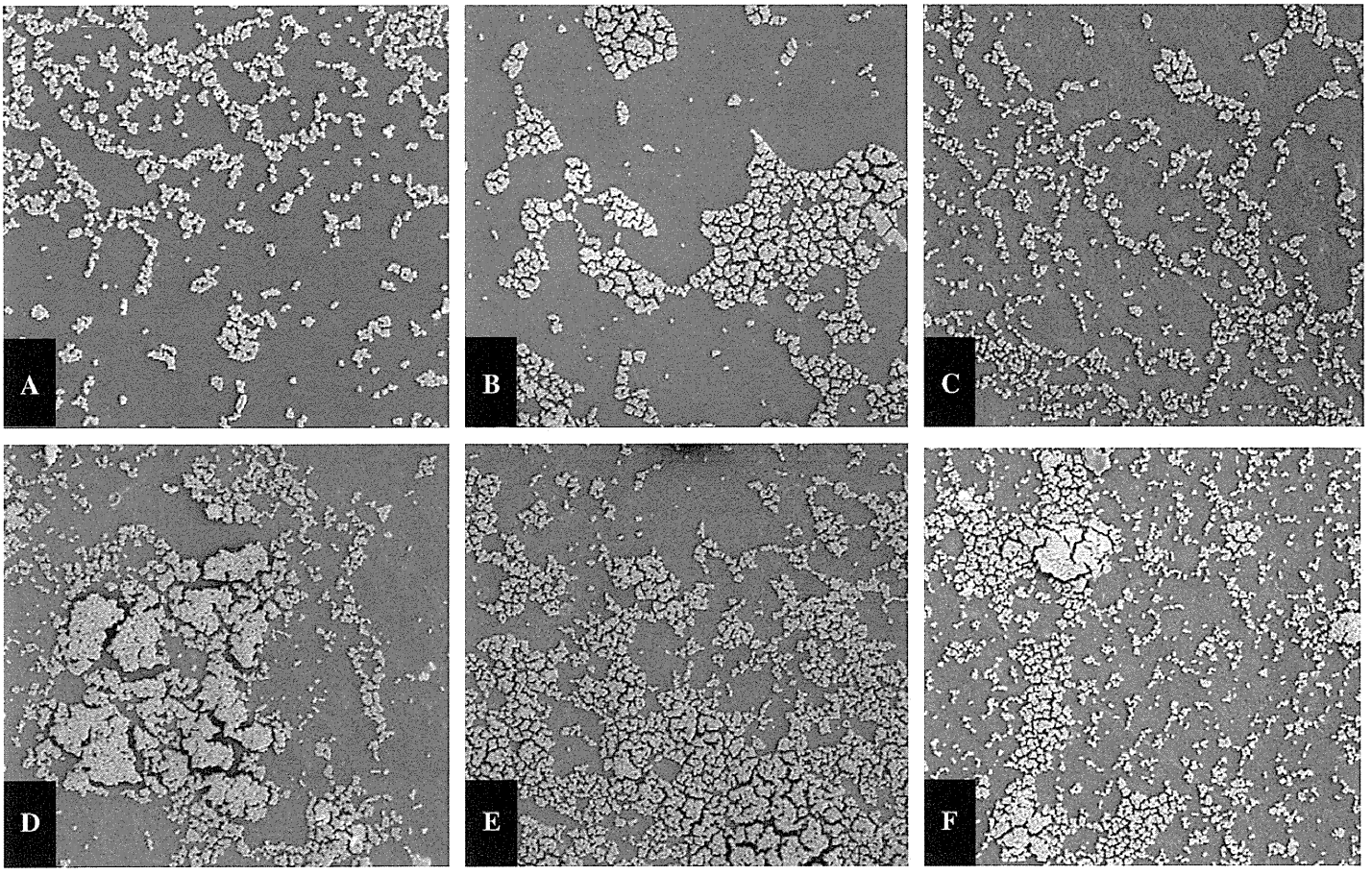

FIG. 5. Effects of MPS on *Staphylococcus epidermidis* (SE) biofilm evaluated by SEM observation (original magnification ×600). (A) EPica Cold; (B) Complete 10 min; (C) ReNu MultiPlus; (D) SoftOne Mois; (E) OPTI-FREE Plus; (F) control (saline).

note that disinfections at 1×10^3 and 1×10^6 CFU resulted in almost the same log reduction in all MPSs as shown in Figure 1. These results suggested that no shortage of preservatives occurred at the 1×10^6 CFU level. Higher inoculums at 1×10^7 or 1×10^8 CFU might cause shortage of preservatives resulting in smaller log reduction. Of the MPSs tested, Epica Cold and ReNu MultiPlus showed relatively high capacities to disinfect CL cases and breakdown

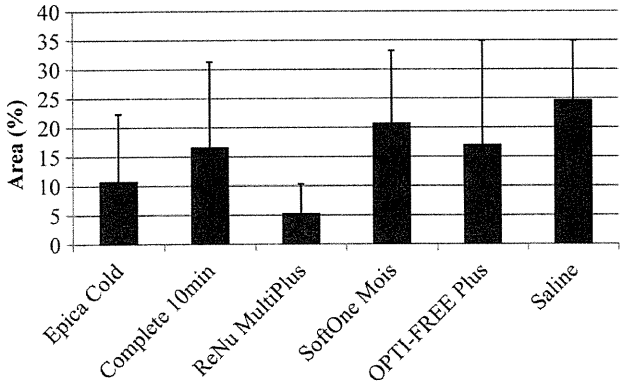

FIG. 6. The results of image analysis of biofilm-firmed area in a lens storage case. The percentage of SE biofilm-covered area in each SEM image (~30,500 μm^2) was analyzed by image analysis. The error bars are SD obtained from four separate lens cases.

SE biofilm compared with the other three MPSs. It was reported that antimicrobial efficacy of polyhexamethylene biguanide (PHMB) reduced when formulated in combination with a phosphate buffer system compared with when formulated in a borate buffer system.[22,23] Low efficacies of Complete 10 min and SoftOne Mois may be associated with combination of PHMB and phosphate buffer in these MPSs; however, the reason SoftOne Mois showed weaker efficacy than Complete 10 min was unknown. Other ingredients, such as surfactants, might be involved in the antimicrobial efficacies.

In a previous study[24] conducted with SCL, we showed that Epica Cold and ReNu MultiPlus were the most effective against CA, whereas OPTI-FREE Pluslost its efficacy after 3 cycle treatments. In this study, we confirmed our previous results by testing SE with the same MPSs used. Epica Cold and ReNu MultiPlus were the most effective compare with OPTI-FREE Plus. On the other hand, Rosenthal et al.[25] showed that PHMB-based MPSs (ReNu MultiPlus, Complete Moisture Plus) decreased fungicidal efficacy more in comparison with POLYQUAD-based MPSs (OPTI-FREE Express, OPTI-FREE RepleniSH), which maintained their high fungicidal activity because of preservative uptake into/or adsorption onto the SCL. The discrepancy between the results of Rosenthal and those of ours may be because of experimental method differences. Rosenthal used a 1 hr to 7 days soaking time, whereas we used a treatment cycle of (lens soiling with serum—bacterial

inoculation—4 hrs of incubation with MPS). Furthermore, the discrepancy between the results of Rosenthal and those of ours may also result from differences between preservative in OPTI-FREE Express, POLYQUAD and Aldox, and that in OPTI-FREE Plus, POLYQUAD only.[26]

The findings presented here highlight the importance of compliant behaviors in preventing CL-related microbial keratitis. It further suggests the inefficacy of certain commercially available MPSs against SE biofilm that may increase the risk of SE-associated microbial keratitis under noncompliance behaviors, such as the lack of mechanical rubbing and subsequent rinsing. Greater efforts to educate patients regarding compliant lens care behavior are needed to reduce the incidence of CL-associated microbial keratitis.

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Assessment of Real-Time Polymerase Chain Reaction Detection of *Acanthamoeba* and Prognosis Determinants of *Acanthamoeba* Keratitis

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Objective: To evaluate the diagnostic value of real-time polymerase chain reaction (PCR) for detecting *Acanthamoeba* in eyes diagnosed with *Acanthamoeba* keratitis (AK) by conventional tests. In addition, to determine the preoperative prognosis-determining factors in eyes with AK.

Design: Retrospective, cross-sectional study.

Participants: A total of 104 eyes of 103 patients who were diagnosed with AK or with bacterial or bacteria-associated keratitis (BK) by conventional tests.

Methods: Twenty-nine eyes with AK and 75 eyes with BK were evaluated for *Acanthamoeba* and bacterial DNA by real-time PCR. The *Acanthamoeba* copy numbers, bacterial load, and clinical parameters in the patients with AK were assessed for those significantly associated with poor outcome, that is, final visual acuity of <20/50 or requiring keratoplasty, by logistic regression analysis.

Main Outcome Measures: Acanthamoeba DNA copy number, bacterial DNA copy number, and odds ratio (OR) for poor prognosis.

Results: The detection of amoebic DNA was 50 times more sensitive by real-time PCR than by conventional cyst counting. The *Acanthamoeba* copy numbers at the first visit (mean: $4.7\times10^5\pm3.2\times10^5$ copies) were significantly correlated with the AK stage, and both were significant risk factors for a poor outcome. The *Acanthamoeba* DNA copy numbers at the first visit and AK stage had a significantly high risk for poor outcome (OR of *Acanthamoeba* DNA copy per logarithm of copy numbers: 3.48, 95% confidence interval [CI], 1.04–111.63, $P<0.05$; OR of AK stage: 2.8 per stage increase, 95% CI, 1.07–7.30, $P<0.05$, after adjustment of age). In the AK cases with poor outcome, the amoebic DNA was not reduced by more than 90% after 1 month of treatment. The weak amoebic reduction was significantly associated with advanced AK stages or previous use of steroids. Bacterial 16S rDNA was detected in 53.6% of the eyes with AK, but it was not associated with any risk for refractoriness.

Conclusions: Real-time PCR was effective in detecting and managing AK. The *Acanthamoeba* copy number and AK stage at the first visit were significantly associated with poor outcome.

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Acanthamoeba keratitis (AK) is a destructive disease process with significant visual morbidity, and prompt diagnosis is important for good visual outcome. However, the available *Acanthamoeba* tests are generally not sensitive enough for precise diagnosis. At present, staining corneal smears with Calcofluor and Fungiflora Y is probably the most effective method of diagnosing AK.[1] However, false negatives can occur, which cause a delay of treatment leading to poor visual outcomes.

Another difficulty with AK is in evaluating the effectiveness of a treatment protocol because of the absence of reliable and quantitative methods of determining the *Acanthamoeba* copy numbers. In refractory cases, the immunologic responses can worsen even after weeks of intensive treatment, and dense infiltrations can then develop in the lesion. Under these conditions, qualitative tests (e.g., culturing and smear staining) do not provide sufficient information on whether the treatment has reduced the *Acanthamoeba* copy numbers. Thus, tests that are more sensitive and provide quantitative values of the *Acanthamoeba* copy numbers will be helpful.

Acanthamoeba is innocuous unless embedded in a diseased cornea or central nervous system. In addition, *Acanthamoeba* can be an opportunistic pathogen in immune-compromised hosts.[2] Free-living *Acanthamoeba* ingest mainly bacteria, and AK can develop in eyes with bacterial keratitis and bacteria-associated keratitis (BK).

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There is also a possibility that AK can develop in eyes with advanced BK, but because of the lack of sensitive and quantitative tests for *Acanthamoeba*, little information is available on whether such a cause is possible. To evaluate such cases, it would be valuable to determine the level of the *Acanthamoeba* copy numbers relative to the bacterial load in the lesion.

Polymerase chain reaction (PCR) measurements are known to have high sensitivity in detecting amoebic DNA.[3–6] Real-time PCR has high sensitivity and the ability to obtain quantitative values of the degree of AK.

Thus, this study determines the sensitivity and specificity of real-time PCR in detecting *Acanthamoeba* DNA in eyes with AK and with BK. In addition, we determined whether the copy numbers of *Acanthamoeba* were correlated with the bacterial load in eyes diagnosed with AK and BK by standard clinical tests. We also used logistic analysis to determine the clinical parameters that were significantly associated with poor outcomes. We shall show that the stage of the AK and the *Acanthamoeba* copy numbers at the initial examination are significantly associated with poor visual outcomes.

Materials and Methods

Diagnosis of *Acanthamoeba* Keratitis and Treatment

A total of 104 eyes of 103 patients with suspected infectious keratitis including AK and BK were studied between January 2006 and December 2010. Of these 103 patients, 49 were men and 54 were women, with a mean age of 48.2±2.2 years. Twenty-nine eyes of 28 patients were diagnosed with AK. Eleven were men and 17 were women, with a mean age of 26.4±1.7 years. Twenty-seven of the patients with AK (96.4%) were contact lens wearers.

Seventeen healthy subjects (9 male and 8 female) with a mean age of 36.4±1.4 years, who were not contact lens wearers, were enrolled for examination of their conjunctival scraping by real-time PCR as normal controls.

The diagnosis of AK was based on a modification of a described method.[4,5] A definitive diagnosis of AK was based on the clinical characteristics and identification of one or more of the following findings in the laboratory tests:[1–4] (1) identification of trophozoites or cysts in corneal scrapings stained with Fungiflora Y,[1] (2) positive *Acanthamoeba* cultures, (3) pathologic identification of *Acanthamoeba* cysts on keratoplasty specimens, and (4) identification of *Acanthamoeba* genome from corneal scrapings by PCR.

To collect tissues for staining or culture, the lesions were scraped or ablated to obtain a sufficient amount of tissue to maximize the amount of *Acanthamoeba* trophozoites or cysts. After this, the corneal bed was swiped, and the swab was processed for *Acanthamoeba* DNA.

The stage of the AK was determined at the first visit and based on the clinical findings observed by slit-lamp biomicroscopy.[4] Briefly, AK was divided into 5 stages of disease severity: 1 = epitheliitis, 2 = epitheliitis with radial neuritis, 3 = anterior stromal disease, 4 = deep stromal keratitis, and 5 = ring infiltrate or extra corneal inflammation.

After a definitive diagnosis of AK, most of the patients were treated with hourly instillations of 0.2% fluconazole, 1% voriconazole, 0.02% polyhexamethylene biguanide, 0.02% chlorhexidine gluconate, and 200 mg of oral itraconazole. The corneas were debrided to reduce the *Acanthamoeba* load and facilitate drug penetration.

The diagnosis of BK was based on positive microbial identification in smear staining or culturing. Cases with positive microbiological results and responsive to appropriate antibiotics were defined as BK. Other cases that did not meet any of the criteria were classified as BK.

The study protocol was approved by the Tottori University Ethics Committee, and the procedures used conformed to the tenets of the Declaration of Helsinki. An informed consent was obtained from all of the participants after an explanation of the procedures to be used.

Real-Time Polymerase Chain Reaction

DNA was extracted from the scrapings of the corneal lesions with the QIAamp DNA mini kit (Qiagen, Hilden, Germany).[6] The 18S rDNA of *Acanthamoeba*, which distinguishes it from other amoeba genera (*Hartmannella, Naegleria, Balamuthia, Nuclearia,* and *Vahlkampfia*), was amplified using reported primers and probe sets.[3]

Forward: 5'-CGACCAGCGATTAGGAGACG-3'

Reverse: 5'-CCGACGCCAAGGACGAC-3'

TaqMan Probe: 5'-FAM-TGAATACAAAACACCACCATCG-GCGC-BHQ

Real-time PCR was performed and analyzed using the Light-Cycler (Roche, Basel, Switzerland) under the following conditions: 95°C for 15 minutes, followed by 50 cycles at 95°C for 0 seconds, and 60°C for 1 minute.

A standard curve was created using a dilution series with known amounts of genomic DNA from *Acanthamoeba castellanii* ATCC30010D. A detection of more than 1 copy was classified as *Acanthamoeba* DNA positive.

The total bacterial load was determined by real-time PCR using a broad-range (universal) probe and primers sets that detect the 16S rDNA from the domain *Bacteria*.[7]

Forward: 5'- TCCTACGGGAGGCAGCAGT-3'

Reverse: 5'- GGACTACCAGGGTATCTAATCCTGTT-3'

TaqMan Probe: 5'-FAM- CGTATTACCGCGGCTGCTG-GCAC-BHQ

The bacterial copy number (i.e., the total bacterial load) was calculated by a standard curve generated by using defined numbers of cloned templates.

Statistical Analyses

Data are presented as the mean ± standard error of the means. To evaluate the significance of the differences between groups, unpaired *t* tests or Mann–Whitney *U* tests were used. Spearman correlation analysis was used to determine the coefficients of correlation (ρ) between factors. Chi-square and Fisher exact tests were used to test the significance of the associations between the 2 kinds of classification. Multivariate logistic regression analysis was carried out to compute the odds ratios (ORs) and the 95% confidence intervals (CIs). In bilateral AK cases, the visually poorer eye was used for the statistical analyses. $P<0.05$ was considered significant.

Results

Sensitivity of Real-Time Polymerase Chain Reaction in Detecting *Acanthamoeba*

Although the detection of microbes by real-time PCR is known to be sensitive, the degree of sensitivity for *Acanthamoeba* has not

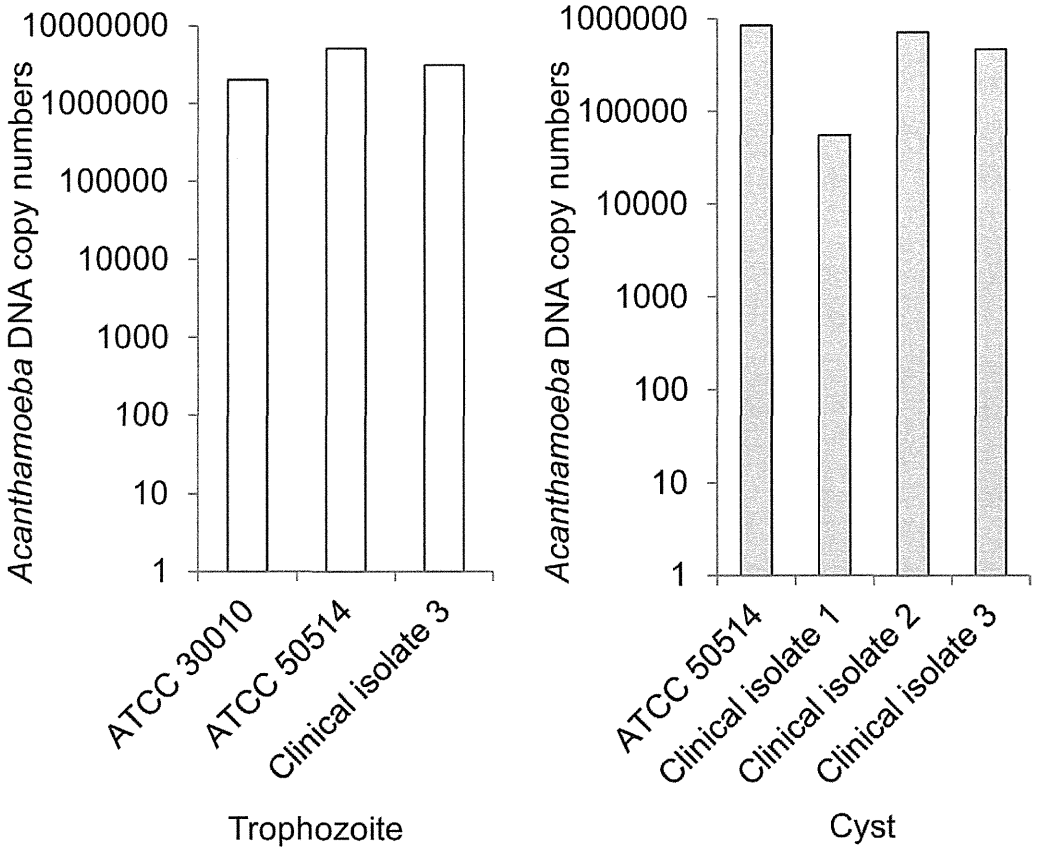

Figure 1. Evaluation of *Acanthamoeba* DNA detection in cultured *Acanthamoeba* cysts and trophozoites. Different strains of *Acanthamoebae* were cultured, and their cysts or trophozoites were diluted to 10 000 counts/ml. The extracted DNA was assessed for the *Acanthamoeba* DNA copy numbers. *Acanthamoeba* DNA detection by real-time polymerase chain reaction had better sensitivity for detecting both cysts and trophozoites. ATCC = American Type Culture Collection.

been determined. Generally, conventional microbiological tests rely on counting the number of amoebic bodies visually. Therefore, we first assessed how many copies of amoebic DNA can be detected for a known number of *Acanthamoeba* trophozoites or cysts (Fig 1). Clinical isolates and American Type Culture Collection strains were used as reference *Acanthamoeba,* and the trophozoites or cysts were diluted to 10 000 counts/ml in suspension.

Amoebic DNA was extracted from the suspension and assessed for the copy numbers using real-time PCR. Our results showed that the copy number detected by real-time PCR had approximately 300 times more sensitivity than visually counting trophozoites and 50 times more sensitivity than counting cysts (Fig 1).

To evaluate the specificity of *Acanthamoeba* real-time PCR in normal eyes, we also examined conjunctival scraping from 17 normal healthy subjects who were not contact lens wearers. No *Acanthamoeba* DNA (<1 copy) was detected in any of the scrapings.

Diagnostic Value of *Acanthamoeba* Real-Time Polymerase Chain Reaction

Acanthamoeba DNA was detected in 25 of the 29 AK eyes (86.2 %) by real-time PCR. The mean *Acanthamoeba* copy number was $4.7\times10^5\pm3.2\times10^5$ copies. The rate of detecting *Acanthamoeba* by smear staining with Fungiflora Y, which we have reported to be sensitive for detecting *Acanthamoeba,* was examined.[1] Among the 29 AK eyes, 26 underwent a Fungiflora test on diagnosis, and 22 eye samples were cultured. With Fungiflora Y staining, *Acanthamoeba* cysts were detected in 20 of 26 eyes (76.9%). In contrast, cultures of corneal specimens were positive in 12 eyes of 22 AK eyes (54.5%).

Acanthamoeba keratitis is generally associated with bacterial infection, but the specificity of identifying *Acanthamoeba* by real-time PCR has not been thoroughly evaluated in corneas diagnosed with BK. Therefore, we determined whether *Acanthamoeba* can be detected in BK cases using real-time PCR and Fungiflora Y staining. In 75 BK cases, none of the eyes was positive for *Acanthamoeba* DNA. Thus, the specificity of misdiagnosing AK by real-time PCR in eyes with BK was 100%.

When Fungiflora Y staining was assessed for specificity using 39 cases of BK, *Acanthamoeba* cyst-like staining was detected in 1 of 39 of the eyes with BK. This positive case was a contact lens user with *Pseudomonas aeruginosa* keratitis. Thus, the specificity of Fungiflora staining for diagnosing AK and not BK was 97.5%.

Evaluation of Bacterial Load by Real-Time Polymerase Chain Reaction

We next evaluated the bacterial load in the corneal lesions of eyes diagnosed as BK at their first visit or as a referral to Tottori University Hospital. For this, we used universal primers and real-time PCR for quantification of the bacterial DNA load. Bacterial

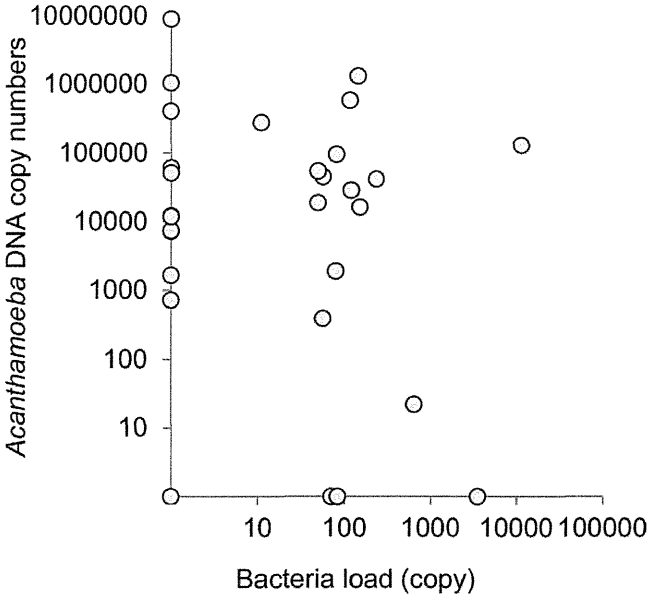

Figure 2. Evaluation of *Acanthamoeba* DNA copy number in *Acanthamoeba* keratitis determined by real-time polymerase chain reaction in relation to bacterial load. *Acanthamoeba* DNA copy number at first visit is not significantly correlated with the bacterial load in the lesion.

DNA was detected in 55 of 75 eyes (73.3%), and the mean bacterial DNA load was $8.8\times10^4\pm4.0\times10^4$ copies.

In the AK cases, 53.6% (15/28 eyes) were positive for bacterial DNA. The mean bacterial DNA copy number was $5.8\times10^2\pm4.0\times10^2$ copies. As expected, this was significantly lower than that in the BK eyes ($P<0.005$) by 10^2-fold.

To determine whether the development of AK was significantly associated with the bacterial load, we used Spearman correlation analysis to determine the relationship between the amoebic DNA copy number and the bacterial load in eyes with AK at the first visit. Amoebic DNA appeared inversely correlated with bacterial load (Fig 2); however, this was not statistically significant (Fig 2).

Correlation of *Acanthamoeba* DNA Copy Number and Stage of *Acanthamoeba* keratitis and Visual Acuity at First Visit

We next determined whether the stage of the AK was significantly associated with the copy number of *Acanthamoeba*. The *Acanthamoeba* copy number in the corneal specimens at the first visit was classified into 5 groups: 0 = not detected, 1 =≤1000, 2 =>1000 but ≤10 000, 3 =>10 000 but ≤100 000, and 4 =>100 000 copies. The relationship between the copy numbers of *Acanthamoeba* for each corneal specimen at the first visit to the stage of the AK was determined by Spearman correlation analysis. The stage of the AK was significantly correlated with the *Acanthamoeba* copy number ($\rho=0.53$, $P<0.05$; Fig 3A). In addition, the Acanthamoeba copy number was significantly correlated with the visual acuity in logarithm of the minimum angle of resolution units at the first visit ($\rho=0.37$, $P<0.05$; Fig 3B).

Next, the bacterial load was divided into 5 groups (0 = not detected, 1 =≤10, 2 =>10 but ≤100, 3 =>100 but ≤1000, 4 =>1000 but ≤10 000, and 5 =>10 000 copies). *Acanthamoeba* keratitis cases in the early stages were not significantly associated with the bacterial load (Fig 3C), and the correlation between the visual acuity and the bacterial load was not significant (Fig 3D). Thus, the *Acanthamoeba* copy number but not bacterial load was significantly associated with the disease severity and visual acuity before treatment.

We also determined whether the *Acanthamoeba* copy numbers at the first visit were significantly associated with the use of steroid or contact lens use. No significant associations were found (Mann–Whitney *U* test).

Parameters Associated with Poor Visual Outcome

We sought to determine factors that were significantly associated with poorer visual outcome in the AK cases after treatment of more than 2 months. A poor outcome was defined as a visual acuity <20/50 at the last visit or a requirement of keratoplasty. In AK cases with poor outcome, the *Acanthamoeba* copy numbers were 20 times higher than AK cases with good outcomes ($P<0.05$, Fig 4A). All of the cases with poor outcome had >10 000 copies of *Acanthamoeba*. When we evaluated the differences of the bacterial load between the AK cases with poor and good outcome, no significant difference was observed (Fig 4B). Thus, high *Acanthamoeba* DNA copy numbers at the first visit were associated with poor outcome.

We next evaluated the pretreatment parameters of the AK eyes that were significantly associated with poorer visual outcomes. By using logistic regression analysis, we calculated the risk of AK stage, *Acanthamoeba* DNA copy number at the first visit, bacterial load, previous use of steroids, and contact lens use (Table 1). We found that the *Acanthamoeba* DNA copy number and the stage of the AK at the first visit were the highest risk factors. The *Acanthamoeba* DNA copy numbers at the first visit had the highest risk for poor outcome (OR per category, 3.48; 95% CI, 1.04–111.63, $P<0.05$, after adjustment of age; Table 1). The AK stage had the second highest OR of 2.8 per stage increase (95% CI, 1.07–7.30, $P<0.05$, after adjustment of age). The previous use of steroids was not a significant risk (OR 8.84) for poor outcome ($P=0.07$). Other factors, including bacterial load and contact lens use, were not significant risk factors.

Parameters Associated with Unresponsive Reduction of *Acanthamoeba* Copy Numbers

Our findings indicate that the *Acanthamoeba* DNA copy number was significantly associated with the visual outcome. When we examined the *Acanthamoeba* copy number, all favorable outcome cases had a reduction in the copy number by >90% after 1 month of treatment (Fig 5A). In the cases with poor outcome, only 28.6% responded favorably to the treatment, and poor outcome was significantly associated with unresponsive reduction in the copy number.

To understand the cause of the outcome-related factors, an *Acanthamoeba* copy reduction was defined as a >90% reduction of *Acanthamoeba* copy numbers within 1 month of treatment. When the stage of the AK was compared between the responsive and unresponsive cases for *Acanthamoeba* copy reduction, the stage before the treatment of the unresponsive cases was significantly more advanced (Mann–Whitney *U* test, $P<0.01$, Fig 5B). The unresponsive *Acanthamoeba* copy reduction and previous steroid use were significantly associated ($P<0.05$, Fisher exact test). Five of the 23 unresponsive AK cases were previous steroid users, and the bacterial load in the unresponsive cases was not significantly associated with a decrease of *Acanthamoeba* copy numbers (unresponsive: 99±44 copies, responsive: 223±183 copies).

Finally, we determined which factors (e.g., the AK stage, amoebic DNA copy number, and bacterial load at the first visit) were significantly associated with an unresponsive DNA reduction. Logistic regression analysis was used to calculate the risks (Table 2). The AK stage was significantly associated with the DNA reduction with an OR of 8.00 per stage (95% CI, 1.06–

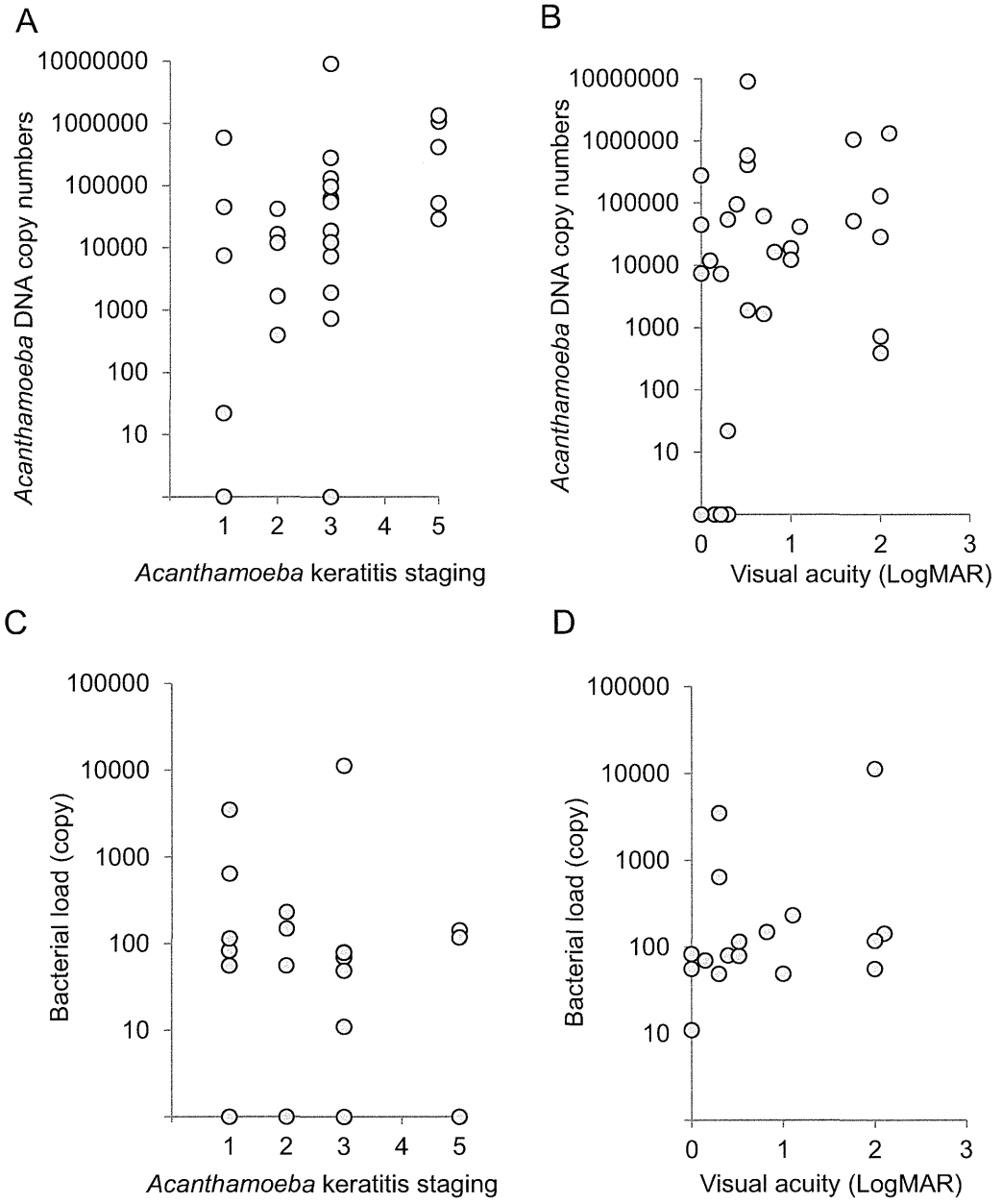

Figure 3. Significant correlation of *Acanthamoeba* DNA copy number with stage of *Acanthamoeba* keratitis (AK) and visual acuity. *Acanthamoeba* DNA copy number at first visit was significantly correlated with AK stage (ρ=0.53) **(A)** and logarithm of the minimum angle of resolution visual acuity (ρI=0.37) **(B)** by Spearman correlation analysis (P<0.05). Bacterial load in the lesion is not significantly correlated with the AK stage **(C)** or visual acuity **(D)**. logMAR = logarithm of the minimum angle of resolution.

58.82, P<0.05, after adjustment of age). The amoebic DNA copy number at the first visit also had a similar risk. The bacterial load at the first visit was not significantly associated with the DNA reduction.

Discussion

Acanthamoeba keratitis is a persistent infection and generally requires prolonged intensive treatment. However, our understanding of how to treat this disease remains undetermined. Presumably, the clinical presentation of AK (i.e., the stage of the AK) reflects the *Acanthamoeba* load, and the immunologic responses significantly affect its prognosis. Culturing, smear staining, and confocal microscopy are widely used for diagnosing AK. However, their qualitative nature and low sensitivity limit the amount of information that can be obtained to determine the cause of the AK. Thus, our initial aim was to determine how real-time PCR can be used for the diagnosis and management of AK.

Earlier studies with multivariate analysis of AK classified AK into 5 stages according to the corneal depth of the lesion and the severity of the corneal involvement as as-

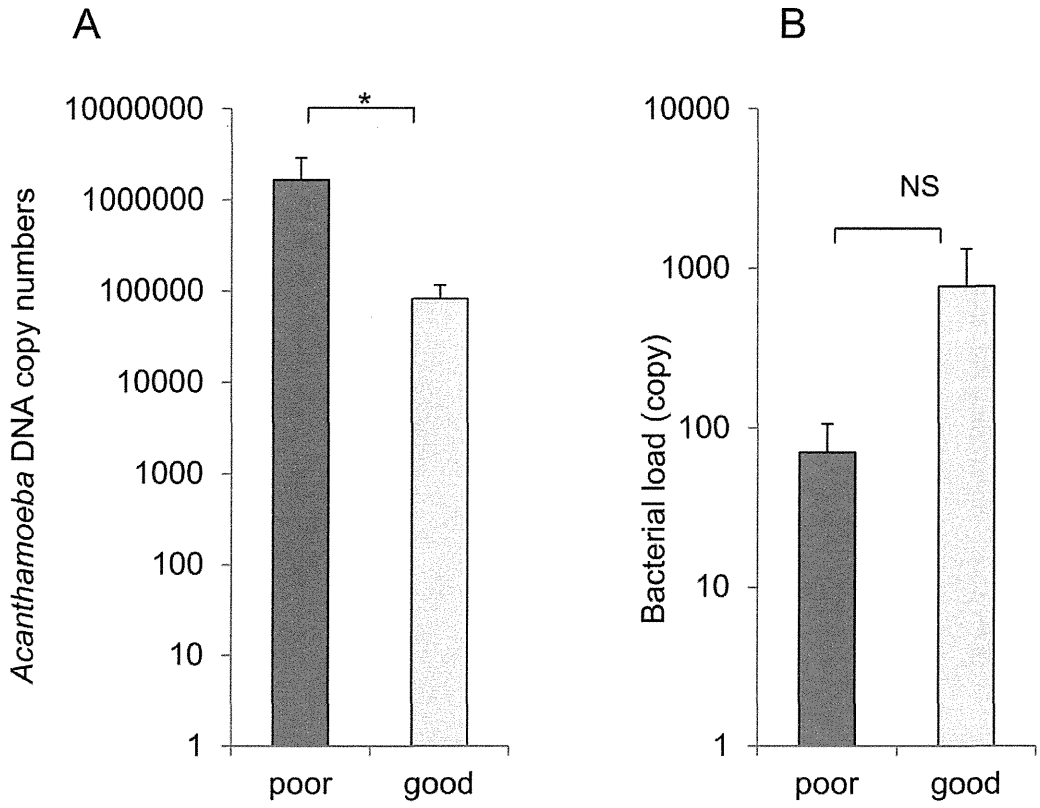

Figure 4. Elevation of *Acanthamoeba* DNA copy number in *Acanthamoeba* keratitis (AK) cases with poor outcomes. The AK cases with poor outcome, defined as visual acuity <20/50 at last visit or requiring keratoplasty, have a significantly higher number of *Acanthamoeba* DNA copies at the first visit compared with the AK eyes with good outcomes **(A)**. Bacterial load at the first visit is not significantly different between them **(B)**. *P<0.05. NS = not significant.

sessed by slit-lamp examinations.[4] The disease staging at presentation was predictive of worse outcomes and shown to help identify patients who might benefit from more aggressive therapy. Of note, advanced-stage AK with deep stromal involvement and ring infiltrates was associated with worse outcomes.[4,8,9]

Then, the important question arose on how much *Acanthamoeba* load is present at each stage. Our results showed that there was a strong and positive correlation of each stage with the *Acanthamoeba* DNA copy number, that is, more advanced stages of AK had higher *Acanthamoeba* DNA copy numbers. This explains why more advanced stages of AK were so refractory to treatment. Although the living amoebic bodies were fewer (Fig 1), this means that destroyed amoeba-derived DNA is abundant. Thus, the strong immunologic responses of the host to *Acanthamoeba*, manifested as ring infiltrate, are still not effective in eliminating the high *Acanthamoeba* copy numbers.

The most obvious advantage of real-time PCR is its high sensitivity.[3,10,11] Real-time PCR for *Acanthamoeba* genomic DNA will detect both live and destroyed amoeba. This was shown in our analysis of cultured *Acanthamoeba* cysts or trophozoites (Fig 1). Cultured *Acanthamoeba* samples generally are accompanied by fragments of the dead bodies and DNA. Real-time PCR attains its greater sensitivity in detecting

Table 1. Parameters Associated with Poor Outcome of *Acanthamoeba* Keratitis by Logistic Regression Analysis

	Odds Ratio					
	Lowest Category	*Second Category*	*95% CI*	*Highest Category*	*95% CI*	*P Value*
Acanthamoeba DNA copy number at the first visit	1.0	≤1000:3.48	1.04–111.63	>100 000:147.39	1.18–18 281.3	0.04*
AK stage	1.0	Stage 2:2.8	1.07–7.30	Stage 5:61.56	1.31–2838.69	0.04*
Bacterial load at the first visit	1.0	≤10:0.88	0.47–1.64	>10 000:0.53	0.02–11.86	0.69
Previous use of steroids	(-):1	8.84	0.852–91.68			0.07
Contact lens use	(-):1					0.996

AK = *Acanthamoeba* keratitis.
*P≤0.05.

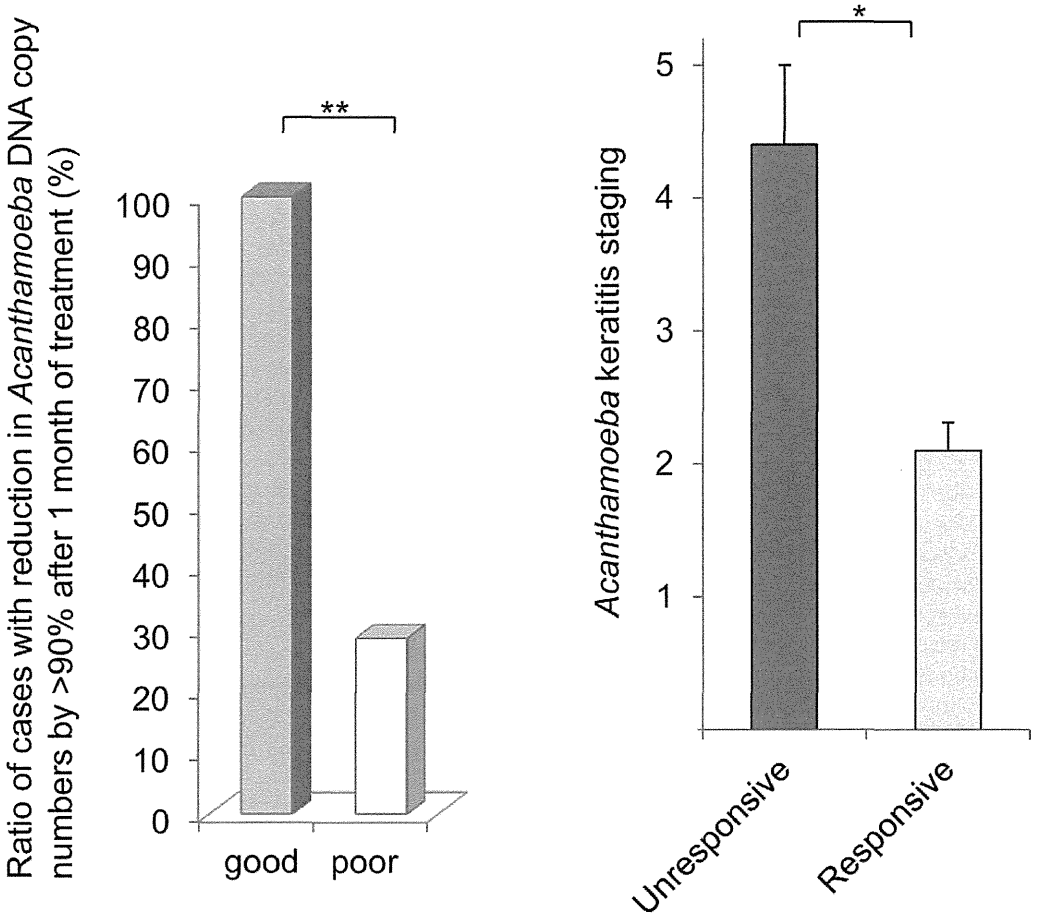

Figure 5. Association of poor outcomes with unresponsive *Acanthamoeba* copy reduction after treatment. Ratio of *Acanthamoeba* keratitis (AK) cases with unresponsive *Acanthamoeba* copy reduction after 1 month of treatment was significantly higher in AK with adverse prognosis **(A)**. In the unresponsive 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.

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

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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

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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

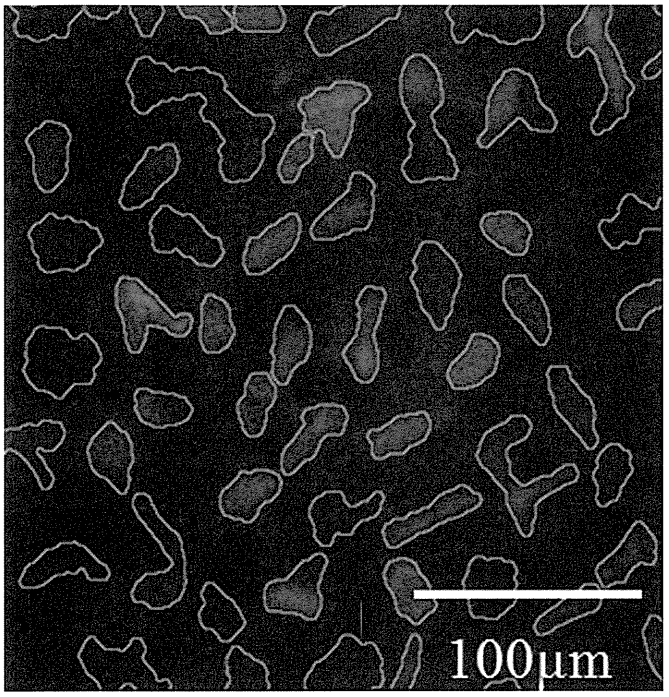

FIGURE 1. Digital images of corneal stromal cells. The digital images were imported into a computer with image analysis software, and the KD was found by automatically tracing the nuclei of corneal stromal cells in the central portion of the image (260 × 260 μm).

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,

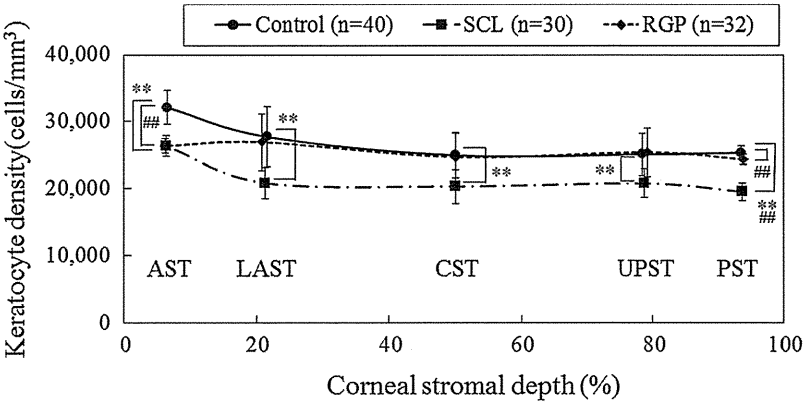

FIGURE 2. KD for each group of CL wearers at different corneal stromal depths (study 1). The corneal stroma was divided into 5 layers starting from the region closest to the corneal epithelium, and the KDs for the control group, RGP lens group, and SCL group are plotted. The KD is lower in all layers 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/mm^3) : 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

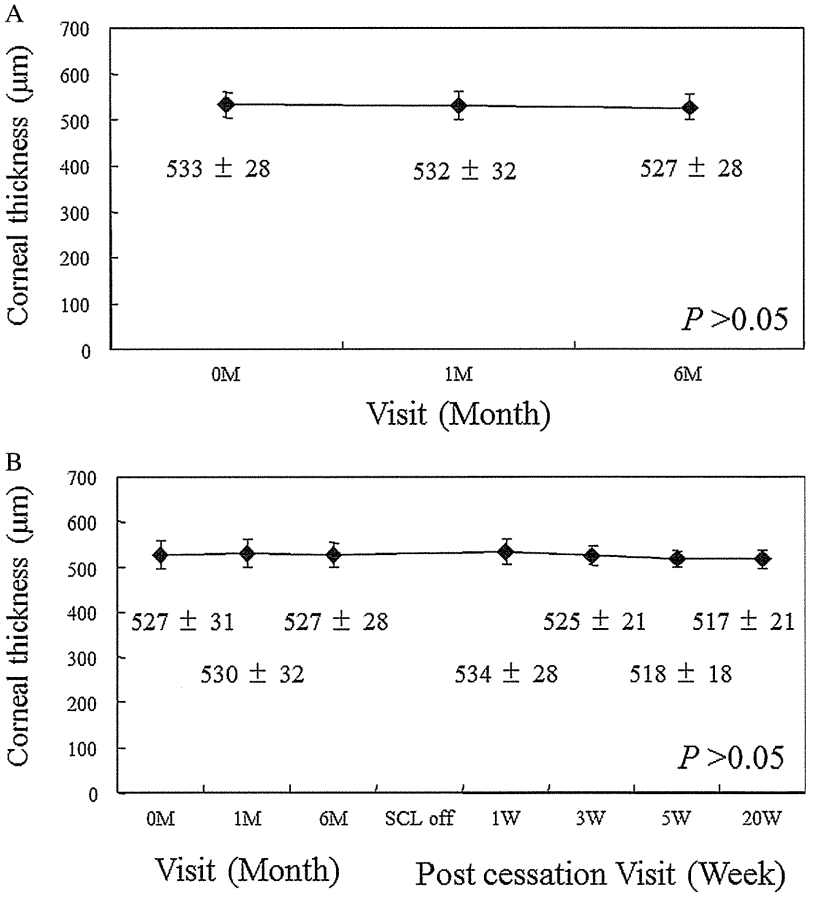

FIGURE 3. Corneal thickness for RGP lens (A) and SCL (B) wearers (study 2). During the 6-month prospective study (study 2), no changes in the corneal thickness are observed in either the RGP lens (A) or the SCL (B) wearers over the course of the observation period ($P > 0.05$). Error bars represent the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

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