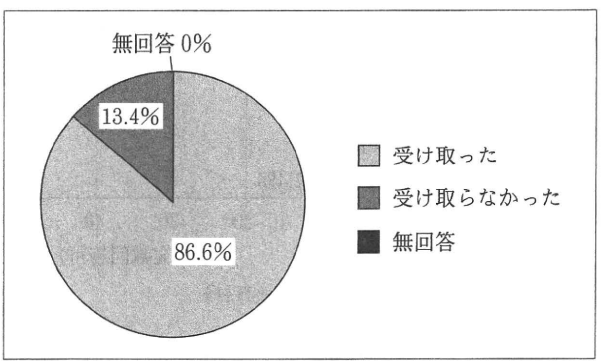

図15　取扱説明書

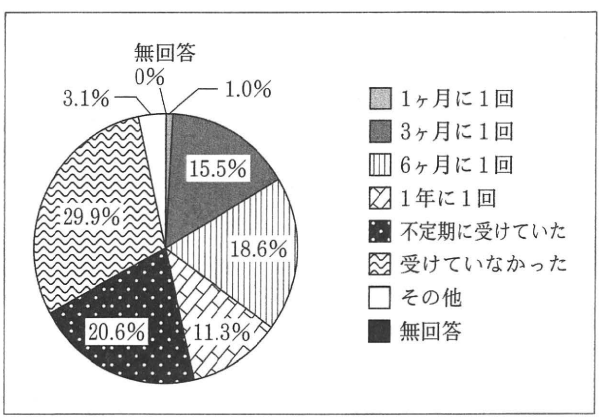

図16　定期検査

答が16.7%であった（図20）。

19）装用日数（1日および1週間連続装用使い捨てSCL・2週間頻回交換SCLについて）

1～7日が38.9%，8～14日が22.2%，15～30日が9.3%，31日以上が3.7%，無回答が25.9%であった（図21）。

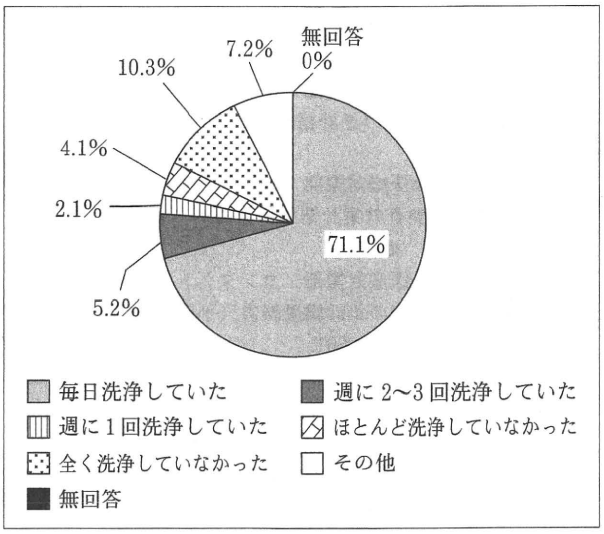

図17　洗　浄

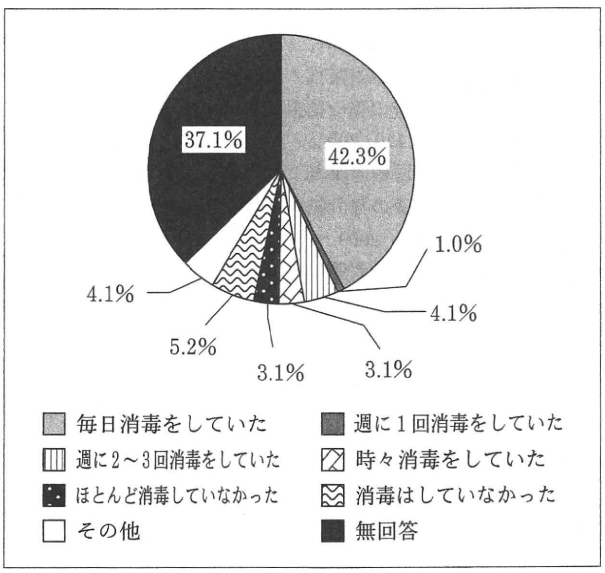

図18　SCLの消毒

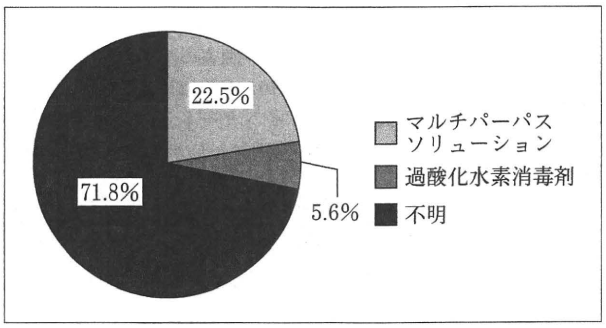

図19　SCLの消毒剤

考　察

これまでの本調査の報告件数を表1に示すが，アンケートに協力いただいた方は，日本眼科医会，日本コンタクトレンズ学会，日本コンタクトレンズ協会のホームページを自ら見たということで，CLへの関心が強いといえる。

CLは両眼に装用する場合が多いので，トラブルを生じるのも両眼であることが多い[10-13]。本調査の結果でも両眼のトラブルが52.6%と半数以上であった。自覚症状としては，充血，目の痛み，乾燥感，かゆみなどが多く，診断名としても結膜充血，角膜上皮びらん（角膜上皮剥

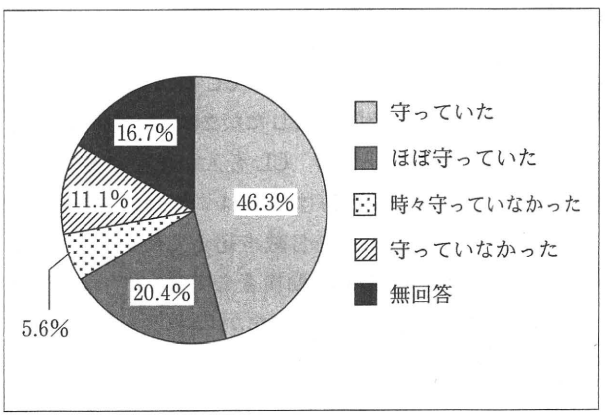

図20　装用方法の遵守
（1日および1週間連続使い捨てSCL，2週間頻回交換SCL）

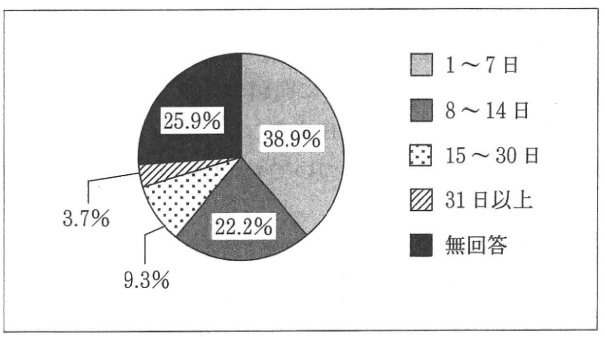

図21　装用日数
（1日および1週間連続使い捨てSCL，2週間頻回交換SCL）

表1　眼障害アンケート調査の報告件数

調査期間	報告件数
平成14年1月～ 平成18年5月31日	330件
平成18年6月20日～ 平成19年7月4日	203件
平成19年7月5日～ 平成20年7月4日	137件
平成20年7月5日～ 平成21年7月4日	141件

離を含む），点状表層角膜症が多かった。CLの合併症としてもっとも重篤と考えられる角膜潰瘍も10%以上（右眼10.9%，左眼11.1%）に認められたが，これまでの日本眼科医会の調査結果と同等の割合であった[1-9]。

日本眼科医会が平成20年に9,904名のCL使用者に行ったアンケート調査結果[14]によると，これまでにCLの使用を一時中止しなければならないようなトラブルを経験した者は49.9%（1回が19.5%，2〜5回が25.9%，6回以上が4.5%）で，これらのトラブルのうち，もっともひどいトラブルが良くなるまでに要した日数は4〜7日が33.6%ともっとも多く，2〜3日が28.9%，1週間〜1ヶ月が25.2%，1ヶ月以上が5.2%，1日が4.3%，治っていないが0.5%であった。多くのCL使用者がトラブルを経験しており，それも比較的ひどいトラブルであることが明らかになった。CL使用者が考えるトラブルの原因は，長時間装用したが24.8%，レンズケアが不適切であったが17.0%，CLを入れたまま寝た（連続装用）が13.5%，CLをすぐにはずさなかったが9.2%，使用期限があるCLを日数を超えて使用したが6.8%，眼科医に指示された定期検査を受けなかったが2.4%，添付文書をよく読まなかったが0.3%であった。

この調査結果と今回の調査結果を比較すると，CLの休止期間が4日以上は今回の調査が29.9%と少なかった。CLの装用状況は連続装用が8.2%で，1日の装用時間は16時間以上が20.6%と，長時間装用している者が多かった。1日および1週間連続使い捨てSCLや2週間頻回交換SCLの装用方法を守っていたのは46.3%であったことから，CLの使用方法に問題のあることや，毎日洗浄していたが71.1%，毎日消毒していたが42.3%であった結果からレンズケアについても問題があることがわかる。

CLの処方を受けていない者は11.3%で，インターネットや通信販売で購入している者は9.3%であった。CLの購入にあたっては必ず眼科専門医の診察を受けて，信頼のおける販売店で入手するように指導することが大切である。

これまでの調査から，2週間頻回交換SCLを装用している者でMPSを使用している場合の眼障害例が多いことが指摘されている[15]が，本調査でも2週間頻回交換SCLが43.3%ともっとも多く，SCL消毒についても過酸化水素消毒剤が5.6%に対して，MPSは22.5%と多かった。使用している消毒剤が不明が71.8%であることは問題で，レンズケアに関心がないことを裏づける結果である。

定期検査についても1ヶ月あるいは3ヶ月に1回定期検査を受けているものは16.5%に対して，全く受けていないものが29.9%であることから，定期検査を受けていないことも問題であったことがわかる。

日本眼科医会が行った他の調査結果[16]からも，CL使用者のCLの使い方やレンズケア，定期検査などに問題があることが指摘されているが，あらためて本調査でもこれらの問題が浮き彫りになった。

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In Vivo and In Vitro Laser Confocal Microscopy to Diagnose *Acanthamoeba* Keratitis

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Purpose: To determine the effectiveness of laser confocal microscopy in identifying *Acanthamoeba* cysts and trophozoites in the cornea of patients with *Acanthamoeba* keratitis (AK) and to evaluate its effectiveness in following AK after treatment.

Methods: The corneas of 9 patients clinically diagnosed with AK were monitored periodically with the Heidelberg Retina Tomograph II-Rostock Cornea Module (HRT II-RCM) to examine for *Acanthamoeba* cysts and trophozoites during the clinical course.

Results: Seven of 9 patients had positive corneal smears, and 5 of 9 patients had positive laboratory cultures. HRT II-RCM demonstrated the presence of highly reflective polygonal shadows with lower reflective borders in the cornea of all patients. In 1 patient, a highly reflective pleomorphic shadow with small less-reflective areas was detected inside the cell. The former finding resembled the image of *Acanthamoeba* cysts in culture as observed by HRT II-RCM, and the latter observation with that of *Acanthamoeba* trophozoites in culture. After treatment, the number of highly reflective inflammatory cells decreased and the number and morphology of the corneal epithelial cells with highly reflective nuclei recovered to normal levels.

Conclusion: These results indicate that in vivo laser confocal microscopy can be a useful method to make a diagnosis and to follow patients with AK.

Key Words: *Acanthamoeba* keratitis, in vivo confocal microscopy, trophozoite

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Acanthamoeba keratitis (AK) is an intractable sight-threatening infection of the cornea seen predominantly in contact lens (CL) wearers. The incidence of AK has been increasing in the past few decades with the widespread use of CLs.[1] The problems encountered in the patients with AK include the difficulty in making a correct diagnosis at the early stage and the lack of effective medications. The early clinical signs of AK are subepithelial infiltrates, pseudodendritic keratitis, and radial neurokeratitis, but AK is often misdiagnosed as herpetic keratitis or fungal keratitis. This then leads to a delay in initiating appropriate treatments. In addition, the effectiveness of isolating *Acanthamoeba* in cultures has been reported to be between 30% and 60%,[2,3] and it required 1–2 weeks to obtain the results from the laboratory. To make a proper or tentative diagnosis of AK as rapidly as possible, examination by confocal microscopy has been enthusiastically conducted in the past decade.[4–8] However, it has been somewhat difficult to differentiate the *Acanthamoeba* cysts from other cell populations because of the resolution limitation. The Heidelberg Retina Tomograph II-Rostock Cornea Module (HRT II-RCM) is a relatively new confocal microscope with high resolution.

The purpose of this study was to determine whether the HRT II-RCM could be helpful in detecting *Acanthamoeba* cysts and trophozoites in patients with AK. In addition, we examined whether the HRT II-RCM can be helpful in following the clinical response to the treatment.

MATERIALS AND METHODS

An informed consent for the examination was obtained from all subjects, and this study was approved by Institutional Review Board of Ehime University. The procedures used conform to the tenets of the Declaration of Helsinki.

Nine patients clinically diagnosed with AK were examined with the HRT II-RCM (Heidelberg Engineering, Heidelberg, Germany). The HRT II-RCM was manipulated in the *xyz* axes to observe the entire cornea. After the confocal microscopic examination was completed, the corneal epithelium was scraped. A part of the sample was stained using Fungiflora Y staining (Biomate, Tokyo, Japan),[9] and the rest of the sample was cultured on nonnutrient *Escherichia coli* agar. After AK was diagnosed, in vivo HRT II-RCM examinations were performed every week to assess the changes in the morphology of AK. In addition, to validate the in vivo findings, in vitro images of cultured *Acanthamoeba* cysts and trophozoites were obtained with the HRT II-RCM and compared with the images obtained in vivo.

RESULTS

The clinical profiles of the patients are summarized in Table 1. All 9 patients (5 men and 4 women; mean age: 32.0

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TABLE 1. Clinical Details of Each Patient

Case Number	Age	Sex	BCVA Before Treatment (Wk)	CL	Duration Before Diagnosis (Wk)	Immunosuppressive Treatment Before Diagnosis	Culture	Histopathological Analysis
1	26	M	20/40	FRSCL	3	—	−	+
2	58	F	6/120	HCL	15	SS	+	+
3	25	F	20/25	FRSCL	2	—	−	−
4	22	M	20/50	FRSCL	4	TS	−	−
5	37	M	20/125	FRSCL	12	TS	−	+
6	24	M	20/40	FRSCL	3	—	+	+
7	25	F	20/40	FRSCL	3	TS	+	+
8	35	M	20/32	DSCL	3	—	+	+
9	18	F	20/600	FRSCL	2	TS	+	+

Case Number	HRT II-RCM Cyst	Clinical Signs: Dendriform Epitheliopathy	Clinical Signs: Subepithelial Infiltration	Clinical Signs: Radial Keratoneuritis	Clinical Signs: Ring Infiltrate	Treatment	BCVA After Treatment	Follow-up Periods (Mo)
1	+	+	+	+	−	CH, FC, MC	20/16	22
2	+	−	−	−	+	CH, VC	20/20	12
3	+	+	+	+	−	CH, VC	20/16	9
4	+	+	+	+	−	CH, VC	20/16	7
5	+	+	+	+	−	CH, VC	20/50	12
6	+	−	+	+	−	CH, VC	20/16	8
7	+	−	+	+	−	CH, VC	20/16	8
8	+	+	+	+	−	CH, VC	20/20	8
9	+	+	+	+	−	CH, VC	20/25	3

BCVA, best-corrected visual acuity; CH, 0.05% chlorhexidine; DSCL, disposable soft CL; FC, 0.2% fluconazole; FRSCL, frequent replacement soft CL; HCL, hard CL; MC, 0.1% miconazole; SS, systemic steroid; TS, topical steroid; VC, 1% voriconazole.

years; and range 18–58 years) were CL wearers and presented with unilateral severe ocular pain and blurred vision. All patients were referred to us because of uncontrollable corneal infection. The average duration of the symptoms before visiting our hospital was 5–6 weeks with a range of 2–15 weeks. One patient was treated with 0.05% chlorhexidine, 0.2% fluconazole, and 0.1% miconazole eyedrops every hour and, 8 of 9 patients were treated with 0.05% chlorhexidine and 1% voriconazole eyedrops every hour. Corneal epithelial scraping was performed once or twice a week in all patients during the hospitalization at the Ehime University Hospital.

Slit-lamp examinations showed a ring-shaped corneal infiltrate in 1 case (Table 1), dendriform epitheliopathy in 6 of 9 cases, subepithelial infiltration in 8 of 9 cases, and radial keratoneuritis in 8 of 9 cases (Table 1). In all cases, the HRT II-RCM examinations of the cornea showed highly reflective polygonally shaped spots surrounded by a lower reflective area of 15–25 μm (Figs. 1C–E). These spots were assumed to be *Acanthamoeba* cysts by the morphological characteristics and the size.[10,11] Images resembling *Acanthamoeba* trophozoites were detected in vivo by HRT II-RCM on the cornea of case 2 (Fig. 1H).

The corneal epithelia were scraped for histopathological and cultural analyses. Fungiflora Y staining of corneal scraping revealed *Acanthamoeba* organisms in 7 of 9 cases, and 5 of 9 cases were positive by culture.

To confirm that the images obtained by confocal microscopy in vivo were *Acanthamoeba* cysts or trophozoites, we examined the surface of the culture plate by HRT II-RCM in which *Acanthamoeba* cysts and trophozoites isolated from case 2 were grown. The *Acanthamoeba* cysts on the culture plates are shown in Figure 1A. The HRT II-RCM images of the cysts can be seen as highly reflective polygonal spots surrounded by a lower reflective area (Fig. 1B). *Acanthamoeba* trophozoites were detected on the culture plates by light microscopy, and they could be seen to be moving. The trails where *E. coli* had disappeared by being phagocytized by the *Acanthamoeba*, trophozoites were also visible (Fig. 1F, winding arrow). The HRT II-RCM images of the *Acanthamoeba* trophozoites on the culture plates were of highly reflective pleomorphic organisms with acanthopodia (arrowhead) and a small low-reflective internal area (Fig. 1G). This low-reflective area probably is the nucleus, and the highly reflective area inside the nucleus is presumably the karyosome (Fig. 1G). Trails were also discovered next to the *Acanthamoeba* trophozoites (Fig. 1G, winding arrow). Similar images of *Acanthamoeba* cysts and trophozoites were detected in vivo.

HRT II-RCM examinations were performed during the clinical course in all cases. Representative slit-lamp photographs and HRT II-RCM images at the level of the basal cell layer and the upper stromal layer during the clinical course of case 4 are shown in Figure 2. Although both histopathological examinations and cultures were negative, this case was diagnosed with AK because of typical clinical signs of AK, namely, dendriform epitheliopathy, epithelial and subepithelial infiltrates, and radial keratoneuritis (Fig. 2A). A photograph of the *Acanthamoeba* cysts obtained by HRT II-RCM at the initial examination is shown in Figures 1E, 2E, and 2I. These

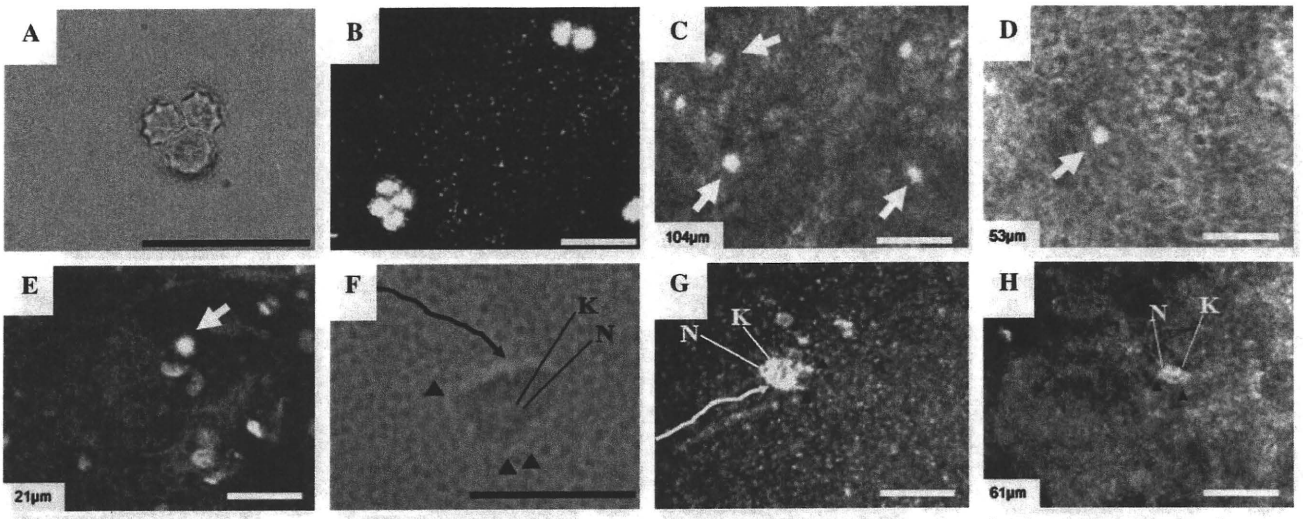

FIGURE 1. Images of *Acanthamoeba* cysts in vivo and in vitro. A, Light microscopic observation of cultured *Acanthamoeba* cysts isolated from case 2. B, HRT II-RCM image of cultured *Acanthamoeba* cysts showing highly reflective polygonally shaped cysts surrounded by a lower reflective area. In vivo HRT II-RCM images of *Acanthamoeba* cysts (arrows) of case 2 (C), case 3 (D), and case 4 (E). F, Light microscopic observation of cultured *Acanthamoeba* trophozoites isolated from case 2 showing acanthopodia (arrowhead), nucleus (N), karyosome (K), and trails (winding arrow). G, HRT II-RCM image of cultured *Acanthamoeba* trophozoites showing highly reflective pleomorphic form with acanthopodia (arrowhead) and small low-reflective internal area representing the nucleus (N). The highly reflective area inside the nucleus was probably the karyosome (K). Trails of the trophozoites can also be seen (winding arrow). H, In vivo HRT II-RCM images of *Acanthamoeba* trophozoite in case 2 showing acanthopodia (arrowhead), nucleus (N), and karyosome (K). The depth at which each of the HRT II-RCM images of C, D, E, and H were taken is indicated on the bottom left corner. All bars = 50 μm.

HRT II-RCM images show many highly reflective inflammatory cells in the epithelium and stroma (Figs. 2E, I). Two weeks after the beginning of treatment, the dendriform epitheliopathy was not detected and the epithelial and subepithelial opacities and radial keratoneuritis were reduced in slit-lamp examinations (Fig. 2B).

These findings indicated a considerable improvement in the inflammatory response. The HRT II-RCM images also showed that the number of inflammatory cells was reduced, although many inflammatory cells and Langerhans cells were still present. In addition, *Acanthamoeba* cysts were still detected in the basal cell layer of the epithelium (Fig. 2F). The density of basal epithelial cells was reduced, and the cells had highly reflected nuclei (Fig. 2F). In the stroma, the number of inflammatory cells had decreased, but many inflammatory cells were still present (Fig. 2J). Therefore, the treatment regime was kept for 2 more weeks. Four weeks after the initial treatment, radial keratoneuritis and dendriform epitheliopathy were not observed but subepithelial opacities were still present by slit-lamp examination (Fig. 2C). The HRT II-RCM examination showed that the number of inflammatory cells and Langerhans cells had decreased and the normal reflective pattern of the basal epithelial cells had returned (Fig. 2G). As a result of the improvements, the frequency of eyedrops was reduced to 6 times per day and the patient was discharged. The patient returned 4 weeks later, slit-lamp and HRT II-RCM examinations showed fewer inflammatory and Langerhans cells, and the density and reflection of the basal epithelial cells were normal (Fig. 2H).

In the stroma, a decrease in the number of inflammatory cells was detected during the clinical course; after 4 weeks, highly reflective nuclei were not observed inside the keratocytes (Figs. 2I–K). Eight weeks after beginning the treatment, nonuniform keratocytes without highly reflective nuclei were detected (Fig. 2L). The degree and duration of inflammation in each case varied, but the overall findings were consistent in all cases.

DISCUSSION

In vivo confocal microscopy was recently introduced, and it was found to overcome the clinical and microbiological difficulties in diagnosing AK.[4–6,12,13] Parmar et al[3] reported that *Acanthamoeba* cysts/trophozoites were identified by in vivo confocal microscopy in 54 of 63 cases of clinically diagnosed AK in a 10-year study. We used the newly developed confocal microscope HRT II-RCM to search for *Acanthamoeba* cysts and trophozoites in eyes diagnosed with AK. Our results showed that it was possible to detect *Acanthamoeba* cysts in all cases, including cases 3 and 4 in which both light microscopy and cultures of corneal scrapings were negative for *Acanthamoebae*.

We also detected *Acanthamoeba* trophozoites in vivo in 1 patient. In addition, we were able to detect *Acanthamoeba* cysts and trophozoites on the culture plates by confocal microscopy in this patient. The in vitro HRT II-RCM images were very helpful in identifying the images of *Acanthamoeba* cysts obtained by in vivo confocal microscopic examination. The polygonally shaped *Acanthamoeba* cysts were clearly

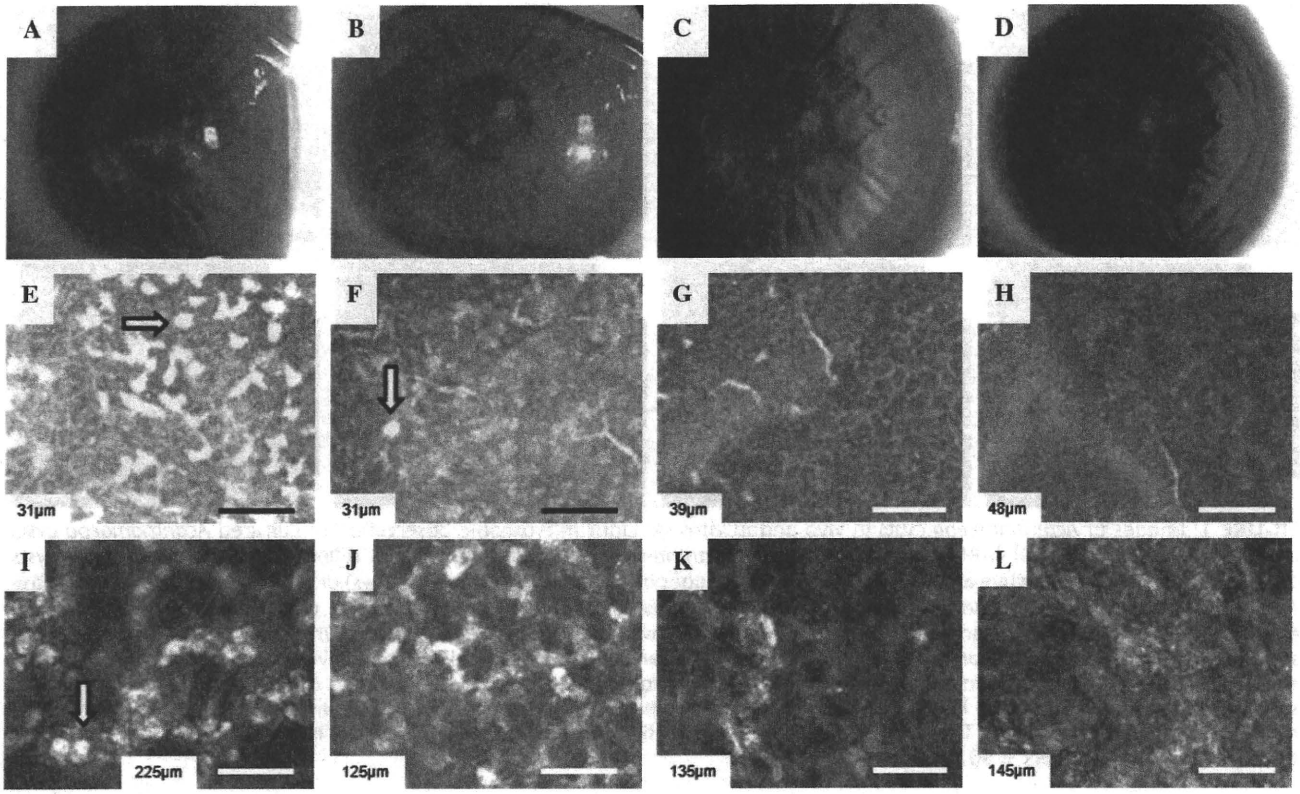

FIGURE 2. Slit-lamp photographs (A–D) and HRT II-RCM images of basal cell layer of corneal epithelium (E–H) and upper layer of stroma (I–L) during clinical course in case 4 (A, E, and I: first examination; B, F, and J: 2 weeks after treatment; C, G, and K: 4 weeks after treatment; D, H, and L: 8 weeks after treatment). E–H, At the first examination, highly reflective numerous inflammatory cells and *Acanthamoeba* cysts (arrow) can be seen in the epithelium. At 2 weeks after treatment, number was decreased but still many inflammatory cells and Langerhans cells can be seen and *Acanthamoeba* cysts (arrow) were still detected. Basal epithelial cells can be seen at lower density with highly reflective nuclei. At 4 weeks, the number of inflammatory cells and Langerhans cells was reduced. At 8 weeks, fewer inflammatory cells and Langerhans cells were present and basal epithelial cells were detected at normal density and reflection. I–L, At the first examination, many highly reflective inflammatory cells and *Acanthamoeba* cysts (arrow) can be seen in the stroma, and the number of inflammatory cells is reduced during the clinical course. After 4 weeks, highly reflective nuclei were not observed inside the keratocytes, and 8 weeks after treatment, nonuniform keratocytes without highly reflective nuclei can be seen. The depth at which all of the HRT II-RCM images were taken is indicated on the bottom left corner. All bars = 50 μm.

detected by HRT II-RCM in vitro (Fig. 1B) and appeared very similar to the images obtained in vivo (Figs. 1C–E). All *Acanthamoeba* cysts detected in vivo and in vitro were polygonally shaped and were surrounded by a lower reflective area of 15–25 μm. These morphological findings are in good agreement with the microbiological findings.[10,11]

We also found that the HRT II-RCM images of *Acanthamoeba* trophozoites on the culture plates had shown highly reflective pleomorphic forms with acanthopodia. It was also possible to differentiate the nuclei and karyosomes inside the *Acanthamoeba* trophozoites not only in vitro but also in vivo in the HRT II-RCM images (Figs. 1G, H). Many investigators have presented images and descriptions of *Acanthamoeba* cysts obtained by in vivo confocal microscopy; however, they were not able to demonstrate either the polygonally shaped cysts or the pleomorphic forms of the trophozoites because of the limited resolution. HRT II-RCM images of the *Acanthamoeba* cysts have been published, and the improved resolution has made it possible to obtain high-resolution images of *Acanthamoeba* cysts.[13] However, the HRT II-RCM images were still in black and white. Thus, it was still difficult to differentiate *Acanthamoeba* cysts from inflammatory cells because their sizes were similar and they both were highly reflective. In addition, it was not easy to find *Acanthamoeba* cysts and trophozoites in the corneal scrapings because *Acanthamoeba* cysts and trophozoites may not be abundant in the corneal epithelium of eyes with AK. Thus, a better understanding of the morphological characteristics is necessary to recognize *Acanthamoeba* cyst and trophozoites by in vivo confocal microscopy.

The HRT II-RCM is a noninvasive and repeatable diagnostic tool that can be used to follow the clinical course and morphological changes of AK (Fig. 2). However, it is still difficult to state that there is a complete absence of *Acanthamoeba* cysts during the clinical course because of the small field of view and low density of cysts. HRT II-RCM examinations can determine the density of inflammatory cells and Langerhans cells and the morphology of epithelial cells and

keratocytes. Thus, it is possible to determine the effectiveness of the treatment at any time. In fact, we did change the treatment in some of the cases because the HRT II-RCM and the slit-lamp images, and the changes led to successful treatments in all 9 cases of AK (Table 1).

In conclusion, the newly developed in vivo confocal microscope, HRT II-RCM, with improved resolution can be used to obtain high-resolution images of *Acanthamoeba* cysts and trophozoites in vivo. These images resembled very closely the images observed by the HRT II-RCM of *Acanthamoeba* cysts and trophozoites in culture. These findings with the HRT II-RCM will help in the diagnosis of AK, and the noninvasive repeated assessments should make this instrument a powerful tool for following the clinical course of AK.

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Effectiveness of In Vivo Confocal Microscopy in Detecting Filamentous Fungi During Clinical Course of Fungal Keratitis

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Purpose: To determine the effectiveness of laser confocal microscopy in detecting filamentous fungi in the cornea of patients with fungal keratitis (FK) and in evaluating the effectiveness of the treatment.

Methods: The corneas of 6 patients clinically diagnosed with FK were examined with the Heidelberg Retina Tomograph II-Rostock Cornea Module (HRT II-RCM). Three of these patients were also monitored periodically with the HRT II-RCM after antifungal treatment.

Results: The HRT II-RCM examination showed interlocking and branching, white, septated, hyphae-like lines in the cornea of all patients. All 6 patients had positive corneal smears and/or laboratory cultures. Three patients were monitored with HRT II-RCM after antifungal treatment. One patient, whose initial smear was negative, was diagnosed by HRT II-RCM before the positive culture results. In another case, the epithelial regeneration was impaired even 3 weeks after the initial treatment and HRT II-RCM revealed a mass of hyphae in the corneal ulcerated lesion. These findings indicated the necessity of surgical debridement. After the surgical debridement, the corneal epithelial defect was healed. HRT II-RCM was able to detect the morphological changes of hyphae after antifungal treatment and helped in the treatment modifications during the clinical course in all 3 patients.

Conclusions: These results indicate that HRT II-RCM can be used to diagnose FK and to monitor the effect of therapy on FK.

Key Words: fungal infection, keratitis, in vivo confocal microscopy, cornea

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Fungal keratitis (FK) is a vision-threatening corneal disease and is still a difficult disease to diagnose and treat. The most common microorganisms that cause FK are *Fusarium solani* and other *Fusarium* sp. and *Aspergillus* sp.[1–3] The infections are usually caused by a prior ocular injury with organic contamination. A correct diagnosis and the prevention of a decrease of visual acuity are challenging clinical problems of eyes with FK.

Although the classical clinical signs of FK, including dry white cotton–wool infiltrates with feathery edges, are helpful for a diagnosis, these signs may not be present in all cases. Corneal smears and cultures are the standard diagnostic methods for infectious keratitis including fungal infections. However, it takes several days to weeks to obtain the results of fungal cultures, and the results could be negative in some cases such as those with deep infiltrates.

Confocal microscopy has been used to try to make a diagnosis of FK as early as possible, and the results to date are good.[4–7] The Heidelberg Retina Tomograph II-Rostock Cornea Module (HRT II-RCM) is a relatively new confocal microscope with high resolution, and a growing number of studies has reported on its effectiveness in investigating infectious corneal diseases, for example, *Acanthamoeba* keratitis and FK.[8–11]

At present, slit-lamp biomicroscopy is used to diagnose FK and also to follow the clinical course after treatment. However, it is difficult to judge whether the treatment is effective or when the antifungal therapy could be tapered or terminated by only a slit-lamp examination.

One of the advantages of confocal microscopy is that it is noninvasive, and it can be repeatedly performed to follow the corneal disease during its clinical course. It can be used along with slit-lamp examinations. The purpose of this study was to determine whether the HRT II-RCM could help in detecting fungal filaments in patients with FK and whether it can be used to follow the clinical course after antifungal treatment.

MATERIALS AND METHODS

An informed consent for the examination was obtained from all subjects, and this study was approved by Institutional Review Board of Ehime University. The procedures used conformed to the tenets of the Declaration of Helsinki.[12]

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Six patients clinically suspected of having FK were examined with the HRT II-RCM (Heidelberg Engineering, Heidelberg, Germany). The HRT II-RCM was moved in the *x*, *y*, and *z* axes to observe the entire cornea. After the confocal microscopic examination, patients underwent laboratory investigations including a direct microscopic evaluation of corneal smears and cultures of corneal scrapings. After a FK was diagnosed, in vivo HRT II-RCM examinations were performed every week to assess the changes in the morphology of the FK in 3 of the 6 patients.

RESULTS

The clinical profiles of the patients are summarized in Table 1. All 6 patients (3 men and 3 women; mean age: 62.7 years; range: 33–81 years) were referred to us because of uncontrollable corneal infections.

Slit-lamp examinations showed focal stromal infiltrates in 5 cases and infiltrates into the entire cornea in 1 case (case 4) (Table 1). In all cases, the HRT II-RCM examinations of the cornea showed a mass of highly reflective, interlocking and branching, white lines in the area of the infiltrates (Figs. 1–4). These hyphae-like structures were about 3–5 μm wide and hundreds of micrometer long. The filaments were assumed to be filamentous fungal hyphae by the morphological characteristics and size. The corneal smears revealed filamentous fungi in 5 of 6 cases, and 5 of 6 cases were positive by culture (Table 1).

HRT II-RCM examinations were performed after the antifungal treatment in 3 cases (case 1–3). Unfortunately, we were not able to perform follow-up examinations by HRT II-RCM on the other 3 cases because 1 case had a therapeutic penetrating keratoplasty (case 4) and 2 cases were followed in other hospitals after our diagnosis of FK (case 5 and 6).

We present the findings in the 3 cases that were followed by HRT II-RCM.

Case 1

The patient was a 33-year-old man who was a soft contact lens wearer and visited a private ophthalmological clinic because of unilateral irritation and blurred vision on October 31, 2008. He was referred to the Ehime University Hospital on the same day. On the initial examination, his best-corrected visual acuity was 20/16 OD and 20/1000 OS. Slit-lamp examination of the left eye revealed a grayish stromal infiltrate with indistinct margins and stromal edema. The corneal smears were negative. We diagnosed the patient with bacterial keratitis and treated him with antibacterial eyedrops. Four days later, the corneal infiltration worsened (Fig. 1A) and he was examined with the HRT II-RCM. The HRT II-RCM examination showed highly reflective, hyphae-like, interlocking and branching white lines in the area of the infiltrates (Fig. 1B). Therefore, we suspected FK and changed the therapy to antifungal drugs on November 4, 2008. The laboratory culture results were returned 4 days later with the presence of *Penicillium* sp. on November 8, 2008. The corneal infiltrate and edema was reduced at 7 days after beginning the antifungal treatment (Fig. 1C). The HRT II-RCM images at 7 days after antifungal treatment showed a decrease in the density of the hyphae (Fig. 1D), which indicated that the treatment was effective. Seventeen days after antifungal treatment, slit-lamp microscopy showed a resolution of the infiltrates (Fig. 1E) and HRT II-RCM images showed no hyphae (Fig. 1F). The treatment was tapered thereafter, and the patient has had no recurrence.

Case 2

The patient was an 80-year-old man who felt dust particles blow into his right eye during farm work on January 6, 2007. He visited a private ophthalmological clinic on January 9, 2007 because his right eye felt irritated and his vision was blurred. He was referred to the Ehime University Hospital on January 11, 2007 because of a severe corneal infection.

On the initial examination, his best-corrected visual acuity was 20/1000 OD and 20/16 OS. Slit-lamp examination of the right eye revealed a white cotton–wool stromal infiltrate with feathery edges (Fig. 2A). Examination of the cornea with the HRT II-RCM showed a mass of interlocking and branching white lines in the area of the infiltrate (Fig. 2B). The corneal smears showed filamentous fungi (Fig. 2C), and antifungal treatment was begun. One week later, slit-lamp examination showed very little change in the corneal stromal infiltrate (Fig. 2D). However, the HRT II-RCM images showed a distinct

TABLE 1. Clinical Details of Each Patient

Case Number	Age	Sex	BCVA Before Treatment	Risk Factor	Diagnosis			Organism	Treatment	BCVA After Treatment
					Smear	Culture	HRT II-RCM			
1	33	M	20/1000	FRSCL	(−)	(+)	(+)	*Penicillium* sp.	VC, PM, GFLX	20/20
2	60	M	20/1000	DM, trauma with vegetative	(+)	(−)	(+)	Filamentous fungi	MC, VC, PM, GFLX	20/20
3	81	F	20/25	Trauma with vegetative	(+)	(+)	(+)	*Fusarium* sp.	VC, PM, GFLX, SD	20/25
4	62	F	HM	Trauma with vegetative	(+)	(+)	(+)	*Fusarium* sp.	VC, PM, GFLX, PKP (2 times)	20/25
5	81	F	20/2000	Trauma with vegetative	(+)	(+)	(+)	*Colletotrichum gloeosporioides*	VC, PM, LVFX	20/25
6	59	M	20/300	RA, immunosuppressive drugs	(+)	(+)	(+)	*Penicillium* sp. *Fusarium* sp.	VC, PM, GFLX	20/50

BCVA, best-corrected visual acuity; DM, diabetes mellitus; FC, 0.2% fluconazole; FRSCL, frequent replacement soft contact lens; GFLX, gatifloxacin; HM, hand motions; LVFX, levofloxacin; MC, 0.1% miconazole; PKP, penetrating keratoplasty; PM, pimaricin eye ointment; RA, rheumatoid arthritis; SD, surgical debridement; VC, 1% voriconazole.

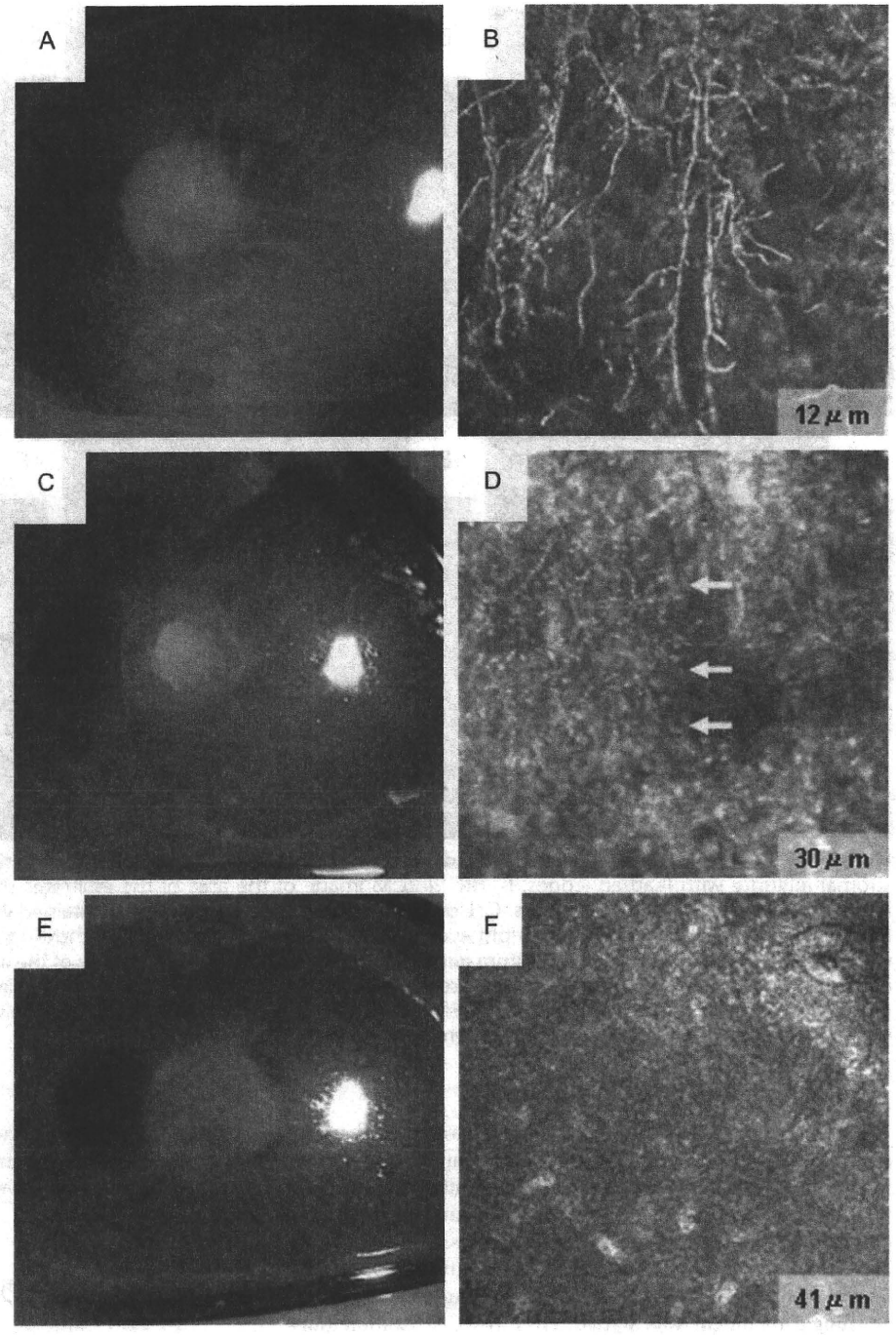

FIGURE 1. Observations of the clinical course of FK in case 1. A, Slit-lamp photograph of the left eye showing a grayish stromal infiltrate with indistinct margins and stromal edema. B, HRT II-RCM image of the area of the infiltrates showing highly reflective, hyphae-like, interlocking and branching, white lines. C, Slit-lamp photograph 7 days after antifungal treatment showing a reduction of the corneal infiltrate and edema. D, HRT II-RCM image of the area of the infiltrates at 7 days after antifungal treatment showing a decrease in the density of hyphae (arrows). E, Slit-lamp photograph 7 days after antifungal treatment showing resolved corneal infiltrate. F, HRT II-RCM image of the area of the infiltrates at 17 days after initial treatment showing no hyphae. The depth at which all of the HRT II-RCM images were taken is indicated on the bottom right corner.

decrease in the density of the hyphae indicating that the antifungal treatment was effective (Fig. 2E). Although the improvement of corneal infiltrate was slow, the HRT II-RCM images recorded 2 weeks after the beginning of the treatment revealed a further decrease in the density of the hyphae (Fig. 2F). HRT II-RCM examinations were performed periodically, and the treatment was tapered after the disappearance of the hyphae.

Case 3

The patient was an 81-year-old woman who had been diagnosed with bacterial keratitis or herpetic keratitis in her right eye and was treated for these conditions at another hospital for 2 months. She was referred to the Ehime University Hospital on June 9, 2009 because of uncontrollable corneal infection.

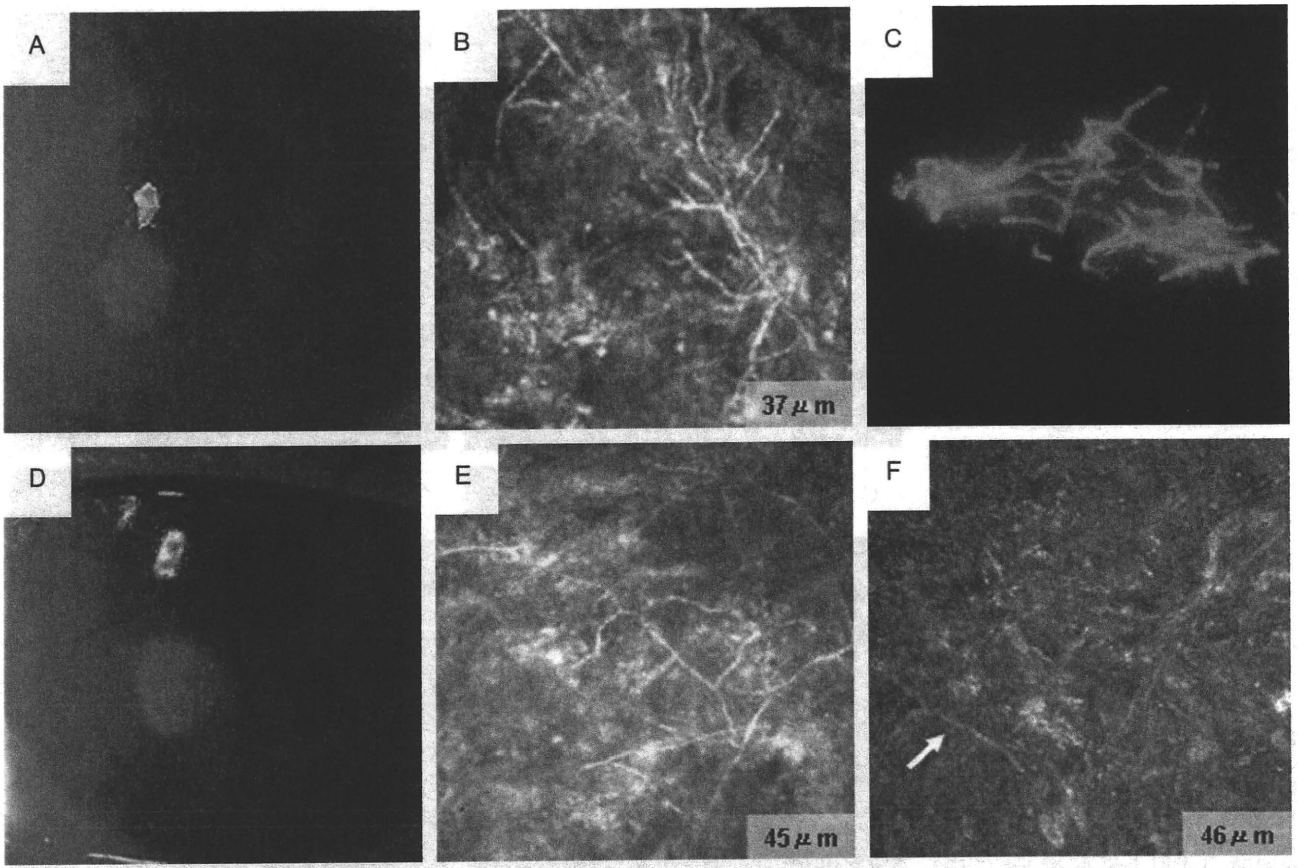

FIGURE 2. Observations of the clinical course of FK in case 2. A, Slit-lamp photograph of the right eye showing a white cotton–wool stromal infiltrate with feathery edges. B, HRT II-RCM image of the area of the infiltrates showing highly reflective, hyphae-like, interlocking and branching, white lines. C, Light microscopic observation of smear stained with Fungi flora Y showing filamentous fungi. D, Slit-lamp photograph of the right eye at 7 days after initial treatment still showing a white cotton–wool stromal infiltrate with feathery edges with little change from initial examination. E, HRT II-RCM image of the area of the infiltrates at 7 days after the initial antifungal treatment showing a decrease in the density of the hyphae. F, HRT II-RCM image of the area of the infiltrates at 14 days after the initial treatment showing a decrease in the density of hyphae (arrow). The depth at which all of the HRT II-RCM images were taken is indicated on the bottom right corner.

On the initial examination, her best-corrected visual acuity was 20/25 OD and 20/16 OS. Slit-lamp examination of the right eye revealed a white stromal infiltrate with a dry texture and feathery edges (Fig. 3A). Examination of the cornea with the HRT II-RCM showed a mass of interlocking and branching white lines in the area of the infiltrate (Fig. 3B). The corneal smears showed filamentous fungi (Fig. 3C), and antifungal treatment was started. HRT II-RCM examinations were performed weekly thereafter. Although there was a decrease in the density of hyphae at the borders of the infiltrate, the regeneration of the epithelium was delayed even 3 weeks after beginning the treatment (Fig. 3D). HRT II-RCM examinations of the corneal ulcerated lesion revealed many hyphae 3 weeks after beginning the treatment (Fig. 3E). Therefore, a surgical debridement was performed at 3 and 5 weeks after the initial treatment. The debrided tissues were examined histochemically, and many hyphae were seen in the necrotic tissues (Fig. 3F). After the surgical debridement, the regeneration of the epithelium was accelerated, and at 7 weeks after the initial treatment, the corneal epithelial defect was healed and hyphae were not detected by HRT II-RCM examinations.

DISCUSSION

In vivo confocal microscopy was recently introduced and found to overcome the clinical and microbiological difficulties in diagnosing FK.[4–8,13] We used a new confocal microscope, HRT II-RCM, to search for hyphae in 6 eyes diagnosed with FK. Our examinations showed hyphae in all 6 cases, and the presence of fungi was confirmed by positive corneal smears in 5 of the 6 cases and the presence of hyphae in cultures of the cornea in 5 of the 6 cases. The high rate of detection of hyphae by HRT II-RCM is comparable to that reported by Kanavi et al[7] who reported that hyphae-like structures were identified by in vivo confocal microscopy

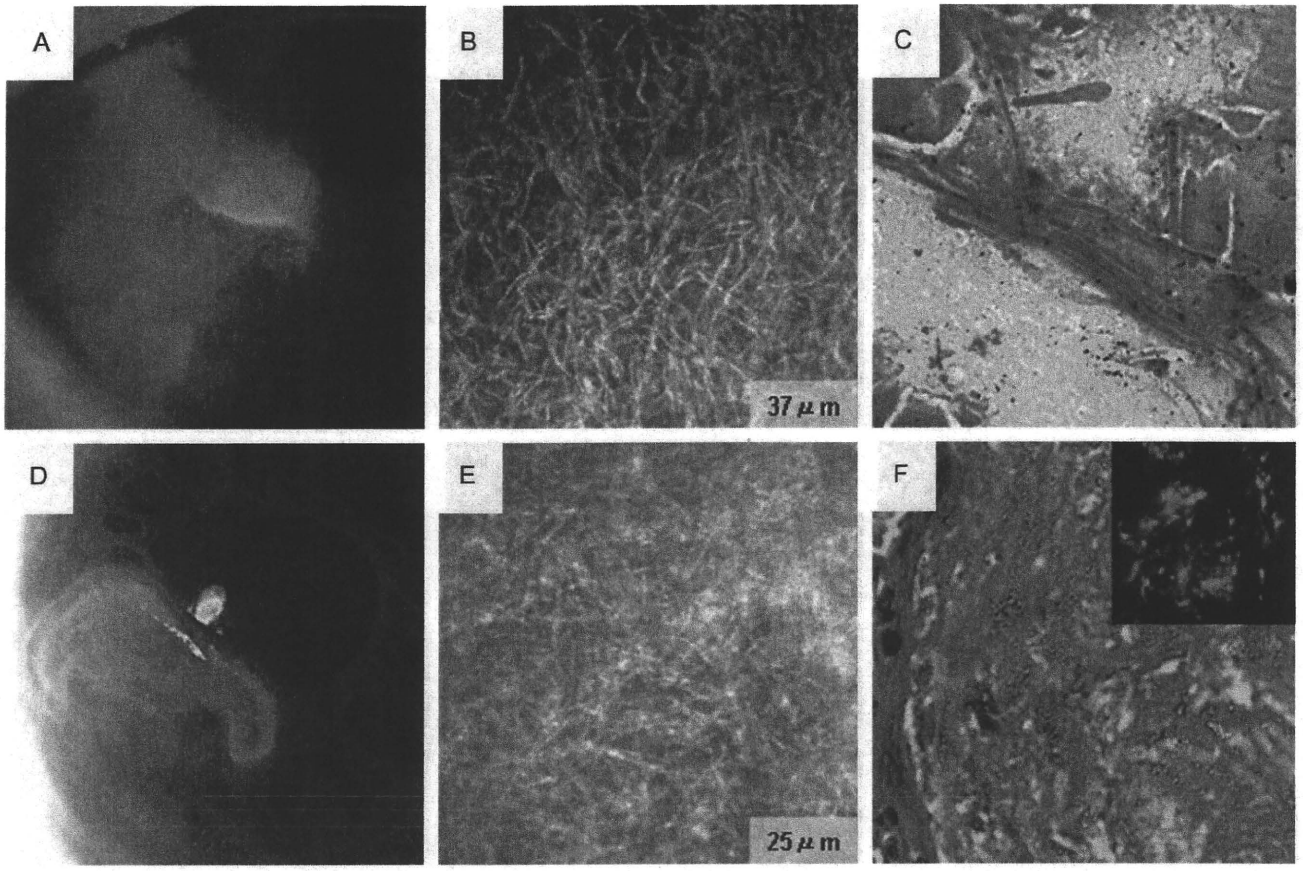

FIGURE 3. Observations of the clinical course of FK in case 3. A, Slit-lamp photograph of the right eye showing a white stromal infiltrate with a dry texture and feathery edges. B, HRT II-RCM image of the area of the infiltrates showing highly reflective, hyphae-like, interlocking and branching, white lines. C, Light microscopic observation of smear with Giemsa staining showing filamentous fungi. D, Slit-lamp photograph of the right eye showing a large epithelial defect with deposited tissues at ulcerated lesion at 3 weeks after the initial treatment. E, HRT II-RCM image of the corneal ulcerated lesion at 3 weeks after the initial treatment showing a mass of hyphae. F, Histochemical examination of debrided tissues showing many hyphae in the necrotic tissues. The inserted photograph in right above shows filamentous fungi stained with Fungi flora Y. The depth at which all of the HRT II-RCM images were taken is indicated on the bottom right corner.

(confoscan 3.0; Nidek Technology) in 27 of 28 cases of clinically diagnosed FK. The HRT II-RCM images obtained in our study were morphologically similar to the HRT II-RCM images of *F. solani* and *Aspergillusfumigatus*.[8] These findings demonstrated the usefulness of HRT II-RCM for early diagnosis of FK.[8] Our HRT II-RCM examinations detected hyphae in the case 1, which had been treated as bacterial keratitis because the corneal smear was negative for fungi. Disorganized collagen bundles can be seen in the corneal stroma during the healing stage of keratitis. Although the collagen bundles were also detected as highly reflective lines, they did not appear to be interlocking or branching. Thus, the filamentous fungal hyphae can be easily differentiated from collagen bundles by the morphological characteristics.

In addition to diagnosing FK, we found that HRT II-RCM was valuable in following the clinical course and documenting the morphological changes of the FK after antifungal treatments.[13,14] Earlier models of the confocal microscope, for example, Confoscan 2.0 (Nidek) used by Shi et al and the Confoscan 3.0 (NIdek) used by Miller et al, were able to detect hyphae, but the images of the hyphae after treatment were not clear because of the lower resolution of these models. On the other hand, the HRT II-RCM can clearly detect the changes of hyphae during the clinical resolution of FK as shown in this study. Thus, HRT II-RCM examination is more reliable for showing that such hyphae are decreasing in density.

FK often responds slowly over a period of weeks to antifungal therapy; therefore, the changes in the clinical signs may not be detected by slit-lamp examinations.[2,3] In fact, little change was found by slit-lamp microscopy 1 week after initial treatment in case 2, but the HRT II-RCM images clearly showed a decrease in the density of hyphae. Thus, the HRT II-RCM images were helpful in judging the effectiveness of the treatment, and the treatment regimen was continued thereafter.

FK occasionally requires surgical debridement to remove the infectious agents, necrotic tissue, and other debris that may hinder the regeneration of the epithelium.[1–3]

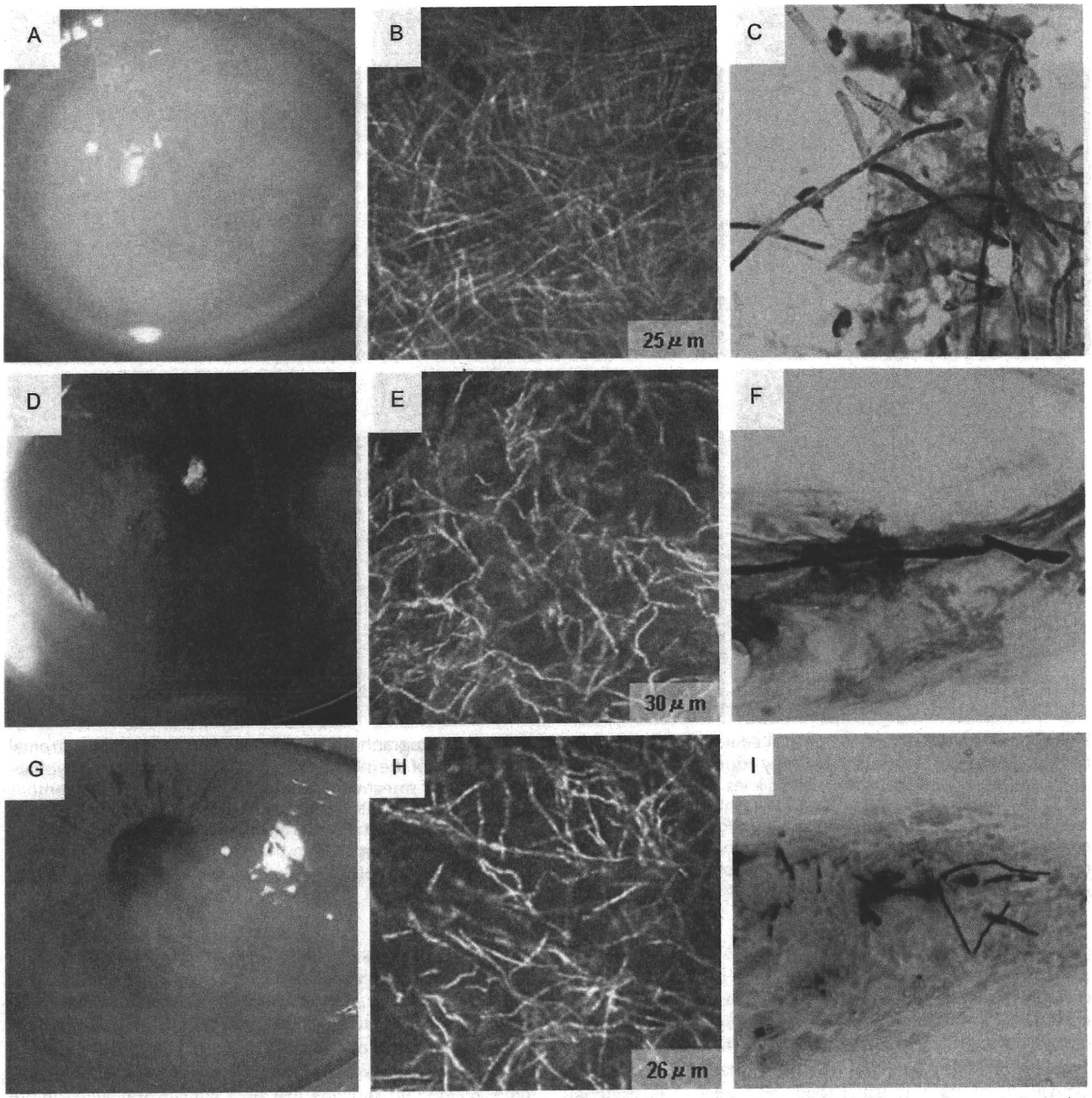

FIGURE 4. Slit-lamp photographs of cases 4, 5, and 6 (A, D, G). HRT II-RCM image of the area of the infiltrates of case 4, 5, and 6 showing highly reflective, hyphae-like, interlocking and branching, white lines (B, E, H). Light microscopic observation of smear with Giemsa staining of case 4, 5, and 6 showed filamentous fungi (C, F, I). The depth at which all of the HRT II-RCM images were taken is indicated on the bottom right corner.

However, it is not easy to determine the indications for surgical debridement by slit-lamp microscopy because slit-lamp microscopy may not be able to differentiate the morphology of ulcerated lesion and infiltrates. In case 3, the HRT II-RCM examination clearly revealed the mass of hyphae in the necrotic tissue in the corneal ulcerated lesion, suggesting that surgical debridement was necessary. We performed surgical debridement twice under HRT II-RCM monitoring, and these procedures resulted in a complete epithelial healing along with the decrease in the density of hyphae.

In summary, our findings showed that the HRT II-RCM will help in the diagnosis of FK and its noninvasiveness allows repeated assessments that should make this instrument a useful tool for judging the effectiveness of the treatments.

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Connective Tissue Growth Factor Cooperates with Fibronectin in Enhancing Attachment and Migration of Corneal Epithelial Cells

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Corneal wound healing is a complex process involving the integrated actions of various growth factors, cytokines and extracellular matrix produced by corneal cells and inflammatory cells. Connective tissue growth factor (CTGF) has been linked to wound healing, and fibronectin (FN) is a major component of the extracellular matrix. However, the functions of CTGF and FN in corneal epithelial cells are not well understood. We therefore investigated the coordinated function of CTGF and FN in the attachment and migration of corneal epithelial cells. Treatment of human corneal epithelial cells (HCECs) with transforming growth factor (TGF) β1 up-regulated the expression of CTGF, but did not noticeably affect FN expression, as judged by immunoblot analysis of cell lysates. In contrast, the amount of FN accumulated in the cultured media was increased in a time-dependent manner, but CTGF was undetectable in the cultured media. The expression level of FN was decreased by the knockdown of CTGF expression with a specific short hairpin RNA, indicating that CTGF acts as an upstream mediator of FN expression. CTGF augmented the FN-mediated increase in the attachment of HCEC by about twofold, although CTGF alone did not influence the attachment. Moreover, the migration assay with rabbit corneal blocks revealed that CTGF (390 nM) alone or in combination of FN (10 μg/mL) promoted corneal epithelial migration; the mean migration distances of control, CTGF, and CTGF + FN were 272, 325, and 626, μm, respectively. In conclusion, CTGF cooperates with FN in enhancing the attachment and migration of corneal epithelial cells.

Keywords: connective tissue growth factor; fibronectin; corneal epithelial cells; wound healing; migration
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The cornea is directly challenged to the exterior environments. Therefore, corneal epithelium plays a pivotal role as a barrier to keep the integrity of the ocular surface. When epithelial defects occur on the cornea, rapid re-epithelialization is critical in order to prevent invasion of pathogens into the corneal stroma. The corneal epithelium maintains its homeostasis and physiological characteristics by repeating a cycle of proliferation, migration and surficial cell loss (Thoft and Friend 1983). Smooth corneal epithelial cell migration plays a central role in corneal epithelial wound healing; various growth factors, cytokines and extracellular matrices are all necessary for this cell migration (Wilson et al. 1992; Li and Tseng 1995). Furthermore, stromal-epithelial interactions occur during corneal wound healing (Wilson et al. 1999) and activated keratocytes undergo myofibroblastic transformation (Jester et al. 1995; Funderburgh et al. 2003).

Connective tissue growth factor (CTGF) is a downstream mediator of transforming growth factor (TGF)-β and is found in abundance in the lacrimal fluid (Grotendorst 1997). CTGF is thought to play a role in maintenance of fibrosis (Bradham et al. 1991). It was reported that CTGF expression is increased in fibrotic diseases of the lung (Allen et al. 1999), kidney (Ito et al. 1998), and skin (Igarashi et al. 1995); this mediator may be involved in the storage of the extracellular matrix. CTGF also exerts effects on cell attachment and migration (Fan et al. 2000). In the cornea, CTGF promotes transdifferentiation of the corneal fibroblast into the myofibroblast, resulting in excess production of collagen and fibronectin (FN). CTGF is also involved in the wound healing process of the corneal stroma (Folger et al. 2001; Blalock et al. 2003; Garrett et al. 2004). One of the reasons why CTGF has so many functions may be due to its binding to other cytokines and the extracellular matrix to modulate their actions (Frazier et al. 1996).

We reported that CTGF binds to FN (Yoshida and

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Munakata 2007). Although the action of CTGF on the corneal epithelium is not well known, it has been shown that FN promotes corneal epithelial cell migration in culture (Watanabe et al. 1987) as well as in vivo (Nishida et al. 1983b). Therefore, we examined the role of CTGF in corneal epithelial wound healing using cultured corneal epithelial cells and focusing on the interaction between FN and CTGF. We also used an organ culture method to investigate corneal epithelial cell migration.

Materials and Methods

Cell Culture

A human corneal epithelial cell (HCEC) line established using a simian virus 40 (SV40)-adenovirus recombinant vector was kindly provided by K. Araki-Sasaki. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) (Gibco; Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco) at 37°C under humidified 5% CO_2 and 95% air.

Animals

Female albino rabbits weighting 2 to 3 kg were obtained from Hokusetsu Sangyo (Settsu, Osaka, Japan). This study was performed in compliance with the Rules and Regulations of the Animal Care and Use Committee, Kinki University School of Medicine, and followed the Guide for the Care and Use of Laboratory Animals, Kinki University School of Medicine.

Proteins

Human plasma fibronectin was purchased from Sigma-Aldrich (St. Louis, MO, USA). TGF-β1 was purchased from R & D Systems (Minneapolis, MN, USA). The recombinant His-tagged human CTGF was purchased from Biovendor Laboratory Medicine, Inc. (Brno, Czech Republic).

Antibodies

The goat anti-human CTGF polyclonal antibody was purchased from R & D Systems. The rabbit anti-human FN polyclonal antibody and anti-human β-actin monoclonal antibody were purchased from Sigma-Aldrich. Horseradish peroxidase-conjugated donkey anti-rabbit and sheep anti-mouse IgG antibodies were purchased from GE Healthcare Bio-Sciences (Piscataway, NJ, USA). Horseradish peroxidase-conjugated rabbit anti-goat IgG antiboby was purchased from MBL (Nagoya, Japan). For the cell attachment assay, the mouse IgG anti-human FN was purchased from Takara Bio Inc. (Shiga, Japan) and normal mouse IgG was purchased from Sigma-Aldrich.

Plasmids and Transfection

Four independent short hairpin RNA (shRNA) constructs which were targeting four different exons of CTGF and one control plasmid were purchased from SABiosciences Corporation (Frederick, MD, USA). The sequences included CTGFshRNA1:5′-CCAGACCCA-ACTATGATTAGA-3′; CTGFshRNA2:5′-AGACATACCGAGCTA-AATTCT-3′; CTGFshRNA3:5′-TACCGACTGGAAGACACG-TTT-3′; CTGFshRNA4:5′-TGACCTGGAAGAGAACATTAA-3′; and shRNA:5′-TGACCTGGAAGAGAACATTAA-3′ (negative control). HCECs were transfected with CTGFshRNA and control plasmids using lipofectamine 2000 transfection reagent (Invitrogen; Carsbad, CA, USA) following the manufacturer's instructions. Briefly, HCEC-coated 12-well plates were grown to 95% confluence. Four microliters of lipofectamine 2000 regent was diluted in 96 μL Opti-MEM medium (Invitrogen) and then mixed with 100 μL Opti-MEM which included 1.6 μg of shRNA plasmids. After incubating at 37°C for 4 hours, the medium was changed to DMEM containing 10% FBS and the plates were incubated for 48 hours under the same conditions.

Immunoblot Analysis

To examine the effects on CTGF and FN expression in HCECs by stimulating TGF-β1, HCECs were cultured in DMEM containing 10% FBS. Prior to stimulation, cells were rendered quiescent by being maintained in serum-starved conditions for 24 hours. They were subsequently treated with 10 ng/mL of TGF-β1 for up to 72 hours. Mean TGF-β1 concentration in tear fluid is approximately 10-20 ng/mL (Gupta et al. 1996); therefore, TGF-β1 concentration of 10 ng/mL was used in this experiment. The cell lysates and the conditioned media were collected at 0, 12, 24, 48, and 72 hours after TGF-β1 stimulation. To investigate the role of CTGF in FN expression, HCECs transfected with CTGFshRNA were prepared as stated above. Cell lysates were prepared as described previously (Yoshida and Munakata 2007). The lysates were examined by Western blot using antibodies against polyclonal goat anti-human CTGF, polyclonal rabbit anti-human FN, monoclonal anti-β-actin and peroxidase-linked secondary antibodies. The immunoblotted membrane was developed using an enzyme-linked chemiluminescence (ECL) kit (GE Healthcare Bio-Sciences) according to the manufacturer's instructions.

Cell Attachment Assay

Two experiments were performed to examine the interaction between CTGF and FN. In the first experiment, 1% bovine serum albumin (BSA) (Roche Diagnostics, Switzerland), CTGF, FN or CTGF+FN were added to the media and incubated for 2 hours. After the 2-hour incubation, HCECs in the wells (1,000 cells/well) were incubated in the treated media at 37°C for 45 minutes. In the second experiment, 100 μL of HCEC suspension (1,000 cells/well) was placed in plates with FN-coated or uncoated wells. CTGF, CTGF + goat IgG anti-human FN, CTGF + normal goat IgG or 1% BSA , 1% BSA + mouse IgG anti-human FN, or 1% BSA + normal mouse IgG were added to the medium to investigate FN-dependant mechanisms. The cells were fixed and stained with 1% crystal violet in 95% ethanol, and the attached cells were counted under a phase-contrast microscope. Quadruplicate samples per treatment were tested and their mean average was obtained. Data were expressed as mean ± standard error of the mean (SEM) of the number of attached cells/well in three independent experiments.

Corneal Epithelial Migration Assay

The distance of rabbit corneal epithelial cell migration in culture was measured by a previously described method (Nishida et al. 1983a). Briefly, rabbits were anesthetized with an intravenous injection of sodium pentobarbital (25 mg/kg body weight) and both eyes were enucleated. The sclerocorneal rim was excised, and the corneas were removed and then cut into small blocks with a razor blade. Six blocks (approximately 2 mm × 4 mm) were obtained from each cornea. The blocks were placed in 24-well culture plates and incubated for 24 hours in the medium containing either 1) CTGF at concentrations of 0, 390 nM or 3.9 μM, or 2) CTGF at the same concentration

plus FN (10 μg/mL). Multiple blocks from the same cornea were used in each experiment. The blocks were paraffin-embedded. Four thin sections (3 μm thick) were cut at 200 μm intervals from each block and stained using hematoxylin-eosin. The length of the path of epithelial migration down both sides of each section was measured on the micrographs. Results obtained from the four sections of each side of each block were averaged as one measurement. Each value is the average ± SEM in three independent experiments.

Statistical Analysis

Statistical comparisons between two groups were performed by unpaired Student's *t*-test. ANOVA was used to compare three or more conditions with post hoc comparisons using the Tukey-Kramer procedure; p values < 0.05 were considered significant. Significant differences between groups are noted by *, † and ‡. Single symbol stands for $p < 0.05$, two symbols, for $p < 0.01$.

Results

Effects of TGF-β1 on expression of CTGF and FN in HCECs

TGF-β1 is known as a potent stimulator for connective tissue formation during wound repair and abundantly present in human tear fluid. It is also known that TGF-β1 regulates CTGF expression and stimulates FN synthesis. Therefore, we examined whether the addition of TGF-β1 promotes the expression levels of CTGF and FN in HCECs. The expression levels of CTGF and FN were analyzed in HCECs treated with TGF-β1 by Western blotting. As shown in Fig. 1, treatment with TGF-β1 up-regulated CTGF expression at 48 and 72 hours, but did not affect FN expression at any time point up to 72 hours in cell lysates. Western blot analysis of the conditioned media was used to characterize CTGF and FN released to the conditioned medium. The amount of FN accumulated in the cultured media was increased in a time-dependent manner (Fig. 1), but we could not detect CTGF in the cultured media at any time point up to 72 hours (data not shown).

Suppression of FN expression with knockdown of CTGF expression

Because CTGF is involved in wound healing and fibrosis, CTGF may play a role in FN synthesis. To test this hypothesis, CTGF-specific shRNAs were introduced into HCECs and their effects on the expression of CTGF and FN were examined. As shown in Fig. 2, CTGF-specific shRNA1 and shRNA2 suppressed CTGF and FN expression in HCECs. The negative control of shRNA and lipofectamine 2000 solution had no inhibitory effects on either CTGF or FN production. CTGF-specific shRNA3 and shRNA4 had weak inhibitory effects on the expression of CTGF and FN compared with shRNA1 and shRNA2.

Cell Attachment Assay

Although FN (10 μg/mL) itself facilitated the HCEC attachment to wells much more than control (1% BSA), CTGF significantly increased the number of HCECs attached in the presence of FN in the conditioned media compared with FN alone. However, CTGF alone had little effect on cell attachment (Fig. 3). There were no significant differences in the number of attachment cells between the CTGF concentration of 1.3 μM and 3.9 μM.

To further characterize the interaction between the CTGF and FN, FN-coated wells were prepared and the effects of CTGF, BSA or anti-FN antibody on cell attachment were examined. The media containing CTGF significantly promoted the attachment of HCECs compared with control (BSA), whereas anti-FN antibody completely inhibited CTGF-stimulated cellular adhesion to FN in comparison with control (IgG antibody) (Fig. 4). These results suggest that CTGF-stimulated cellular adhesion would be a FN-dependant pathway.

The Effect of CTGF in the Presence or Absence of FN on Corneal Epithelial Cell Migration

Epithelial cell migration over cultured corneal blocks showed that CTGF alone stimulated epithelial cell migration in a dose-dependent manner (Fig. 5). Mean values of control, CTGF (390 nM), CTGF (390 nM) + FN, and CTGF (3.9 μM) were 272, 325, 626, and 720 μm, respectively. CTGF (390 nM) plus FN (10 μg/mL) promoted significantly greater epithelial cell migration compared to CTGF alone (Fig. 5). When the CTGF concentration in the media was 3.9 μM, no significant difference in migration distance was seen between CTGF plus FN and CTGF alone (Fig. 5).

Discussion

This report documents the interaction between CTGF and FN in corneal epithelial adhesion and cell migration. Little is known about the effects of CTGF on corneal epithelial cells. Recently, Secker et al. (2008) demonstrated that CTGF regulates corneal re-epithelialization stimulated by TGF-β1. Our results concur with their report that CTGF promotes corneal epithelial migration and expand on their findings by demonstrating that CTGF promoted corneal epithelial cell adhesion and migration in the presence of FN, even in the absence of TGF-β1.

To the best of our knowledge, there are no reports where protein level fluctuation of FN stimulated by TGF-β1 in cultured human corneal epithelial cells was investigated, although there are many papers reporting that TGF-β1 stimulates an increase in the expression of FN in the corneal fibroblasts (Ohji et al. 1993; Sharma et al. 2009). As shown in Fig. 1, TGF-β1 promoted CTGF expression, but did not affect FN production in the cell lysates. However, Western blot analysis of the conditioned media from HCECs treated with TGF-β1 showed that the amount of FN released by HCECs were increased in a time-dependent manner. This is probably that FN is constitutively expressed in the corneal epithelial cells and the accumulation of secreted FN was increased in the media. On the other hand, CTGF could be detected in the cell lysates, while could not be detected in the supernatant of the cultured media (Fig. 1). This is prob-