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分担研究報告書

ヒト角膜内皮細胞培養技術の確立

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研究要旨：全層角膜移植以外の角膜内皮機能不全の治療法として，角膜内皮部分移植や培養角膜内皮細胞移植，人工角膜移植，角膜再生医療などが研究されている。角膜内皮部分移植は角膜全体ではなく角膜の実質深層とデスメ膜，内皮細胞層のみを移植する方法である。培養角膜内皮細胞移植は患者自身の角膜内皮を取り出して培養技術で増殖させ，再び眼内に戻せれば理想的な治療となりうると考えられるが，角膜内皮細胞は培養が難しいために現在は実験段階にとどまっている。そこで本研究では、同意を得た患者から提供をうけた眼組織手術検体を用いて、眼組織が持つ幹細胞のプロファイリングを採取部位ごとにあきらかにし、最適な培養条件を検討した。

A．研究目的

眼組織中に幹細胞が存在し、その眼組織由来幹細胞が増殖させることが可能になれば、角膜内皮系幹細胞の採取および利用が可能になる。本研究は、ヒト眼組織由来幹細胞の存在を明らかにし、その採取・分離・培養のための条件を検討するものである。

B．研究方法

小児眼科手術検体組織をそれぞれの部位を目視で分離し、コラゲナーゼ処理後、間葉系細胞培地にて培養を行い、その至適培養条件を検討した。また、その細胞を用いて、マイクロアレイによる網羅的遺伝子解析を行った。

（倫理面への配慮）
当センターにおいては、ヒト細胞の培養に関し、研究面において既に倫理審査を受け、承認を受けている（国立成育医療センター研究所、受付番号25、26 及び27、平成15年1月承認、受付番号49、平成15年10月承認、受付番号55、平成15年11月承認、受付番号88、89、90、91平成16年7月承認、受付番号55、平成16年11月追加承認、受付番号146、平成17年4月承認、受付番号156、平成17年7月承認)。また、倫理的な手続きおよび考え方が年次毎に異なると予想され、最新の社会的な影響を十分に考慮し研究を行った。なお、研究協力者に倫理専門家を加え、本研究遂行にあたって新たな倫理的問題が生じないよう、常にモニタリングを行い、必要に応じて意見交換を行っている。

C．研究結果

小児眼科手術検体組織内には間葉系細胞と同様の培養条件で分離可能であり、増殖する細胞が存在することを明らかにした。しかしながらこれら小児眼組織由来細胞は増殖能力は旺盛ながら、組織を細かく分離した後に培養を開始すると増殖が悪くなることから、細胞の培養条件を詳細に検討する必要がある。また、マイクロアレイの結果より、眼組織特異的な遺伝子発現パターンを検討中である。

D．考察

本研究において、小児眼科手術検体組織内には間葉系細胞と同様の培養条件で増殖する細胞が存在することが示された。今後これらの増殖する細胞群のプロファイリングを詳細に明らかにすることで、角膜内皮細胞培養の手法を確立していきたい。

E．結論

角膜内皮機能不全の患者自身から角膜内皮細胞を採取し，シート状に培養して増殖させることができれば，自己の細胞を用いた自家角膜内皮移植術が可能になり，拒絶反応の問題が解決される。自己移植，同種移植のいずれの場合にも，提供眼不足に苦慮する本邦の角膜移植医療の現状を根本から変えていくことが可能となる。本研究を遂行していくことで、眼科領域における再生医療の安全性、有効性を基盤研究・臨床研究の両側面から社会に提示できることは医療行政の面からも非常に有意義なものである。

F．健康危険情報

なし

G．研究発表

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(予定を含む。)
1. 特許取得
なし
2. 実用新案登録
なし
3. その他
なし

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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Ⅳ. 研究成果の刊行物・別刷

Corneal penetration of simultaneously applied topical levofloxacin, norfloxacin and lomefloxacin in human eyes

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ABSTRACT.

Purpose: **This study was performed to assess the corneal penetration of three topically applied fluoroquinolones (levofloxacin, norfloxacin and lomefloxacin) in corneal buttons obtained from patients undergoing penetrating keratoplasty.**

Methods: **Fourteen patients received three drops each of 0.5% levofloxacin, 0.3% norfloxacin and 0.3% lomefloxacin (the standard clinically available preparations) over a 30-min interval beginning 90 mins before their scheduled keratoplasty. Corneal samples obtained from excised buttons at the time of surgery were stored at − 80 ° until analysis. The concentration of the administered fluoroquinolones was measured using high-performance liquid chromatography.**

Results: **The mean corneal concentration of levofloxacin (4.6 ± 3.5 μg/g, mean ± standard deviation) was significantly higher than that of lomefloxacin (2.7 ± 1.8 μg/g, p = 0.0018) and norfloxacin (1.3 ± 1.2 μg/g, p = 0.00012).**

Conclusion: **Levofloxacin achieves a higher mean corneal concentration than norfloxacin and lomefloxacin in the human cornea.**

Key words: cornea – fluoroquinolones – high-performance liquid chromatography – levofloxacin

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Introduction

Bacterial keratitis is a common, potentially sight-threatening ocular infection caused by various species of bacteria (O'Brien 1997). The causative organism is rarely identified in time for the initial antibiotic regimen to be organism-specific, and is sometimes not identified despite extensive efforts. Thus, successful treatment of presumed bacterial keratitis often requires empirical selection of an effective broad-spectrum antibiotic regimen.

The fluoroquinolones are the newest family of antibacterial agents used in the treatment of bacterial keratitis (Leibowitz 1991; Neu 1991; O'Brien et al. 1995; Hyndiuk et al. 1996; O'Brien 1997). Double-blind, randomized clinical trials have shown that single-agent fluoroquinolone therapy with ofloxacin (O'Brien et al. 1995) or ciprofloxacin (Hyndiuk et al. 1996) is comparable in efficacy to therapy combining fortified beta-lactam agents with aminoglycosides. Their bactericidal activity against the most frequently observed gram-positive and gram-negative ocular pathogens is generally excellent and their high potency has made fluoroquinolones a common choice for the topical therapy of bacterial keratitis. Currently, four topical fluoroquinolones (ofloxacin, norfloxacin, lomefloxacin and levofloxacin) have been approved for clinical use in Japan. In addition to these compounds, ciprofloxacin has been used clinically in the USA and Europe. While fluoroquinolones have similar spectra of activity in general, they are not equally potent against a number of bacteria and their pharmacokinetic properties differ (Neu 1991; Ogawa & Hyndiuk 1993; Ernst et al. 1997; Wimer et al. 1998). The clinical usefulness of fluoroquinolones is thought to depend on several factors, including their *in vitro* bactericidal activity, their ability to penetrate the site of infection and their relative toxicities.

Levofloxacin, the L-isomer of the racemate ofloxacin, is significantly more potent than the D-isomer and represents the active component of ofloxacin (Ernst et al. 1997; Wimer

et al. 1998). As the purified potent isomer of ofloxacin, levofloxacin is roughly twice as biologically active as ofloxacin (Ernst et al. 1997; Wimer et al. 1998). Another important benefit of levofloxacin is its high solubility in water; the concentration of the clinically available topical levofloxacin preparation is 0.5%, while that of other fluoroquinolones is 0.3% (Ross & Riley 1990). It might be expected that the higher concentration of topical levofloxacin would aid in achieving higher corneal and aqueous humour penetration. Kawashima et al. (1995) reported that 0.5% levofloxacin achieved a significantly higher maximum concentration in aqueous humour than 0.3% ofloxacin after topical application of three drops of each agent at 15-min intervals in a rabbit model. We also recently reported that topical levofloxacin achieves better penetration in human aqueous humour than lomefloxacin and norfloxacin (Yamada et al. 2003). However, there are currently no data in the literature that assess the corneal concentration of levofloxacin after topical administration. The present study was designed to assess the relative topical penetration of three fluoroquinolones (levofloxacin, norfloxacin and lomefloxacin) in the human cornea.

Methods

Fourteen patients (10 women and four men; mean age 64 ± 17 years) who underwent penetrating keratoplasty at Keio University Hospital were enrolled (Table 1). Indications for penetrating keratoplasty included leukoma ($n = 5$), graft failure ($n = 5$), bullous keratopathy ($n = 3$) and keratoconus ($n = 1$). Exclusion criteria included the presence of apparently non-intact corneal epithelium, active corneal inflammation, and topical antibiotic use within 1 week of enrolment. Each subject received thorough written and oral explanations of the study and provided written informed consent prior to enrolment. The study was approved by the Committee for the Protection of Human Subjects at Keio University School of Medicine.

Topical preparations of 0.5% levofloxacin (Santen Pharmaceutical Co. Ltd, Osaka, Japan), 0.3% norfloxacin (Banyu Pharmaceutical Co. Ltd, Tokyo, Japan), and 0.3% lomefloxacin (Senju Pharmaceutical Co. Ltd, Osaka, Japan) were obtained from each manufacturer. All patients received three drops of each of the three study drugs over a 30-min period, with one drop of each administered at 0, 15 and 30 mins. At each dosing interval, patients first received one drop of one drug, followed by a 2-min delay, then one drop of the second drug, and finally one drop of the third drug after another 2-min delay. The order of drug administration varied across patients in a crossover fashion, with each patient randomly assigned to one of three regimens (Table 1).

Drops were administrated by a nurse to ensure strict compliance with the administration regimen.

Table 1. Patients were randomly assigned to one of three treatment regimens. At 0, 15- and 30-mins, all patients received one drop each of 0.5% levofloxacin, 0.3% norfloxacin and 0.3% lomefloxacin in the sequence listed.

Patient no.	Age (years)	Gender	Diagnosis	Treatment sequence
1	73	Female	Bullous keratopathy	LVFX/NFLX/LFLX
2	79	Male	Regraft	NFLX/LFLX/LVFX
3	51	Male	Keratoconus	LFLX/LVFX/NFLX
4	56	Female	Regraft	LVFX/NFLX/LFLX
5	71	Female	Leukoma	NFLX/LFLX/LVFX
6	70	Female	Regraft	LFLX/LVFX/NFLX
7	62	Female	Leukoma	LVFX/NFLX/LFLX
8	76	Female	Leukoma	NFLX/LFLX/LVFX
9	66	Female	Bullous keratopathy	LFLX/LVFX/NFLX
10	85	Male	Regraft	LVFX/NFLX/LFLX
11	44	Female	Leukoma	NFLX/LFLX/LVFX
12	54	Male	Regraft	LFLX/LVFX/NFLX
13	21	Female	Leukoma	LVFX/NFLX/LFLX
14	82	Female	Bullous keratopathy	NFLX/LFLX/LVFX

LVFX = levofloxacin, NFLX = norfloxacin, LFLX = lomefloxacin.

Patients received concurrent surgical preparation with miotic/mydriatic drops, and surgery commenced approximately 1 hour after application of the last antibiotic drop. After excision, the host corneal button was divided into two halves using a razor blade and the halves were blotted to dry using a cellulose sponge. The tissue was stored at − 80 ° until analysis. At the time of analysis, corneal specimens were weighed, pulverized and homogenized in 0.8 ml of 0.1 M phosphate buffer. The drugs were eluted by adding 6 ml of chloroform. After brief centrifugation, the supernatant layer was removed. The elute was concentrated by evaporation under nitrogen gas, and reconstituted to 0.5 ml of an 85 : 15 mixture of 0.05 M phosphoric acid (pH 3.0) and acetonitrile. Preliminary experiments using rat corneas showed > 90% recovery of calibrator solution processed in the presence of drug-free corneas (data not shown).

Drug levels in the corneal buttons were determined using high-performance liquid chromatography (HPLC) (Yamada et al. 2002). An HPLC system consisting of an L-7100 solvent delivery system (Hitachi, Tokyo, Japan), an L-7480 fluorescence detector (Hitachi), and a C-21 chromatography workstation (System Instrument, Tokyo, Japan) was used. Elution was performed using an ODS-80 column (Tosoh Inc., Tokyo, Japan) at 50 ° with a mobile phase consisting of an 85 : 15 mixture of 0.05 M phosphoric acid (pH 3.0) and acetonitrile. The flow rate was 0.7 ml/min and detection was performed by fluorescence (excitation 290 nm, emission 470 nm). Drug concentrations were determined using a calibration line constructed using preparations of known concentration ranging from 0.025 μg/ml to 1.25 μg/ml. Drug concentrations were calculated from the peak height on the chromatogram and are expressed as micrograms of drug per gram of corneal tissue.

Results are presented as the mean ± standard deviation (SD). Statistical significance was calculated by comparing results by Wilcoxon signed rank test. A level of $p < 0.05$ was considered statistically significant.

Results

A typical chromatogram is shown in Fig. 1. The retention times of norfloxacin,

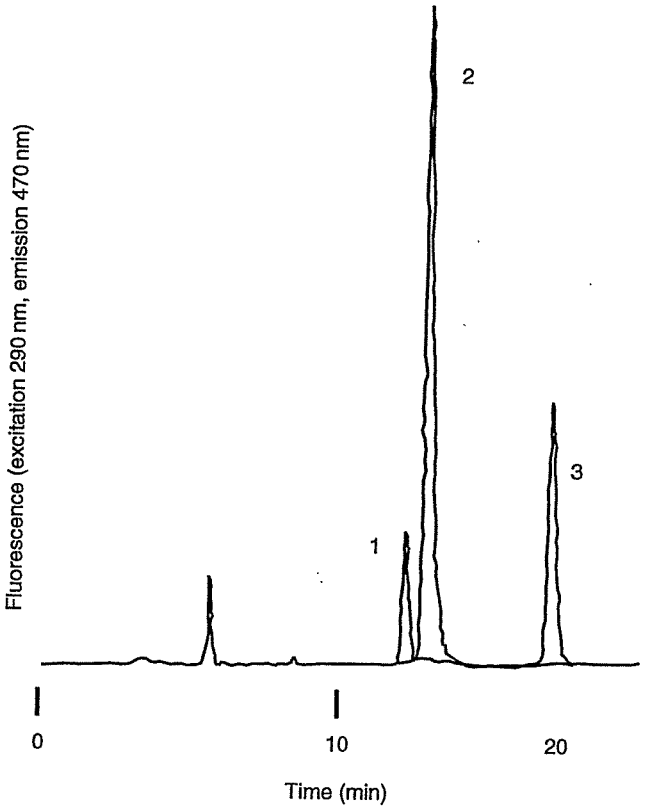

Fig. 1. High performance liquid chromatogram of fluoroquinolones. The retention times of (1) norfloxacin, (2) levofloxacin and (3) lomefloxacin were 14.1 mins, 15.0 mins and 19.6 mins, respectively.

levofloxacin and lomefloxacin were 14.1, 15.0 and 19.6 mins, respectively.

The fluoroquinolone concentrations measured in the corneal buttons are shown in Table 2.

The mean concentration ± SD of norfloxacin, levofloxacin and lomefloxacin were 1.3 ± 1.2 μg/g, 4.6 ± 3.5 μg/g, and 2.7 ± 1.8 μg/g, respectively. The difference in corneal concentration was significant between norfloxacin and levofloxacin ($p = 0.00012$, Wilcoxon signed rank test), and lomefloxacin and levofloxacin ($p = 0.0018$).

Table 2. Intracorneal concentrations of norfloxacin, levofloxacin and lomefloxacin.

Patient no.	Drug concentration (μg/g)		
	Norfloxacin	Levofloxacin	Lomefloxacin
1	0.7	4.0	3.3
2	0.9	1.5	0.8
3	0.3	1.4	0.4
4	4.9	11.9	6.4
5	1.1	4.2	3.1
6	1.6	2.8	1.5
7	0.2	0.6	0.4
8	0.6	4.2	3.0
9	0.8	12.1	5.9
10	1.4	1.9	0.9
11	2.6	4.4	3.2
12	1.9	8.5	3.1
13	0.5	2.8	3.0
14	1.3	4.6	3.5
Average	1.3	4.6	2.7
SD	1.2	3.5	1.8

Discussion

In this study, corneal fluoroquinolone concentration demonstrated great (approximately 20-fold) variability, although all the patients were given identical amounts of each drug. Similar variability has been observed in previous studies of fluoroquinolone penetration into the cornea and aqueous humour (McDermott et al. 1993; Diamond et al. 1995; Donnenfeld et al. 1997; Price et al. 1997; Yamada et al. 2003). Multiple factors including tear turnover rate, blinking frequency and completeness, timing of sampling, and epithelial continuity are thought to contribute to this large interpatient variability. In the present study, the condition of the corneal epithelium may have been the most important factor, given that all subjects were patients undergoing penetrating keratoplasty (McDermott et al. 1993; Diamond et al. 1995; Donnenfeld et al. 1997; Price et al. 1997).

The presence of significant interpatient variability required that a large sample size be sought to attempt to detect significant differences among the three fluoroquinolones. For this purpose, we used the analytical method reported by Diamond et al. (1995). Any factors that promote or inhibit the penetration of one drug would be expected to have an essentially identical effect on each agent, because all three fluoroquinolones were administered to each eye virtually simultaneously. All three fluoroquinolones were assayed simultaneously in each corneal sample in order to increase the effective sample size. However, the instillation of multiple eyedrops at one time has potential drawbacks (DeSantis 1994). When a drop of one medication is followed closely by drops of other medications, substantial washout (decreasing drug penetration) and change in pH (which may increase or decrease drug penetration) may occur. We set 2-min intervals between the administration of each eyedrop to minimize the above effects (DeSantis 1994). However, in a previous study, we found that the mean concentration of levofloxacin in the aqueous humour was 0.6 ± 0.3 μg/ml when three drops each of 0.5% levofloxacin, 0.3% norfloxacin and 0.3% lomefloxacin were administered (Yamada et al. 2003). This value was lower than the concentration of levofloxacin achieved in the aqueous humour (1.0 ± 0.5 μg/ml) when levofloxacin alone was administered (Yamada et al. 2002). These

results suggest that the instillation of multiple eyedrops, even using 2-min intervals between drops, results in reduced drug penetration. Therefore, these data cannot be directly compared with the results of other studies in which only a single drug was administered.

This study demonstrated distinct differences in corneal drug concentrations achieved among the three fluoroquinolones. Levofloxacin had the highest corneal concentration, followed by lomefloxacin, with norfloxacin showing the lowest mean concentration. The concentration of each fluoroquinolone administered in this study was not uniform, given that the levofloxacin preparation was a 0.5% solution, while lomefloxacin and norfloxacin were administered as 0.3% solutions. The high solubility of levofloxacin in water at neutral pH, which permits the higher 0.5% concentration of the levofloxacin preparation, has been noted to represent an advantage of this drug over other fluoroquinolones (Ross & Riley 1990).

Antibiotics used for the treatment of bacterial keratitis should demonstrate excellent corneal penetration and sufficient *in vitro* activity against a broad spectrum of bacteria (O'Brien 1997). The mean intracorneal concentration of levofloxacin achieved in the present study was greater than the MIC_{90} of levofloxacin against most common pathogens of bacterial keratitis, although the mean concentration was lower than the MIC_{90} of some *Pseudomonas* strains (Table 3) (Une et al. 1988; Ernst et al. 1997; Wimer et al. 1998). The mean intracorneal concentration of lomefloxacin exceeded the MIC_{90} of the above-mentioned pathogens, excluding *Streptococcus pneumoniae*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* (Une et al. 1988; Neu 1991). The mean intracorneal concentration of norfloxacin, however, did not exceed the MIC_{90} of most pathogens (Chin et al. 1988).

Some caution should be exercised when interpreting the data presented. The success or failure of therapy is not necessarily predicted by the mean intracorneal concentration exceeding the MIC_{90} value or not. Resistance or susceptibility as determined through MIC90 assays might not correlate with clinical response because these *in vitro* assays are based on clinical response to systemic infections in media that differ from the corneal stroma. The large interpatient variability observed in this study and in previous studies (McDermott et al. 1993; Diamond et al. 1995; Donnenfeld et al. 1997; Price et al. 1997) indicates that drug penetration may occasionally be quite low even if the average achieved concentration is satisfactory. Penetration may be improved in patients with inflamed corneas (such as in the setting of bacterial keratitis) and/or non-intact corneal epithelium (Ozturk et al. 1999). More frequent drug application and/or longer duration of drug application may increase intracorneal drug concentrations (McDermott et al. 1993; Price et al. 1997). Notably, some *Staphylococcus* and *Pseudomonas* strains are highly resistant to all fluoroquinolones (Kowalski et al. 2001). The data in the present study, however, may be appropriately used to attempt to predict relative *in vivo* potency among the fluoroquinolones.

In this study, topically applied levofloxacin achieved higher corneal concentrations than lomefloxacin or norfloxacin. These data may favour the selection of levofloxacin, rather than norfloxacin or lomefloxacin, for the initial treatment of bacterial keratitis. However, the precise role of levofloxacin in clinical practice will need to be determined by investigations assessing clinical outcomes.

Table 3. Norfloxacin, levofloxacin and lomefloxacin MIC_{90} values for bacteria associated with stromal keratitis.

Organism	Norfloxacin MIC_{90} (μg/ml)*	Levofloxacin MIC_{90} (μg/ml)†‡	Lomefloxacin MIC_{90} (μg/ml)‡
Staphylococcus aureus	2	0.25–0.5	0.78
Staphylococcus epidermidis	1	0.19–0.41	1.56
Streptococcus pneumoniae	16	0.06–3.13	12.5
Enterococcus faecalis	32	1–3.13	12.5
Proteus mirabilis	0.5	0.06–0.25	0.39
Klebsiella pneumoniae	0.5	0.1–3.13	1.56
Pseudomonas aeruginosa	2–8	1–8	50

* * Chin et al. (1988)
† Une et al. (1988)
‡ Wimer et al. (1998)

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Phospholipids and Their Degrading Enzyme in the Tears of Soft Contact Lens Wearers

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Purpose: Low tear phospholipids levels are associated with tear film instability in soft contact lens wearers. We assayed levels of phospholipids and their degrading enzyme secretory phospholipase A_2 ($sPLA_2$) both in tears and deposited on contact lenses composed of 2 hydrophilic materials after 1 day of routine use.

Methods: Polymacon (Medalist; FDA group 1, low water/nonionic) and Etafilcon A (One Day Acuvue; group 4, high water/ionic) contact lenses were worn for 12 hours by 16 experienced contact lens wearers. Phospholipids in tear fluids and deposited on contact lenses were estimated by phosphorus determination with ammonium molybdate through enzymatic digestion. Double-antibody sandwich ELISA was used to determine group IIa $sPLA_2$ concentrations, and $sPLA_2$ activity was assayed using 1,2-diheptanoyl thiophosphatidylcholine as substrate.

Results: Phospholipids concentrations in tears with Polymacon and Etafilcon A were 186 ± 39 and 162 ± 33 μg/mL, respectively. The latter concentration was significantly lower than that observed in the same subjects when not wearing contact lenses ($P = 0.0023$). In tears, both group IIa $sPLA_2$ concentrations and enzymatic activity remained unchanged, regardless of lens wearing. However, Etafilcon A (0.57 ± 0.09 μg/lens) showed more group IIa $sPLA_2$ deposition than Polymacon (0.01 ± 0.01 μg/lens; $P < 0.001$). Furthermore, group IIa $sPLA_2$ deposited on Etafilcon A but not on Polymacon lenses retained its enzymatic activity.

Conclusion: Significant differences of group IIa $sPLA_2$ deposition were found in the 2 lenses tested. Such deposition might induce phospholipid hydrolysis in tears and thereby promote tear film instability in hydrophilic contact lens wearers.

Key Words: contact lens, dry eye, phospholipids, secretory phospholipase A_2, tears

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Contact lens–induced dry eye is a major cause of contact lens intolerance.[1–3] According to 1 survey conducted in Japan, more than 80% of soft contact lens wearers occasionally experience a dry sensation in the eyes. This feeling of dryness can cause many patients to reduce their contact lens wearing time or may render them intolerant of contact lens wear.[3] Tear film stability, clinically estimated by tear film break-up time (BUT), is especially compromised in soft contact lens wearers.[2,3] Contact lens intolerance is best described by tear film stability and tear volume. Thus, although an association between contact lens wear and tear film instability is well recognized, the mechanism is not fully understood.

Normal tears are characterized by low surface tension that is intimately associated with tear film stability.[4] Recent studies have shown that phospholipids play crucial roles in maintaining low surface tension of tears.[4–9] In the most widely accepted model of tear film structure, the aqueous-mucin layer is coated by 2 thin lipid layers: polar lipids such as phospholipids lie adjacent to the aqueous layer, whereas nonpolar lipids are present at the tear–air interface.[5,6] Phospholipids are important for maintaining stable tear film by linking the nonpolar hydrophobic outer lipid layer and the aqueous layer of tear film.

It has been shown that low levels of phosphatidylcholine and phosphatidylethanolamine in meibum are linked to the severity of meibomian gland dysfunction.[10] We recently reported that concentrations of these phospholipids in the tears of patients with marginal and moderate dry eye were significantly lower than those in subjects without dry eye.[11] Guillon et al[12] reported that low levels of tear phospholipids are associated with short tear film BUT in soft contact lens wearers. Recent studies have also shown that low surface tension of tears is not caused by phospholipids alone but to a complex of phospholipids and tear proteins.[4] Moreover, some proteins such as tear lipocalin and secretory phospholipase A_2 ($sPLA_2$) may regulate phospholipids levels in tear fluids. Tear lipocalin, which comprises 15% to 33% of total protein mass in tears,[13,14] binds various lipids including phospholipids.[15–17] Although the physiological roles of lipocalin are not fully understood, tear lipocalin may adjust the lipid composition of tears by binding lipids from tear fluid and/or releasing lipids into tear fluid. We recently reported that concentrations of tear lipocalin in patients with meibomian gland dysfunction are significantly lower than in normal controls.[18] Tear lipocalin concentration correlated positively with tear film BUT and negatively with fluorescein staining scores in these patients. On the other hand, Grus et al[19]

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reported that lipocalin concentrations are somewhat elevated in the tears of contact lens wearers versus in normal controls, and Glasson et al[20] reported that contact lens–intolerant subjects had significantly higher amounts of lipocalin in tears compared with subjects who were able to tolerate contact lens wear.

$sPLA_2$ is a lipolytic enzyme that catalyzes hydrolysis of phospholipids at the *sn*-2 position, yielding free fatty acid and lysophospholipid.[21] Group IIa $sPLA_2$ is the most abundant form of $sPLA_2$ in tears. It has been found in tears in concentrations averaging from 1.45 to 54.5 μg/mL, levels that exceed normal serum group IIa $sPLA_2$ concentrations by 4 orders of magnitude.[22,23] Group IIa $sPLA_2$ in tears is purported to serve a bacteriocidal function.[24] However, as suggested by Song et al,[25] excess amounts of this enzyme may compromise tear film stability through hydrolysis of tear phospholipids. Glasson et al[20] reported that contact lens–intolerant subjects had slightly but significantly higher tear concentrations of group IIa $sPLA_2$ than contact lens–tolerant individuals and hypothesized that decreased tear phospholipids and tear film instability might be caused by the action of group IIa $sPLA_2$ in tear fluids.

As described above, phospholipids levels in the tears of hydrophilic contact lens wearers are lower than in normal controls, which might contribute to tear film instability. We posited 2 hypotheses to explain this phenomenon: (1) phospholipids deposition occurs on contact lenses and (2) tear phospholipids are degraded by group IIa $sPLA_2$ deposition on contact lenses. To test these 2 hypotheses, we assayed phospholipid, protein, and $sPLA_2$ content in tears and on contact lenses composed of 2 frequently replaced hydrophilic materials after 1 day of routine use.

MATERIALS AND METHODS

Subjects and Contact Lenses

Sixteen experienced contact lens wearers (4 men and 12 women) ranging in age from 26 to 44 years, with no history of eye disease except for refractive errors, participated in this study. Each subject wore Polymacon (Medalist; Bausch & Lomb Japan, Tokyo, Japan; FDA group 1, low water/non-ionic) and Etafilcon A (One Day Acuvue; Johnson & Johnson Japan, Tokyo, Japan; group 4, high water/ionic) contact lenses for 12 hours (from 7:00 AM to 7:00 PM) on different days. At the end of daily wear, tears were collected using a Schirmer test strip from the right eye of each subject. The length of wet portion of each strip was recorded. Then, Schirmer test strips and worn contact lenses from all subjects' right eyes were stored at −80°C until analysis. On a different day, subjects were instructed not to wear contact lenses for 1 day; in the evening, tears in the right eye of all subjects were collected using a Schirmer test strip. The protocol was approved by our institutional review board, and all subjects provided written informed consent before participation.

Phospholipids Analysis

Schirmer test strips and contact lenses were cut in half and used for lipids analysis and protein analysis. Lipids were extracted by a modified Bligh and Dyer procedure.[26] In short, samples were placed in a test tube with 1.0 mL of 2:1 chloroform:methanol extraction solvent (Wako, Osaka, Japan) for 16 hours. After adding 0.2 mL of water, the tubes were vortexed for 30 seconds. The aqueous layer was discarded, and the lipid layer was subjected to analysis.

Phospholipids levels were estimated by phosphorus determination with ammonium molybdate through enzymatic digestion. After lipid extracts were evaporated to dryness under nitrogen gas, 50 μL of 10 mmol/L TRIS hydrochloride (Sigma Chemical, St. Louis, MO) buffer (pH 7.8) containing 2.0 U/mL phospholipase C (from *Bacillus cereus*; Sigma Chemical) was added, and samples were incubated at 37°C for 20 minutes.[27] Samples were incubated at 37°C for an additional 30 minutes after adding 50 μL of 175 mmol/L diethanolamine hydrochloride (Sigma) buffer (pH 9.6) containing 2.0 U/mL alkaline phosphatase (human placental origin; Sigma). Then, 50-μL aliquots of each sample were placed in a 96-well microplate and mixed with molybdate-malachite green reagent (BIOMOL, Plymouth Meeting, PA). Absorbance of solutions at 620 nm was measured by spectrophotometer.

Total Protein and $sPLA_2$ Analysis

Solvent consisting of a 50:50 mixture of 0.2% trifluoroacetic acid and acetonitrile (Wako) was used to extract proteins. Strips and lenses were placed in extraction solution for 16 hours, and extraction solution was subsequently analyzed.[28] The total protein content of extraction solution was measured by bicinchoninic acid (BCA) analysis. Sample solution (10 μL) was mixed with 300 μL of protein assay regent (Cytoskeleton, Denver, CO), which was comprised of bicinchoninic acid and cupric sulfate, in a 96-well microplate. Absorbance of the solution at 595 nm was measured by spectrophotometer. A standard curve established with bovine serum (Sigma) was used to quantify protein contents of the lens extract.

Double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) was used to determine group IIa $sPLA_2$ concentrations in protein samples. For this purpose, a commercial ELISA kit (Cayman Chemicals, Ann Arbor, MI) was used according to the manufacturer's instructions. Samples were diluted to 1/500 or 1/5000 concentrations, and absorbance was measured at 420 nm by spectrophotometer.

Measurement of $sPLA_2$ activity was performed with a commercial $sPLA_2$ activity assay kit (Cayman Chemicals). This assay uses a 1,2-dithio analog of heptanal phosphatidylcholine, which serves as substrate for most PLA_2, with the exception of cytosolic PLA_2. On hydrolysis of the thio ester bond at the *sn*-2 position by PLA, free thiols were detected using 5,5-dithio-*bis*-(2-nitorobenzonic acid). Absorbance was measured at 420 nm by spectrophotometer.

RESULTS

Phospholipids

Results of the determination of phospholipids in tears and deposited on contact lenses are detailed in Table 1 and Figure 1. Phospholipids concentrations in tears with Polymacon were 186 ± 39 μg/mL, which is lower but not

TABLE 1. Phospholipids and their Degrading Enzyme $SPLA_2$ in Tears and from Worn Contact Lenses (CL)

	Tears			Worn CL	
	Without CL	Polymacon	Etafilcon A	Polymacon	Etafilcon A
Phospholipids (tears: μg/mL; CL: μg/lens)	220 ± 35	186 ± 39	162 ± 33*	2.1 ± 0.4	1.8 ± 0.4
Total protein (tears: mg/mL; CL: mg/lens)	5.93 ± 1.49	6.59 ± 2.11	5.64 ± 2.69	0.04 ± 0.04	0.48 ± 0.06†
Group IIa $sPLA_2$ (tears: μg/mL; CL: μg/lens)	5.02 ± 0.68	5.13 ± 0.33	5.02 ± 0.51	0.01 ± 0.01	0.57 ± 0.09†
$sPLA_2$ activity (tears: mmol/minute/mL; CL: mmol/minute/lens)	0.47 ± 0.18	0.52 ± 0.18	0.47 ± 0.18	ND	0.13 ± 0.04

*P = 0.0023, †P < 0.0001; Mann-Whitney test.
ND, not detected.

significantly different versus concentrations observed without contact lens wear (P = 0.059; Mann-Whitney U test). On the other hand, phospholipids concentrations in tears with Etafilcon A were 162 ± 33 μg/mL, which is significantly lower than concentrations without contact lenses (P = 0.0023; Mann-Whitney U test). Phospholipid content recovered from contact lenses was 2.1 ± 0.4 and 1.8 ± 0.4 μg/lens in the Polymacon and Etafilcon A groups, respectively, which is not significantly different.

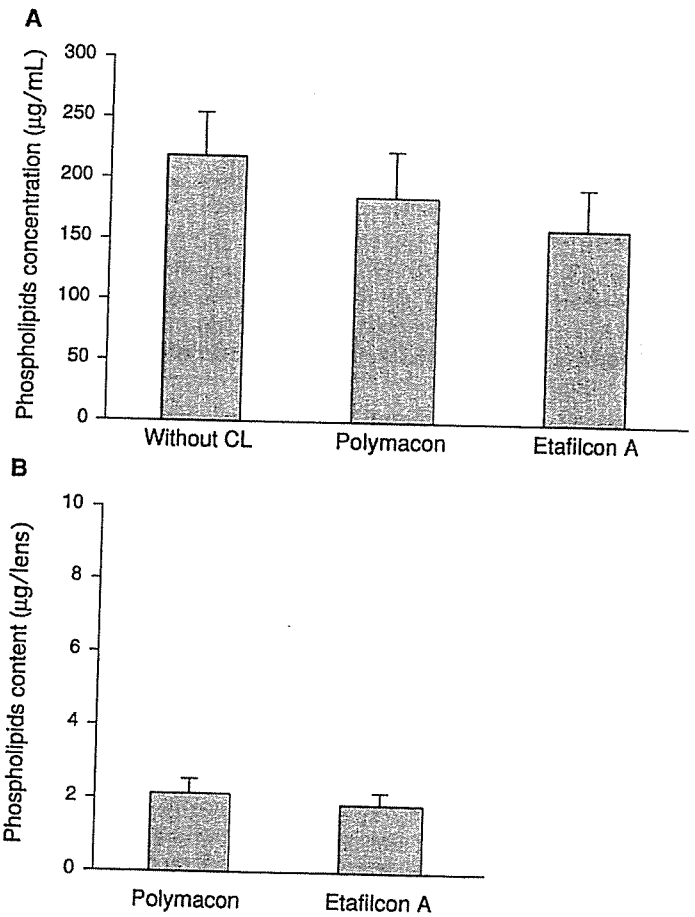

FIGURE 1. Phospholipids in tears (A) and deposited on hydrophilic contact lenses (B). Phospholipids concentrations in tears with Polymacon and Etafilcon A were 186 ± 39 and 162 ± 33 μg/mL, respectively. The latter concentration was significantly lower than that observed in the same individuals without contact lens wear (P = 0.0023; Mann-Whitney U test).

Total Protein

Total protein concentrations in tears are listed in Table 1. Total protein concentrations in tears were not significantly different among the 3 groups. In contrast, tears in the Etafilcon A group (0.48 ± 0.06 mg/lens) revealed significantly (P < 0.0001; Mann-Whitney U test) higher total protein contents compared with the Polymacon group (0.04 ± 0.04 mg/lens).

Group IIa $sPLA_2$ and $sPLA_2$ Activity

Group IIa $sPLA_2$ concentrations and enzymatic activities in tears and deposited on contact lenses are detailed in Figures 2 and 3. In tears, both group IIa $sPLA_2$ concentrations and enzymatic activities were not significantly different among the 3 groups. However, lens deposition in the Etafilcon A group (0.57 ± 0.09 μg/lens) showed significantly (P < 0.0001; Mann-Whitney U test) higher group IIa $sPLA_2$ content compared with the Polymacon group (0.01 ± 0.01 μg/lens). Group IIa $sPLA_2$ deposited on Etafilcon A lenses retained enzymatic activity: $sPLA_2$ activity in this group was 0.13 ± 0.04 mmol/min/lens. $sPLA_2$ activity was not detected in the Polymacon group.

No significant quantity of proteins and lipids was recovered from unworn Etafilcon A and Polymacon contact lenses.

DISCUSSION

In this study, phospholipid concentrations in tears were estimated by phosphorus determination with ammonium molybdate through enzymatic digestion.[27] Although this method is not suitable for analyzing the class of phospholipids, it enabled us to determine the quantity of phospholipids in small volumes of tears, because phospholipids of all classes contain a single inorganic phosphate. Phospholipids concentrations in tears with Etafilcon A wear were significantly lower than in those without contact lenses. Although it has been reported that phospholipids levels in the tears of soft contact lens wearers are lower than in normal controls, our result shows for the first time that lipids composition of tears is altered by contact lens wear.[12] To explore the mechanism of this phenomenon, we also analyzed the amounts of phospholipids deposited on contact lenses. However, these amounts of phospholipids recovered from worn lenses were small and considered insignificant. Lipid deposition patterns observed in this study are consistent with previously published work.[29–31] Therefore, it is not likely that decreased phospholipids levels

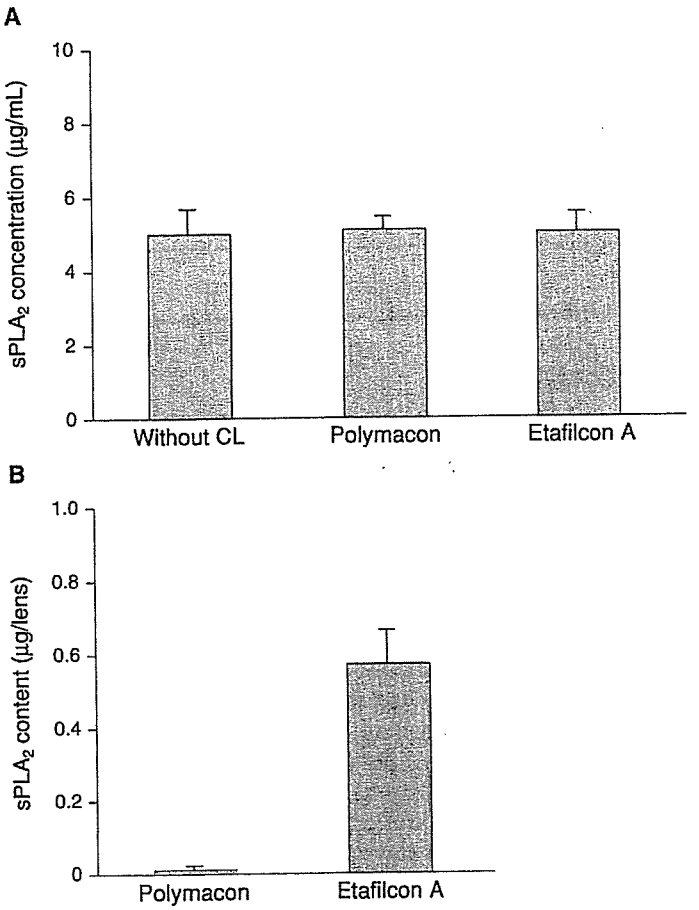

FIGURE 2. Group IIa $sPLA_2$ concentration in tears (A) and content deposited on contact lenses (B). Group IIa $sPLA_2$ concentrations in tears were not significantly different among 3 groups. Etafilcon A (0.57 ± 0.09 μg/lens) showed significantly ($P < 0.0001$; Mann-Whitney U test) higher group IIa $sPLA_2$ deposition than Polymacon (0.01 ± 0.01 μg/lens).

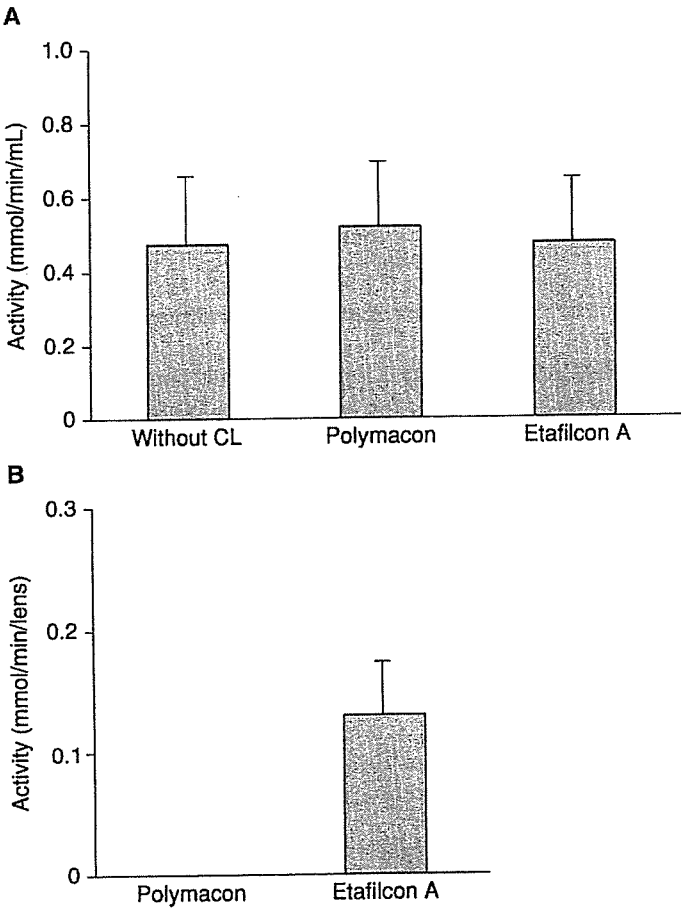

FIGURE 3. $sPLA_2$ activities in tears (A) and deposited on contact lenses. $sPLA_2$ activity in tears was not significantly different among 3 groups. B, $sPLA_2$ activity in the Etafilcon A group was 0.13 ± 0.04 mmol/min/lens, whereas no significant $sPLA_2$ activity was detected in the Polymacon group.

in tears of hydrophilic contact lens wearers result from deposition of phospholipids on the lenses.

It is widely recognized that contact lens adsorption of proteins is a complex process influenced by many variables such as water content and surface charge on lenses.[29–34] Significant differences were found in protein deposition profiles of the 2 lens groups tested in this study. Etafilcon A lenses accumulated significantly more protein including group IIa $sPLA_2$ than Polymacon lenses. Protein deposition is thought to be predominantly influenced by the ionic charge of the lens material.[30] Ionic materials such as methacrylic impart a negative charge to the lens and thus favor deposition of positively charged species such as lysozymes. Group IIa $sPLA_2$ is also highly cationic in tears,[35] resulting in electrostatic attraction to negatively charged lens material.

Glasson et al[20] reported that contact lens–intolerant wearers had significantly higher tear concentrations of group IIa $sPLA_2$ than subjects who were able to tolerate contact lens wear. These researchers suggested that decreased tear phospholipids and tear film instability might be caused by enzymatic action of group IIa $sPLA_2$ in tears. In this study, however, there was no significant difference of group IIa $sPLA_2$ concentration in the tears of subjects regardless of contact lens wear. Instead, we found significant deposition of group IIa $sPLA_2$ on Etafilcon A contact lenses. Aho et al[36] reported that contact lens wearers had statistically lower group IIa $sPLA_2$ content in tears at noon and at 4:00 PM versus healthy controls. They proposed that transient low levels of group IIa $sPLA_2$ observed in contact lens wearers might be caused by contact lens absorption of group IIa $sPLA_2$. Our results seem to support this hypothesis. Assuming that total tear volume of normal subjects is 10 μL and concentration of group IIa $sPLA_2$ in normal tears is 54.5 μg/mL,[23] tears of normal subjects would be expected to contain a total of 0.55 μg of group IIa $sPLA_2$. Therefore, the amount of group IIa $sPLA_2$ deposited on Etafilcon A contact lenses in this study is as high as the total amount expected in normal tears. We also showed that group IIa $sPLA_2$ deposited on contact lenses retained its activity as a lipolytic enzyme. Retention of enzymatic activity by group IIa $sPLA_2$ seems to be of functional significance.

Our results suggest a novel additional mechanism for contact lens–induced dry eye: the activity of group IIa $sPLA_2$ deposited on contact lenses may play a role in the development of tear film instability. It should be noted, however, that

concentrations of phospholipids in the tears of subjects in this study were lower than those of normal control subjects in our previous study, even when they had not worn contact lenses for at least 1 day.[11] In that study, we reported that phospholipid concentration in tears from patients with moderate dry eye was 182.3 ± 89.2 μg/mL, which is significantly lower than those in normal subjects (379.0 ± 97.8 μg/mL). There might be other changes in the ocular surface of contact lens wearers to decrease phospholipid levels in their tears.

In summary, we found significant group IIa $sPLA_2$ deposition on Etafilcon A contact lenses. Contact lens enzyme deposition may promote phospholipid hydrolysis in tears, resulting in decreases of tear phospholipids and increases of free fatty acids. These biochemical alterations may lead to tear film instability and thereby contribute to contact lens intolerance.

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Lattice Corneal Dystrophy Type III in Patients with a Homozygous L527R Mutation in the *TGFBI* Gene

Late-onset lattice corneal dystrophy (LCD) is associated with decreasing vision, minor recurrent epithelial erosions or no erosions at all, and lattice lines much thicker than those usually observed in LCD types I and II. A patient with this type of LCD is classified as LCD type III.[1] Most LDC type III cases have been reported in Japanese patients, and the inheritance pattern is proposed to be autosomal recessive. However, Stock et al.[2] reported that although LCD type IIIA resembles type III clinically, it differs in that type IIIA has an autosomal dominant inheritance pattern. A later study reported that LCD type IIIA is caused by mutations in the transforming growth factor beta-induced (*TGFBI*) gene.

More recently, a heterozygous L527R mutation in the *TGFBI* gene has been reported to be the cause of late-onset LCD in six Japanese patients.[3] Interestingly, only two of these had a family history of LCD. Hirano et al.[4] reported that two Japanese patients with late-onset LCD also had a heterozygous L527R mutation and no family history. They clinically diagnosed LCD type III in these two patients.

We present the characteristics of two patients with late-onset LCD who were homozygous for the L527R mutation.

Case Reports

Patient 1 was a 78-year-old man who presented with decreased vision in both eyes. His corrected visual acuity was 0.3 OD and 0.1 OS. He is the younger brother of patients 2 and 3 in the family with LCD type III reported by Hida et al.[1] (their Fig. 2). His corneal opacities were bilateral and observed as grayish nodular deposits in the midstroma with relatively thick lattice lines that extended from limbus to limbus (Figs. 1A, B). He had no history of corneal erosions. Penetrating keratoplasty was performed on his left eye in March 1996 and on his right eye in December 2001. Histologic findings of the excised corneas showed large deposits of amyloid in the stroma, predominantly midway between the epithelium and the endothelium.

Patient 2 was a 78-year-old man who presented with decreased vision in both eyes. His corrected visual acuity was 0.3 OD and 0.01 OS. The corneal opacities consisted of grayish nodular deposits in the central midstroma and relatively thick lattice lines that extended from limbus to limbus in both eyes (Fig. 1C). The appearance of the corneal opacities was similar to those of patient 1. His sister had undergone keratoplasty at the age of 60 years, but the details of the condition of her cornea could not be obtained. There was no history of corneal erosions in patient 2. Penetrating keratoplasty was performed on the left eye in January 1993. Histologic findings of the excised cornea showed large stromal deposits of amyloid (Fig. 1D).

A genetic investigation was performed according to the guidelines of the Declaration of Helsinki. Written informed consent was obtained. Direct sequencing[5] of the *TGFBI* gene revealed homozygous L527R mutations (Fig. 2).

Comments

Recently, we reported on five Japanese patients with late-onset LCD who also carried a heterozygous L527R mutation.[5] One (case 3 in reference 5) of the five patients had been previously reported as having LCD type III by Hida et al.[1] (case 5 in reference 1), and only one (case 4 in reference 5) with the L527R mutation had a family history of LCD, an affected sibling. Thus, most patients with late-onset LCD reported in Japan have relatively thick lattice lines and/or tiny, discrete nodular deposits, but no family history or affected siblings. They are heterozygous[3–5] or homozygous for the L527R mutation. These results indicate that the heterozygous L527R mutation in the *TGFBI* gene for LCD has low penetrance in the Japanese population, but the homozygous L527R mutation might have increased penetrance. Interestingly, the corneas in those patients with a homozygous L527*R* mutation appeared very similar to the corneas of the heterozygous patients. The reason for this is unknown.

Mutations in the *TGFBI* gene can cause LCD type IIIA as well as LCD type III, both late-onset LCD. Because the corneal appearance in patients with type III is very similar to that in patients with type IIIA, the two types are diagnosed by the inheritance pattern. However, the difference most likely results from the degree of penetrance of the *TGFBI* gene mutation.

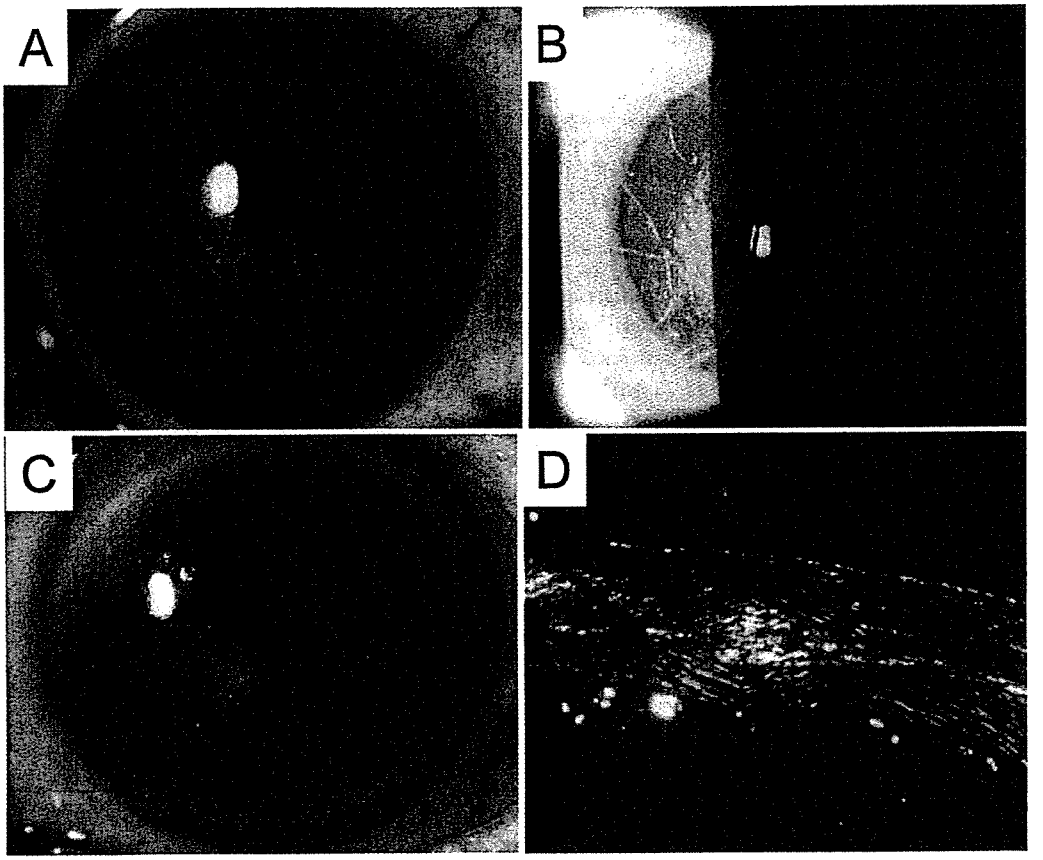

Figure 1A–D. Slit-lamp photographs of corneas of the two patients with late-onset lattice corneal dystrophy. **A** Central opacities with grayish nodular deposits and discrete, thick lattice lines can be seen in patient 1. The patient was reported previously to have LCD type III (case 2 in reference 1). **B** The thick lattice lines can be better seen with retroillumination in patient 1. **C** The central opacities with grayish nodular deposits and discrete thick lattice lines in the left eye in patient 2. The appearance of corneal opacities was similar to the appearance in patient 1. **D** Congo red stain. The deposits in the anterior stromal layer and deposits in the deeper stroma show birefringence and red-green dichroism under polarized light.

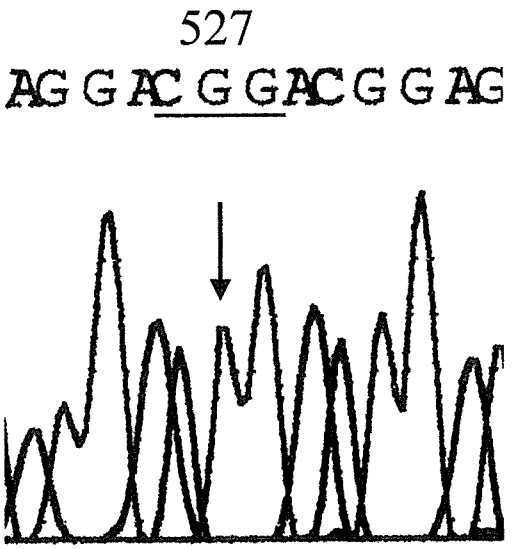

Figure 2. Result of direct sequencing of sense strands around codon 527 in the *TGFBI* gene in patient 1. A homozygous T-to-G base change (CTG to CGG) at codon 527 was detected.

Key Words: L527R mutation, late onset, lattice corneal dystrophy, *TGFBI* gene, type III lattice corneal dystrophy

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A Case of Conjunctival Leech Infestation

Conjunctival leech infestation is a rare type of conjunctival foreign body. Several cases have been reported in Japan.[1–4] However, only one case of human conjunctival infestation has been reported in the English literature, to our knowledge.[5] In this paper, we present a case of conjunctival leech infestation and discuss its clinical significance and differential diagnosis.

Case Report

A 38-year-old woman had been walking in a stream searching for river fish on June 15, 1993. She presented at the Hatanaka Eye Clinic with foreign body sensation in the right eye 1 week later. The best-corrected visual acuity was 20/20 OU. The intraocular pressure was 20 mmHG OD and 19 mmHG OS. Slit-lamp examination of the right eye revealed a black-brown foreign body measuring 7 mm in length in the lower fornical conjunctiva (Fig. 1). Although it seemed like a case of a protruding uvea with associated scleral perforation, there were no inflammatory cells in the anterior chamber or the vitreous cavity. Results of a fundus examination were unremarkable. There was mild subconjunctival hemorrhage in the nasal bulbar conjunctiva. The superior tarsal conjunctiva had marked papillary hypertrophy (Fig. 2). During examination, the foreign body moved onto the nasal bulbar conjunctiva.

The doctor (HO) tried to remove the foreign body with forceps, but it was firmly adhered to the nasal bulbar conjunctival. Next, he administered commercially available bupivacaine eye drops (Benoxil; Santen, Osaka, Japan). A few minutes later, the foreign body was easily extracted with forceps.

The removed foreign body was a long, narrow leech with a single longitudinal line on its back. From this characteristic feature it was identified as *Hirudo nipponia* at the Department of Parasitology, Nara Medical University.

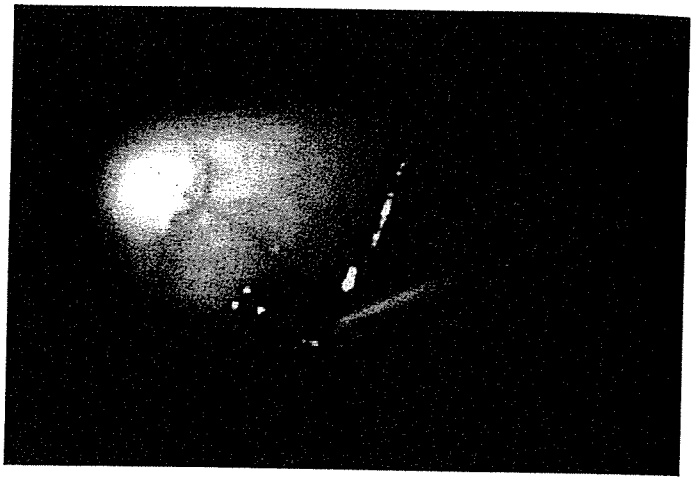

Figure 1. A leech was observed on the inferior fornical conjunctiva of a 38-year-old woman who had been walking in a stream searching for river fish.

Figure 2. The upper tarsal conjunctiva showed marked papillary hypertrophy.

Comments

Conjunctival leech infestation may be rare in foreign countries. Only one patient with a conjunctival leech infestation has been reported in the English literature.[5] The patient (a 7-year-old boy) had a history of swimming in a stream. He had pain, burning, itching, foreign body sensation, and severe blepharospasm. There was a localized lesion of subconjunctival hermorrhage and superficial corneal epithelial defect. The area corresponded to where the leech was adherent.

In the Japanese reports, the subjective symptoms include foreign body sensation,[1] epiphora,[2] itching,[3] and no symptoms.[4] The objective symptoms include mild conjunctival injection[1,3] and no description.[2–4] In none of the reports was follicular or papillary hypertrophy of the conjunctiva