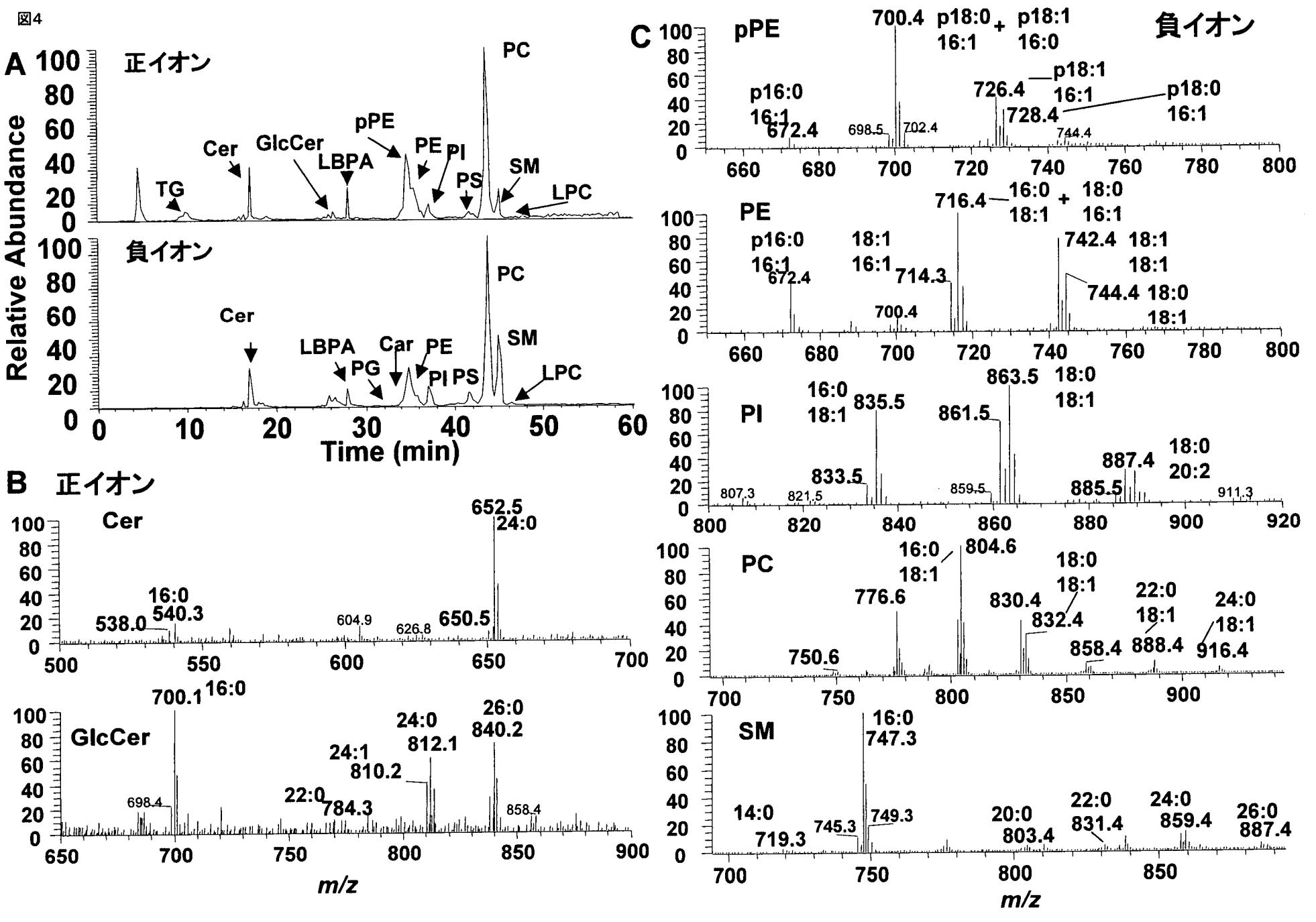
図4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Incidence of endophthalmitis after cataract surgery in Japan

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ABSTRACT.

Purpose: **To estimate the incidence rate of endophthalmitis after cataract surgery performed by Japanese surgeons.**

Methods: **A sample of 20% of members of the Japanese Society of Ophthalmic Surgeons was randomly selected. Each member was sent a postal survey asking for information on the number of cataract surgeries performed in 2003 and the number of postsurgery cases of endophthalmitis.**

Results: **Replies were received from 78.7% (513/652) of survey recipients. The total number of cataract surgeries was 100 539, among which 52 cases of endophthalmitis occurred, resulting in an overall incidence rate of 0.052%. Incidence rates were 0.049% (38/78 170) for scleral incision phacoemulsification and 0.043% (9/20 894) for clear corneal incision phacoemulsification, with no significant difference between groups. The average annual volumes of surgery were 210 and 280 cases for surgeons who preferred scleral incision and clear corneal incision, respectively. There was a significant difference between volumes (Student's *t*-test, $p < 0.01$). The incidence of endophthalmitis was significantly lower with high-volume (> 300 cases/year) surgeons than with low-volume (≤ 300 cases/-year) surgeons (0.040% versus 0.066%, chi-square test, $p < 0.05$).**

Conclusions: **The incidence of endophthalmitis after cataract surgery in Japan as estimated by a postal survey was low (0.052%) and consistent with rates reported previously. Surgeons who preferred clear corneal incision performed significantly more surgeries annually, but the incidence of endophthalmitis was similar between scleral and clear corneal incision phacoemulsification surgery.**

Key words: cataract surgery – endophthalmitis – incidence – infection – survey

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Introduction

The reported incidence of endophthalmitis after cataract surgery varies across studies, depending on factors such as preoperative prophylactic, surgical techniques, occurrence of intraoperative complications, postoperative antibiotic regimen, and so on (Olson 2004). Temporal and geographic variations also exist (Morlet et al. 2003). In a systematic review of the literature from 1963 to 2003, Taban et al. (2005) reported an overall rate of 0.128% for postcataract endophthalmitis during that period. Only a few large-scale, multicentre studies on the incidence of endophthalmitis after cataract surgery have been carried out since this review, with varying results (Wejde et al. 2005; West et al. 2005; Barry et al. 2006). West et al. (2005) analysed US Medicare data for 1994–2001 and found a prevalence of postcataract surgery endophthalmitis of 0.215%. The national prospective survey in Sweden reported an incidence of 0.0595% for postoperative endophthalmitis in 1999–2001 (Wejde et al. 2005). The most recent European multicentre study indicated that the rate of endophthalmitis following

cataract surgery was 0.204% on average (Barry et al. 2006). These incidence rates, however, are significantly higher than the prevalences of postoperative endophthalmitis reported in recent, large, single-centre studies, which range from 0.04% to 0.076% (Wong & Chee 2004; Lalitha et al. 2005; Miller et al. 2005). Considering the increasing emphasis on the prevention of endophthalmitis after cataract surgery and the great variation in the few data that exist, we conducted a retrospective study to estimate the rate of endophthalmitis following modern cataract surgery in Japan. As this was a postal survey, the obtained incidence may underestimate the actual incidence of endophthalmitis.

Materials and Methods

A sample of 20% of the 3290 members of the Japanese Society of Ophthalmic Surgeons was randomly selected. A survey form asking for the total number of cataract surgeries each surgeon had performed in 2003 and the number of endophthalmitis cases that occurred among these was mailed to 656 members in March 2004. The surgeons were asked to report all endophthalmitis cases, including culture-proven cases and presumed cases with a negative culture. They were also asked to give details of the type of cataract surgery and incision used.

Results

Four survey forms were returned because of an unknown address, member withdrawal or member death. Of the remaining 652 recipients, 513 surgeons (78.7%) returned the survey form. The enclosed data revealed that a total of 100 539 cataract surgeries had been performed during the study period, including 78 170 cases of phacoemulsification through scleral incision, 20 894 cases of phacoemulsification through clear corneal incision, and 1475 cases of extracapsular cataract extraction (ECCE).

The surgeons reported 52 cases of endophthalmitis, resulting in an overall rate of 0.052% (Table 1). Incidence rates were 0.049% for scleral incision phacoemulsification, 0.043% for clear corneal incision phacoemulsification and 0.203% for ECCE. The rate was significantly higher in the ECCE group than in either of the phacoemulsification groups ($p < 0.05$, chi-square test). The ECCE group apparently represented not only planned but also unplanned ECCE procedures. Two cases were excluded from these statistics because they underwent combined cataract surgery with trabeculectomy and keratoplasty.

Table 1. Incidence of endophthalmitis after cataract surgery.

Procedure	Rate
Overall	0.052%
Scleral incision phacoemulsification	0.049%
Clear corneal incision phacoemulsification	0.043%
Extracapsular cataract extraction	0.203%

The average annual volumes of surgery for surgeons who preferred scleral incision and clear corneal incision were 210 and 280 cases, respectively. There was a significant difference between these rates ($p < 0.01$).

During 2003, 119 surgeons performed > 300 cataract surgeries and 330 surgeons performed ≤ 300 cataract surgeries. 64 surgeons did not perform cataract surgery. Incidence rates for cases of endophthalmitis with high- (> 300) and low-volume (≤ 300) surgeons were 0.040% and 0.066%, respectively. There was a significant difference between the two groups ($p < 0.05$).

Discussion

The main purpose of this study was to estimate the incidence of endophthalmitis in Japan. Towards this end, we made the questionnaire as simple as possible so that recipients could fill in the sheet easily, and obtained a very high response rate (78.7%). If the survey form had been more complicated, the surgeons would have had to spend considerable time answering the questions and the response rate would have been compromised. It is likely that the non-respondents included doctors who did not do cataract surgery. Taking that possibility into account, the actual response rate may have been even higher.

In this study, the overall incidence of endophthalmitis after cataract surgery was approximately 0.05%. Previous large-scale, multicentre studies have tended to report higher prevalences of postcataract surgery endophthalmitis (West et al. 2005; Barry et al. 2006), whereas recent large-scale, single-centre studies have reported similar incidences of postoperative endophthalmitis, ranging from 0.04% to 0.076% (Wong & Chee 2004; Lalitha et al. 2005; Miller et al. 2005). In the most recent European study, the group that was treated with the most effective prophylactic regimen yielded an incidence rate of 0.058% (Barry et al. 2006). Thus, given incidence rates following modern cataract surgery in developed countries, the rate obtained in the current study seems reasonable.

Controversy exists regarding the relationship between the risk of endophthalmitis after cataract extraction and type of incision. Some authors report that clear corneal incisions are associated with an increased incidence of postoperative endophthalmitis (Schmitz et al. 1999; Lertsumitkul et al. 2001; Cooper et al. 2003; Nagaki et al. 2003), whereas others have shown an equal distribution of endophthalmitis incidence between surgeries using different types of incision (Colleaux & Hamilton 2000; Miller et al. 2005). Interestingly, there are no reports indicating that clear corneal incision is associated with a lower incidence of postoperative endophthalmitis than scleral incision. In a systematic review of the literature, Taban et al. (2005) indicated that the incidence of endophthalmitis associated with cataract extraction had increased over the last decade, and this upward trend in endophthalmitis frequency coincided temporally with the development of sutureless clear corneal incisions. In a survey of 340 633 cataract surgeries performed in Germany, Schmitz et al. (1999) used multivariate analysis to identify clear corneal incision as a risk factor for postoperative endophthalmitis. Corneoscleral incision was associated with a 65% reduction in relative risk of developing endophthalmitis after cataract surgery (Schmitz et al. 1999). In a retrospective case-control study in Australia, logistic regression showed an increased risk of endophthalmitis with clear corneal temporal incisions (Lertsumitkul et al. 2001). In another retrospective case-control

study, Cooper et al. (2003) reported a three-fold higher risk of endophthalmitis after cataract surgery with clear corneal incision compared with superior scleral tunnel incision. In a prospective, randomized study, Nagaki et al. (2003) demonstrated a 4.6-fold higher relative risk of endophthalmitis with clear corneal incision against superior scleral tunnel incision. Miller et al. (2005) reported the incidence of acute-onset endophthalmitis after temporal clear corneal incision phacoemulsification to be higher (0.05%) than that after phacoemulsification by other types of incision (0.02%), but the difference was not statistically significant. Colleaux & Hamilton (2000) found that the incidence of endophthalmitis was higher in clear corneal (0.129%) versus scleral tunnel (0.05%) incision, although the difference was not statistically significant.

In our study, incidence rates of postoperative endophthalmitis were similar in scleral and clear corneal incision phacoemulsification. However, the details of the data reveal an interesting point. The average annual volume of cataract surgery of surgeons who preferred to make a clear corneal incision was significantly larger than that of surgeons who opted for scleral incision. By contrast, the rate of endophthalmitis was significantly lower after surgery performed by high-volume surgeons than after that carried out by low-volume surgeons, a result that agrees with the findings of a previous prospective cohort study (Fang et al. 2006). Considering the significantly larger surgical volume of surgeons who preferred to use clear corneal incision, it would appear that the incidence of endophthalmitis in clear corneal incision cases should have been significantly lower than that in scleral incision cases. The fact that this was not the case implies that clear corneal incision may be associated with a higher risk of postoperative endophthalmitis than scleral incision. The validation of this speculation awaits further studies.

Fang et al. (2006) also reported that provider volume (hospital and surgeon volume) was associated with risk for postoperative endophthalmitis. Patients who underwent cataract surgery at low-volume hospitals or with low-volume surgeons had a significantly higher risk of postoperative endophthalmitis than those undergoing treatment at high-volume hospitals or with high-volume surgeons. The authors postulated that the lower risk of postoperative endophthalmitis in the high-volume hospital group may be due to a greater proportion of younger patients than in the low-volume hospital group. Younger patients may tend to seek out high-volume hospitals for surgery in the belief that such hospitals produce better outcomes. Fang et al. (2006) also mentioned that the lower risk of postoperative endophthalmitis in the high-volume surgeon group may be due to a lower proportion of patients undergoing surgery carried out by doctors who are under 35 years old than in the low-volume surgeon group.

Our results indicate that ECCE is associated with a significantly higher incidence of endophthalmitis than phacoemulsification. Conflicting re ports exist on this issue. Some argue that ECCE is associated with increased risk of endophthalmitis relative to phacoemulsification (Kalpadakis et al. 2002; Haapala et al. 2005; Lalitha et al. 2005), but others claim that phacoemulsification is associated with a higher risk (Wong & Chee 2004). Some reports have found no difference in rates of endophthalmitis between ECCE and phacoemulsification (Somani et al. 1997; Semmens et al. 2003; Trinavarat & Atchaneeyasakul 2005). Our study population included only a small number of cases treated with ECCE, and this group presumably comprised both planned and unplanned procedures, a fact that reflects one of the drawbacks of our study, as mentioned below. We would therefore like to withhold any conclusion on this issue.

There are potential limitations to the present study, some of which are inherent to any postal survey and some of which are particular to our study. Firstly, definitions of endophthalmitis may be inconsistent. Surgeons were requested to report all endophthalmitis cases, including culture-proven cases and presumed cases with a negative culture. In some cases, cultures were not performed. Inaccurate coding of endophthalmitis is not unusual in practice, which represents a serious concern for the data quality of any epidemiological analysis (Li et al. 2003). Secondly, in order to achieve a high response rate, our study form was so simple that many important data were not collected. For instance, ECCE may include both planned and unplanned procedures, which may involve cases that converted from phacoemulsification due to intraoperative complications. No data are available on pre-, peri- or postoperative prophylactics. Thirdly, this was a retrospective, survey-based study and thus control of data quality was not easy. The quality of the outcome depends on the quality of the inputs (Taban et al. 2005). Fourthly, it is possible that surgeons who experienced higher rates of endophthalmitis were disinclined to respond to the survey. It is not possible, however, to trace those who did not return the survey.

Nonetheless, the high response rate of the current survey is an advantageous feature of this study. The prevalence of postoperative endophthalmitis obtained in this study is at the lower end of the spectrum of incidence currently reported in the developed world. Wejde et al. (2005) reported a low nationwide incidence of postcataract surgery endophthalmitis in Sweden and attributed the results to the administration of intracameral antibiotics. In Japan, administration of intracameral antibiotics is not common practice. Thus, further studies are needed to determine the factors associated with increased or decreased rates of endophthalmitis and to establish an effective regimen to prevent the occurrence of this harmful complication.

References

Barry P, Seal DV, Gettinby G, Lees F, Peterson M, Revie CW & ESCRS Endophthalmitis Study Group (2006): ESCRS study of prophylaxis of postoperative endophthalmitis after cataract surgery: preliminary report of principal results from a European multicentre study. J Cataract Refract Surg **32**: 407–410.

Colleaux KM & Hamilton WK (2000): Effect of prophylactic antibiotics and incision type on the incidence of endophthalmitis after cataract surgery. Can J Ophthalmol **35**: 373–378.

Cooper BA, Holekamp NM, Bohigian G & Thompson PA (2003): Case-control study of endophthalmitis after cataract surgery comparing scleral tunnel and clear corneal wounds. Am J Ophthalmol **136**: 300–305.

Fang YT, Chien LN, Ng YY, Chu HF, Chen WM, Cheng CY & Wu SC (2006): Association of hospital and surgeon operation volume with the incidence of postoperative endophthalmitis: Taiwan experience. Eye **20**: 900–907.

Haapala TT, Nelimarkka L, Saari JM, Ahola V & Saari KM (2005): Endophthalmitis following cataract surgery in southwest Finland from 1987 to 2000. Graefes Arch Clin Exp Ophthalmol **243**: 1010–1017.

Kalpadakis P, Tsinopoulos I, Rudolph G, Schebitz K & Froehlich SJ (2002): A comparison of endophthalmitis after phacoemulsification or extracapsular cataract extraction in a socioeconomically deprived environment: a retrospective analysis of 2446 patients. Eur J Ophthalmol **12**: 395–400.

Lalitha P, Rajagopalan J, Prakash K, Ramasamy K, Prajna NV & Srinivasan M (2005): Postcataract endophthalmitis in South India: incidence and outcome. Ophthalmology **112**: 1884–1889.

Lertsumitkul S, Myers PC, O'Rourke MT & Chandra J (2001): Endophthalmitis in the western Sydney region: a case-control study. Clin Exp Ophthalmol **29**: 400–405.

Li J, Morlet N, Semmens J, Gavin A, Ng J & EPSWA Team (2003): Coding accuracy for endophthalmitis diagnosis and cataract procedures in Western Australia. The Endophthalmitis Population Study of Western Australia (EPSWA): second report. Ophthalmic Epidemiol **10**: 133–145.

Miller JJ, Scott IU, Flynn HW Jr, Smiddy WE, Newton J & Miller D (2005): Acute-onset endophthalmitis after cataract surgery (2000–04): incidence, clinical settings and visual acuity outcomes after treatment. Am J Ophthalmol **139**: 983–987.

Morlet N, Li J, Semmens J, Ng J & EPSWA Team (2003): The Endophthalmitis Population Study of Western Australia (EPSWA): first report. Br J Ophthalmol **87**: 574–576.

Nagaki Y, Hayasaka S, Kadoi C et al. (2003): Bacterial endophthalmitis after small-incision cataract surgery. Effect of incision placement and intraocular lens type. J Cataract Refract Surg **29**: 20–26.

Olson RJ (2004): Reducing the risk of postoperative endophthalmitis. Surv Ophthalmol **49**: S55–S61.

Schmitz S, Dick HB, Krummenauer F & Pfeiffer N (1999): Endophthalmitis in cataract surgery: results of a German survey. Ophthalmology **106**: 1869–1877.

Semmens JB, Li J, Morlet N, Ng J & EPSWA Team (2003): Trends in cataract surgery and postoperative endophthalmitis in Western Australia (1980–98): the Endophthalmitis Population Study of Western Australia. Clin Exp Ophthalmol **31**: 213–219.

Somani S, Grinbaum A & Slomovic AR (1997): Postoperative endophthalmitis: incidence, predisposing surgery, clinical course and outcome. Can J Ophthalmol **32**: 303–310.

Taban M, Behrens A, Newcomb RL, Nobe MY, Saedi G, Sweet PM & McDonnell PJ (2005): Acute endophthalmitis following cataract surgery: a systematic review of the literature. Arch Ophthalmol **123**: 613–620.

Trinavarat A & Atchaneeyasakul LO (2005): Surgical techniques of cataract surgery and subsequent postoperative endophthalmitis. J Med Assoc Thai **88**: S1–S5.

Wejde G, Montan P, Lundstrom M, Stenevi U & Thorburn W (2005): Endophthalmitis following cataract surgery in Sweden: national prospective survey 1999–2001. Acta Ophthalmol Scand **83**: 7–10.

West ES, Behrens A, McDonnell PJ, Tielsch JM & Schein OD (2005): The incidence of endophthalmitis after cataract surgery among the US Medicare population increased between 1994 and 2001. Ophthalmology **112**: 1388–1394.

Wong TY & Chee SP (2004): The epidemiology of acute endophthalmitis after cataract surgery in an Asian population. Ophthalmology **111**: 699–705.

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Presence of adipose differentiation-related protein in rat meibomian gland cells

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Abstract

Adipose differentiation-related protein (ADRP) is an intrinsic lipid storage protein found in lipid droplets of different type of cells. ADRP has been recognized to be a specific marker of lipid accumulation and a marker of differentiated adipocytes. The purpose of this study was to determine whether ADRP was present in the cells of the meibomian gland. The expression of the mRNA of ADRP was determined by RT–PCR and Northern blot analysis of the meibomian gland and other rat tissues. A newly generated polyclonal antibody against rat ADRP was used for Western blot analysis and immunohistochemical staining to determine whether ADRP was expressed in the rat meibomian gland. Meibomian gland acinar cells were isolated to determine when ADRP was expressed during cell differentiation in vitro. Northern blot analysis and Western analysis showed that ADRP was expressed in the meibomian gland. Immunoreactivity to ADRP was observed in the lobules of acinar cells in the meibomian gland, and was preferentially located adjacent the vacuolated cytoplasm. In culture, the meibocytes began to store lipid droplets in the cytoplasm as they became confluent, and the immunoreactivity for ADRP was found at the margins of the oil droplets. Our results suggest that ADRP can serve as a new marker for the identification of differentiated meibocytes containing lipid droplets.

Keywords: meibomian gland; adipose differentiation-related protein (ADRP); lipid droplet; adipose differentiation; cultured meibomian gland acinar cells; ocular surface

1. Introduction

The precorneal tear film is a tri-layered structure comprised of a lipid layer, an aqueous layer, and a mucin layer. The outermost layer of the tear film is 0.1 μm thick, and is made up primarily of a lipid fraction secreted from the meibomian glands lying within the tarsal plates (Holly, 1980). It is well known that this outer layer produces a smooth optical surface, retards tear evaporation, prevents tear from spilling over the lid margin, and provides structural and refractive integrity to the ocular surface (Tiffany, 1985, 1987; Mathers et al., 1996).

The meibomian glands consist of a number of lipid-producing acini and lipid-secreting ducts. Acinar cells differentiate from flat basal cells located at the margin of each lobule, begin storing lipid droplets as they move inwards, and finally release the lipids into the lumen in a holocrine fashion (Mathers et al., 1996). Abnormalities of lipid synthesis or secretion lead to meibomian gland dysfunction (MGD) which is the most common cause of posterior marginal blepharitis. When MGD is present in older subjects or in association with different ocular surface disorders, the quality and quantity of lipids secreted are subnormal (Shine and McCulley, 2004; Tsai et al.,

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2006). This can lead to superficial punctate keratopathy accompanied by instability of the tear film (McCulley and Sciallis, 1977; Mathers, 1993; Shimazaki et al., 1995; Driver and Lemp, 1996; Bron and Tiffany, 1998).

The cDNAs of a number of proteins involved in fat metabolism in the adipocytes have been cloned, and the mechanisms of the synthesis and storage of lipids have been extensively studied (Ailhaud et al., 1992; Masuda et al., 2006; Yamaguchi et al., 2006a,b). However, the precise mechanism of the synthesis and storage of lipids in the meibomian glands has not been determined. Among the lipid droplet associated proteins, the most prominent protein is perilipin, which is a phosphoprotein that is located at the margins of the lipid droplets in adipocytes and steroidogenic cells. It is believed that perilipin is involved in the regulation of lipid metabolism (Greenberg et al., 1991; Londos et al., 1995). Perilipin is a member of the PAT family of proteins along with adipose differentiation-related protein (ADRP), TIP47, and S3-12 protein (Miura et al., 2002; Wolins et al., 2003). In contrast to perilipin and S3-12 whose tissue distribution is restricted to fat and steroidogenic cells, ADRP and TIP47 are widely distributed throughout the body (Wolins et al., 2001).

ADRP, a 50 kDa protein, was originally identified by differential screening of an adipocyte cDNA library (Jiang and Serrero, 1992). ADRP is not detected in undifferentiated cells but increases rapidly to high levels when adipocyte precursors differentiate into adipocytes. These properties of ADRP have allowed it to serve as a marker of the differentiation of adipocytes precursors (Brasaemle et al., 1997; Heid et al., 1998). In addition, Heid et al. reported that ADRP is present in secretory acinar cells of the lactating mammary gland, suggesting that ADRP may play an important role in the lipid-secreting exocrine glands (Heid et al., 1996). Recently, Schirra et al. listed ADRP as one of the genes that is up-regulated in mouse meibomian gland by androgen (Schirra et al., 2005). It has been documented that androgen regulates the quality and/or quantity of lipids produced by sebaceous glands and meibomian glands, and that androgen deficiency causes meibomian gland dysfunction (Sullivan et al., 1998, 1999, 2000a,b, 2002).

Because of these findings, we hypothesized that ADRP is a constituent of the lipid droplets in the cells of the meibomian gland, and is produced after the differentiation of adipocytes in the meibomian gland. To test our hypotheses, we generated a polyclonal antibody against rat ADRP and used this to examine whether ADRP is expressed in the meibomian gland tissues and to determine the site of ADRP in the meibomian gland tissues. We also isolated meibomian gland acinar cells to determine when ADRP is expressed in the meibomian acinar cells during cell differentiation.

2. Materials and methods

2.1. Animals

Six-week-old, male Sprague–Dawley rats (Nihon Clea Inc., Osaka, Japan), weighing approximately 150 g, were used. The rats were housed at Ehime University School of Medicine, Animal Care Facility, and cared for according to Animal Experiment Guideline. The experiments were carried out in compliance with the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23). To obtain tissues, the rats were killed with an overdose of intravenous pentobarbital.

2.2. Culture of primary rat meibomian gland acinar cells

Meibomian gland cells were isolated from the rat meibomian glands by the method of Maskin et al. with slight modifications (Maskin and Tseng, 1991, 1992). Briefly, rat eyelids were removed and disinfected with 70% ethanol solution. The tarsal plates were carefully excised under an operation microscope, avoiding contamination of the cells of the sebaceous gland, and immediately placed into Dulbecco's modified Eagle's medium (DMEM). Individual meibomian glands were dissected out and finely minced into 1 mm^3 pieces, followed by enzymatic digestion in 0.25% collagenase A (Roche, Basel, Switzerland) and 0.6 U/ml dispase II (Invitrogen, Carlsbad, CA), at 37 °C for 4 h. After digestion of the tarsal connective tissue, the enzyme solution was replaced with culture medium consisting of DMEM with Ham's F12, supplemented with 10% fetal bovine serum (Invitrogen), 10 ng/ml mouse epidermal growth factor (Upstate, Lake Placid, NY), 0.4 μg/ml hydrocortisone, 10^{-9} M cholera toxin (Invitrogen), and antibiotic and antimyotic solutions (Sigma–Aldrich, St. Louis, MO). Fragments were cultured on Type I collagen-coated culture dishes. All cultures were incubated in humidified 5% CO_2 + 95% air at 37 °C, and the medium was changed every 2 days.

When the cells had formed large colonies around the explants, they were treated with 0.25% trypsin and 0.05% EDTA in PBS(−) and subcultured. To determine whether the cultured cells produced lipids, random colonies were fixed in 10% neutral buffered formaldehyde and stained with Oil-red O.

2.3. Total RNA extraction and reverse transcription–polymerase chain reaction (RT–PCR) for ADRP

Meibomian glands were carefully dissected from the tarsal plates of experimental animals under an operation microscope. Total RNA was extracted from the meibomian glands using Isogen (Nippon Gene, Japan), and then reverse-transcribed using Omniscript Reverse Transcriptase (Qiagen, Valencia, CA) according to the manufacturer's protocol. PCR amplification was performed using TaKaRa Ex Taq (TaKaRa, Kusatsu, Japan) under the following conditions: 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 20 s, and extension at 72 °C for 30 s. The specific primer pairs for rat ADRP used for the RT–PCR were: forward 5′-AACCTGCCCTTGGTGAGCTC-3′, and reverse 5′-GCCATT GACCACAGACTTGG-3′ (GenBank accession no. BC085861). The absence of DNA contamination in the RNA preparations was verified by PCR amplification in the absence of reverse transcription.

The PCR fragments were subcloned into pBluescript SKα (Stratagene, La Jolla, CA) and sequenced in both directions by the DNA Sequencer ABM Prism-310 (Perkin–Elmer, Wellesley, MA). The subcloned ADRP cDNA was used for probe synthesis for Northern blot analysis.

2.4. Northern blot analysis for ADRP in different tissues

Total RNA was isolated from rat meibomian glands, skeletal muscle, heart, adipose tissue, liver, and kidney. Ten micrograms of total RNA from these tissues were electrophoresed in 1% agarose gel containing 0.4 M formaldehyde and transferred onto a Hybrand-N+ nylon membrane (Amersham Pharmacia Biotech, Tokyo, Japan). The membrane was hybridized with ^{32}P-labeled rat ADRP cDNA probes using Quick Hyb Hybridization Solution (Stratagene, La Jolla, CA) according to the manufacturer's protocol. The hybridization signals were detected by BAStation (Fuji film, Tokyo, Japan).

2.5. Production of anti-ADRP antibody in rabbits

A peptide consisting of 26 amino acid residues (MASVAVDPQPSVVTRVANLPLVSSTY) at the N-terminus of rat ADRP (GenBank accession no. BC085861) was synthesized using a peptide synthesis column. The synthesized peptide was mixed with Freund's complete adjuvant and injected into albino rabbits weekly for 2 months to produce an anti-ADRP polyclonal antibody. The increase in the serum anti-ADRP antibody level was monitored every 2 weeks by ELISA with the synthesized peptide.

2.6. Western blot analysis for ADRP

Western blot analysis was performed according to the method of Than et al. to confirm the presence of ADRP in the rat meibomian gland tissues, rat liver and the cultured second generation cells (Than et al., 1999). Briefly, the samples were homogenized in buffer containing 10 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 1 mM EDTA, and 1 mM phenylmethyl sulfonyl fluoride. After centrifugation, the amount of soluble proteins in the supernatant was determined with a Dc protein assay kit (Bio Rad, Hercules, CA). Then the samples were mixed in a buffer (25% glycerol, 2% SDS, 0.01% bromophenol blue, Tris–HCl, pH 6.8), and electrophoresed on 10% SDS–polyacrylamide, followed by transfer onto PVDF membrane (Bio Rad, Hercules, CA). Following incubation with anti-ADRP antibody (1:300 dilution, 1 h at room temperature), the membrane was incubated with peroxidase-conjugated anti-rabbit IgG (Invitrogen). Then, antigen-antibody complexes were made visible with 3,3′-diaminobenzidine tetrahydrochloride (DAB).

2.7. Immunohistochemistry with rabbit anti-ADRP antibody

Rat eyelids were excised and fixed in 10% buffered formaldehyde solution, embedded in paraffin, and cut into 5 μm sections. Prior to immunohistochemical staining, the sections were incubated in sodium citrate buffer at 120 °C for 20 min to activate the antigen (Bankfalvi et al., 1994). Then, immunohistochemical staining was carried out with anti-ADRP antibody (1:50 dilution, 16 h at 4 °C) using Histofine Simple Stain Max-Po (Nichirei, Tokyo, Japan), according to the manufacturer's protocol. All sections were developed with DAB and counterstained with hematoxylin.

For the fluorescein staining of cultured cells, second passaged cultured cells that were 70–80% confluent were fixed in 100% methanol. Following blocking with 1% BSA and 10% donkey serum, the sample were exposed to anti-ADRP antibody (1:100 dilution, 16 h at 4 °C) followed by incubation with FITC-labeled donkey anti-rabbit IgG. Finally, the slides were coverslipped using anti-fading mounting medium containing propidium iodide (Vector, Burlingame, CA).

2.8. Analysis of meibomian lipid composition by thin-layer chromatography with flame ionization detection (TLC/FID)

The identification of the lipids synthesized by the cultured cells was determined by thin-layer chromatography with flame ionization detection (TLC/FID) using the method of Ackman (1981). Briefly, the lipids were extracted from the dissected meibomian gland tissues, and cultured cell pellets were sonicated in chloroform/ methanol (2:1) for 1 min. After centrifugation, the supernatants were evaporated at 40 °C under nitrogen flow, and then the lipid residues were dissolved in chloroform. The lipid samples were applied to Chromarod S-III which had been activated by passing through the flame. The chromarods were developed in hexane/benzene (1:1), dried with a cold air fan and redeveloped in hexane/diethyl ether/formic acid (70:30:1). Then the chromarods were scanned through the FID at a speed of 2.3 cm/s, hydrogen flow rate of 180 ml/min, and an air flow rate of 2000 ml/min.

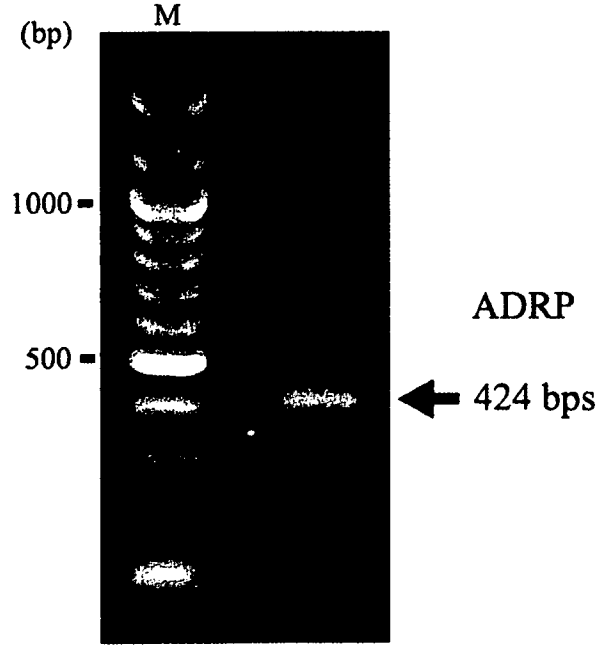

Fig. 1. RT–PCR products of ADRP in the rat meibomian gland tissues. A PCR product can be seen at 424 bp.

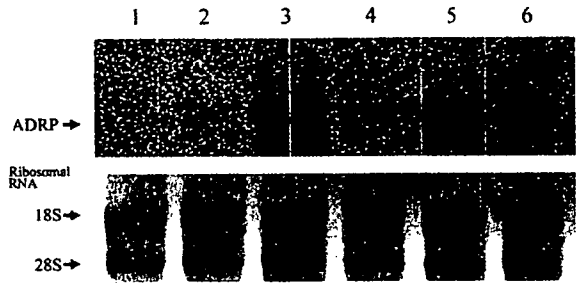

Fig. 2. Northern blot analysis of the mRNA of ADRP expressed in rat meibomian gland tissues. Ribosomal RNA (28 S and 18 S) in the gel were made visible with ultraviolet light. Expression of ADRP can be seen in the meibomian gland, liver, kidney, heart, and skeletal muscle. The level of ADRP expression in meibomian glands is almost as high as in liver and kidney in comparison with 18s or 28s rRNA in the same samples. 1, skeletal muscle; 2, heart; 3, meibomian gland; 4, adipose tissue; 5, liver; 6, kidney.

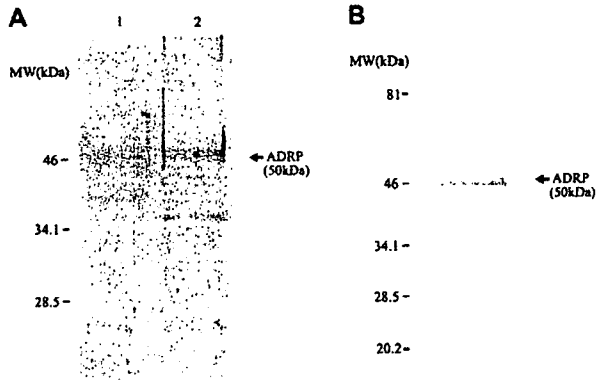

Fig. 3. Expression of ADRP proteins in rat meibomian gland tissues, rat liver and cultured cells. Western blot analysis of proteins extracted from rat meibomian gland tissues, rat liver (A) and cultured cells isolated (B). A unique band with a molecular weight of 50 kDa was detected, and the molecular weight agrees with rat ADRP. (A) 1, rat meibomian gland tissues; 2, rat liver. (B) Cultured cells.

3. Results

3.1. Expression of ADRP gene in rat meibomian tissues

RT–PCR was used to determine whether ADRP is expressed in the rat meibomian glands, and subsequent electrophoresis revealed a single RT–PCR product of 424 bp (Fig. 1). Northern blot analysis confirmed that the band was the mRNA of ADRP. The expression of ADRP was also detected in the liver, kidney, heart, and skeletal muscle. The relative level of ADRP expressed in meibomian glands was almost as high as in liver and kidney in comparison with 18s or 28s rRNA in the same samples (Fig. 2).

3.2. Localization of ADRP in meibomian gland tissues

Western blot analysis with the anti-ADRP antibody was performed on the soluble extract of the rat meibomian gland and liver. A unique bands were identified in both tissues at a molecular weight of 50 kDa that matched the molecular weight of rat ADRP (Fig. 3A) (Brasaemle et al., 1997; Heid et al., 1998). To determine the cellular sites of ADRP in the rat eyelid, we performed immunohistochemical staining. Immunoreactivity to ADRP was observed in the lobules of acinar cells of the meibomian glands as well as in the sebaceous glands in the dermis (Fig. 4A). A higher magnification of lobules of the meibomian glands showed that the immunoreactivity was specifically located adjacent to the vacuolated cytoplasm (Fig. 4B). Some of the cells in the basal cell layer of the acini and ductal epithelium did not react to the anti-ADRP antibody (Fig. 4B). For controls, sections were exposed to normal rabbit immunoglobulin G (IgG), and no positive staining was detected with any of these antibodies (Fig. 4C).

3.3. Growth and morphology of cultured meibomian gland cells

Two to three days after the primary culture of excised fragments of rat meibomian glands, a monolayer of cells was detected growing out from the meibomian gland tissue fragments. When the primary cultures had grown to a subconfluent monolayer, they were subcultured. The cells were confluent by day 6 and appeared cobblestone-like (Fig. 5A). After 2 weeks, these cells had a frothy appearance (Fig. 5B), contained lipid droplets in the cytoplasm, and were positively stained with oil red O (Fig. 5C). This cellular appearance has been reported as a characteristic of meibomian gland cultures (Zouboulis et al., 1991; Maskin and Tseng, 1992; Ito et al., 1998).

Fig. 4. Immunohistochemical localization of ADRP in the rat meibomian gland tissues. (A) Lobules of secretary cells both in the meibomian glands (MG) and sebaceous gland (SG) are immunostained with anti-ADRP antibody. (B) Higher magnification of lobules of the meibomian gland. Positive staining can be seen at the margins of the vacuolated cytoplasm. (C) For controls, no positive staining was detected with normal rabbit immunoglobulin G (IgG). All bars = 100 μm.

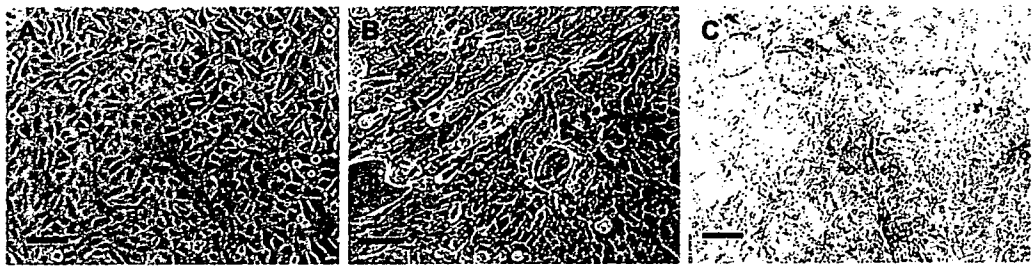

Fig. 5. Culture of rat meibomian gland cells. (A) Confluent secondary meibomian gland cells consisting of monomorphic cells arranged in a cobble-stone pattern by day 6. (B) Lipid droplets are seen in the cytoplasm of the differentiated cells by day 10. (C) Stained droplets are observed in the cytoplasm with Oil red O. All bars = 100 μm.

3.4. Localization of ADRP in cultured meibomian gland cells

Western blots were then used to determine whether the cultured rat meibomian gland cells express ADRP. A single band was detected at a molecular weight of 50 kDa (Fig. 3B). To examine the relationship between the expression of ADRP in meibomian gland cells and the stage of differentiation of the cells in more detail, immunohistochemical studies were performed at various stages of the cultured rat meibomian gland cells (Fig. 6A). In contrast to the negative staining at the cobblestone-like stage, positive staining was observed at the margins of the lipid droplets of the frothy cells at the center of the culture (Fig. 6B).

3.5. Lipid analysis of meibomian gland tissues and cultured cells

Lipids from cultured meibomian gland cells and meibomian gland tissues were quantitatively examined by TLC/FID, and typical results are shown in Fig. 7. TLC/FID of meibomian gland tissues showed that wax and cholesteryl ester were the most significant lipid components, and cholesteryl ester was the major component of cultured meibomian gland cells with a trace amount of wax ester (Fig. 7). Small amounts of fatty acid, triglyceride, cholesterol, and polar lipids were also detected in the cultured cells and in meibomian gland tissues (Fig. 7) (Nikkari, 1974).

4. Discussion

Our Northern and Western blot analyses showed that ADRP was expressed in the meibomian glands. The mRNA of ADRP was originally identified in a C3H mouse teratoma-derived adipogenic cell line during the early stages of differentiation of the adipocytes, and has been thought to be a marker of differentiated adipocytes (Jiang and Serrero, 1992; Brasaemle et al., 1997; Heid et al., 1998). Northern blot analysis also revealed that ADRP was expressed widely in rat tissues including skeletal muscle, heart, adipose tissue, liver and kidney along with the meibomian glands. These results are good agreement with previous reports (Gao and Serrero, 1999; Gao et al., 2000).

ADRP was also demonstrated immunohistochemically at the margin of each lipid droplet that was present in the cytoplasm of the rat meibomian gland cell. Positive staining was also observed in the lipid droplets in the sebaceous gland at the lid margins, indicating that ADRP is widely stored as a lipid reservoir in rat tissues. However, some of the cells in the basal cell layer of the acini and ductal epithelium were negative for ADRP.

There have been several reports describing the differentiation of meibomian acinar cells (Driver and Lemp, 1996). For example, Sirigu et al. (1992) showed histochemically that undifferentiated cells were situated at the periphery and the differentiated cells were present more centrally in the acinus. These observations would support our conclusion that ADRP was preferentially expressed in differentiated meibomian gland cells.

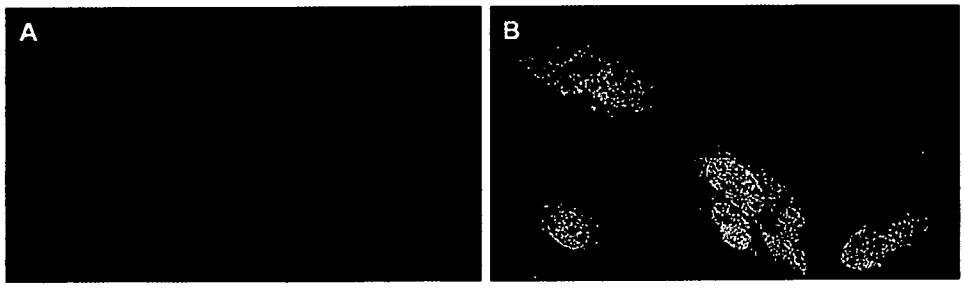

Fig. 6. Immunohistochemical localization of ADRP in cultured rat meibomian gland cells. (A) No positive staining is detected in the cobblestone-like cells. (B) Positive staining can be seen at the margins of the lipid droplets of the froth-like cells at the center of the culture. Nuclei of cultured cells are stained with propidium iodide. Bar = 50 μm.

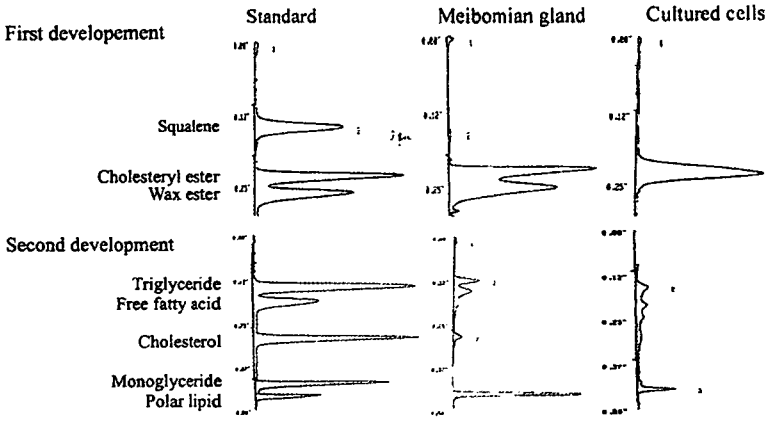

Fig. 7. Lipid analysis of meibomian gland tissues and cultured cells. Lipids from cultured meibomian gland cells as well as meibomian gland tissues were quantitatively measured by TLC/FID. While meibomian gland tissues show that wax and cholesteryl ester are the major lipid constituents, cholesterol ester was the major component with a trace amount of wax ester in cultured meibomian gland cells.

To investigate what role of ADRP plays in the meibomian gland in more detail, rat meibomian gland cells were grown in culture, as was used for rabbit meibocytes. The cultured cells were most likely meibocytes because the cells were polygonal in shape and contained numerous secretory vesicles which were stained with Oil-red O even after several passages. This staining is consistent with the Type I morphology in acinar cells (Maskin and Tseng, 1991). In addition, lipid analysis showed that the main lipid constituent of these cells was cholesteryl esters, and the wax esters were detected in only small amounts. These results suggested that these cultured cells were not fully differentiated in spite of the abundant lipid accumulation in the cytoplasm (Greene et al., 1970; Tiffany, 1978; Nicolaides et al., 1981).

We used cultured meibocytes for immunostaining with anti-ADRP antibody at the different stages of differentiation. In contrast to the absence of staining for ADRP in the cobblestone-like cells which did not stain with oil red O, positive staining was observed at the margins of the lipid droplets in the froth-like cells at the center of the culture. Thus, ADRP was selectively present in the cells that functioned as fat storage cells. Along with the immunohistochemical results, these in vitro results suggested that ADRP may be used as a marker of meibocyte differentiation.

The high homology of the N-terminus of the ADRP genes in rats and humans suggests a strong cross-reaction would be expected of our rat antibody with that of human tissues. This would then indicate that this antibody can be used in the clinic. The standard lipid staining methods used now, such as SudanIII, Oil red O, and Nile blue staining, have some problems in paraffin sections, because of the loss of lipids during the processing for paraffin embedding. For instance, the correct diagnosis of sebaceous carcinoma is only 22.5% according to Doxanas and Green (1984) and only 38% according to Takagi et al. (1988). However, the anti-ADRP antibody showed distinct immunohistochemical staining in the vacuolated cytoplasm in the paraffin sections of rat meibomian glands. We have applied this antibody on human specimens removed by surgery, and a strong and reliable staining for ADRP was detected in 6 of 6 sebaceous carcinomas in paraffin sections (unpublished data). Thus, our anti-ADRP antibody may be valuable for the differential pathological diagnosis of lid tumors.

In summary, we have shown that ADRP is present in rat meibomian gland cells, possibly in the lipid storage organelles. Our findings suggest that ADRP could be a candidate marker for meibocyte differentiation.

References

Ackman, R.G., 1981. Flame ionization detection applied to thin-layer chromatography on coated quartz rods. Methods Enzymol. 72, 205–252.

Ailhaud, G., Grimald, P., Negrel, R., 1992. Cellular and molecular aspects of adipose tissue development. Annu. Rev. Nutr. 12, 207–233.

Bankfalvi, A., Navabi, H., Bier, B., Bocker, W., Jasani, B., Schmid, K.W., 1994. Wet autoclave pretreatment for antigen retrieval in diagnostic immunohistochemistry. J. Pathol. 174, 223–228.

Brasaemle, D.L., Barber, T., Wolins, N.E., Serrero, G., Blanchette-Mackie, E.J., Londos, C., 1997. Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. J. Lipid Res. 38, 2249–2263.

Bron, A.J., Tiffany, J.M., 1998. The meibomian glands and tear film lipids. Structure, function, and control. Adv. Exp. Med. Biol. 438, 281–295.

Doxanas, M.T., Green, W.R., 1984. Sebaceous gland carcinoma. Review of 40 cases. Arch. Ophthalmol. 102, 245–249.

Driver, P.J., Lemp, M.A., 1996. Meibomian gland dysfunction. Surv. Ophthalmol. 40, 343–367.

Gao, J., Serrero, G., 1999. Adipose differentiation related protein (ADRP) expressed in transfected COS-7 cells selectively stimulates long chain fatty acid uptake. J. Biol. Chem. 274, 16825–16830.

Gao, J., Ye, H., Serrero, G., 2000. Stimulation of adipose differentiation related protein (ADRP) expression in adipocyte precursors by long-chain fatty acids. J. Cell Physiol. 182, 297–302.

Greenberg, A.S., Egan, J.J., Wek, S.A., Garty, N.B., Blanchette-Mackie, E.J., Londos, C., 1991. Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. J. Biol. Chem. 266, 11341–11346.

Greene, R.S., Downing, D.T., Pochi, P.E., Strauss, J.S., 1970. Anatomical variation in the amount and composition of human skin surface lipid. J. Invest. Dermatol. 54, 240–247.

Heid, H.W., Schnolzer, M., Keenan, T.W., 1996. Adipocyte differentiation-related protein is secreted into milk as a constituent of milk lipid globule membrane. Biochem. J. 320 (Pt 3), 1025–1030.

Heid, H.W., Moll, R., Schwetlick, I., Rackwitz, H.R., Keenan, T.W., 1998. Adipophilin is a specific marker of lipid accumulation in diverse cell types and diseases. Cell Tissue Res. 294, 309–321.

Holly, F.J., 1980. Tear film physiology. Am. J. Optom. Physiol. Opt. 57, 252–257.

Ito, A., Sakiguchi, T., Kitamura, K., Akamatsu, H., Horio, T., 1998. Establishment of a tissue culture system for hamster sebaceous gland cells. Dermatology 197, 238–244.

Jiang, H.P., Serrero, G., 1992. Isolation and characterization of a full-length cDNA coding for an adipose differentiation-related protein. Proc. Natl. Acad. Sci. USA. 89, 7856–7860.

Londos, C., Brasaemle, D.L., Gruia-Gray, J., Servetnick, D.A., Schultz, C.J., Levin, D.M., Kimmel, A.R., 1995. Perilipin: unique proteins associated with intracellular neutral lipid droplets in adipocytes and steroidogenic cells. Biochem. Soc. Trans. 23, 611–615.

Maskin, S.L., Tseng, S.C., 1991. Culture of rabbit meibomian gland using collagen gel. Invest. Ophthalmol. Vis. Sci. 32, 214–223.

Maskin, S.L., Tseng, S.C., 1992. Clonal growth and differentiation of rabbit meibomian gland epithelium in serum-free culture: differential modulation by EGF and FGF. Invest. Ophthalmol. Vis. Sci. 33, 205–217.

Masuda, Y., Itabe, H., Odaki, M., Hama, K., Fujimoto, Y., Mori, M., Sasabe, N., Aoki, J., Arai, H., Takano, T., 2006. ADRP/adipophilin is degraded through the proteasome-dependent pathway during regression of lipid-storing cells. J. Lipid Res. 47, 87–98.

Mathers, W.D., 1993. Ocular evaporation in meibomian gland dysfunction and dry eye. Ophthalmology 100, 347–351.

Mathers, W.D., Lane, J.A., Sutphin, J.E., Zimmerman, M.B., 1996. Model for ocular tear film function. Cornea 15, 110–119.

McCulley, J.P., Sciallis, G.F., 1977. Meibomian keratoconjunctivitis. Am. J. Ophthalmol. 84, 788–793.

Miura, S., Gan, J.W., Brzostowski, J., Parisi, M.J., Schultz, C.J., Londos, C., Oliver, B., Kimmel, A.R., 2002. Functional conservation for lipid storage droplet association among Perilipin, ADRP, and TIP47 (PAT)-related proteins in mammals, Drosophila, and Dictyostelium. J. Biol. Chem. 277, 32253–32257.

Nicolaides, N., Kaitaranta, J.K., Rawdah, T.N., Macy, J.I., Boswell 3rd, F.M., Smith, R.E., 1981. Meibomian gland studies: comparison of steer and human lipids. Invest. Ophthalmol. Vis. Sci. 20, 522–536.

Nikkari, T., 1974. Comparative chemistry of sebum. J. Invest. Dermatol. 62, 257–267.

Schirra, F., Suzuki, T., Richards, S.M., Jensen, R.V., Liu, M., Lombardi, M.J., Rowley, P., Treister, N.S., Sullivan, D.A., 2005. Androgen control of gene expression in the mouse meibomian gland. Invest. Ophthalmol. Vis. Sci. 46, 3666–3675.

Shimazaki, J., Sakata, M., Tsubota, K., 1995. Ocular surface changes and discomfort in patients with meibomian gland dysfunction. Arch. Ophthalmol. 113, 1266–1270.

Shine, W.E., McCulley, J.P., 2004. Meibomianitis: polar lipid abnormalities. Cornea 23, 781–783.

Sirigu, P., Shen, R.L., Pinto da Silva, P., 1992. Human meibomian glands: the ultrastructure of acinar cells as viewed by thin section and freeze-fracture transmission electron microscopies. Invest. Ophthalmol. Vis. Sci. 33, 2284–2292.

Sullivan, D.A., Rocha, E.M., Ullman, M.D., Krenzer, K.L., Gao, J., Toda, I., Dana, M.R., Bazzinotti, D., da Silveira, L.A., Wickham, L.A., 1998. Androgen regulation of the meibomian gland. Adv. Exp. Med. Biol. 438, 327–331.

Sullivan, D.A., Wickham, L.A., Rocha, E.M., Krenzer, K.L., Sullivan, B.D., Steagall, R., Cermak, J.M., Dana, M.R., Ullman, M.D., Sato, E.H., Gao, J., Rocha, F.J., Ono, M., Silveira, L.A., Lambert, R.W., Kelleher, R.S., Tolls, D.B., Toda, I., 1999. Androgens and dry eye in Sjogren's syndrome. Ann. N.Y. Acad. Sci. 876, 312–324.

Sullivan, B.D., Evans, J.E., Krenzer, K.L., Reza Dana, M., Sullivan, D.A., 2000a. Impact of antiandrogen treatment on the fatty acid profile of neutral lipids in human meibomian gland secretions. J. Clin. Endocrinol. Metab. 85, 4866–4873.

Sullivan, D.A., Sullivan, B.D., Ullman, M.D., Rocha, E.M., Krenzer, K.L., Cermak, J.M., Toda, I., Doane, M.G., Evans, J.E., Wickham, L.A., 2000b. Androgen influence on the meibomian gland. Invest. Ophthalmol. Vis. Sci. 41, 3732–3742.

Sullivan, D.A., Sullivan, B.D., Evans, J.E., Schirra, F., Yamagami, H., Liu, M., Richards, S.M., Suzuki, T., Schaumberg, D.A., Sullivan, R.M., Dana, M.R., 2002. Androgen deficiency, Meibomian gland dysfunction, and evaporative dry eye. Ann. N.Y. Acad. Sci. 966, 211–222.

Takagi, T., Tsuda, N., Shimada, O., Hori, M., 1988. Review of 21 cases of sebaceous adenocarcinoma in Nagasaki University over a 25-year period. Jpn. Clin. Ophthalmom. 42, 517–519.

Than, N.G., Sumegi, B., Than, G.N., Kispal, G., Bohn, H., 1999. Cloning and sequencing of human oncodevelopmental soluble placental tissue protein 17 (PP17): homology with adipophilin and the mouse adipose differentiation-related protein. Tumour Biol. 20, 184–192.

Tiffany, J.M., 1978. Individual variations in human meibomian lipid composition. Exp. Eye Res. 27, 289–300.

Tiffany, J.M., 1985. The role of meibomian secretion in the tears. Trans Ophthalmol. Soc. UK. 104 (Pt 4), 396–401.

Tiffany, J.M., 1987. The lipid secretion of the meibomian glands. Adv. Lipid Res. 22, 1–62.

Tsai, P.S., Evans, J.E., Green, K.M., Sullivan, R.M., Schaumberg, D.A., Richards, S.M., Dana, M.R., Sullivan, D.A., 2006. Proteomic analysis of human meibomian gland secretions. Br. J. Ophthalmol. 90, 372–377.

Wolins, N.E., Rubin, B., Brasaemle, D.L., 2001. TIP47 associates with lipid droplets. J. Biol. Chem. 276, 5101–5108.

Wolins, N.E., Skinner, J.R., Schoenfish, M.J., Tzekov, A., Bensch, K.G., Bickel, P.E., 2003. Adipocyte protein S3-12 coats nascent lipid droplets. J. Biol. Chem. 278, 37713–37721.

Yamaguchi, T., Matsushita, S., Motojima, K., Hirose, F., Osumi, T., 2006a. MLDP, a novel PAT family protein localized to lipid droplets and enriched in the heart, is regulated by peroxisome proliferator-activated receptor alpha. J. Biol. Chem. 281, 14232–14240.

Yamaguchi, T., Omatsu, N., Omukae, A., Osumi, T., 2006b. Analysis of interaction partners for perilipin and ADRP on lipid droplets. Mol. Cell Biochem. 284, 167–173.

Zouboulis, C.C., Xia, L.Q., Detmar, M., Bogdanoff, B., Giannakopoulos, G., Gollnick, H., Orfanos, C.E., 1991. Culture of human sebocytes and markers of sebocytic differentiation in vitro. Skin Pharmacol. 4, 74–83.

Dendritic Keratitis Caused by an Acyclovir-Resistant Herpes Simplex Virus With Frameshift Mutation

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Purpose: To report a case of acyclovir-resistant herpes simplex virus (HSV) keratitis after long-term, inconsistent use of topical acyclovir and fluorometholone.

Methods: A 70-year-old man with dendritic keratitis caused by an acyclovir-resistant HSV strain was examined. The 50% inhibitory concentration of different antiviral agents against the isolated virus and the DNA sequence of viral thymidine kinase were determined.

Results: The 50% inhibitory concentration of acyclovir and trifluorothymidine for the isolated HSV strain was 13.75 and 0.28 μg/mL, respectively, indicating that the virus was resistant to acyclovir. DNA sequencing of the viral thymidine kinase revealed that this virus had a frameshift mutant with a G insertion in the 7Gs homopolymer. Topical trifluorothymidine was effective, and the epithelial lesion was completely resolved within 2 weeks.

Conclusion: A typical form of dendritic keratitis was caused by an acyclovir-resistant HSV with frameshift mutation in a 7Gs homopolymer region.

Key Words: acyclovir resistance, keratitis, frameshift mutation, herpes simplex virus, thymidine kinase

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Herpes simplex keratitis is still 1 of the leading causes of infectious corneal blindness in the world. Acyclovir (ACV) ointment has been extensively used as the first choice drug of herpes simplex keratitis in Japan because of its high antiviral efficacy and low cytotoxicity. This treatment has resulted in a significant reduction of patients who develop necrotizing stromal keratitis and corneal perforations.

However, the widespread use of ACV has invariably led to the emergence of herpes simplex virus (HSV) strains resistant to ACV, especially in severely immunocompromised patients. During the past several years, ACV-resistant HSV strains have been isolated from various sites including the eye. Three mechanisms may be involved in the development of ACV-resistant HSV: a loss of thymidine kinase (TK) activity (TK-deficient virus), an alteration of TK activity or substrate specificity (TK-altered virus), and an alteration of DNA polymerase activity. In 95% of the cases, ACV resistance is associated with a mutation in the *TK* gene.[1]

We report a case of dendritic keratitis that did not respond to topical ACV ointment. A novel frameshift mutation was detected in this ACV-resistant, ocular HSV isolate.

CASE REPORT

A 70-year-old, immunocompetent man was referred from a local hospital in March 2005 for recurrent dendritic keratitis that had been treated unsuccessfully with topical ACV. He complained of redness, irritation, pain, and blurred vision in his right eye. His best-corrected visual acuity was reduced to 20/25 in the affected eye. Slit-lamp examination showed dendritic keratitis with slight stromal edema but no significant signs of anterior chamber inflammation.

The epithelial lesion was found to develop at the lower portion of the cornea, a part of which was geographic in appearance (Fig. 1). Suspecting poor compliance, we gave the patient strict instructions on the use of ACV ointment 5 times a day for 1 week, but no improvement was observed. The patient had a 10-year history of recurrent dendritic and disciform keratitis and had been given topical ACV with fluorometholone for a long time, but his compliance was poor.

We considered the possibility of an ACV-resistant HSV; corneal epithelial debridement was performed under local anesthesia for virus isolation. Typical cytopathic effect was observed on the next day, and the isolated virus was identified as HSV-1 by indirect immunofluorescence. As reported previously,[2] antiviral plaque reduction assay was performed with ACV, trifluorothymidine (TFT), foscarnet (FOS), and idoxuridine (IDU) to determine the 50% viral inhibitory concentration (IC_{50}). As a result, the IC_{50} of this strain against ACV, TFT, and FOS was 13.75, 0.28, and 7.14 μg/mL, respectively. No plaque reduction occurred at any concentration of IDU. Generally, in an antiviral plaque reduction assay, HSV with IC_{50} exceeding 2 μg/mL to ACV is considered resistant.[3] The IC_{50} of this strain was 13.75 μg/mL, indicating that the dendritic keratitis was caused by an ACV-resistant HSV strain.

We therefore discontinued topical ACV and switched to 1% topical TFT, which this strain was sensitive to. The patient was given 1% topical TFT 8 times per day, and the 1% TFT solution was prepared in our facility from TFT (Sigma, St. Louis, MO). The epithelial lesion healed completely within 2 weeks.

The analysis of the *TK* gene of the isolated virus strain was performed as described[4] and compared with the *TK* gene of an ACV-sensitive clinical isolated from a patient with dendritic keratitis. A G insertion in a homopolymer harboring 7 Gs was detected in the TK

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FIGURE 1. Slit-lamp photograph of right eye with fluorescein staining showing typical dendritic keratitis with a part of geographic in appearance at the lower portion of the cornea.

sequence. This insertion was located at nucleotides 429 to 436 (codon 144–146), causing a premature stop codon at 227 (Table 1).

TABLE 1. TK Gene Sequence

	Nucleotide Change	Amino Acid Change
Isolated virus	125: C→T	42: Pro→Leu
	256: G→A	89: Arg→Gln
	437: ins G	146: frameshift
		227: stop codon

DISCUSSION

Among the mutations that are associated with ACV resistance, one half are nucleotide insertions or deletions and the other half are nucleotide substitutions. Generally, such mutants have low virulence. For example, Yao et al[2] have reported 2 ACV-resistant HSV-1 mutants with a nucleotide substitution, and both had low virulence in the cornea and had a low incidence of establishing a latent infection. One had a point mutation in the ATP binding site (codon 55) of the *TK* gene, whereas the other was in codon 125.

Nucleotide insertions or deletions are usually responsible for a frameshift resulting in the synthesis of a truncated, nonfunctional TK. A mutant strain with a frameshift mutation within the 7 Gs homopolymers, however, could synthesize a part of a full-length TK because of an unusual net +1 frameshift during translation. This outcome first reported in a patient with progressive esophagitis that had developed after bone marrow transplantation. It was noted that the mutant retained some TK activity and had the ability to reactivate from latent infections in the mouse trigeminal ganglia.[5] In fact, recurrences have occurred in a bone marrow transplant patient.[6] Thus, net +1 frameshift mutants have stronger virulence and higher rate of recurrences than the more common mutants because of their specific process of translation. Our mutant also possessed strong virulence, causing typical dendritic keratitis and the ability to establish latency[5] (Zhang et al, unpublished data).

ACV-resistant mutants of HSV emerge predominantly in severely immunocompromised patients (eg, bone marrow transplants, cancer, and AIDS).[1] Although resistance to ACV rarely occurs among immunocompetent patients, there have been several reports of ACV-resistant herpes simplex keratitis in otherwise healthy individuals.[4] This finding is probably attributable to the inherent immunocompromised status of the cornea because of its avascularity. Our patient was immunocompetent according to his medical history. Therefore, we presume that the long-term, irregular concomitant use of a low dose of ACV and steroids is responsible for the emergence of this ACV-resistant mutant. Although sensory ganglia harboring latent HSV-1 are not generally reinfected with a second strain of HSV-1, superinfection has been reported to occur.[7,8] Because the strain we reported here can establish latency[5] (Zhang et al, unpublished data), a possibility of recurrence, albeit remote, should be kept in mind.

REFERENCES

1. Morfin F, Thouvenot D. Herpes simplex virus resistance to antiviral drugs. *J Clin Virol.* 2003;26:29–37.
2. Yao YF, Inoue Y, Kase T, et al. Clinical characteristics of acyclovir-resistant herpetic keratitis and experimental studies of isolates. *Graefes Arch Clin Exp Ophthalmol.* 1996;234:126–132.
3. Englund J, Zimmerman ME, Swierkosz EM, et al. Herpes simplex virus resistant to acyclovir: a study in a tertiary care center. *Ann Intern Med.* 1990;112:416–422.
4. Bodaghi B, Mougin C, Michelson S, et al. Acyclovir-resistant bilateral keratitis associated with mutations in the HSV-1 thymidine kinase gene. *Exp Eye Res.* 2000;71:353–359.
5. Hwang CB, Horsburgh B, Pelosi E, et al. A net +1 frameshift permits synthesis of thymidine kinase from a drug-resistant herpes simplex virus mutant. *Proc Natl Acad Sci USA.* 1994;91:5461–5465.
6. Morfin F, Thouvenot D, Aymard M, et al. Reactivation of acyclovir-resistant thymidine kinase-deficient herpes simplex virus harbouring single base insertion within a 7 Gs homopolymer repeat of the thymidine kinase gene. *J Med Virol.* 2000;62:247–250.
7. Landry ML, Zibello TA. Ability of herpes simplex virus (HSV) types 1 and 2 to induce clinical disease and establish latency following previous genital infection with the heterologous HSV type. *J Infect Dis.* 1988;158:1220–1226.
8. Remeijer L, Maertzdor J, Buitenwerf J, et al. Corneal herpes simplex virus type 1 superinfection in patients with recrudescent herpetic keratitis. *Invest Ophthalmol Vis Sci.* 2002;43:358–363.

DNA of Cytomegalovirus Detected by PCR in Aqueous of Patient With Corneal Endotheliitis After Penetrating Keratoplasty

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Purpose: Corneal endotheliitis often leads to severe endothelial dysfunction and can be caused by herpes simplex virus (HSV), varicella zoster virus (VZV), and other viruses (eg, the mumps virus). We report a case of corneal endotheliitis caused by cytomegalovirus (CMV) that developed after a penetrating keratoplasty.

Methods: A complete ophthalmologic examination was performed on a patient with corneal endotheliitis that developed after a penetrating keratoplasty. To determine the cause of the endotheliitis, polymerase chain reaction (PCR) was used to amplify the DNA of HSV, VZV, and CMV in samples of the aqueous humor.

Results: Slit-lamp biomicroscopy showed a moderate stromal edema in the upper temporal part of the transplanted cornea along with keratic precipitates (KPs) arranged in a coin-shaped pattern. Repeated treatments with steroids and acyclovir were only temporarily successful. PCR detected the DNA of CMV in an aqueous sample, and the treatment was switched to topical and systemic application of ganciclovir. This resulted in the disappearance of the KPs and resolution of the stromal edema within 2 weeks.

Conclusions: From the PCR results and the favorable response to ganciclovir, the corneal endotheliitis was most likely caused by cytomegalovirus in this case.

Key Words: cytomegalovirus, corneal endotheliitis, ganciclovir, penetrating keratoplasty, polymerase chain reaction

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A case characterized by progressive corneal edema and keratic precipitates of unknown origin was first described by Khodadoust and Attarzadeh[1] and designated as idiopathic immune corneal endotheliopathy in 1982. This clinical entity, now called corneal endotheliitis, is recognized as an inflammatory disorder with different clinical appearances, but with the corneal endothelium being primarily affected. Evidence was gathered by detecting virus DNA in samples of the aqueous and positive responses to appropriate antiviral drugs showed that herpes simplex virus (HSV), varicella zoster virus (VZV), and mumps virus are the principal etiologic agents.[2–5] However, whether other viruses can cause the endotheliitis has not been conclusively determined.

Recently, Koizumi et al[6] reported a case of corneal endotheliitis in which the DNA of cytomegalovirus (CMV) was found in the aqueous humor. We report another case of corneal endotheliitis of CMV origin that developed after penetrating keratoplasty (PK).

CASE REPORT

A 77-year-old healthy man had his left eye struck with a stone that resulted in a corneal perforation and severe iridal damage. Seven months later, he underwent a PK for the corneal opacity with simultaneous extracapsular cataract extraction and iridoplasty. He received topical corticosteroids (0.1% dexamethasone, 4 times per day) and levofloxacin 0.5% postoperatively. The recovery was uneventful.

Three months after surgery, he returned, complaining of blurred vision in his left eye. Slit-lamp biomicroscopy revealed a moderate degree of stromal edema in the upper temporal part of the corneal allograft accompanied by white keratic precipitates (KPs) arranged in a unique, coin-shaped pattern (Fig. 1). There were no cellular infiltrations or opacities in the corneal stroma. The conjunctiva was not hyperemic, and the anterior chamber was essentially silent. The right eye was completely normal.

From the clinical findings, we strongly suspected that this patient had corneal endotheliitis and started treatment with topical acyclovir (3% eye ointment, 5 times per day) and systemic acyclovir (1500 mg IV, daily for 2 weeks). The coin-shaped KPs and stromal edema were improved by the therapy but reappeared when the treatment with acyclovir was lessened.

Polymerase chain reaction (PCR) of an aqueous humor sample was performed to search for the DNA of HSV and VZV.[7] The results were negative for these viruses. Because the success of acyclovir was not maintained, we aspirated more aqueous humor and searched for the DNA of CMV, HSV, and VZV. PCR for the immediate-early antigen 1 gene of CMV was carried out using 2 primers; 5′-TTAGTGAACCGT CAGATCGC-3′ (forward) and 5′-GCATGCATAAGAAGCCAAGG-3′ (reverse). For the PCR, we used an initial cycle at 94°C for 4 minutes followed by 40 cycles at 94°C for 1 minute, 58°C for 1 minute, and 72°C for 2 minutes, with a final extension period at 72°C for 10 minutes. A 186-bp fragment of CMV DNA was isolated, and DNA for HSV and VZV was not found (Fig. 2). Using the same PCR protocol, we did not detect CMV DNA from the aqueous humor of 1 healthy patient and 3 patients with nonviral uveitis. We also determined the quantity of CMV DNA by using real-time PCR[8] and detected 2.5×10^2 copies/mL.

From these findings, we concluded that the disease was most likely caused by CMV and began treatment with topical ganciclovir (0.5% eye drops, 8 times per day) and systemic ganciclovir (500 mg

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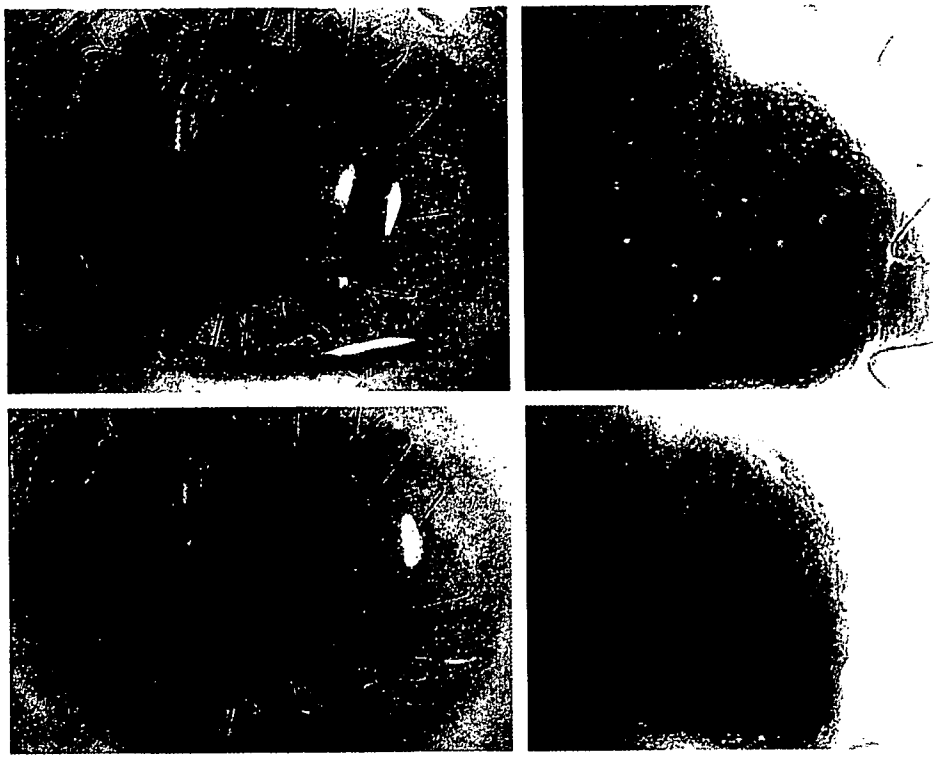

FIGURE 1. Cornea of patient before and after ganciclovir treatment. Top left, Three months after PK, the corneal stroma in the upper temporal area is edematous. Top right, Three months after penetrating keratoplasty, coin-shaped KPs can be seen in the cornea. Bottom left, After ganciclovir treatment, the corneal stromal edema has reduced in size. Bottom right, After ganciclovir treatment, the KPs have resolved.

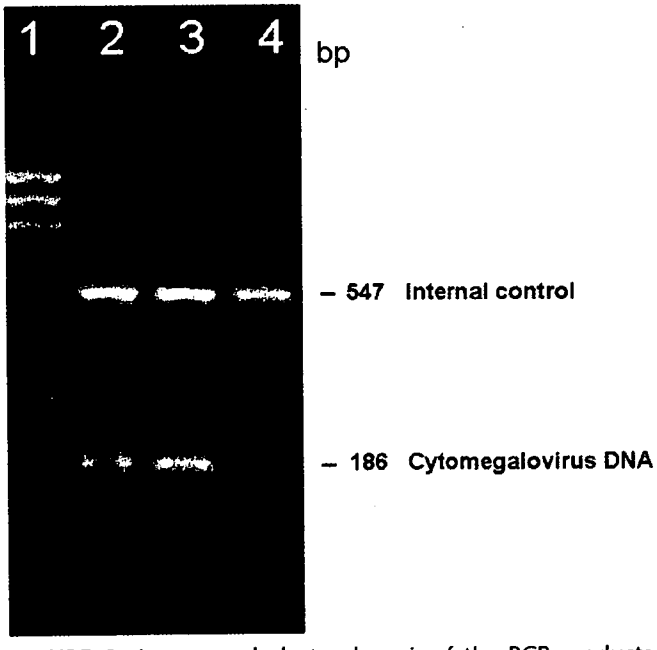

FIGURE 2. Agarose gel electrophoresis of the PCR products for the DNA of CMV. Lane 1, Molecular marker (ϕX174 DNA/*Hae*III markers). Lane 2, DNA extracted from the patient's aqueous humor. Lane 3, CMV DNA-positive sample. Lane 4, CMV DNA-negative sample. Lanes 2 and 3 contain the 186-bp band diagnostic for CMV DNA and the 547-bp internal control band. Lane 4 contains only the internal control band.

IV, daily for 2 weeks) after obtaining an informed consent. Within 2 weeks, the coin-shaped KPs disappeared with the resolution of the stromal edema (Fig. 1). The patient continued the use of topical ganciclovir and had no recurrences.

DISCUSSION

The identification of the DNA of CMV in the aqueous humor and the extremely favorable response to ganciclovir indicated that this endotheliitis was caused by a CMV infection. In typical cases of corneal endotheliitis of HSV origin, the stromal edema develops from the peripheral cornea, and KPs form a line as observed in corneal endothelial rejection.[3] In our case, the characteristic clinical finding was the arrangement of the KPs in a coin-shaped pattern resembling a virus-induced plaque in a cell culture. The presence of these coin-shaped lesions was also identified by Koizumi et al[6] and might be a specific manifestation of CMV-related corneal endotheliitis.

Systemic acyclovir was effective in this patient only during the period of continuous use; the lesion reappeared soon after the acyclovir was discontinued. This finding is not surprising when we consider the marked difference in the inhibitory concentrations of acyclovir and ganciclovir against CMV. In fact, Michel et al[9] reported that the 50% inhibitory concentration of acyclovir was more than 10 times higher than that of ganciclovir when used to treat clinical CMV isolates. The discrepancy in clinical response to acyclovir and ganciclovir further suggested that the endothelial lesion of this patient was of CMV etiology. CMV has also been shown to be sensitive to trifluridine, so trifluridine ophthalmic solution could have been tried in our case.[10]

CMV infections generally occur in immunocompromised hosts (eg, CMV retinitis in AIDS patients), and one may ask why this disease developed in a 77-year-old, otherwise healthy, man. However, de Schryver et al[11] have reported CMV iridocyclitis without retinal necrosis in immunocompetent patients. Thus, CMV can enter the anterior chamber in healthy immunocompetent individuals. The anterior chamber is an immune-privileged site and would be the optimal site for an opportunistic pathogen such as CMV to reactivate. Zheng et al[12] showed that the anterior chamber–associated immune deviation as proposed by Streilein[13] plays a critical role in the pathogenesis of HSV-induced corneal endotheliitis in a rabbit model. The continuous presence of viral antigens leads to an antigen-specific suppression of cellular immunity. Under the suppression of cell-mediated immunity, CMV was presumably able to proliferate efficiently in the anterior chamber. In addition, the suppression of local immunity by the long-term use of topical steroids may have facilitated the development of endotheliitis.

The route by which CMV entered the eye to infect the corneal endothelium is of great concern. CMV is generally harbored in mononuclear cells in latent form and is carried to systemic organs via the bloodstream. The virus in our patient could have entered the anterior chamber through the iris vessels to produce the corneal endothelial lesion; alternatively, it is possible that CMV might be latently harbored in the iris or in the corneal or trabecular endothelial cells.

From these findings, CMV should be added to the list of etiologic pathogens causing corneal endotheliitis.

REFERENCES

1. Khodadoust AA, Attarzadeh A. Presumed autoimmune corneal endotheliopathy. *Am J Ophthalmol.* 1982;93:718–722.
2. Maudgal PC, Missotten L, De Clercq E, et al. Varicella-zoster virus in the human corneal endothelium: a case report. *Bull Soc Belge Ophtalmol.* 1980;190:71–86.
3. Ohashi Y, Yamamoto S, Nishida K, et al. Demonstration of herpes simplex virus DNA in idiopathic corneal endotheliopathy. *Am J Ophthalmol.* 1991;112:419–423.
4. Robin JB, Steigner JB, Kaufman HE. Progressive herpetic corneal endotheliitis. *Am J Ophthalmol.* 1985;100:336–337.
5. Singh K, Sodhi PK. Mumps-induced corneal endotheliitis. *Cornea.* 2004; 23:400–402.
6. Koizumi N, Yamasaki K, Kawasaki S, et al. Cytomegalovirus in aqueous humor from an eye with corneal endotheliitis. *Am J Ophthalmol.* 2006; 141:564–565.
7. Cao M, Xiao X, Egbert B, et al. Rapid detection of cutaneous herpes simplex virus infection with the polymerase chain reaction. *J Invest Dermatol.* 1989;92:391–392.
8. Tanaka N, Kimura H, Iida K, et al. Quantitative analysis of cytomegalovirus load using a real-time PCR assay. *J Med Virol.* 2000; 60:455–462.
9. Michel D, Hohn S, Haller T, et al. Aciclovir selects for ganciclovir-cross-resistance of human cytomegalovirus in vitro that is only in part explained by known mutations in the UL97 protein. *J Med Virol.* 2001;65:70–76.
10. Wingard JR, Stuart RK, Saral R, et al. Activity of trifluorothymidine against cytomegalovirus. *Antimicrob Agents Chemother.* 1981;20: 286–290.
11. de Schryver I, Rozenberg F, Cassoux N, et al. Diagnosis and treatment of cytomegalovirus iridocyclitis without retinal necrosis. *Br J Ophthalmol.* 2006;90:852–855.
12. Zheng X, Yamaguchi M, Goto T, et al. Experimental corneal endotheliitis in rabbit. *Invest Ophthalmol Vis Sci.* 2000;41:377–385.
13. Streilein JW. Ocular immune privilege and the Faustian dilemma. The Proctor lecture. *Invest Ophthalmol Vis Sci.* 1996;37:1940–1950.

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OCULOPLASTICS AND ORBIT

Effects of adrenergic and cholinergic antagonists on diameter of nasolacrimal drainage system

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Abstract
Background To determine the effect of an adrenergic and a cholinergic antagonist on the diameter of the lumen of the nasolacrimal drainage system.
Methods The asymptomatic side of 38 patients (29 women, nine men) with unilateral stenosis/obstruction of the nasolacrimal drainage system was studied. The tear meniscus height (TMH) of the asymptomatic side was normal, and the lacrimal drainage system was patent as revealed by dacryocystography. The nasolacrimal drainage system of the asymptomatic side was infused with 100 μl of 0.01% bunazosin hydrochloride, a selective α-1 adrenergic antagonist, or 100 μl of 0.4% tropicamide, a muscarinic and cholinergic antagonist. Dacryocystography was performed to determine the diameter of the lumen of the nasolacrimal drainage system before and after the antagonists.
Results Bunazosin reduced the diameter of the lumen significantly, and the changes were more marked in the nasolacrimal duct (NLD), especially the middle and the lower regions. The diameter of the lumen of the lacrimal sac was not changed significantly. In contrast, tropicamide did not cause any significant change in the diameter of the lumen of the nasolacrimal drainage system.
Conclusions The alterations of the size of the lumen of the nasolacrimal drainage system, especially the NLD, by an adrenergic antagonist suggest that the lumen diameter is under continuous sympathetic tone, and the parasympathetic tone is weak.

Keywords Nasolacrimal drainage system · Adrenergic antagonist · Cholinergic antagonist · Dacryocystography · Nasolacrimal duct

Introduction

A recent study from our institute showed that phenylephrine, an α-1 adrenergic agonist, increased the diameter of the lumen of the nasolacrimal drainage system, especially the lumen of the nasolacrimal duct (NLD) [19]. Pilocarpine, a cholinergic agonist, on the other hand, reduced its diameter [19]. These findings suggested that the size of the lumen can be changed by the autonomic nervous system, and the changes occur because of the thick cavernous wall of the NLD [19]. However, the autonomic innervation to the nasolacrimal drainage system has not been studied in detail. Unlike the nasolacrimal drainage system, the physiological and pharmacological influences of the autonomic nervous system on the nasal mucosa have been widely investigated. Thus, Ishibe and associates [13] presented evidence for the existence of α-1 adrenergic and muscarinic cholinergic receptors in the human nasal mucosa.

Stimulation of the sympathetic nervous system constricts the blood vessels in the nasal mucosa, which leads to a shrinkage of the cavernous tissue of the nasal mucosa [17]. This vasoconstrictive action of adrenergic agents on the human nasal mucosa maintains the patency of the nasal passage [6] and is almost exclusively mediated through α-1

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receptors, and the α-2 receptors are of only a minor importance [14]. Kawarai and associates [14] reported that prazosin, a selective α-1 adrenergic antagonist, blocked the evoked nasal vasoconstrictor response.

In contrast, stimulation of the parasympathetic nervous system leads to vasodilation and a swelling of the mucosa [17], and this mechanism plays an essential role in the "protective" nasal cleaning reflex [6]. This vasodilation is not blocked by atropine, and thus appears to be due to a non-cholinergic mechanism [4]. Because the NLD has characteristics similar to that of the nasal mucosa [16, 24], we hypothesized that α-1 adrenergic antagonists will induce a decrease in the diameter of the lumen of the NLD via vasodilation, and that cholinergic antagonists will not alter its size.

The purpose of this study was to determine whether the diameter of the lumen of the lacrimal drainage system is altered by sympathetic and parasympathetic antagonists. To accomplish this, dacryocystogaphy was performed before and after the infusion of sympathetic and parasympathetic antagonists.

Patients and methods

Subjects

Thirty-eight patients (29 women and 9 men, age: 63.4± 14.5 years; mean±SD) were studied. Because X-rays are used for dacryocystography, we could not ethically use subjects without disease of the nasolacrimal drainage system as controls. Instead, we selected patients who had a unilateral stenosis/obstruction of the nasolacrimal drainage system or dacryocystitis with epiphora and mucoid discharge from the medial canthal region. These patients required dacryocystography on the symptomatic side to make a definitive diagnosis, and on the asymptomatic side to be certain that the lacrimal drainage system was patent and normal anatomically.

All subjects were examined in the Department of Ophthalmology at Saijo City Shuso Hospital between October 2005 and September 2006. Approval for this study was obtained from the Institutional Review Board of the Ehime University School of Medicine. The procedures used conformed to the tenets of the Declaration of Helsinki, and informed consent was obtained from all subjects after the nature and possible consequences of the study were explained.

Patients with any ocular surface disease, glaucoma, nasal inflammatory disease, abnormal eyelid position, recent ocular trauma, acute/chronic upper respiratory inflammation, or eye surgery were excluded. Patients with heart disease or bronchial asthma were also excluded because of the possible adverse reaction to the drugs.

The lacrimal drainage system on the asymptomatic side was carefully examined in all patients, and no signs of epiphora and mucoid discharge were recognized. Dacryocystography showed no stenosis/obstruction or dilatation of the nasolacrimal system on this side. In addition, the tear meniscus height (TMH) was normal, and the lacrimal system was patent on nasolacrimal irrigation.

Dacryocystography

Dacryocystography was performed as reported [19], and all examinations were conducted by one of the authors (JN). Briefly, dacryocystography was performed on both sides to compare the differences between the asymptomatic and symptomatic sides and to be certain that stenosis/obstruction was not present bilaterally. Dacryocystography was performed with a digital radiographic system (ADR-1000A; Toshiba Medical Systems, Tokyo, Japan), and the images were stored electronically as a 1,024×1,024-pixel matrix in the 6-inch image intensifier (II) mode. Under these conditions, one pixel corresponded to approximately 0.118 mm.

To record the images, the patient was placed in a supine position and both eyes were anesthetized topically with 0.4% oxybuprocain hydrochloride. Then, 0.5 to 1.0 ml of 61.2% iopamidol (Iopamiron 300, Nihon Schering K.K., Osaka, Japan), a water-soluble contrast medium, was infused slowly and steadily under fluoroscopic guidance from the upper punctum into the nasolacrimal drainage system through the canaliculus with a 27-guage lacrimal cannula (outer diameter=0.4 mm, inner diameter=0.15 mm; Inami, Tokyo, Japan). The cannula was attached to a 2.5-ml syringe.

Dacryocystography was performed from two directions; anteroposterior and 45° lateral oblique. Images were obtained before and after the application of the autonomic drugs.

Effect of adrenergic and cholinergic antagonists on nasolacrimal drainage system

The effects of adrenergic and cholinergic antagonists were determined as we reported [19]. Bunazosin was assigned to the patients whose clinical record ended with an odd number (Group A), and tropicamide to those with an even number (Group B). Group A consisted of 14 women and five men with a mean age of 62.6±13.7 years (range 24–80 years), and each received 100 μl of 0.01% bunazosin hydrochloride (Detantol Ophthalmic Solution; Santen Pharmaceutical, Osaka, Japan). Bunazosin hydrochloride is a potent and selective α-1 adrenergic antagonist that is used clinically as an anti-hypertensive drug. Recently, bunazosin was registered as a topical ocular hypotensive drug in Japan [12].

Group B consisted of 15 women and four men with a mean age of 64.2±15.7 years (range 25–85 years), and each

received 100 μl of 0.4% tropicamide, a muscarinic and cholinergic antagonist, commonly used clinically as mydriatic and cycloplegic agents (Mydrin-M; Santen Pharmaceutical, Osaka, Japan).

Both drugs contained benzalkonium chloride as a preservative agent.

After the control dacryocystography images were recorded, approximately 100 μl (corresponding to two or three drops) of bunazosin or tropicamide was infused into the asymptomatic side from the upper punctum into the nasolacrimal drainage system through the canaliculus, using a procedure similar to that used to infuse the contrast medium. During the infusion, the lower punctum was compressed to prevent a reflux of the antagonists. The patients were asked not to blink and to keep their eyes closed for 15 minutes in order to reduce the outflow of the drug by lacrimal pumping. Fifteen minutes later, dacryocystography was performed with the digital radiographic system aimed in the anteroposterior or the oblique direction to obtain the post-treatment images.

The diameter of the lumen width of the nasolacrimal drainage system was measured from the dacryocystographic images before and after the autonomic antagonists. All measurements were made by one of the authors (JN) in a masked way. The names and clinical record numbers of the patients were concealed from the analyzer. For all measurements, the electronic images were magnified six times which showed the margins of the nasolacrimal drainage system clearly, and the measurements were based on the scale required to give 1-cm squares that were non-transparent to radiation. The diameter of the lumen was measured by identifying one edge of the duct and anchoring one end of an adjustable line at this point. Then the other end of the line was dragged to the other edge of the duct. The length of the line was calculated automatically based on the number of pixels, and the data were displayed. Thus, at the measurement points, the edges of both sides of the nasolacrimal drainage system were dragged manually, and then the sizes were automatically calculated and displayed on the monitor screen of the digital radiographic system. In addition to the absolute size, the ratio of the lumen width before and after drug administration was calculated using the following formula:

$$\text{ratio}(\%)=(\alpha-\beta)/\beta \times 100$$

α=width after drug administration

β=width before drug administration

The diameter of the lumen of the nasolacrimal drainage system was measured at five points: point 1, at the level of the internal common punctum which is approximately the upper lacrimal sac (LS); point 2, 5 mm below point 1, which is approximately the middle of the LS; point 3, 10 mm below point 1, which is approximately the lower LS or upper membranous NLD; point 4, 15 mm below point 1, which is approximately the middle membranous NLD; and point 5, 20 mm below point 1, which is approximately the lower membranous NLD.

Statistical analyses

Student's *t*-tests were used to compare the values obtained from the two groups before drug administration. Paired *t*-tests were used to compare the diameters of the lumen before and after administration at each of the five points, and one-way analysis of variance with a *post-hoc* Tukey test was used for within-group analysis at each measurement point and for the analysis of the ratio of the lumen width after drug administration. A *P*-value of <0.05 was taken to be significant. In these calculations, the actual measured pixel-based width was used.

Results

Except for a mild and temporary mydriasis caused by a reflux of a small amount of the drugs from the punctum in Group B, no other complications were seen during the study. After the measurements, there were no complications, such as ocular hypertension, nasolacrimal stenosis/obstruction, or dacryocystitis. The results of dacryocystography on the symptomatic side are shown in Table 1.

Dacryocystography before antagonists

The mean pretreatment diameters of the lumen obtained from the anteroposterior images of Group A were; 1.7±1.0, 1.9±1.2, 1.3±0.7, 1.7±0.8, and 2.2±1.2 mm at points 1 through 5 respectively. In the oblique images, the widths were; 2.0±1.2, 1.8±1.3, 1.4±0.9, 1.8±1.0, and 2.5±1.3 mm at points 1 through 5 respectively (Table 2). The mean diameter of the pretreatment lumen obtained from the anteroposterior images of the Group B were: 1.6±0.7,

Table 1 Results of dacryocystography on the symptomatic side

Variable	*N* (%)
Chronic dacryocystitis due to nasolacrimal obstruction	13(34)
Nasolacrimal stenosis	10(26)
Functional nasolacrimal obstruction	7(18)
Internal common punctal obstruction	4(11)
Upper and lower punctal obstruction	2(5)
Upper and lower canalicular obstruction	1(3)
Upper punctal obstruction + lower canalicular stenosis	1(3)
Total	38(100)