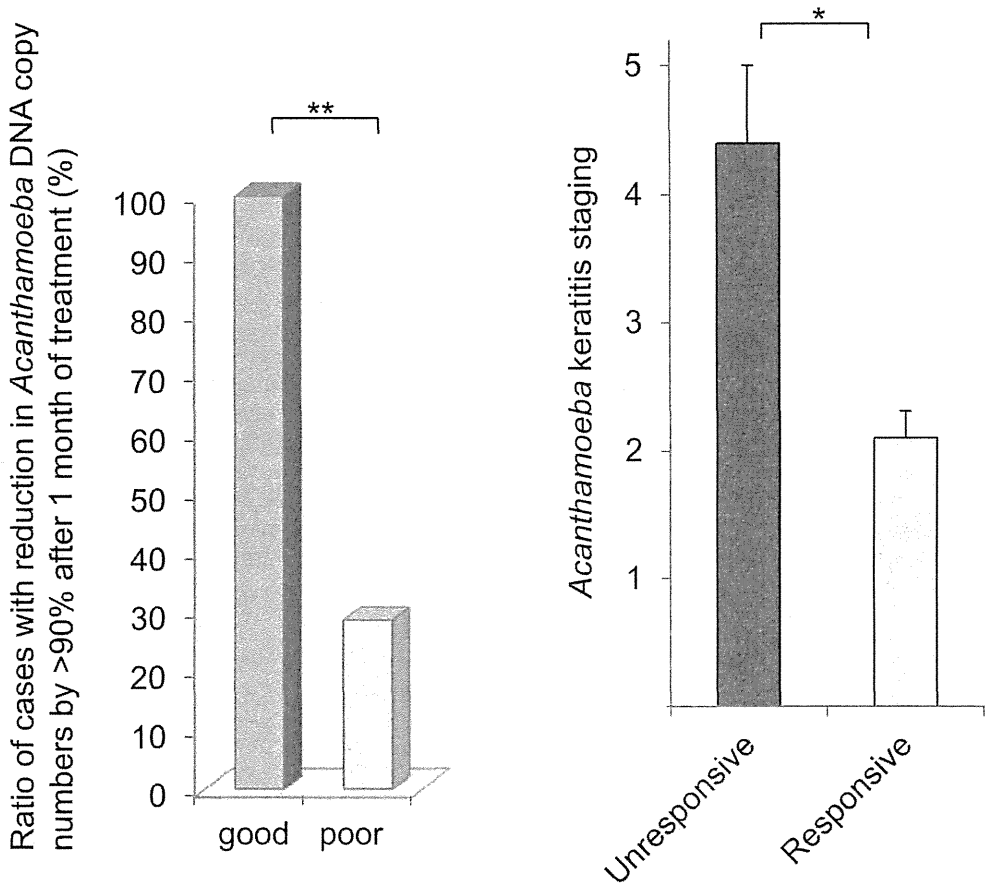

Figure 5. Association of poor outcomes with unresponsive *Acanthamoeba* copy reduction after treatment. Ratio of *Acanthamoeba* keratitis (AK) cases with unresponsive *Acanthamoeba* copy reduction after 1 month of treatment was significantly higher in AK with adverse prognosis (**A**). In the unresponsive AK cases, AK stage was significantly advanced compared with the responsive cases for amoebic copy reduction (**B**). *$P<0.01$. **$P=0.0005$.

Acanthamoeba because it does not rely on the functional integrity of the amoeba as do the conventional methods. The high sensitivity of the real-time PCR is also derived from the specificity of the TaqMan probe method[3] and the precise regression to the predetermined amount of amoebic DNA standards.

Even though *Acanthamoeba* is environmentally ubiquitous, our real-time PCR did not detect amoebic DNA in conjunctival smears from normal subjects. Although the number of subjects tested was limited, our findings indicate that amoebic trace is most likely absent in healthy eyes.

Acanthamoeba preys mainly on bacteria but also on fungi and other protozoans. Thus, *Acanthamoeba* might be observed as coinfectants in infectious keratitis cases. When we determined the specificity of *Acanthamoeba* PCR in BK cases, *Acanthamoeba* DNA was not detected in any of the BK cases, but 53.6% of the AK cases had low levels of bacterial DNA. This supports the concept of a bacterial involvement in the cause of AK, although the stage of the AK was not significantly correlated with the bacterial load (Fig 3). Thus, once AK is established, the bacterial load probably plays a limited role in its progression.

Table 2. Parameters Associated with Unresponsive *Acanthamoeba* DNA Reduction after 1 Month by Logistic Regression Analysis

	Odds Ratio					
	Lowest Category	*Second Category*	*95% CI*	*Highest Category*	*95% CI*	*P Value*
AK stage	1.0	Stage 2:8.00	1.06–58.82	Stage 5:4096	1.28–11 973 037	0.04*
Acanthamoeba DNA copy number at the first visit	1.0	≤1000:2.79	0.98–8	>100 000:60.88	0.92–4096	0.055
Bacterial load at the first visit	1.0	≤10:1.30	0.60–2.85	>10 000:6.52	2.99–14.25	0.51

AK = *Acanthamoeba* keratitis.
*$P\leq0.05$.

In the course of lengthy treatments for AK, clinicians are often frustrated when a chosen treatment regimen is ineffective. In refractory cases, *Acanthamoeba* is sometimes resistant to antifungal drugs or antiseptic drugs. Indeed, in our case series, multidrug-resistant *Acanthamoeba* were detected especially in the refractory cases. Moreover, drug-sensitivity testing of *Acanthamoeba* in vitro takes weeks for completion and does not necessarily mirror the sensitivity to the drugs in vivo, especially in refractory cases.[12] This suggests that the proliferation of *Acanthamoeba* seems to depend on both an impaired immune response of the host and the virulence of the *Acanthamoeba.* Consistent with this, the AK outcome was significantly correlated with an unresponsive reduction of amoeba copy numbers after anti-amoeba treatment.

Previous multivariate analysis of AK showed that the duration of the symptoms before diagnosis was a risk factor for a more advanced stage of the disease, and the more advanced stage at presentation was a risk factor for worse outcome.[4] Consistent with these findings, advanced disease stage was one of the significant risk factors for poor outcomes. Furthermore, we found that the detected *Acanthamoeba* copy numbers at the first visit were another risk factor. Advanced AK stage was also a risk factor for unresponsive reduction of amoebic DNA.

Our findings should help clinicians make earlier decisions on when to switch to surgical intervention after treatment. Of note, risk assessments for poor outcomes do not necessarily require real-time amoebic PCR. We suggest that conventional PCR or even smear staining would be sufficient for this purpose. For example, careful sampling of AK lesions during the course of treatment and evaluations by conventional Calcofluor or Fungiflora Y staining will determine whether more than 90% of amoebic bodies have been cleared after 1 month of treatment.

The sensitivity of real-time PCR in patients with AK did not reach the theoretic 100% sensitivity that real-time PCR should have achieved, perhaps because the sampled amount was not sufficient and the sampled location was not correct. The staining of corneal lesions usually requires more tissues, and therefore staining samples were collected before sampling for PCR. When AK is at an early stage and has low amoebic numbers, the sampling may remove even trace amounts of *Acanthamoeba.* In this case, smear staining would be positive but PCR would be negative. The location or depth of the lesion may also affect its outcome. For example, when samples are obtained from inflammatory-prone lesions at the early stage, but without amoeba, real-time PCR would be negative.

The strong immune responses of the host also affect the amoebic DNA load. Aggressive AK treatment or presumably host factors would exacerbate the *Acanthamoeba* copy numbers. This can present as dense inflammatory opacities that are difficult to differentiate from AK with high levels of *Acanthamoeba.* Indeed, we had a case with low visual acuity due to severe corneal and anterior chamber inflammation, in which the small amount of amoebic DNA was readily eradicated after a few weeks of treatment, and treatment was successfully switched to topical steroid therapy to reduce the inflammatory responses.

Refractory AK cases sometimes require therapeutic keratoplasty. The management of post-keratoplasty cases requires intensive use of steroids because they are susceptible to rejection because of the larger graft size and strong inflammatory environment provoked by the AK.

Real-time PCR for *Acanthamoeba* is also useful for confirmation of the complete removal of *Acanthamoeba.* It is a great relief for surgeons to know that the amoebic DNA becomes negative after surgical intervention in cases with advanced-stage AK with a million copies. *Acanthamoeba* real-time PCR requires only a minute amount of sample and is useful for confirming the absence of *Acanthamoeba.* Amoebic PCR ensures the validity of aggressive treatment or surgical intervention and would support the proper timing for the use of steroids for better visual outcome.

In conclusion, collectively, *Acanthamoeba* real-time PCR is effective in diagnosing AK. Real-time PCR detection does not provide information on virulence of *Acanthamoeba* or immunologic responses of the host, but it does provide useful information in managing AK.

References

1. Shiraishi A, Kobayashi T, Hara Y, et al. Rapid detection of *Acanthamoeba* cysts in frozen sections of corneal scrapings with Fungiflora Y. Br J Ophthalmol 2009;93:1563–5.
2. MacLean RC, Hafez N, Tripathi S, et al. Identification of *Acanthamoeba* sp. in paraffin-embedded CNS tissue from an HIV+ individual by PCR. Diagn Microbiol Infect Dis 2007;57:289–94.
3. Riviere D, Szczebara FM, Berjeaud JM, et al. Development of a real-time PCR assay for quantification of *Acanthamoeba* trophozoites and cysts. J Microbiol Methods 2006;64:78–83.
4. Tu EY, Joslin CE, Sugar J, et al. Prognostic factors affecting visual outcome in *Acanthamoeba* keratitis. Ophthalmology 2008;115:1998–2003.
5. Kandori M, Inoue T, Takamatsu F, et al. Two cases of *Acanthamoeba* keratitis diagnosed only by real-time polymerase chain reaction. Cornea 2010;29:228–31.
6. Kakimaru-Hasegawa A, Kuo CH, Komatsu N, et al. Clinical application of real-time polymerase chain reaction for diagnosis of herpetic diseases of the anterior segment of the eye. Jpn J Ophthalmol 2008;52:24–31.
7. Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology 2002;148:257–66.
8. Chew HF, Yildiz EH, Hammersmith KM, et al. Clinical outcomes and prognostic factors associated with *Acanthamoeba* keratitis. Cornea 2011;30:435–41.
9. Nguyen TH, Weisenthal RW, Florakis GJ, et al. Penetrating keratoplasty in active *Acanthamoeba* keratitis. Cornea 2010; 29:1000–4.
10. Thompson PP, Kowalski RP, Shanks RM, Gordon YJ. Validation of real-time PCR for laboratory diagnosis of *Acanthamoeba* keratitis. J Clin Microbiol 2008;46:3232–6.
11. Chang CW, Wu YC, Ming KW. Evaluation of real-time PCR methods for quantification of *Acanthamoeba* in anthropogenic water and biofilms. J Appl Microbiol 2010;109:799–807.
12. Perez-Santonja JJ, Kilvington S, Hughes R, et al. Persistently culture positive *Acanthamoeba* keratitis: in vivo resistance and in vitro sensitivity. Ophthalmology 2003;110:1593–600.

Footnotes and Financial Disclosures

Originally received: August 11, 2011.
Final revision: November 5, 2011.
Accepted: December 15, 2011.
Available online: March 3, 2012. Manuscript no. 2011-1207.

[1] Division of Ophthalmology and Visual Science, Faculty of Medicine, Tottori University, Tottori, Japan.

[2] Department of Ophthalmology, Ehime University School of Medicine, Ehime, Japan.

[3] Department of Ophthalmology, Kinki University Faculty of Medicine, Osaka, Japan.

[4] Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan.

Financial Disclosure(s):
The author(s) have no proprietary or commercial interest in any materials discussed in this article.

Supported by a grant for research on emerging and reemerging infectious diseases from the Ministry of Health, Labor, and Welfare of Japan: H23-Shinko-Ippan-014. The funding organization had no role in the design or conduct of this research.

Correspondence:
Yoshifumi Ikeda, 81-859-38-6617, Division of Ophthalmology and Visual Science, Tottori University Faculty of Medicine, 36-1 Nishi-cho, Yonago 683-8504, Japan. E-mail: yoshifumiikeda@hotmail.com.

Confocal Microscopic Observations of Stromal Keratocytes in Soft and Rigid Contact Lens Wearers

Kiyohiko Ohta, BS,† Ichiro Shimamura, MD,* Atsushi Shiraishi, MD, PhD* and Yuichi Ohashi, MD, PhD**

Purpose: To determine the density of corneal stromal cells in wearers of soft contact lenses (SCLs) and rigid gas-permeable (RGP) contact lenses (CLs).

Methods: The keratocyte density (KD) was measured at different depths of the stroma by confocal microscopy. In study 1, 32 wearers of rigid gas-permeable (RGP) lenses and 30 wearers of SCLs were studied. Forty volunteers with no history of CL wear were studied as controls. In study 2, 16 volunteers with no history of CL wear were divided into 2 groups; 7 subjects wore RGP lenses (oxygen transmissibility, Dk/L, 35) and 9 subjects wore SCLs (Dk/L, 34). All subjects were asked to wear the CLs daily for 6 months.

Results: In study 1, the KDs in the anterior stroma (AST) and the posterior stroma (PST) of the cornea were significantly lower in the RGP lens group than in the control group. The KD in the SCL group was significantly lower at all depths of the cornea than that of the control group. In study 2, the KD in the AST of the RGP lens group was significantly lower after 1 month of CL wear. The KD in the AST and PST of the SCL group was decreased significantly at 1 month, and all layers were decreased by 10% to 20% 6 months after wearing CLs. At 5 weeks after discontinuation of SCL wear, the KD in all layers was not significantly different from that at the baseline.

Conclusions: The change in the KD was greater in CL wearers than in volunteers with no history of CL wear and also greater in SCL wearers than in RGP lens wearers. Analysis of the KD by confocal microscopy may be a useful method for evaluating the effect of CL wear.

Key Words: confocal microscopy, contact lens wear, keratocytes, hypoxia, Dk/L

(*Cornea* 2012;31:66–73)

Wearing contact lenses (CLs) causes large changes in the environment of the corneal cells. A decrease in the level of oxygen is the most important environmental change, and the decrease leads to acute reactions, including corneal epithelial defects and formation of corneal endothelial blebs. Subacute reactions, such as corneal neovascularization and pigment slide, and chronic reactions, such as abnormal cell morphology, also arise from the hypoxic conditions. The abnormal cell morphologies have been studied by specular microscopy, and it is widely accepted that the corneal endothelial cell density decreases after prolonged use of hard CLs made of polymethyl methacrylate and also after wearing conventional soft contact lens (CSCL) with low water content.[1–8] It has also become clear that the use of these lenses causes the area of corneal epithelial cells to increase and their barrier function to decrease.[9–13]

Because observing the cornea by specular microscopy is noninvasive and relatively simple, the corneal epithelial cells and corneal endothelial cells have been extensively studied. However, it is difficult to observe the uniform collagen fibrils of the stromal layer of the cornea by specular microscopy, and little progress has been made in investigating the corneal stromal cells. Recent advancements in confocal microscopy have made it possible to observe the corneal stromal cells relatively easily, and this has led to a series of studies on the morphological changes in the corneal stromal cells in CL wearers.[14–24]

Since the 1996 report by Kaufman et al,[14] the confocal microscopic findings of the morphological changes in the keratocytes in CL wearers have been steadily accumulating.[14–24] It has been reported that wearers of soft contact lenses (SCLs) have lower keratocyte density (KD) than that of the control group and that long-term use of rigid gas-permeable (RGP) CLs affects the keratocytes in the superficial layer of the stroma. However, each of these reports had a different experimental design, studied different types of CLs, and used different methods to analyze the KD. For example, Bansal et al[15] and Hollingsworth and Efron[21] evaluated the KD in the anterior stroma (AST) layer, and Jalbert and Stapleton[16] evaluated the KD in the AST and posterior stroma (PST) layers of the corneal stroma. However, their studies only compared the AST or the AST and PST layers, but the investigators did not define the exact depth of these layers. On the other hand, Patel et al[20] measured the thickness of the corneal stroma with the Z-scan mode and divided the cornea into 5 layers, which enabled them to compare the KD in the different stromal layers. Their study had a different experimental design, studied different types of CLs, and used different methods to analyze the KD. Thus, a direct comparison of the morphological changes of the KD in

Received for publication March 8, 2010; revision received March 7, 2011; accepted March 18, 2011.
From the *Department of Ophthalmology, Ehime University School of Medicine, To-on, Ehime; and †Clinical Research Laboratories, Menicon Co, Ltd, Nagoya, Aichi, Japan.
The authors state that they have no financial or conflicts of interest to disclose.
Reprints: Kiyohiko Ohta, Clinical Research Laboratories, Menicon Co, Ltd, 21-19, Aoi 3, Naka-ku, Nagoya, Aichi 460-0006, Japan (e-mail: k-oota@menicon-net.co.jp).

RGP lens wearers and SCL wearers could not be made, and the morphological changes in the keratocytes in CL wearers have still not been determined.

The purpose of this study was to determine the effect of RGP lens and SCL wear on the KD. To accomplish this, we measured the KD in the different layers of the stroma by confocal microscopy in long-time CL wearers. We also conducted a prospective investigation on the effect of RGP lens and SCL wear in a group of subjects who had never worn CLs.

MATERIALS AND METHODS

Informed consent for the examination was obtained from all subjects, and the procedures used conformed to the tenets of the Declaration of Helsinki.

Study 1

Sixty-two patients who were healthy and had worn CLs for a mean duration of 7.6 ± 3.0 years (±SD) with a range from 1.5 to 15 years were studied. Of these 62 patients, there were 32 subjects who had worn an RGP lens for a mean duration of 8.2 ± 3.2 years and whose mean age was 25.8 ± 2.9 years. There were 22 men and 10 women in this group. There were 30 patients who had worn an SCL for a mean duration of 7.1 ± 2.7 years and whose mean age was 24.3 ± 1.8 years. There were 17 men and 13 women in this group. The specific types of CLs worn by these subjects are shown in Tables 1 and 2, respectively. A group of 40 subjects (26 men and 14 women) with a mean age 24.9 ± 2.2 years who had not worn CLs was studied as the control group (Table 3).

None of the subjects had anterior eye disease or a history of trauma to the eyes, and none was currently using any types of eye drops. After informed consent was obtained, the subjects were interviewed regarding their age, type of CL worn, history of CL wear, and manner in which they wore their CLs.

Study 2

All participants had expressed an interest in wearing CLs and agreed to participate in the research with the understanding that CL wear may lead to a decrease in the KD. No harmful events were detected during the CL wear at the 6-month examination period. The 16 healthy volunteers (9 men and 7 women) with no history of CL wear and a mean age of 22.4 ± 2.3 years were randomly separated into 2 groups. The 7 subjects in group 1 were prescribed RGP lenses. The mean age of this group was 24.3 ± 2.3 years, and there were 4 men and 3 women. The 9 subjects in group 2 were prescribed SCLs. The mean age of this group was 20.9 ± 0.8 years, and there were 5 men and 4 women. None of the subjects had anterior eye disease or a history of trauma to the eyes, and none of the subjects were using eye drops.

Subjects in both groups were asked to wear their CLs daily. The RGP lens group used lenses created from the same material as Menicon EX lenses [Menicon Co, Ltd, Aichi, Japan; oxygen permeability (Dk) 64; ISO method], and the SCL group used Menicon Soft S lenses [Menicon Co, Ltd.; Dk 34, oxygen transmissibility (Dk/L) 34; ISO method]. The design of the RGP lens was identical to the commercially available Menicon EX lenses, and the CLs were specially produced so that the central portion of the lens was thicker. Thus, the lenses in the 2 groups had the same Dk/L of 35. Subjects were instructed to wear the CLs daily for 6 months, and medical interviews were performed before the initial CL wear and for 6 months after discontinuing the CL wear. The medical interview was conducted in a manner similar to that used in study 1.

Ultrasound Pachymetry

The CL wearers were examined by slit-lamp biomicroscopy, and no abnormalities of the anterior segment were found in all subjects. Eyes were anesthetized with 0.4% oxybuprocaine hydrochloride (benoxinate hydrochloride; Santen Pharmaceutical Co, Ltd, Osaka, Japan), and the central corneal thickness was measured with an ultrasonic pachymeter (SP-2000; Tomey Co, Ltd, Aichi, Japan). Measurements were performed 5 times on each eye, and the average and SDs were determined and used for the statistical analyses.

Confocal Microscopy

The ConfoScan2 (CS2; Nidek Technologies, Vigonza, Italy) was used for confocal microscopy. The corneal stromal and the corneal endothelial cells were photographed by the same examiner (I.S.) between 10:00 AM and 12:00 PM. After determining the absence of abnormalities in the anterior segment by slit-lamp biomicroscopy, the eyes were anesthetized with benoxinate hydrochloride, and photographs

TABLE 1. RGP Lens Data (Study 1)

Proprietary Name	Wear (No. Eyes)	Manufacturer	US Adopted Name	Subjects (No. Eyes)	Dk	Dk/L
Menicon O_2-32	DW (2)	Menicon	—	2 (2)	31	21
Menicon EX	DW (15)	Menicon	Tolofocon A	10 (17)	64	43
	EW (2)					
Menicon Super EX	DW (7)	Menicon	Melafocon A	5 (7)	126	70
Hard EX	DW (4)	HOYA	—	3 (4)	125	83
Breath-O Hard CL	DW (5)	TORAY	—	4 (7)	150	88
	EW (2)					
Menicon Z	DW (14)	Menicon	Tisilfocon A	8 (14)	163	125
Total	DW (51)	—	—	32 (51)	—	—

Dk, oxygen permeability, $\times 10^{-11}$ (cm^2/s) ($mLO_2/(mL \times mm\ Hg)$); Dk/L, oxygen transmissibility, $\times 10^{-9}$ (cm/s) ($mLO_2/(mL \times mm\ Hg)$); DW, daily wear; EW, extended wear.

TABLE 2. SCL Data (Study 1)

SCL Types	Proprietary Name	Wears	Manufacturer	US Adopted Name	US FDA Class	Subjects (No. Eyes)	Dk	Dk/L	Water Content (%)
DSCL	1Day ACUVUE	1 d	Johnson & Johnson	Etafilcon A	Group 4	8 (14)	28	33.3	58
FRSCL	SUREVUE	2 wk	Johnson & Johnson	Etafilcon A	Group 4	2 (3)	28	26.7	58
	Medalist	2 wk	Bausch & Lomb	Polymacon	Group 1	3 (6)	9.5	27.1	38.6
	Focus 2WEEK LENSES	2 wk	CIBA VISION	Vilfilcon A	Group 4	2 (4)	16	26.7	55
	ACUVUE 2	2 wk	Johnson & Johnson	Etafilcon A	Group 4	6 (11)	28	33.3	58
	Monthly Fine	1 mo	SEED	Polymacon	Group 1	1 (2)	8	11.5	38.1
CSCL	Menicon Soft S	Conventional	Menicon	Mipafilcon A	Group 2	3 (5)	34	34	72
	Aime Super Soft	Conventional	Aime	—	Group 1	1 (2)	12	34	40
	PLENO	Conventional	HOYA	Polymacon	Group 1	1 (2)	11	22	38.6
	Menicon Soft MA	Conventional	Menicon	Govafilcon A	Group 1	1 (2)	9	10	37.5
	Menicon Soft 72	Conventional	Menicon	Mipafilcon A	Group 2	1 (1)	34	23	72
	SOFT α	Conventional	Nichicon	Polymacon	Group 1	1 (1)	9.3	12	38
	Total	—	—	—	—	30 (53)	—	—	—

DSCL, disposable soft contact lens; FDA, Food and Drug Administration; FRSCL, frequent replacement soft contact lens.

were taken while the patient looked straight ahead. To improve the resolution, a viscous gel (Viscotears Liquid Gel; CIBA Vision Ophthalmics, Rome, Italy) with a refractive index similar to that of the cornea was used to couple the objective lens optically to the cornea. The lens did not come in contact with the cornea. The cornea was photographed with the full automatic scan mode of the confocal microscope. The scanning system was mechanically moved along the *z* axis, obtaining successive equally spaced frames of the corneal layers. The corneal thickness interval was set to 20 μm, which then determined the time between the photographed images in a sequence. The CS2 Z-scan mode was used to measure and evaluate the depth in the corneal stroma at which each image was taken.

Examination of Stromal and Endothelial Cell Morphology

Images of each of the corneal layers were collected from each subject. The thickness of the corneal stromal layer was measured using the CS2 Z-scan mode. Digital images were photographed at a depth of 0% (superficial layer), which was defined as being directly below Bowman's membrane, and 100% of the depth of the corneal stromal layer defined as being directly above the corneal endothelium. The AST was approximately 5% below the 0% depth, the lower anterior stroma (LAST) was approximately 20% below the 0% depth, the central stroma (CST) was approximately 50% below the 0% depth, the upper posterior stroma (UPST) was approximately 80% below the 0% depth, and the PST was approximately 95% below the 0% depth. There were usually 3 to 12 (mean, 6) extracted digital images that were suitable for analysis from each layer.

Next, the digital images were imported into an image analysis program (Win ROOF; Mitani Co, Ltd, Fukui, Japan). The average KD value for each layer was determined by an automatic analysis of the corneal stromal cell nuclei in the central region of each image (260 × 260 μm; Fig. 1). The automatic image analysis program was created using a Fourier transform and image binarization program before this study, and the reproducibility was confirmed. The focal depth of the CS2 is 25.9 μm.[25] The KD in each image was calculated as cells per cubic millimeter by the focal depth of 25.9 μm. The central region of randomly chosen images of the corneal endothelial cells was analyzed by the CS2's automatic analysis function. The data analysis was performed by the same examiner (K.O.) who was masked to the type of CLs worn by the subjects whose images were being examined.

TABLE 3. Subject Demographic and Lens Wearing Data (Study 1)

	Control	RGP	SCL
Subjects (No. Eyes)	40 (75)	32 (51)	30 (53)
Age (yr)			
Mean ± SD	24.9 ± 2.2	25.8 ± 2.9	24.3 ± 1.8
Range	22–30	22–35	22–30
Men:women	26:14	22:10	17:13
Wear duration (yr)			
Mean ± SD		8.2 ± 3.2	7.1 ± 2.7
Range		3.0–15	1.5–12
Corneal thickness (μm)			
Mean ± SD	532 ± 16	550 ± 31	550 ± 22
Corneal endothelial cell (cells/mm^2)			
Mean ± SD	2676 ± 225	2739 ± 264	2760 ± 243
	Not significant	Not significant	Not significant

Statistical Analyses

Statistical analyses of the differences in the KD were done by the SAS Ver. 8.2 (SAS Institute, Tokyo, Japan). For study 1, the differences in the KD among the RGP lens group, SCL group, and control group for each layer (AST, LAST, CST, UPST, and PST) were evaluated by using a mixed effect model, with the KD values as the response variable, the groups as the fixed effect, and the cases as random effects. The least squares of the means were calculated for the differences in the

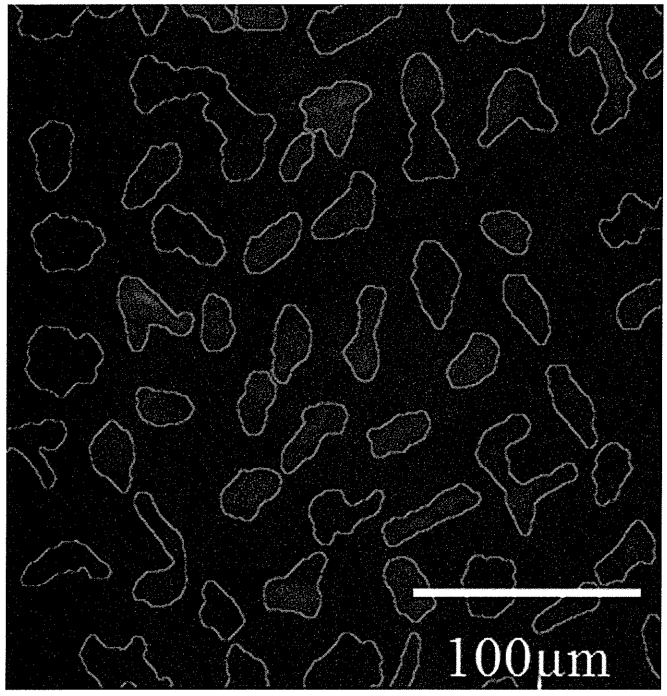

FIGURE 1. Digital images of corneal stromal cells. The digital images were imported into a computer with image analysis software, and the KD was found by automatically tracing the nuclei of corneal stromal cells in the central portion of the image (260 × 260 μm).

KD among the different SCL types [CSCL, frequent-replacement SCL (FRSCL), and disposable SCL (DSCL)] using the mixed effect model. The age, corneal and stromal thickness, and corneal endothelial cell density were compared among the 3 groups and tested by the Fisher protected least significant difference test with the StatView Ver. 5.0 for windows. The relationship between the KD and the duration of SCL use was evaluated by correlation coefficients.

For study 2, the least squares of the means of the changes in the KD from baseline in each layer and for the RGP lens group and SCL group were determined by using a mixed effect model, with the change as a response variable, the baseline value of the KD (after initial CL wearing) and the duration of CL as fixed effects, and the cases as random effects. The changes of corneal thickness and corneal endothelial cell density from the baseline values were evaluated by Dunnett tests, and the difference of lens power between RGP lens and SCL was evaluated by the Mann–Whitney test, with the StatLight 2000 for Windows.

RESULTS

Study 1

In the RGP lens group, the KD was significantly lower than that of the control group in the AST and PST layers ($^{\#\#}P < 0.01$; Fig. 2). The KD in all stromal layers of the cornea in the SCL group was significantly lower than that of the control group ($^{**}P < 0.01$; Fig. 2). In the PST layer, a significant difference was found in the KD between the RGP lens and SCL groups ($^{\#\#}P < 0.01$; Fig. 2).

No significant difference was found in the KD among the CSCL, FRSCL, and DSCL groups in any of the layers ($P > 0.05$; Tables 4, 5). In addition, when the RGP lens and SCL groups were divided into 3 groups based on the Dk/L of the individual CLs, no significant difference in the KD was found among the 3 groups of RGP lens wearers (Dk/L ≤ 50, 50 < Dk/L < 100, and Dk/L ≥ 100; $P > 0.05$) and the 3 groups of SCL wearers (Dk/L ≤ 20, 20 < Dk/L < 30, and Dk/L ≥ 30; $P > 0.05$) in any of the layers (data not shown). In addition, the duration of SCL wear was not significantly correlated with the changes in the KD (AST, $r = -0.39$; LAST, $r = -0.10$; CST, $r = -0.03$; UPST, $r = 0.19$; PST, $r = -0.22$). No significant difference was found in the corneal thickness and the corneal endothelial cell density among the control,

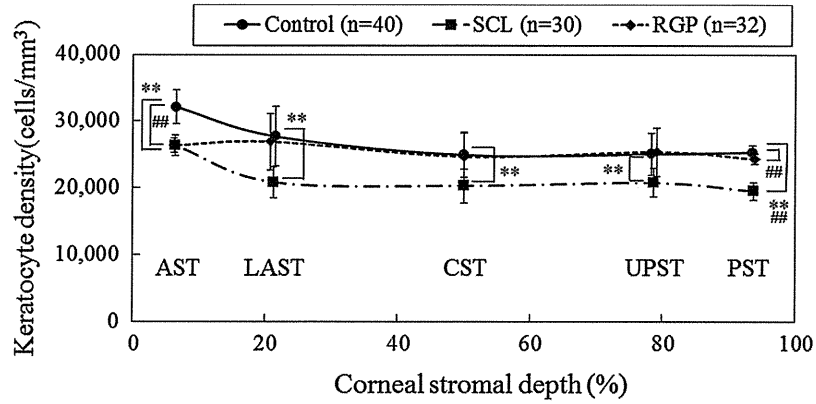

FIGURE 2. KD for each group of CL wearers at different corneal stromal depths (study 1). The corneal stroma was divided into 5 layers starting from the region closest to the corneal epithelium, and the KDs for the control group, RGP lens group, and SCL group are plotted. The KD is lower in all layers of the SCL group than in the control group ($^{**}P < 0.01$). The KD in the AST and PST layers in the RGP lens group is lower than that of the control group ($^{\#\#}P < 0.01$). Error bars represent the SDs.

CL	Keratocyte density (cells/mm³) : Mean ± SD				
	Anterior stroma (AST)	Lower anterior stroma (LAST)	Central stroma (CST)	Upper posterior stroma (UPST)	Posterior stroma (PST)
Control	32135 ± 2506	27705 ± 4459	24910 ± 3320	25055 ± 3202	25305 ± 975
SCL	26340 ± 1494**	20842 ± 2337**	20317 ± 2546**	20745 ± 2171**	19524 ± 1295**
RGP	26359 ± 1120##	26900 ± 4221	24672 ± 3661	25377 ± 3596	24337 ± 833##

TABLE 4. Subject Demographic and SCL Types (Study 1)

	CSCL	FRSCL	DSCL
Subjects (No. Eyes)	8 (13)	14 (26)	8 (14)
Age (yr)			
Mean ± SD	24.6 ± 0.9	23.9 ± 1.7	24.9 ± 2.6
Men:women	4:4	10:4	4:4
Wear duration (yr)			
Mean ± SD	7.9 ± 3.5	6.9 ± 2.2	6.5 ± 2.8

RGP lens, and SCL groups after the CL wear ($P > 0.05$; Table 3).

Study 2

Two subjects in the RGP lens group dropped out in the middle of the study, and we were able to follow-up only 5 subjects until the completion of the study [mean age, 24.2 ± 2.8 years; 2 men and 3 women; average lens power, −2.30 ± 1.7 diopter (D)]. In the RGP lens group, the KD of the AST layer was significantly lower at 1 month after beginning the CL wear than at baseline ($P < 0.01$). In addition, the KD in the AST and PST layers was significantly lower at 6 months after beginning the CL wear ($P < 0.01$). There was no significant difference in the KD in the LAST, CST, and UPST layers at the 6-month examination ($P > 0.05$; Table 6). In addition, no significant difference was found in corneal thickness ($P > 0.05$; Fig. 3A) and the corneal endothelial cell density ($P > 0.05$).

The KDs of the AST, PST ($P < 0.01$), and UPST ($P = 0.02$) layers in the SCL group (average lens power, −2.08 ± 2.0 D) were significantly decreased at 1 month after beginning the CL wear. Six months later, the KD in all layers was reduced to approximately 10% to 20% ($P < 0.01$) of the baseline values (Table 7). No significant difference was found in corneal thickness ($P > 0.05$; Fig. 3B) and the corneal endothelial cell density ($P > 0.05$). When SCL wear was discontinued, the KD recovered to the baseline values in all layers within 5 weeks ($P > 0.05$; Table 7). There was no significant difference between the average lens power of the RGP lens and SCL groups ($P = 0.66$).

DISCUSSION

We used the Z-scan mode of the CS2 to evaluate the KD in 5 stromal layers of the cornea in wearers of SCLs and RGP lenses. The investigation was conducted retrospectively in study 1 and prospectively in study 2. To the best of our knowledge, this is the first study that made a direct comparison of the KD in all layers of cornea stroma in RGP lens and SCL users. Although several studies have evaluated the KD in CL wearers,[14–24] the exact changes in the KD in RGP lens wearers and SCL wearers are inconclusive. Our retrospective and prospective studies showed results that were consistent with earlier studies, and the results of the 6 months prospective study strongly supported the results of the retrospective study.

It has been reported that the KD decreases with increasing age,[26] and a significant difference ($P = 0.02$; Table 3) was found between the ages of the RGP lens group (mean age, 25.8 ± 2.9 years) and the SCL group (mean age, 24.3 ± 1.8 years). Thus, the difference in the KD between the RGP lens and SCL groups was more likely due to the slightly younger (1.5 years) subjects in the SCL group because the KD was reported to decrease by 0.3% per year.[26]

Our overall findings showed that RGP lens wear initially affected the KD in the AST layer of the cornea, and with longer wear, the decrease extended to the AST and PST layers of the cornea. SCL wear initially affected the KD in the AST, UPST, and PST layers of the cornea and then extended to all layers of the cornea. Of interest is that after a discontinuation of CL wear, there was a relatively rapid recovery of the KD. Thus, our results probably might resolve some of the controversy regarding the morphological changes of the KD in RGP lens wearers and SCL wearers. However, the mechanism of CL-induced keratocyte loss has still not been determined. There are many investigators who have suggested that the KD is affected by hypoxia, mechanical stress, or cytokine-mediated activity. In a prospective study, Kallinikos et al[24] examined subjects who wore a 30-day continuous wear RGP lens for 12 months, and they examined the KD in 5 corneal stromal layers. They found a significant decrease in the KD in the anterior to middle layers of the corneal stroma. They suggested that the decrease in the KD was not due to insufficient oxygen but due to mechanical stress from the CL wear. A similar phenomenon has been observed before the long-term use of polymethyl methacrylate lenses and has been reported by Bansal et al[15] and Hollingsworth et al.[21] Our results were quite similar to those of Kallinikos et al,[24] although their results showed that the decrease in the KD was found in the LAST and not in the AST layer in RGP lens wearers.

In study 2, the KD in the AST layer was found to be significantly reduced even after only 1-month wear of both RGP lenses and SCLs. Edelhauser[27] and Efron[28] reported that mechanical stimulation by CL wear releases inflammatory mediators, which have been shown to affect cell function and density in the cornea. Thus, accumulating evidence supports the hypothesis that the decrease in the KD in the AST in RGP

TABLE 5. KD in SCL Types (Study 1)

	KD (cells/mm^3), Mean ± SD				
CL	AST	LAST	CST	UPST	PST
CSCL	25,965 ± 1673	21,352 ± 2973	20,298 ± 3125	21,616 ± 2531	19,300 ± 1463
FRSCL	26,269 ± 1406	20,908 ± 2041	20,520 ± 2341	20,429 ± 1733	19,663 ± 1192
DSCL	26,836 ± 1440	20,236 ± 2281	19,927 ± 2503	20,569 ± 2535	19,503 ± 1367

TABLE 6. KD in RGP Lens Wears (Study 2)

	KD (cells/mm^3), Mean ± SD				
CL Wear	**AST**	**LAST**	**CST**	**UPST**	**PST**
0 mo	33,681 ± 1746	25,302 ± 663	25,531 ± 853	25,531 ± 764	24,365 ± 1979
1 mo	31,956 ± 956*	25,359 ± 295	25,188 ± 950	25,416 ± 903	24,440 ± 1263
6 mo	29,751 ± 1567*	25,645 ± 568	25,473 ± 723	25,473 ± 817	22,944 ± 628*

*Decreased KD ($P < 0.01$).
The KD after beginning the RGP lens wear was compared with initial density.

lens and SCL wearers may be due to mechanical stress from wearing CLs.

However, our results demonstrated that a significant decrease in the KD was found in the PST layers and not in the middle layers of the stroma after 1 month in SCL wearers. Also, a significant decrease in the KD was found in the PST layers, but not in the middle layers of stroma in 6 months in the retrospective study of RGP lens wearers. The fact that the decrease in the KD in the AST and PST without middle layers of stroma, cannot be simply explained by the mechanical stress hypothesis.

Exposure of the cornea to low oxygen concentrations may be another reasonable cause for the decrease in the KD. In general, a Dk/L of at least 24 is necessary to avoid corneal edema during daily wear.[29] In study 2, both types of CLs had a Dk/L > 24, which had no effect on the corneal thickness, endothelial cell density, and the epithelial cells. However, when both the RGP lens and the SCL had similar Dk/Ls, a significant decrease in the KD was found in the PST layer in the early period only in the SCL group. With longer wearing times, a significant decrease in the KD in all corneal layers was observed in the SCL group. In study 1, a comparison of the corneas of SCL and RGP lens wearers with Dk/L of approximately 40 showed that the decrease in the KD in all layers of the cornea excluding the AST and PST were significant only in the SCL group. Thus, it seems that the effect of CL wear on the density of the corneal stroma cannot be adequately explained by a simple comparison of the Dk/L values.

The main oxygen supply to the corneal tissue is from the air through the lacrimal fluid–mediated pathways. Because

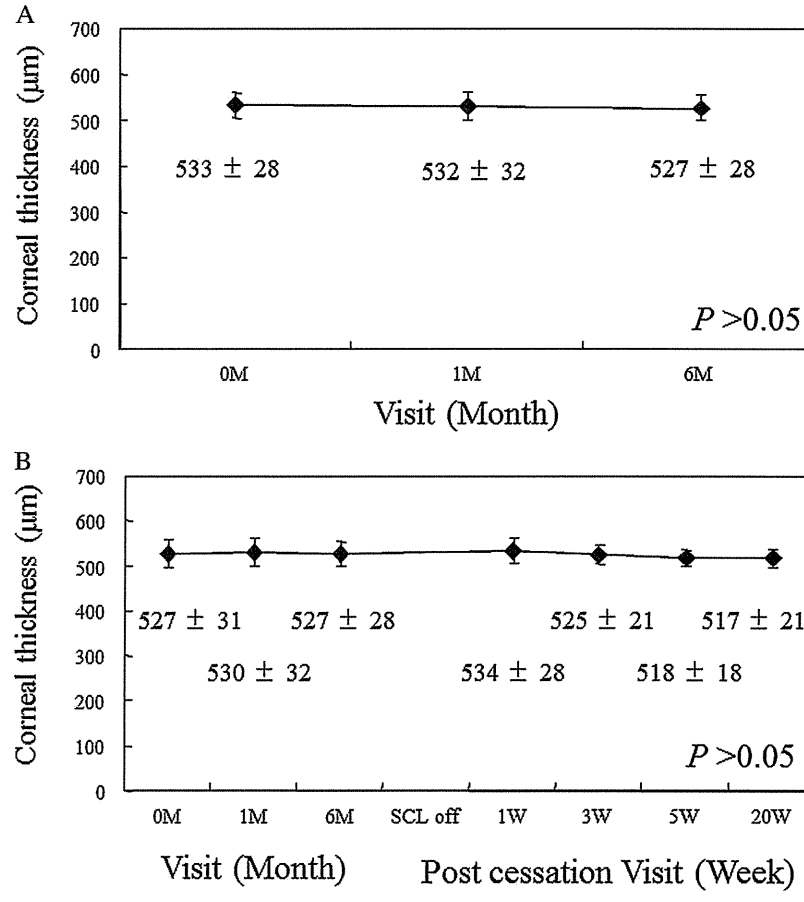

FIGURE 3. Corneal thickness for RGP lens (A) and SCL (B) wearers (study 2). During the 6-month prospective study (study 2), no changes in the corneal thickness are observed in either the RGP lens (A) or the SCL (B) wearers over the course of the observation period ($P > 0.05$). Error bars represent the SDs.

TABLE 7. KD in SCL Wears (Study 2)

	KD (cells/mm^3), Mean ± SD				
	AST	LAST	CST	UPST	PST
CL wear					
0 mo	32,411 ± 1707	26,885 ± 1727	25,784 ± 1776	26,640 ± 1219	24,220 ± 1283
1 mo	30,795 ± 881*	26,368 ± 668	25,988 ± 1239	25,511 ± 1335†	22,713 ± 1381*
6 mo	26,182 ± 967*	23,955 ± 1285*	23,720 ± 1179*	24,090 ± 1631*	20,270 ± 745*
After discontinuing CL wear					
1 wk	26,930 ± 1030*	24,446 ± 1469*	24,103 ± 1394*	24,903 ± 978*	19,893 ± 969*
3 wk	31,485 ± 894	25,345 ± 1180†	25,059 ± 1199	25,916 ± 1010†	21,875 ± 1549
5 wk	32,206 ± 1336	25,893 ± 1968	24,846 ± 1069	25,416 ± 599	23,067 ± 1028
20 wk	32,464 ± 1250	27,015 ± 1112	26,445 ± 895	27,073 ± 1297	23,126 ± 673

*Decreased KD ($P < 0.01$).
†Decreased KD ($P < 0.05$).
The KD after beginning the SCL wears and discontinuing SCL wear was compared with initial density.

oxygen obtained from the air is gradually consumed by the corneal epithelial cells, the partial pressure of oxygen is lower in the deeper regions of the corneal stroma. Therefore, these regions of the corneal stroma seem to be more likely influenced by decreases in the oxygen supply from the corneal surface. The corneal endothelium is the farthest from the corneal surface, but it is supplied with oxygen from the anterior aqueous, where the partial pressure of oxygen is 40 to 60 mm Hg.[30–32] Thus, the endothelial cells are less likely to be affected by low levels of oxygen on the surface of the eye. When a SCL wearer blinks, the lacrimal fluid under the SCL is reduced to 1/10 to 1/20 of that of RGP lens wearers even if the SCL fits well.[30,33] Key[34] reported that the minimal tear exchange capacity and larger diameters may explain why a higher rate of corneal infiltrates, sterile ulcers, and irregular staining patterns is seen in SCL wearers than RGP lens weares. Therefore, even if the Dk/L values of SCL are identical to those of RGP lens, the supply of oxygen to the cornea may not be the same.

It has been reported that corneal thinning occurs in CL wearers.[35–37] Holden et al[35,36] reported a 2.3% (11 μm) stromal thinning in subjects who wore extended wear SCL over a 5-year period, but it was manifested only after discontinuing CL wear for 7 days. Recently, Liu and Pflugfelder[37] observed a 30 to 50 μm decrease in corneal thickness in subjects who had worn SCL for an average of 13.5 years. All the corneal cells in SCL wearers undergo gradual metabolic adaptations in response to chronic oxygen deficiency in order to survive over a long period of time in an unfavorable environment. This can be thought of as a remodeling. Despite these suggestions, statistical analyses of the data of our study 1 and study 2 showed no significant difference in the corneal thickness. The discrepancy of the results from previous reports and ours may be because most of the previous studies examined extended CL wearers, whereas we examined daily CL wearers. Thus, extended CL wear may be a risk factor for corneal thinning.

Although corneal thinning and morphological abnormalities in the corneal endothelial cells were not observed in our study, a decrease in the KD occurred in the AST and PST layers after only 1 month of SCL wear. Thereafter, the decrease in the KD gradually extended to all layers of the cornea. Surprisingly, within 5 weeks of discontinuing SCL wear, the KD in all layers of the cornea returned to baseline. These results suggest that the corneal stromal cells may be more sensitive than either the corneal endothelial cells or the corneal epithelial cells to the level of oxygen and mechanical stress caused by CL wear.

Other methods to evaluate the oxygen supply to the corneal tissue have not been published except by the slit-lamp findings, CL fitting, and Dk/L. Measuring the partial pressure of oxygen in the lacrimal fluid beneath the CL with an oxygen pressure–monitoring system[38–43] would certainly be the most accurate method of determining the oxygen levels. However, this procedure is invasive and complicated and is not suitable for clinical testing. Therefore, the measurement of the KD using confocal microscopy may be useful as a noninvasive and simple way to obtain an indirect index of the oxygen supply in corneal tissues, especially at PST.

CONCLUSIONS

We found that the KD of CL wearers decreased as early as 1 month after wearing. The decrease in the KD may provide proof of stress on the cornea by CL wear. The findings of earlier studies combined with our results indicate that the abnormalities that occur in the AST layer of the cornea are most likely due to mechanical stress to the corneal tissue and that the abnormalities occurred in the PST layer of the cornea seem most likely due to insufficient oxygen supply to the corneal tissue. Analysis of the KD using confocal microscopy may be a useful method of evaluating the morphological changes of the corneal tissue during CL wear.

REFERENCES

1. Caldwell DR, Kastl PR, Dabezies OH, et al. The effect of long-term hard lens wear on the corneal endothelium. *Contact Intraocul Lens Med J.* 1982;8:87–91.
2. MacRae SM, Matsuda M, Shellans S, et al. The effects of hard and soft contact lenses on the corneal endothelium. *Am J Ophthalmol.* 1986;102: 50–57.
3. MacRae SM, Matsuda M, Shellans S. Corneal endothelial changes associated with contact lens wear. *CLAO J.* 1989;15:82–87.

4. MacRae SM, Matsuda M, Phillips DS. The long-term effects of polymethylmethacrylate contact lens wear on the corneal endothelium. *Ophthalmology.* 1994;101:365–370.
5. McMahon TT, Polse KA, McNamara N, et al. Recovery from induced corneal edema and endothelial morphology after long-term PMMA contact lens wear. *Optom Vis Sci.* 1996;73:184–188.
6. Setala K, Vasara K, Vesti E, et al. Effects of long-term contact lens wear on the corneal endothelium. *Acta Ophthalmol Scand.* 1998;76:299–303.
7. Bourne WM, Hodge DO, McLaren JW. Estimation of corneal endothelial pump function in long-term contact lens wearers. *Invest Ophthalmol Vis Sci.* 1999;40:603–611.
8. Lee JS, Park WS, Lee SH, et al. A comparative study of corneal endothelial changes induced by different durations of soft contact lens wear. *Graefes Arch Clin Exp Ophthalmol.* 2001;239:1–4.
9. Yokoi N, Kinoshita S. Clinical evaluation of corneal epithelial barrier function with the slit-lamp fluorophotometer. *Cornea.* 1995;14:485–489.
10. McNamara NA, Polse KA, Bonanno JA. Fluorophotometry in contact lens research: the next step. *Optom Vis Sci.* 1998;75:316–322.
11. McNamara NA, Polse KA, Fukunaga SA, et al. Soft lens extended wear affects epithelial barrier function. *Ophthalmology.* 1998;105:2330–2335.
12. Ichijima H, Yokoi N, Nishizawa A, et al. Fluorophotometric assessment of rabbit corneal epithelial barrier function after rigid contact lens wear. *Cornea.* 1999;18:87–91.
13. Lin MC, Graham AD, Fusaro RE, et al. Impact of rigid gas-permeable contact lens extended wear on corneal epithelial barrier function. *Invest Ophthalmol Vis Sci.* 2002;43:1019–1024.
14. Kaufman SC, Hamano H, Beuerman RW, et al. Transient corneal stromal and endothelial changes following soft contact lens wear: a study with confocal microscopy. *CLAO J.* 1996;22:127–132.
15. Bansal AK, Mustonen RK, McDonald MB. Higt resolution in vivo scanning confocal microscopy of the cornea in long term contact lens wear. *Invest Ophthalmol Vis Sci.* 1997;38:S138.
16. Jalbert I, Stapleton F. Effect of lens wear on corneal stroma: preliminary findings. *Aust N Z J Ophthalmol.* 1999;27:211–213.
17. Bourne WM. The effect of long-term contact lens wear on the cells of the cornea. *CLAO J.* 2001;27:225–230.
18. Efron N, Mutalib HA, Perez-Gomez I, et al. Confocal microscopic observations of the human cornea following overnight contact lens wear. *Clin Exp Optom.* 2002;85:149–155.
19. Efron N, Perez-Gomez I, Morgan PB. Confocal microscopic observations of stromal keratocytes during extended contact lens wear. *Clin Exp Optom.* 2002;85:156–160.
20. Patel SV, McLaren JW, Hodge DO, et al. Confocal microscopy in vivo in corneas of long-term contact lens wearers. *Invest Ophthalmol Vis Sci.* 2002;43:995–1003.
21. Hollingsworth JG, Efron N. Confocal microscopy of the corneas of long-term rigid contact lens wearers. *Contact Lens Anterior Eye.* 2004;27:57–64.
22. Kallinikos P, Efron N. On the etiology of keratocyte loss during contact lens wear. *Invest Ophthalmol Vis Sci.* 2004;45:3011–3020.
23. Trittibach P, Cadez R, Eschmann R, et al. Determination of microdot stromal degenerations within corneas of long-term contact lens wearers by confocal microscopy. *Eye Contact Lens.* 2004;30:127–131.
24. Kallinikos P, Morgan P, Efron N. Assessment of stromal keratocytes and tear film inflammatory mediators during extended wear of contact lenses. *Cornea.* 2006;25:1–10.
25. McLaren JW, Nau CB, Kitzmann AS, et al. Keratocyte density: comparison of two confocal microscopes. *Eye Contact Lens.* 2005;31:28–33.
26. Moller-Pedersen T. A comparative study of human corneal keratocyte and endothelial cell density during aging. *Cornea.* 1997;16:333–338.
27. Edelhauser HF. The resiliency of the corneal endothelium to refractive and intraocular surgery. *Cornea.* 2000;19:263–273.
28. Efron N. Contact lens-induced changes in the anterior eye as observed in vivo with the confocal microscope. *Prog Retin Eye Res.* 2007;26:398–436.
29. Holden BA, Mertz GW. Critical oxygen levels to avoid corneal edema for daily and extended wear contact lenses. *Invest Ophthalmol Vis Sci.* 1984;25:1161–1167.
30. Liesegang TJ. Physiologic changes of the cornea with contact lens wear. *CLAO J.* 2002;28:12–27.
31. Bonanno JA, Polse KA. Effect of rigid contact lens oxygen transmissibility on stromal pH in the living human eye. *Ophthalmology.* 1987;94:1305–1309.
32. Bonanno JA. Contact lens induced corneal acidosis. *CLAO J.* 1996;22:70–74.
33. Polse KA. Tear flow under hydrogel contact lenses. *Invest Ophthalmol Vis Sci.* 1979;18:409–413.
34. Key JE II. Are hard lenses superior to soft? Arguments in favor of hard lenses. *Cornea.* 1990;9(suppl 1):S9–S11; discussion S15.
35. Holden BA, Sweeney DF, Vannas A, et al. Effect of long-term extended contact lens wear on the human cornea. *Invest Ophthalmol Vis Sci.* 1985;26:1489–1501.
36. Holden BA, Sweeney DF, Efron N, et al. Contact lenses can induce stromal thinning. *Clin Exp Optom.* 1988;71:109–113.
37. Liu Z, Pflugfelder SC. The effects of long-term contact lens wear on corneal thickness, curvature, and surface regularity. *Ophthalmology.* 2000;107:105–111.
38. Freeman RD. Oxygen consumption by the component layers of the cornea. *J Physiol.* 1972;225:15–32.
39. Freeman RD, Fatt I. Oxygen permeability of the limiting layers of the cornea. *Biophys J.* 1972;12:237–247.
40. Fatt I, Freeman RD, Lin D. Oxygen tension distributions in the cornea: a re-examination. *Exp Eye Res.* 1974;18:357–365.
41. Efron N, Ang JH. Corneal hypoxia and hypercapnia during contact lens wear. *Optom Vis Sci.* 1990;67:512–521.
42. Rivera RK, Polse KA. Corneal response to different oxygen levels during extended wear. *CLAO J.* 1991;17:96–101.
43. Ichijima H, Hayashi T, Mitsunaga S, et al. Determination of oxygen tension on rabbit corneas under contact lenses. *CLAO J.* 1998;24:220–226.