Table 2 Comparison of the lumen diameter of the nasolacrimal drainage system at five points before and after bunazosin (pixels)

	Anteroposterior imaging		Oblique imaging	
	Before	After	Before	After
Internal common punctum	14.2±8.7	14.2±8.2	16.8±9.9	15.9±10.0
5 mm	16.2±10.5	15.0±9.1	15.6±11.1	15.8±10.2
10 mm	10.7±5.9	8.2±4.4*	11.8±7.5	8.6±5.1†
15 mm	14.3±6.5	8.8±5.8‡	15.0±8.3	7.9±5.7§
20 mm	18.6±9.8	10.7±8.9‡	21.0±11.2	11.2±9.7‡

* $P<.05$. † $P<.01$. ‡ $P<.0001$. § $P<.00001$.

1.8±0.7, 1.2±0.6, 1.5±1.0, and 2.3±1.3 mm at points 1 through 5, and in the oblique images they were 1.9±0.8, 1.8±0.8, 1.3±0.5, 1.6±1.0, and 2.3±1.3 mm respectively (Table 2). The pretreatment lumen diameters in Group A were not significantly different from those diameters in Group B for both the anteroposterior and oblique images. Most importantly, the diameters of the lumens in the anteroposterior images did not differ significantly from those measured on the oblique images. This would indicate that the shape of the nasolacrimal drainage system at these points was approximately circular.

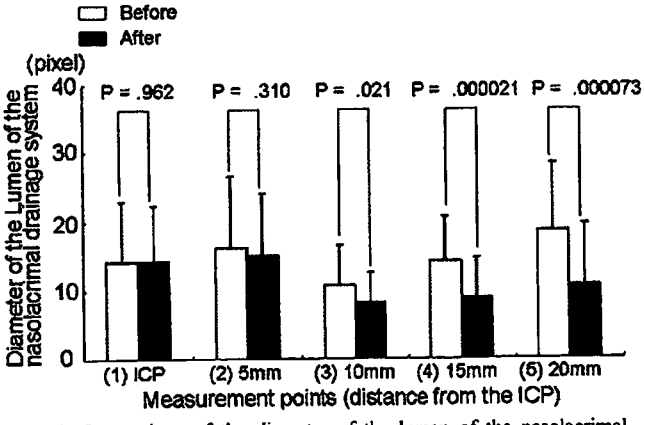
Effects of bunazosin and tropicamide

After the bunazosin infusion, the diameter of the lumen of the nasolacrimal drainage system decreased significantly at points 3 through 5 in the anteroposterior images (Fig. 1, *left panel*; Table 2), and at the same points in the oblique images (Fig. 1, *right panel*; Table 2). The diameter of the lumen after drug administration at point 3 was significantly smaller than at point 2 in the anteroposterior images, and at point 4 than at points 1 and 2 in the oblique images.

The ratio of the diameter of the lumen before and after drug administration was smaller at points 4 and 5 than at points 1 and 2 in the anteroposterior images (Fig. 2, *left panel*). In the oblique images, the ratio was smaller at points 4 and 5 than at points 1 through 3, and at point 3 than at point 2 (Fig. 2, *right panel*).

These findings indicate that bunazosin reduced the diameter of the lumen of the nasolacrimal drainage system significantly, but the changes were not uniform along the NLD; the changes in the diameter of the lumen of the NLD were more marked in the middle and lower region of the NLD, while the lumen diameter of the LS was essentially unchanged (Fig. 3).

In Group B, the diameter of the lumen of the nasolacrimal drainage system was not changed significantly at all points in both the anteroposterior (Fig. 4, *left panel*; Table 3) and oblique images (Fig. 4, *right panel*; Table 3). The diameter of the lumen at point 5 in Group B was significantly larger than at point 3 before tropicamide in both anteroposterior and oblique images. This difference was still present after tropicamide at points 1 and 3 in the anteroposterior images, and at point 3 in the oblique



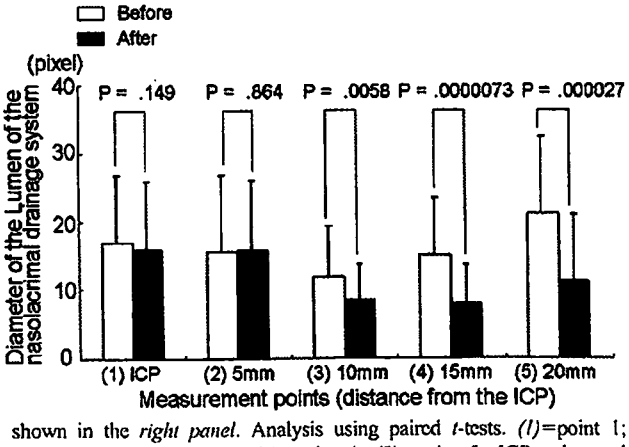


Fig. 1 Comparison of the diameter of the lumen of the nasolacrimal drainage system at five points before and after bunazosin. The anteroposterior images are shown in the *left panel*, and the oblique images are shown in the *right panel*. Analysis using paired *t*-tests. *(1)*=point 1; *(2)*=point 2; *(3)*=point 3; *(4)*=point 4; *(5)*=point 5; *ICP* = internal common punctum

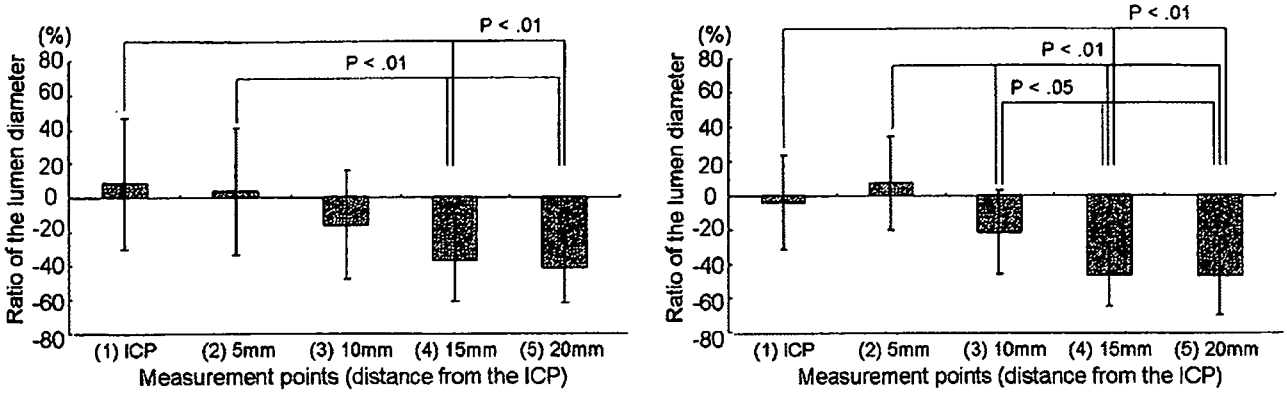


Fig. 2 Comparison of the changes in the ratios of the diameter of the lumen of the before and after bunazosin from anteroposterior images (*left panel*) and oblique images (*right panel*). The significance of the differences was determined by one-way analysis of variance with a *post-hoc* Tukey test. *(1)*=point 1; *(2)*=point 2; *(3)*=point 3; *(4)*=point 4; *(5)*=point 5; *ICP* = internal common punctum

images. There were no significant differences in the ratio of the lumen widths in both anteroposterior (Fig. 5, *left panel*) and oblique images (Fig. 5, *right panel*).

These findings indicate that tropicamide did not alter the diameter of the lumen of the nasolacrimal drainage system significantly (Fig. 6).

Discussion

The blood flow and blood volume of the nasal mucosa are under autonomic control by innervations from sympathetic and parasympathetic nerve fibers [4]. This control of the nasal vessels plays a crucial role in regulating the patency of the nasal passages through erectile tissues [5, 6, 17]. The erectile tissues, the so-called "cavernous tissues" of the nasal mucosa, are involved in the congestion of the nasal passage

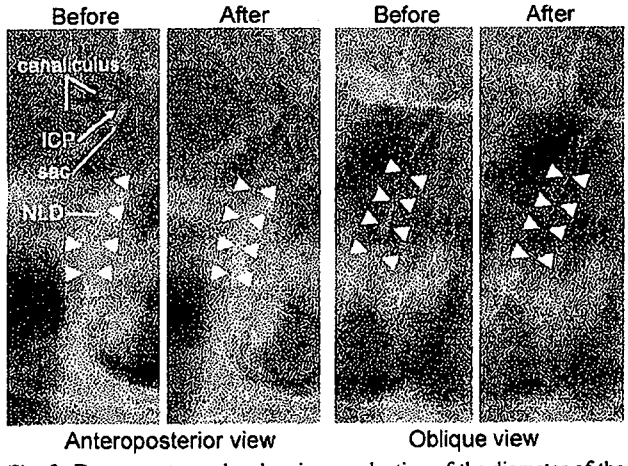


Fig. 3 Dacryocystography showing a reduction of the diameter of the lumen of the nasolacrimal drainage system after bunazosin administration in anteroposterior and oblique images (*arrowheads*). Images are of the right nasolacrimal drainage system. *ICP* = internal common punctum. *NLD* = nasolacrimal duct

[8]. Because the nasal blood volume contributes to the vascular capacitance of the nasal mucosa, vascular dilatation induces a swelling of the nasal mucosa, whereas vascular constriction induces a shrinkage of the mucosa [17].

Only a few investigations have been made on the autonomic innervation to the nasolacrimal drainage system [19, 20], whereas the innervation, physiology and pharmacology of the nasal mucosa have been widely investigated. There are anastomoses between the dense plexus of the nasal mucosa, the LS, and the NLD [22]. Lidström [16] reported that the morphology of the mucosa of the NLD essentially resembles that of the nasal mucosa, and Whitnall [24] reported that the mucosa of the membranous NLD gradually attains the characteristics of the nasal mucosa as it approaches the nasal cavity. Thus, we suggest that the membranous NLD may possess physiological characteristics that are similar to those of the nasal mucosa [19]. Therefore, it is reasonable to discuss the nasolacrimal drainage system based on the findings of the nasal mucosa.

Under normal circumstances, the nasal blood vessels are kept in a state of partial constriction by "continuous sympathetic vascular tone" [5, 6, 14]. That is, under normal conditions, both the resistance and capacitance of the vessels of the nasal mucosa are under the tonic control of the sympathetic system via the cervical sympathetic trunk [17]. Removal of this constrictive tone by cutting the cervical sympathetic nerve allows the nasal blood vessels to relax and leads to a greater blood flow [17].

Ishibe and associates [13] demonstrated the existence of α-1 and β adrenergic and muscarinic cholinergic receptors on the human nasal mucosal cells. It was also demonstrated that the vasoconstrictive action of adrenergic substances is exclusively mediated through α-1 receptors, and α-2 receptors are of minor importance [14]. This sympathetic tone also affects the ocular blood flow [23].

The findings that α-1 adrenergic antagonists reduced the diameter of the lumen of the NLD may indicate the

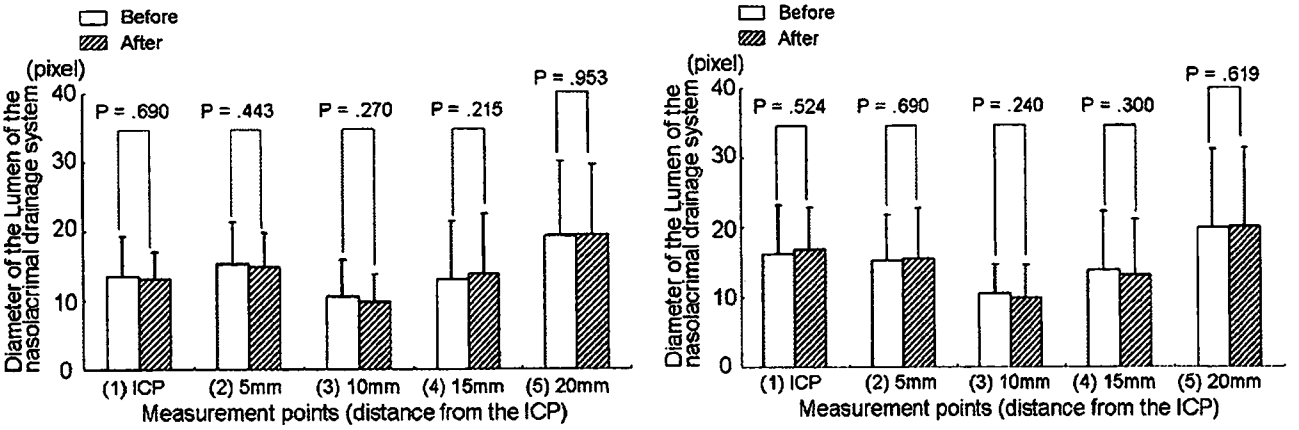


Fig. 4 Comparison of the diameter of the lumen of the nasolacrimal drainage system at five points before and after tropicamide. The anteroposterior images are shown in the *left panel*, and oblique images are shown in the *right panel*. The significance of the differences was determined by paired *t*-tests. *(1)*=point 1; *(2)*=point 2; *(3)*=point 3; *(4)*=point 4; *(5)*=point 5; *ICP* = internal common punctum

presence of a continuous sympathetic tone to the NLD, as in the nasal mucosa and ocular blood vessels. This sympathetic tone is probably important for maintaining the patency of the nasolacrimal drainage system. In addition, the results of our previous study showed that α-1 adrenoceptor agonists increased the diameter of the lumen of the NLD, which strongly supports the existence of α-1 adrenergic receptors in the NLD.

Because the cavernous body is better developed in the NLD than in the LS [19, 20, 24], the diameter of the lumen may be changed only in the NLD. This property is similar to that obtained in our study using α-1 adrenergic and cholinergic agonists [19]. Additional immunohistochemical or/and pharmacological investigations are needed to determine whether α-receptors are present in the nasolacrimal drainage system.

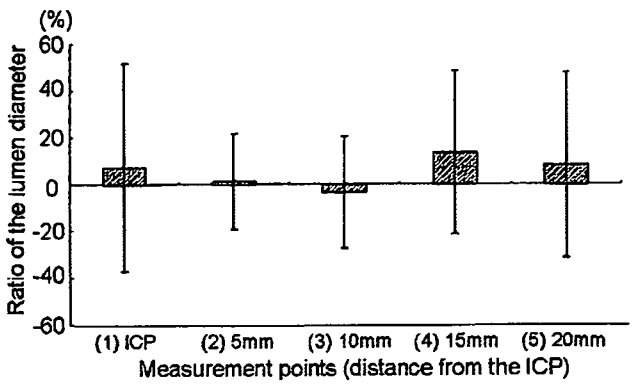
Although a parasympathetic tone is also present in the nasal mucosa [17], it is not as strong as the sympathetic tone [6, 17]. Lung and associates [17] reported that the nasal vessels, and in particular the capacitance vessels, are predominantly under sympathetic control. The key mechanism is the sympathetic system rather than the parasympathetic system, and there is a negligible tonic parasympathetic discharge to the nasal resistance and capacitance vessels. It has been suggested that cholinergic innervations exist because pilocarpine affects the ocular blood flow, but it is not tonically active because topical tropicamide has no effect on the ocular blood flow [3]. The hypothesis that the NLD has similar physiological features, viz., parasympathetic innervation is present but not tonically active, is a good explanation for our findings that cholinergic antagonist did not alter the diameter of the lumen of the NLD, but the cholinergic agonist pilocarpine reduced the lumen [19].

Under normal conditions, the nasal airflow alternates from one side of the nose to the other side over a period of 2–4 hours, and this is controlled by the autonomic nervous system. This phenomenon is termed the nasal cycle [2, 5], and is present not only in the nasal mucosa but also in the pupil size, lacrimal secretion, and nasal secretion [2]. The sympathetic tone regulates the nasal cycle [5], and a hypoactive sympathetic nervous system in the nasal system induces autonomic diseases, such as nasal allergy [13]. Although it is not clear whether the nasolacrimal drainage system has a "cycle" that is controlled by the autonomic nervous system, the disruption of this mechanism may be one of the etiological factors of nasolacrimal obstruction.

There are four pharmacologically recognized classes of muscarinic receptor subtypes, M1 through M4, and 5

Table 3 Comparison of the lumen diameter of the nasolacrimal drainage system at five points before and after tropicamide (pixels)

	Anteroposterior imaging		Oblique imaging	
	Before	After	Before	After
Internal common punctum	13.4±5.9	13.1±3.9	16.2±7.0	16.8±6.0
5 mm	15.2±6.0	14.7±5.1	15.2±6.6	15.5±7.2
10 mm	10.5±5.2	9.6±4.1	10.6±4.1	9.9±4.8
15 mm	13.0±8.4	13.8±8.6	13.9±8.3	13.1±7.9
20 mm	19.3±10.8	19.3±10.2	19.8±11.2	20.1±11.3



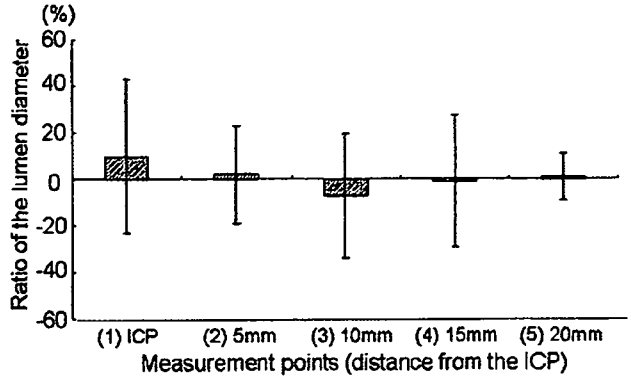


Fig. 5 Comparison of the change in the ratios of the diameter of the lumen after tropicamide from anteroposterior images (*left panel*) and oblique images (*right panel*). The significance of the differences was determined by one-way analysis of variance with a *post-hoc* Tukey test. *(1)*=point 1; *(2)*=point 2; *(3)*=point 3; *(4)*=point 4; *(5)*=point 5; *ICP* = internal common punctum

muscarinic receptor genes, m1 through m5 [11]. The M3 receptor is the predominant muscarinic receptor in the human iris sphincter and ciliary body [7], and the other four subtypes are present at lower levels [7], whereas M2, M4, and M5 receptors are present in the human corneal epithelium and endothelium [9]. In physiological studies, all subtypes were found in the nasal mucosa, and the m3 receptor is probably the most important [18]. Tropicamide is considered to be M4 receptor-specific, although it has blocking effects on M1 and M3 [15]. Therefore, the affinity of tropicamide to muscarinic receptor subtypes may affect the diameter of the lumen of the NLD, and studies are needed to determine which muscarinic receptors are present and dominant in the nasolacrimal drainage system.

Atropine and cyclopentolate are potent non-selective muscarinic antagonists and are used to treat inflammations of the iris. Even though these drugs may be expected to be more effective than tropicamide on the diameter of the lumen of the NLD, we chose not to use them because of the long-lasting side effects, including mydriasis and photophobia.

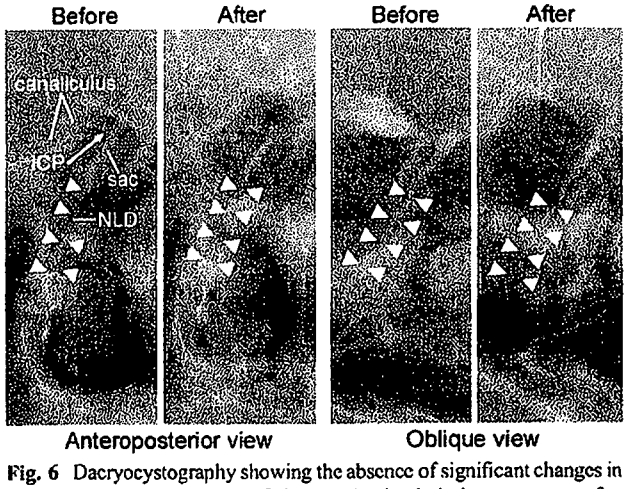


Fig. 6 Dacryocystography showing the absence of significant changes in the diameter of the lumen of the nasolacrimal drainage system after tropicamide administration for anteroposterior and oblique images (*arrowheads*). Images are of the right nasolacrimal drainage system. *ICP* = internal common punctum. *NLD* = nasolacrimal duct

Amanat and associates [1] reported that 30% of the patients with unilateral epiphora had a physiological obstruction in the lower part of the NLD of the asymptomatic side by lacrimal scintigraphy, indicating that the anatomically asymptomatic side may not be completely physiologically normal in our patients. Even though using asymptomatic sides may introduce some bias to our data, we chose not to use healthy volunteers for ethical reasons. We believed that a cross-over design with the use of bunazosin and tropicamide on the same patient would have increased the statistical power of our conclusions. However, repeated X-radiation would have to be performed on the same patient.

It is notable that the antiglaucoma eyedrops, bunazosin and pilocarpine [19], reduced the diameter of the lumen of the NLD. Most of the eyedrop is lost to drainage within 25 to 30 seconds after instillation, which includes the rapid drainage of 80% or more of the volume through the nasolacrimal drainage system [21]. Huang et al. [10] reported that the decrease in the intraocular pressure after antiglaucoma eyedrops was greater after nasolacrimal occlusion by punctal plugs. The mechanism responsible for this increased efficiency is probably that the punctal plugs blocked drainage of the eyedrops through the nasolacrimal drainage system, thus keeping the medication in contact with the ocular surfaces for a longer time [10]. Zimmerman and associates [25] reported that nasolacrimal occlusion increased the drug concentration available to the eye and markedly decreased the systemic absorption. It was thus suggested that one potential benefit of punctal occlusion was the reduction of systemic absorption of medications, which would then result in fewer systemic side-effects [10].

The relationship between the diameter of the lumen of the nasolacrimal drainage system and the kinetics of tear

flow has not yet been clearly determined. If a narrower lumen of the NLD is correlated with a lower tear clearance, the prior application of pilocarpine and/or bunazosin may increase the efficacy and decrease the systemic side effects of anti-glaucomatous drugs.

In summary, our data showed that an adrenergic antagonist decreased the diameter of the lumen of the nasolacrimal drainage system, whereas a cholinergic antagonist had no effect on its diameter. These findings indicate that the nasolacrimal drainage system is under the control of sympathetic tone maintaining the lumen diameter, while the parasympathetic tone is weak.

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CASE REPORTS

Suture-Related Keratitis Caused by *Corynebacterium macginleyi*[▽]

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We report two cases of suture-related keratitis following penetrating keratoplasty. In both cases, *Corynebacterium macginleyi* was isolated from corneal specimens. Scanning electron microscopy revealed that corynebacteria could aggregate and form a biofilm. The MICs of sulbenicillin and fluoroquinolones were high for both isolates. Our findings show that *C. macginleyi* can cause keratitis with biofilm formation.

CASE REPORT

Case 1. A 74-year-old woman underwent penetrating keratoplasty for a corneal opacity. Postoperatively, she was treated with topical corticosteroids (0.1% dexamethasone) and 0.3% gatifloxacin four times daily, and her recovery was uneventful. Four months later, she visited us with a complaint of blurred vision in her right eye. Slit-lamp biomicroscopy revealed an epithelial defect and a moderate degree of stromal infiltration, along with a loose corneal suture thread. We scraped over the surface of the suppurative area of the cornea and removed the loose corneal suture thread. Direct microscopy and bacterial culture of the corneal scraping were performed. The direct microscopy of the corneal scraping demonstrated the presence of gram-positive rods, and confluent growth of corynebacteria occurred after 48 h of incubation at 37°C in a 5% CO_2-enriched atmosphere on Columbia agar plates supplemented with 5% sheep blood (SBA). Colonies were grayish translucent and less than 0.5 mm in diameter. We considered corynebacteria to be the causative agent of the keratitis. We stopped the topical corticosteroids and 0.3% gatifloxacin and started treatment with topical 0.3% tobramycin and 0.5% cefmenoxime every hour. The corneal lesion responded to these agents promptly, and the corneal infiltration healed within 1 week.

Case 2. A 49-year-old man underwent penetrating keratoplasty for bullous keratopathy caused by a birth injury. Postoperatively, he was treated with topical corticosteroids (0.1% dexamethasone) and 0.5% levofloxacin four times daily, and his recovery was uneventful. The antibiotic eye drops were stopped 1 year after surgery. When he visited us 3 years after the surgery, slit-lamp biomicroscopy revealed an epithelial defect and a corneal plaque with a loose corneal suture thread (Fig. 1A). We removed the loose corneal suture thread and performed direct microscopy and bacterial culture of the removed corneal plaque. Direct microscopy demonstrated the presence of numerous gram-positive rods (Fig. 1B), and a large number of small colonies (<0.5 mm in diameter after 48 h of incubation) were observed on SBA. We diagnosed keratitis caused by corynebacteria, stopped the topical corticosteroids, and initiated treatment with topical 0.3% tobramycin and 0.3% gatifloxacin every hour. The epithelial defect and corneal plaque disappeared within 1 week.

Bacteriological findings. The isolates (EC009 in case 1 and EC010 in case 2) were suspected of being lipophilic corynebacteria because small colonies (<0.5 mm in diameter) were found after 48 h of incubation on SBA. In order to identify corynebacteria, biochemical testing and molecular genetic methods were performed. The commercial API Coryne system was used together with the API Coryne database 2.0 (6) according to the instructions of the manufacturer (bioMerieux, Marcy l'Etoile, France). In the API Coryne system, both EC009 and EC010 produced the numerical pattern 5-1-0-0-3-0-5, by which the API Coryne database identified them as *Corynebacterium macginleyi* with 99.5% probability. Furthermore, the complete 16S rRNA (~1.5 kb) and the partial *rpoB* genes were amplified with previously described primers (11, 12). Primers were as follows: for 16S rRNA, 8UA (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1485B (5′-TACGGTTACCTTGTTACGAC-3′), and for *rpoB*, C2700F (5′-CGWATGAACATYGGBCAGGT-3′) and C3130R (5′-TCCATYTCRCCRAARCGCTG-3′). The DNA sequences were compared to published sequences retrieved from the GenBank database (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD). CLUSTAL W software, originally described by Thompson et al. (20), was used to align the sequences, calculate percentages of similarity, and construct a phylogenetic tree. Both the 16S rRNA gene sequences obtained from the clinical isolates had 99.2% similarity to sequences from both *C. macginleyi* CIP 104099 (accession number X80499) and *C. accolens* CIP 104783 (accession number AJ439346). The *rpoB* sequences of both isolates showed 97.3 and 91.6% similarity to the sequences from *C. macginleyi* CIP 104099 (accession number AY492276) and *C. accolens* CIP 104783 (ac-

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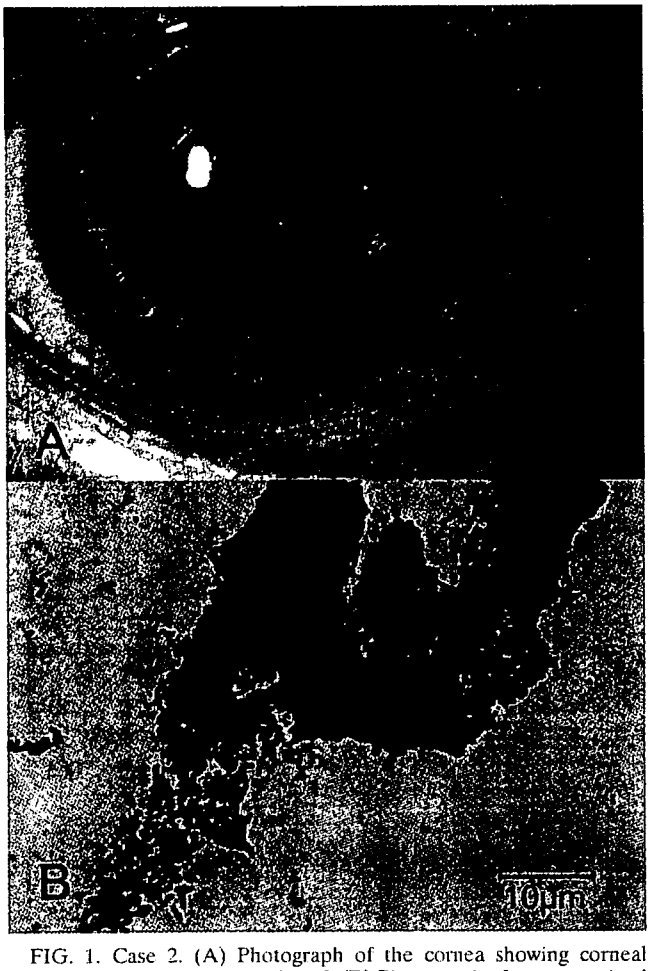

FIG. 1. Case 2. (A) Photograph of the cornea showing corneal plaque with the loose suture thread. (B) Photograph of a gram-stained specimen from the corneal scraping showing an aggregation of gram-positive rods.

cession number AY492242), respectively. Our clinical isolates were identified as *C. macginleyi* by the 16S rRNA and *rpoB* sequences, along with the result from the API Coryne system.

The MICs of various antimicrobial agents used in ophthalmic solutions for these isolates and the type strain (GTC3120 from the Gifu Type Culture Collection) were determined by the microtiter broth dilution method by following specific guidelines from the Clinical and Laboratory Standards Institute (1). All samples were cultured with Mueller-Hinton medium containing 3% lysed horse blood and incubated at 35°C in an ambient atmosphere for 24 and 48 h. We estimated the MICs for both isolates and the type strain because breakpoints have not been established for corynebacteria (1). The MICs of most antimicrobial agents for the type strain were low, while the MICs of sulbenicillin and fluoroquinolones for the clinical isolates were high (Table 1).

SEM of the suture threads. The suture threads removed in cases 1 and 2 were prefixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h, washed with cacodylate buffer, immersed in 1% tannic acid in aqueous solution for 1 h, washed with cacodylate buffer, postfixed with 2% osmium tetroxide in cacodylate buffer for 2 h, washed again with distilled water, dehydrated in a graded ethanol series, and dried using the critical-point drying method. The sutures were observed under a scanning electron microscope (model S-800; Hitachi, Tokyo, Japan). In case 1, the scanning electron microscopy (SEM) findings showed numerous bacilli on the suture knot, with a matrix connecting the bacilli, and the bacilli appeared to form a biofilm on the surface (Fig. 2A and B). In case 2, the SEM findings showed some agents covering the suture, and high magnification confirmed that these agents were a substantial gathering of bacilli (Fig. 2C and D).

Corynebacteria, along with *Staphylococcus epidermidis* and *Propionibacterium acnes*, constitute the major colonizers of the conjunctival sac, eyelids, and meibomian glands (8). Corynebacteria other than *C. diphtheriae* seem to have low virulence against the cornea (18). Therefore, corynebacteria are considered microflora if they are isolated in cases of infectious keratitis. However, several studies have found that some strains of corynebacteria cause keratitis (9, 14). *C. macginleyi* was recently uniquely isolated from the ocular site and found to cause conjunctivitis and endophthalmitis (4, 5, 7, 10). *C. macginleyi* was first identified in 1995 by Riegel et al. during investigations of lipophilic corynebacteria (17). However, it was not clear whether *C. macginleyi* could cause keratitis, and the factors contributing to the virulence of *C. macginleyi* are not well understood. In our cases, corynebacteria were considered causative agents because confluent growth occurred at the site of inoculation on culture plates and the results of the cultures were consistent with direct microscopy findings showing gram-positive pleomorphic rods.

The keratitis in both cases may have been triggered by a loose suture thread adhered to by *C. macginleyi*. A loose suture thread seems to be a risk factor for microbial keratitis following keratoplasty because organisms easily attach to the suture thread and migrate into the cornea. A previous study also reported that 35% of infectious keratitis cases following keratoplasty were related to sutures (2). Thus, biofilm formation on the suture seems to be one mechanism of pathogenicity in infectious keratitis. In our cases, the SEM findings revealed *C.*

TABLE 1. Antibiotic susceptibilities of *C. macginleyi* strains

Antibiotic	MIC (μg/ml)[a] for:		
	EC009[b]	EC010[c]	Type strain[d]
Sulbenicillin	16	16	4
Cefmenoxime	0.5	0.25	≤0.13
Tobramycin	≤0.13	≤0.13	≤0.13
Erythromycin	2	≤0.13	≤0.13
Vancomycin	0.5	0.5	0.5
Ofloxacin	>128	128	0.25
Norfloxacin	128	16	0.5
Ciprofloxacin	128	8	≤0.13
Levofloxacin	>128	64	≤0.13
Gatifloxacin	32	8	≤0.13

[a] Determined using the broth microdilution method with Mueller-Hinton medium containing 3% lysed horse blood.
[b] EC009 was isolated in case 1.
[c] EC010 was isolated in case 2.
[d] The type strain (GTC3120) was obtained from the Gifu Type Culture Collection.

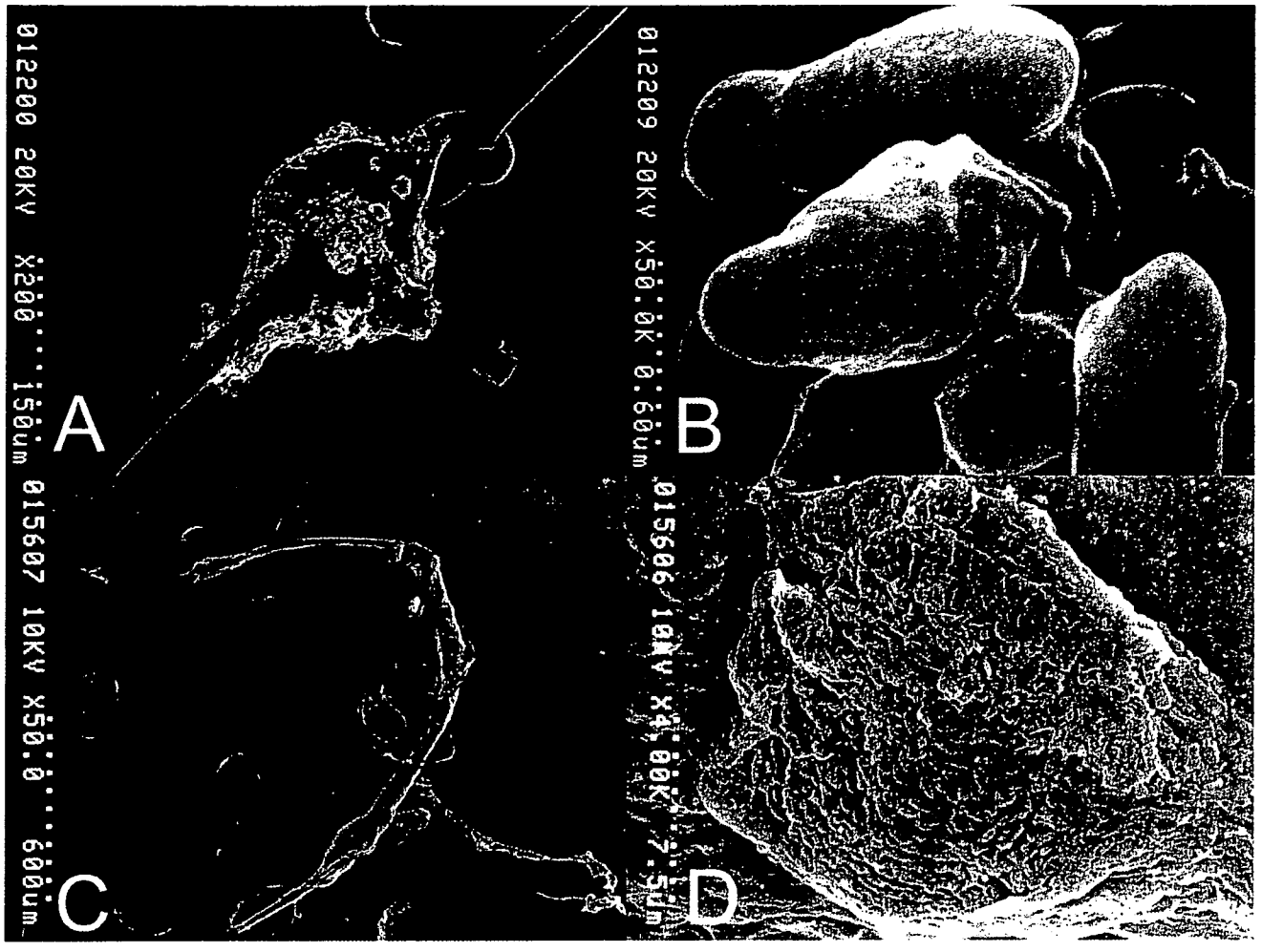

FIG. 2. (A and B) Case 1. (A) Photograph from a scanning electron microscope (taken at low magnification) of the suture showing organisms attached to the suture knot. (B) Photograph from a scanning electron microscope (taken at high magnification) of the suture showing bacilli with the matrix. (C and D) Case 2. (C) Photograph from a scanning electron microscope (taken at low magnification) of the suture showing agents surrounding the suture. (D) Photograph from a scanning electron microscope (taken at high magnification) of the suture showing the aggregation of numerous bacilli.

macginleyi strongly attached to the nylon suture, and an extracellular matrix appeared on the surface of the organisms. Furthermore, in case 2, we detected a plaque consisting of an aggregation of *C. macginleyi*. These facts imply that *C. macginleyi* cells can form a biofilm and aggregate and thereby cause keratitis. Mihara et al. reported that corynebacteria, which could not be identified to the species level, formed a biofilm on the cornea (16). In previous case reports, *C. macginleyi* was identified as the pathogen causing infections associated with intravenous and bladder catheters (3, 21), and SEM has demonstrated that corynebacteria can form biofilms on catheters (15). Kwaszewska et al. showed that 75.6% of lipophilic corynebacteria isolated as natural flora from human skin were able to form biofilms (13). Therefore, biofilm formation seems to be a factor contributing to the virulence of corynebacteria, especially *C. macginleyi*. However, because little is known about the mechanism of biofilm formation by corynebacteria, further investigation is required.

A previous study demonstrated that *C. macginleyi* isolated in cases of conjunctivitis was sensitive to fluoroquinolones (10). However, the two isolates in our cases had high levels of resistance to the fluoroquinolones levofloxacin and gatifloxacin, which are used in ophthalmology. It is likely that the long-term use of topical fluoroquinolones for prophylaxis against infection led to the appearance of fluoroquinolone-resistant *C. macginleyi*. Along with topical antibiotics, we applied topical corticosteroids for prophylaxis against corneal rejection. Therefore, steroids can render the cornea immunocompromised and lead to infections. When the mechanism of resistance to fluoroquinolones in corynebacteria was investigated in a previous study, a mutation in the *gyrA* gene of corynebacteria resulted in high MICs of the fluoroquinolones ciprofloxacin, levofloxacin, and moxifloxacin (19). Therefore, the high concentrations of fluoroquinolones in ophthalmic solutions may create an environment that selects isolates carrying a mutation of *gyrA*. To prevent the appearance of fluoroquinolone-resistant strains, we should avoid the unnecessary use of ophthalmic solutions containing fluoroquinolones.

In conclusion, we found that *C. macginleyi* can cause keratitis and that biofilm formation seems to contribute to the virulence of the bacterium. The use of topical fluoroquinolones and steroids may facilitate keratitis caused by *C. macginleyi*.

Nucleotide sequence accession numbers. The nucleotide sequences of the 16S rRNA genes of EC009 and EC010 have been given GenBank accession numbers AB354691 and AB354692. The sequences of the *rpoB* genes of EC009 and EC010 were deposited under accession numbers AB354693 and AB354694.

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Human corneal epithelial cell proliferation by epiregulin and its cross-induction by other EGF family members

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Purpose: To investigate the effects of epiregulin, a newly identified member of the epidermal growth factor (EGF) family, on the proliferation of human corneal epithelial cells (HCECs).

Methods: The proliferation of HCECs was determined by cell counting and BrdU incorporation assays at specific times after exposure to different concentrations of human recombinant epiregulin (0 to 20 ng/ml). Immunohistochemical staining was used to localize epiregulin in cadaveric corneas. RT-PCR and real-time PCR were used to determine the expression levels of epiregulin in cultured and cadaveric HCECs. To examine the interaction between epiregulin and epidermal growth factor receptors (EGFRs), the phosphorylation of ErbB1 and ERK1/ERK2 (ERK1/2) was estimated by western blot analysis in the presence or absence of AG1478, a specific inhibitor of EGFR kinase activity. To search for cross-induction of epiregulin by other EGF family members, the expressions of EGF, heparin-binding epidermal growth factor-like growth factor (HB-EGF), amphiregulin (AR), and transforming growth factor-alpha (TGF-α) mRNA were determined by real-time PCR in the presence of 10 ng/ml of epiregulin. Conversely, the expression of epiregulin was also determined following the incubation of HCECs with 10 nM of either of EGF, HB-EGF, TGF-α, or AR.

Results: The mRNA of epiregulin was expressed in cultured HCECs and HCECs obtained from cadaveric eyes. Epiregulin was strongly detected in the limbal epithelium and basal epithelium of the peripheral cornea, but it was weakly detected in the central corneal epithelium. HCECs proliferated in the presence of epiregulin in a dose-dependent manner as detected by an increase in cell numbers or in BrdU incorporation. When HCECs were incubated with exogenous epiregulin, the expression of the mRNA of epiregulin was up-regulated as detected by real-time PCR, and the phosphorylation of ErbB1 and ERK1/2 was up-regulated in a dose-dependent manner as shown by Western blot analysis. These up-regulations were inhibited by AG1478, a specific inhibitor of EGFR kinase activity. Epiregulin increased the expression of HB-EGF and AR, while TGF-α, HB-EGF, AR, and EGF increased the expression of epiregulin in HCECs.

Conclusions: These findings indicate that epiregulin played an autocrine role in the proliferation of HCECs presumably through cross-induction with other EGF family members.

The corneal epithelium is a multi-layered stratified epithelium that covers the surface of the cornea and acts as a physical barrier to noxious agents. To maintain an effective barrier, a constant renewal of corneal epithelial cells is necessary, and this was accomplished with the aid of different growth factors [1,2]. Of these growth factors, the members of the epidermal growth factor (EGF) family have been most extensively studied. The EGF family consists of EGF, transforming growth factor-alpha (TGF-α), heparin binding EGF-like growth factor (HB-EGF), amphiregulin (AR), epiregulin, betacellulin (BTC), neuregulin 1, neuregulin 2, and neuregulin 3 [3-12]. Among these, TGF-α and HB-EGF are known to stimulate not only the migration and proliferation of corneal epithelial cells, but also the synthesis of the basement membrane and extracellular components [6,11,13-16].

In general the growth factors of the EGF family consist of a transmembrane domain and one or more EGF domains. Soluble mature peptides are released from the extracellular domains by proteolytic cleavage as ligands for EGF receptors [17]. There are four types of EGF receptors: ErbB1 (EGFR/Her1), ErbB2 (Neu/Her2), ErbB3 (Her3), and ErbB4 (Her4) [18-21]. The members of the EGF family can be divided into two groups according to their binding specificity to transmembrane receptors. The first group, which includes EGF, HB-EGF, TGF-α, AR, BTC, and epiregulin, generally binds to ErbB1 [4,5,12,22-24] and plays a crucial role in epithelial development and wound-healing for skin, lungs, and the gastrointestinal tract [25-27]. The members of the second group, the neuregulins, bind to ErbB3 and ErbB4 [3,7,8,28] and are crucial for the development of cardiac muscle and the central nervous system [29-32].

It has been shown that the first group of the EGF family members plays important roles during the healing of corneal injuries [1,16,33-35]. These binding EGFR ligands activate ErbB1, thus activating subsequent intracellular signals such as extracellular signaling-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K), which lead to corneal epithelial cell proliferation and migration [36-39].

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Epiregulin is a relatively new member of the EGF family and was originally purified from conditioned medium of the NIH3T3 cell line T7 [12]. It is made up of 46 amino acids that form a single-chain polypeptide that exhibits 24-25% amino sequence homology with other EGFR ligands [12,40,41]. Epiregulin is unique due to its affinity to bind to all of the ErbB family members [42]. It has been demonstrated that epiregulin plays a role in the proliferation of epithelial cells, e.g., epidermal and urothelial cells [43,44]. In the eye, Zhou et al. [45] reported that the mRNA of epiregulin was preferentially expressed in limbal basal cells of mice; however, the precise role of epiregulin in human corneal epithelial cells has not been determined. Like other members of the first group of the EGF family, epiregulin also binds to ErbB1, and it is thus reasonable to consider that epiregulin may play a role in corneal epithelial cell proliferation.

The purpose of this study was to determine whether the proliferation of HCECs is up-regulated in the presence of epiregulin, and whether epiregulin can act on the HCECs together with other members of the EGF family including EGF, TGF-α, HB-EGF, and AR.

METHODS

Human subjects: All procedures involving human subjects were conducted in accordance with the tenets of the Declaration of Helsinki (JAMA 1997; 277:925-926). The experimental protocol for these experiments was approved by the Institutional Review Board of Ehime University.

Materials: All reagents used for cell cultures were purchased from Invitrogen (Carlsbad, CA) except for the growth factors. Recombinant human epiregulin, EGF, TGF-α, AR, and HB-EGF were purchased from R&D Systems (Minneapolis, MN).

Cell cultures: Primary HCECs were isolated from human corneoscleral buttons dissected from eyes acquired from an American eye bank. The buttons were carefully denuded of the endothelial cells and adherent iris tissues. After digestion with 1.2 U/ml Dispase at 4 °C for 24 h, the loosened epithelial sheets were removed and dissociated into single cells by enzyme digestion with 0.25% trypsin. The isolated HCECs were cultured in serum-free, modified MCDB153 medium containing insulin (5 μg/ml), hydrocortisone (0.5 μM), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), and bovine hypothalamic extract (50 μg/ml). The medium was changed every 2 days, and cells from the third or fourth passage were used for all experiments.

Expression of epiregulin mRNA in cadaveric corneal epithelium: The corneal epithelium was collected from the cadaveric corneoscleral buttons using a surgical knife, and then total RNA was immediately extracted using an RNeasy kit (Qiagen, Valencia, CA) and measured by spectrophotometry (OD at 260 and 280 nm). Total RNA was reverse-transcribed to cDNA using Omniscript Reverse Transcription Reagents (Qiagen) according to the instructions of the manufacturer. The PCR conditions for the initial denaturation step were 95 °C for 15 min and 40 cycles at 95 °C for 10 s, followed by 64 °C for 20 s for annealing and 72 °C for 20 s for extension.

Proliferation of HCECs by epiregulin: HCECs were seeded in 6 well plates at a density of $8x10^4$ cells/well in serum-free modified MCDB153 medium. On the following day, the cells were subcultured in fresh medium containing serial concentrations of recombinant epiregulin (0-20 ng/ml) in the absence of bovine hypothalamic extract because the cells were still 20% confluent. After 6 days, the cells were collected and counted using a hemocytometer.

To determine whether the HCECs had proliferated, the level of BrdU incorporated into the HCECs was determined. HCECs were seeded in 6 well plates at a density of $8x10^4$ cells/well in serum-free modified MCDB153 medium. After reaching about 50% confluency, the cells were subcultured in a medium without bovine hypothalamic extract. On the following day, the cells were subcultured in the same medium containing serial concentrations of recombinant human epiregulin (0-20 ng/ml) and incubated for 48 h. The cells were then incubated with medium containing BrdU for 2 h. BrdU was detected immunohistochemically using the 5-Bromo-2'-deoxy-uridine Labeling and Detection kit 2 (Roche, Indianapolis, IN) according to the instructions of the manufacturer. The number of BrdU-labeled cells/5 mm^2 were counted (n=4).

Phosphorylation of ErbB1 and ERK1/ERK2 by epiregulin: HCECs were seeded in 24 well plates at a density of $5x10^4$ cells/well in serum-free, modified MCDB153 medium. After reaching about 80% confluency, the cells were subcultured in a medium without bovine hypothalamic extract. On the following day, the cells were subcultured in the same medium containing serial concentrations of recombinant human epiregulin (0-20 ng/ml) for 5 min. The cells were rinsed with cold Ca^{++}- free and Mg^{++}-free phosphate buffer saline (PBS^-) and then harvested on ice with a cell scraper. The cells were lysed in 200 μl of lysis buffer containing 62.5 mM Tris-HCl, 2% SDS, 25% glycerol, 0.01% bromophenol blue, and proteinase inhibitors at pH 6.8. After centrifugation, the cell lysates were separated on 7.5% polyacrylamide gel containing sodium dodecyl sulfate (SDS-PAGE) and transferred to a nitrocellulose membrane (Millipore, Billerica, MA). After blocking with 5% non-fat dry milk, the membrane was incubated

Table 1. Specific primer pairs for human epiregulin, HB-EGF, TGF-α, AR, EGF, and GAPDH are listed

Gene (Accession number)	Forward primer	Reverse primer	Product size (bp)
Epiregulin (NM_001432)	CGTGTGGCTCAAGTGTCAAT	TGGAACCGACGACTGTGATA	235
HB-EGF (NM_001945)	GCTCTTTCTGGCTGCAGTTC	AGCTGGTCCGTGGATACAGT	120
TGF-α (NM_003236)	TGATACACTGCTGCCAGGTC	ATCTCTGGCAGTGCTGTCCT	207
AR (NM_001657)	CGGGAGCCGACTATGACTAC	CCATTTTTGCCTCCCTTTTT	172
EGF (NM_001963)	CAGGGAAGATGACCACCACT	CAGTTCCCACCACTTCAGGT	187
GAPDH (AY340484)	CGACCACTTTGTCAAGCTCA	AGGGGTCTACATGGCAACTG	238

In the table, HB-EGF=heparin-binding epidermal growth factor-like growth factor, TGF-α=transforming growth factor-alpha, AR=amphiregulin, EGF=epidermal growth factor, and GAPDH=glyceral-dehyde-3-phosphate dehydrogenase.

with a 1:1,000 dilution of an anti-phospho-EGFR(ErbB1, Tyr1173) antibody (Upstate Biotechnology, Lake Placid, NY) or an anti-phospho-ERK1/ERK2 (ERK1/2) antibody (R&D systems) at 4 °C for 12 h. After washing with PBS, the membrane was incubated with a 1:2,500 dilution of fluorescein-labeled goat anti mouse IgG (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 h at RT. The signal was amplified with an anti-fluorescein alkaline phosphatase conjugate followed by the addition of a fluorescent substrate, Attophos (Amersham Pharmacia Biotech). The membrane was scanned using a FluorImager (Molecular Dynamics, Sunnyvale, CA), and the expression levels of ErbB1 and ERK1/2 were determined relative to that of beta-actin in the same sample using the NIH Image program. The phosphorylation of ErbB1 and ERK1/2 was also determined by Western blot analysis in HCECs exposed to serial concentrations of AG1478 (Calbiochem, La Jolla, CA), a specific inhibitor of EGFR kinase activity, with 100 ng/ml of epiregulin.

Quantitative real time PCR analysis: To determine whether exogenous epiregulin affected the expression of endogenous epiregulin, i.e., an auto-induction mechanism, or other members of the EGF family, i.e., a cross-induction mechanism, HCECs were stimulated by epiregulin, and the expression of the mRNA of the other growth factors was examined by real time PCR. Briefly, HCECs were seeded in 24 well plates at a density of $5x10^4$ cells/well in serum-free, modified MCDB153 medium. After reaching about 80% confluency, the cells were subcultured in a medium without bovine hypothalamic extract. On the following day, the cells were again subcultured in the same medium containing 10 ng/ml of epiregulin and harvested at 0.5, 1, 2, 3, 6, and 12 h. To determine whether other EGF family members affected the expression of epiregulin (a cross-induction mechanism by other EGF family members), HCECs were exposed to 10 nM of HB-EGF, TGF-α, AR, or EGF. In addition, to determine whether AG1478 affected the expression of endogenous epiregulin by exogenous epiregulin stimulation, HCECs were exposed to 10 ng/ml of epiregulin for 2 h with or without 1 h of preincubation in AG1478 (1 μM). Then the total RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA) and

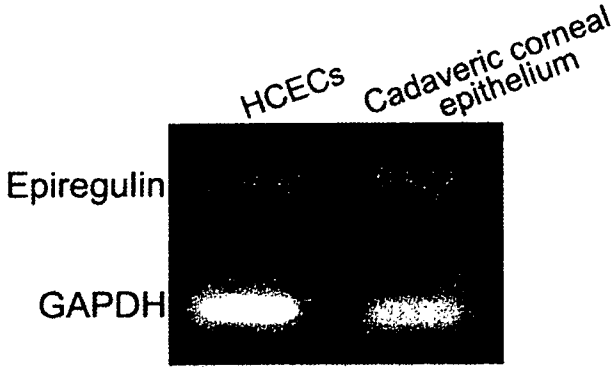


Figure 1. Expression of epiregulin mRNA in HCECs and cadaveric human corneal epithelia. The expression of epiregulin mRNA was detected in HCECs and corneal epithelium collected from cadaveric eyes by RT-PCR.

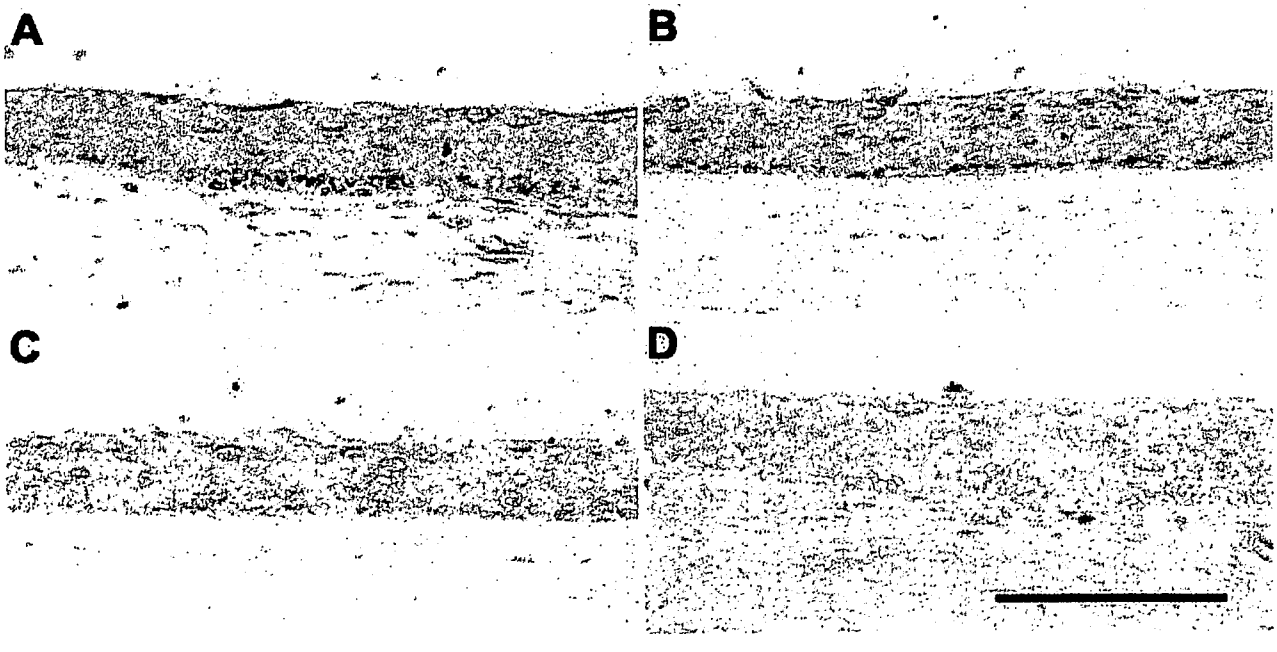
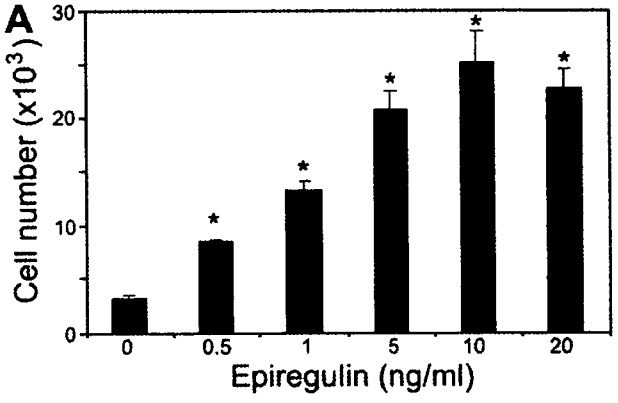


Figure 2. Immunohistochemical localization of epiregulin in human cornea. **A**: Corneal limbus. **B**: Peripheral cornea. **C**: Center of cornea. **D**: Corneal limbus (treated with normal goat Ig G). Immunoreactivity was detected strongly on basal and superficial limbal epithelium, on the basal layer of peripheral corneal epithelium, and some basal cells of central cornea. The scale bar is equal to 100 μm.

measured by spectrophotometry (OD at 260 and 280 nm). Equal amounts (1 μg) of total RNA were reverse-transcribed to cDNA using Omniscript Reverse Transcription Reagents (Qiagen) according to the instructions of the manufacturer. The PCR primers are listed in Table 1. These primers were designed from the full-length cDNA sequence in Genbank, and their specificities were confirmed by BLAST (National Library of Medicine, Bethesda, MD).

Real-time PCR was performed using a DyNAmo SYBR Green qPCR Kit (Finnzymes, Espoo, Finland). Amplifications were performed in a final volume of 20 μl containing 0.5 μM of primer mixture and 2 μl of cDNA. The PCR conditions for the initial denaturation step were 95 °C for 15 min and 40 cycles at 95 °C for 10 s, followed by 60 °C (HB-EGF, AR, EGF and GAPDH) or 64 °C (epiregulin and TGF-α) for 20 s for annealing, and 72 °C for 20 s for extension. All PCR reactions were performed by OPticon2 DNA Engine (BioRad, Hercules, CA), and each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of primer dimers. The comparative cycle threshold (C_t) was calculated for all samples to quantify the relative expression of each mRNA with standardization using that of GAPDH mRNA [46]. All experiments were performed in duplicate for each datum point.



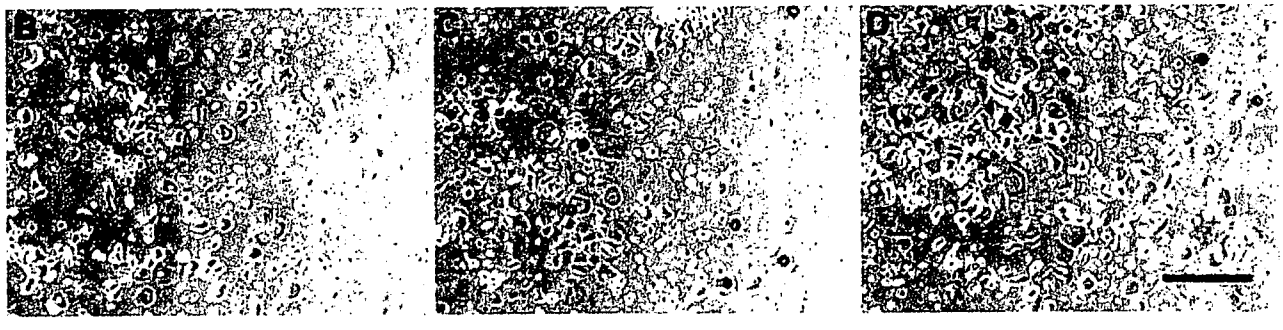
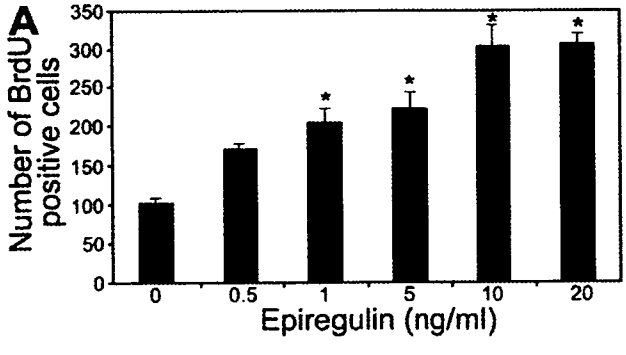


Figure 3. Effects of epiregulin on the proliferation of HCECs. Serial concentrations of recombinant epiregulin were added to the medium, and after 6 days the cell numbers were counted using a hemocytometer. The bottom panel shows representative photographs of HCECs 6 days after the addition of epiregulin. (**B**: 0 ng/ml, **C**: 1 ng/ml, **D**: 10 ng/ml) The scale bar is equal to 400 μm.



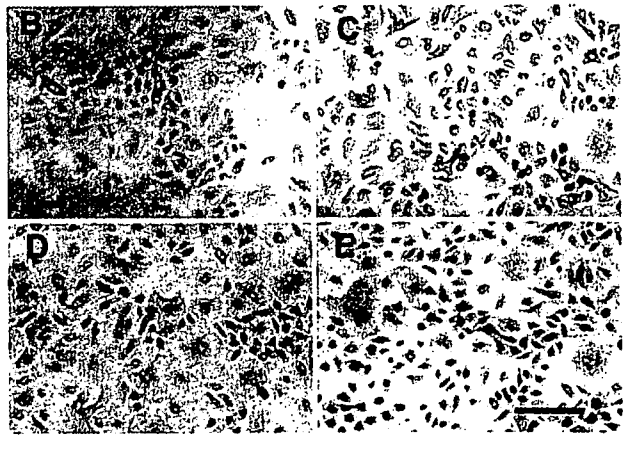


Figure 4. Effect of epiregulin on BrdU incorporation into HCECs. Serial concentrations of epiregulin were added to the medium, and after 48 h the incorporation of BrdU was determined by counting the number of BrdU labeled cells/5 mm^2. The bottom panel shows representative photographs of BrdU labeled cells. (**B**: 0 ng/ml, **C**: 1 ng/ml, **D**: 5 ng/ml, **E**: 10 ng/ml). The scale bar is equal to 200 μm. Asterisks show a significant difference ($p<0.01$) from the corresponding control (no addition of epiregulin). P values were calculated by two-sample t test. Results are representative of four independent experiments.

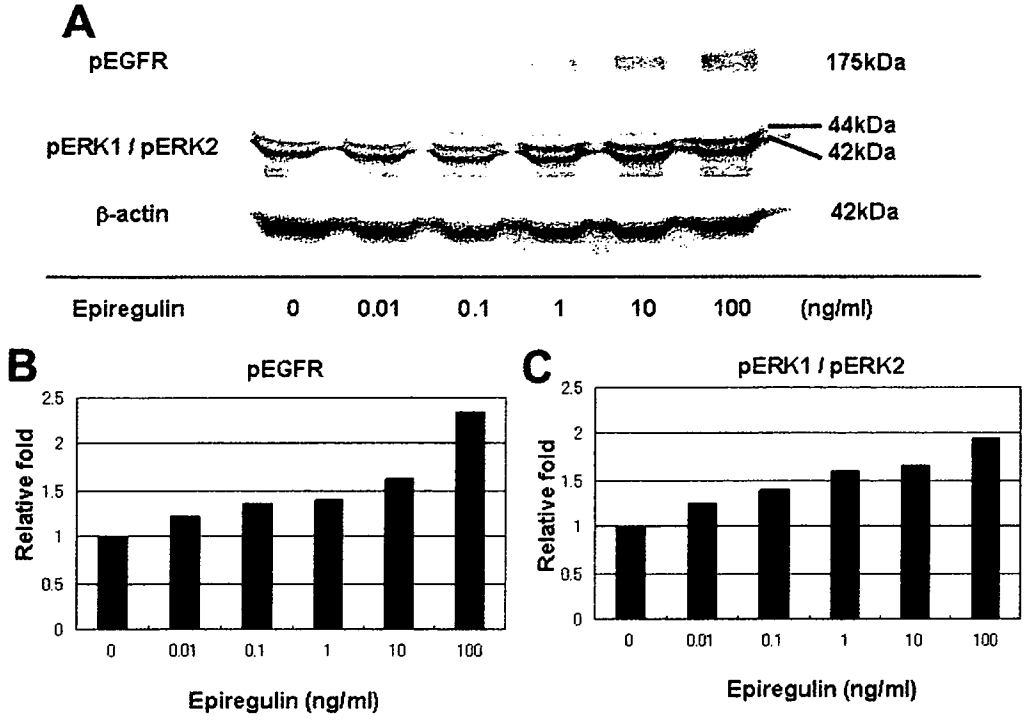


Figure 5. Phosphorylation of ErbB1 (EGFR) and ERK1/2 by epiregulin. HCECs were exposed to serial concentrations of epiregulin for 5 min. The cell lysates were subjected to 7.5% SDS-PAGE and then phosphorylated ErbB1 and ERK1/2 were detected with anti-phosphorylated-ErbB1 or anti-phosphorylated ERK1/2 antibody. The expression levels of ErbB1 and ERK1/2 were measured relative to that of β-actin in the same sample.

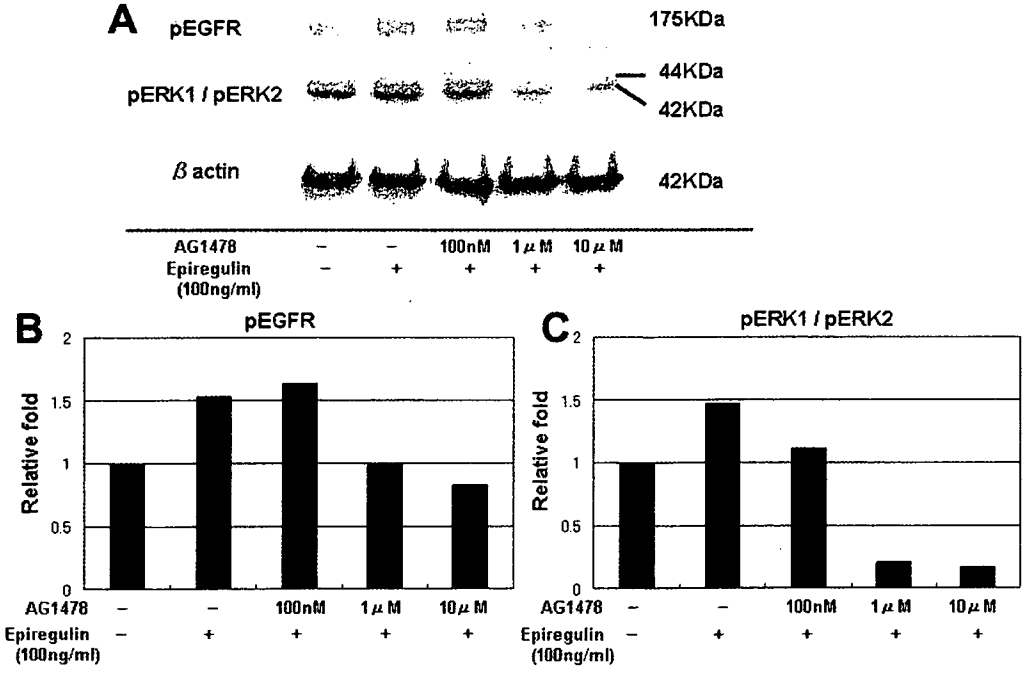


Figure 6. Inhibitory effect of AG1478 on the epiregulin induced phosphorylation of ErbB1 (EGFR) and ERK1/2. HCECs were exposed to serial concentrations of AG1478 for 1 h before incubation with 100 ng/ml of epiregulin for 5 min. The cell lysates were subjected to 7.5% SDS-PAGE, and then phosphorylated ErbB1 and ERK1/2 were detected with anti-phosphorylated-ErbB1 or anti-phosphorylated ERK1/2 antibodies. The expression levels of ErbB1 and ERK1/2 were measured relative to that of β-actin in the same sample.

Immunohistochemical staining: Cadaveric corneas were fixed in methanol, embedded in paraffin, and cut into 5 µm sections. Immunohistochemical staining was carried out with anti-epiregulin antibody (R & D Systems, 1:50 dilution, 16 h at 4 °C) using the VECTASTAIN Elite ABC kit (Vector Lab, Burlingame, CA), according to the manufacturer's protocol for paraffin sections. All sections were developed with AEC and counterstained with hematoxylin. For control, sections were treated with normal goat immunoglobulin G (IgG).

RESULTS

Expression of epiregulin in human cornea and corneal epithelial cells: To determine whether epiregulin is expressed in human corneal epithelial cells, we used RT-PCR to detect epiregulin mRNA in the corneal epithelium collected from the cadaveric eyes and from cultured HCECs. Epiregulin mRNA was detected in both corneal epithelial cells and cultured HCECs (Figure 1). Immunohistochemical staining showed that immunoreactivity was detected in all layers of the corneal epithelium, however, strong staining was detected in the basal and superficial layers of the limbal epithelium and in the basal layer of peripheral corneal epithelium. Immunoreactivity was very weakly detected in the central cornea (Figure 2). Immunoreactivity was also detected in the endothelium and weakly detected in keratocytes. Other than the cornea, epiregulin was detected strongly in the conjunctival epithelium, blood vessel endothelium, and weakly in the subconjunctival fibroblasts.

Recombinant human epiregulin stimulates proliferation of HCECs: To determine whether epiregulin stimulates the proliferation of HCECs, HCECs were cultured in serum-free medium with serially diluted concentrations of epiregulin, and the total number of cells and the number of BrdU-labeled cells were counted. The results showed that epiregulin stimulated the proliferation of HCECs in a dose-dependent manner with an 8.0 fold increase in cell numbers at 10 ng/ml (Figure 3). The number of BrdU labeled cells was also increased by epiregulin in a dose dependent manner with an increase up to 3.0 fold at 10 and 20 ng/ml (Figure 4).

Phosphorylation of ErbB1 and ERK1/2 by epiregulin: Epiregulin is known to bind to ErbB1 and to activate MAP kinase pathways [12]. In the next study, we investigated whether the tyrosine residues of ErbB1 and ERK1/2 in HCECs were phosphorylated in the presence of epiregulin. Western blot analysis revealed that epiregulin enhanced the phosphorylation of ErbB1 and ERK1/2 in a dose-dependent manner (Figure 5). The phosphorylation by 100 ng/ml of epiregulin was suppressed by a 1 µM or higher concentration of AG1478 (Figure 6).

Autoinduction of epiregulin and cross-induction of other EGF family members by epiregulin: We examined whether epiregulin up-regulated the transcription of its own mRNA in HCECs, i.e., an auto-induction mechanism of epiregulin. The results from real-time PCR showed that the expression of epiregulin increased as early as 0.5 h after incubation with 10 ng/ml of epiregulin, reached a peak (4.8 fold increase from 0 h) at 3 h after incubation (Figure 7), and gradually returned to its original level by 12 h. This up-regulation was completely inhibited by AG1478 (Figure 8).

Because EGF-related autocrine growth factors were able to induce other growth factors of the EGF family in a variety of epithelial cells [47], we examined whether epiregulin up-regulated the other members of the EGF family, i.e., a cross-induction mechanism of epiregulin, in HCECs. Real time PCR analysis showed that epiregulin exposure significantly increased the mRNA levels of AR at 2 h and of HB-EGF between 0.5 and 2 h, while the mRNA levels of EGF and TGF-α remained essentially unchanged (Figure 9).

Lastly, we investigated whether the expression of epiregulin was enhanced by other EGF family members, i.e., a cross-induction mechanism by other EGF family members, using real-time PCR analysis. The results showed that the ex-

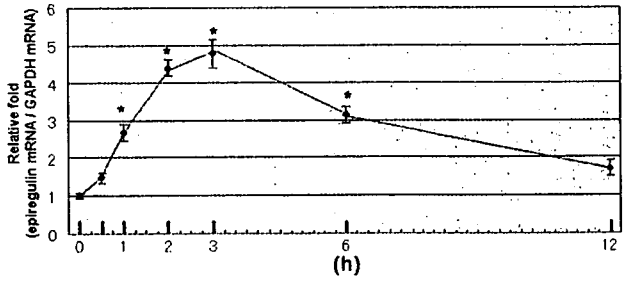


Figure 7. Auto-induction of epiregulin in HCECs. HCECs were incubated with 10 ng/ml of epiregulin for 0.5, 1, 2, 3, 6, and 12 h, and the relative expression of mRNA was determined by real time PCR. The amount of mRNA was calculated relative to the amount of GAPDH mRNA in the same samples. Asterisks indicate a significant difference from the corresponding control (0 h). P values were calculated by the two-sample t test. Results are mean±SEM of five independent experiments.

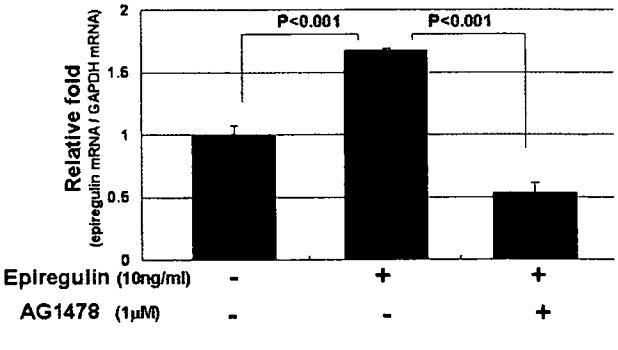


Figure 8. The inhibitory effect of AG1478 on the auto-induction of epiregulin in HCECs. HCECs were exposed to 10 ng/ml of epiregulin for 2 h with or without preincubation of AG1478 (1 µM), and the relative expression of epiregulin mRNA was examined by real-time PCR. The amount of mRNA was calculated relative to the amount of GAPDH mRNA in the same samples. P values were calculated by the two-sample t test. Results are mean±SEM of five independent experiments.

pression of mRNA of epiregulin increased around 2 or 3 h after treatment with all of the EGF family members including TGF-α, HB-EGF, EGF, and AR (Figure 10).

DISCUSSION

The integrity of the EGF receptor-ligand signaling system is of great importance in regulating epithelial cell differentiation and proliferation. Among the members of the EGF family (EGF, TGF-α, AR, and HB-EGF), ligands for ErbB1 are known to be responsible for the migration and proliferation of HCECs and for the synthesis of extracellular matrix during corneal epithelial wound healing [13-16,34,35].

Epiregulin is a relatively new member of the EGF family, and in a previous report on the distribution of epiregulin in human tissues, northern blot analysis showed that epiregulin mRNA was found predominantly in the placenta and peripheral blood leukocytes and to a lesser extent in the heart. A very weak expression was also detected in normal adult bone marrow, ovaries, small intestine, colon, lungs, and liver [41]. Similar to other growth factors belonging to the EGF family, epiregulin has been reported to promote the proliferation of other human epithelial cells such as the urothelium and epidermis [43,44].

We have shown that epiregulin mRNA was expressed in human corneal epithelial cells. Immunohistochemical staining for epiregulin revealed that immunoreactivity was strongly detected in limbal epithelial cells, and in the basal layer of peripheral corneal epithelia in which transient amplifying cells are supposed to exist. Similarly, Zhou et al. [45] reported that the mRNA of epiregulin was preferentially expressed in limbal basal cells of mouse corneal epithelia. The differences in expression patterns may be due to the differences in species and experimental methods. However, in both studies epiregulin expression was strongly detected in the progenitor or proliferating corneal epithelial cells. These results would suggest that epiregulin may contribute to the proliferative capacity of corneal epithelial cells.

We have shown that epiregulin was able to enhance the proliferation of HCECs. It has been suggested that the proliferation of corneal epithelial cell by members of the EGF family was induced through the ErbB1-MAPK pathway [38]. Our results showed that epiregulin was also able to activate ErbB1 following ERK1/2 phosphorylation. Thus, like other EGF family members, epiregulin may induce corneal epithelial cell proliferation as, at least, a ligand for ErbB1.

Most of the EGF-related autocrine growth factors are capable of promoting the expression of other EGF family growth factors in a variety of epithelial cells [47,48], and an increase in the level of EGF family growth factors (EGFR ligand transcripts) is dependent upon signals from their own receptors, "EGFR" [48-51]. Shirakata et al. reported that epiregulin is part of an auto-induction and cross-induction mechanism involving HB-EGF, AR, and TGF-α in keratinocyte proliferation [44]. In this study, we have shown that epiregulin stimulated the induction of its own mRNA as well as the phosphorylation of ErbB1. The significant inhibition of the induction of epiregulin expression by AG1478, a specific inhibitor of EGFR kinase activity, suggests the possible existence of an auto-induction of epiregulin, and phosphorylated ErbB1 may be part of the pathway of the auto-induction mechanism. These results also indicate that epiregulin can be listed as one of the auto- or paracrine growth factors for corneal epithelial cells interaction with ErbB1.

Our results also showed that epiregulin served as an effector of similar auto- and cross-induction mechanisms in corneal epithelial cells. Although an up-regulation by epiregulin was limited to HB-EGF and AR in the case of HCECs, the epiregulin mRNA was up-regulated by EGF, HB-EGF, TGF-α, and AR. These findings suggest that epiregulin most likely

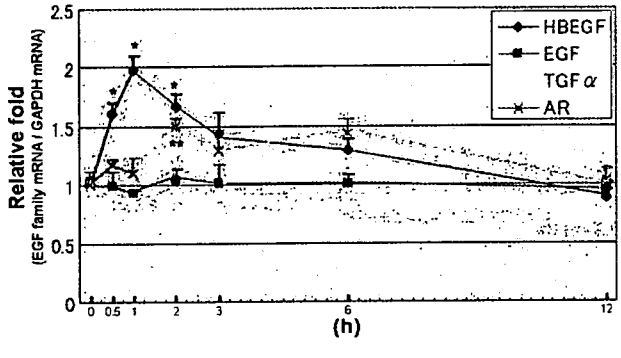


Figure 9. The cross-induction of HB-EGF, TGF-α, EGF and AR mRNA by epiregulin. HCECs were incubated with 10 ng/ml epiregulin for 0.5, 1, 2, 3, 6, and 12 h, and the relative expressions of HB-EGF, TGF-α, EGF, and AR mRNA were examined by real-time PCR. The amount of mRNA was calculated relative to the amount of GAPDH mRNA in the same samples. Asterisks indicate a significant difference from the corresponding control (0 h). P values were calculated by the two-sample t test. Results are mean±SEM of five independent experiments.

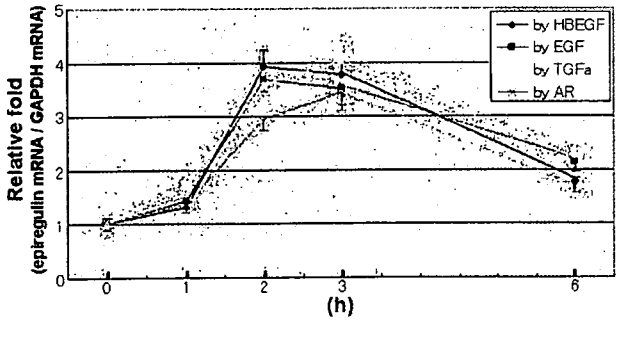


Figure 10. Cross-induction of epiregulin mRNA expression by HB-EGF, EGF, AR, and TGF-α. HCECs were incubated with 10 nM each of EGF, HB-EGF, AR, and TGF-α for 1, 2, 3, and 6 h, and the relative level of expression of epiregulin mRNA was examined by real-time PCR. The amount of mRNA was calculated relative to the amount of GAPDH mRNA in the same samples. Results are the mean±SEM of five independent experiments.

operates in corneal epithelial proliferation in concert with other EGF family members.

The extent of cross-induction differs among the members of the EGF family. It has been reported that each EGF family growth factor has distinct biological functions. The tissue distribution, molecular characteristics, receptor binding, preference to dimerize, and receptor affinity differ among the growth factors [44,47]. Interestingly, Barnard et al. reported results similar to ours that HB-EGF and AR were rapidly induced by EGF-related peptides in the intestinal epithelial cell line, and speculated that differences in the post-receptor processing may contribute to the heterogeneity in the biological responses [47]. TGF-α, HB-EGF, and AR have been reported to be up-regulated on corneal epithelial cells, although the levels of EGF are at trace levels and do not change during the corneal wound healing process [16,52]. Although TGF-α mRNA was not induced by epiregulin under the conditions we employed, it is consistent that HB-EGF and AR were induced in corneal epithelial cells, and these results might be a molecular characteristic of the corneal epithelial cells. Consistent with other reports, EGF was not induced by epiregulin; however, EGF induced the expression of epiregulin in HCECs.

It is known that EGF exists in tear fluid along with HB-EGF and TGF-α, and corneal wounds increase the expression of EGF supplied from lacrimal glands through the sensory nerves [53,54]. Thus, EGF may be able to stimulate corneal epithelial cells, but not be induced in corneal epithelial cells. Together with previous reports, our results suggested that epiregulin may function together with not only endogenously produced EGF family members, but also with those in the tear fluid through cross-induction mechanisms during corneal epithelial wound-healing.

The presence of autocrine or paracrine feedback loops involving EGFR have been documented for the members of the EGF family in different tissues [51]. When it comes to these pathways, an increase in the level of EGFR ligand transcripts is dependent upon the signal from their own receptors, "EGFR" [48-51]. As shown in this study, the phosphorylation of ErbB1 by epiregulin can lead to an increase in transcripts of the other EGF family members. It would be highly efficient for corneal epithelial cells to utilize a common pathway when the integrity of the corneal epithelium is seriously compromised.

In conclusion, epiregulin is present in human corneal epithelial cells and is able to induce HCECs to proliferate as an autocrine growth factor. The intricate cross-induction mechanism involving epiregulin certainly plays a role in a variety of events which occur in the corneal epithelium.

ACKNOWLEDGEMENTS

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Effects of Beta-Adrenergic Antagonist on Width of Nasolacrimal Drainage System Lumen

JUNJI NARIOKA,[1,2] and YUICHI OHASHI[1]

ABSTRACT

Aims: **The aims of this study were to determine the effect of a β-adrenergic antagonist on the width of the nasolacrimal drainage system lumen.**

Methods: **The asymptomatic sides of 18 patients (14 women and 4 men) with unilateral stenosis or obstruction of the nasolacrimal drainage system were studied. The tear meniscus height on the asymptomatic side was normal and the nasolacrimal drainage system was patent, as reveeled by dacryocystography. The nasolacrimal drainage system on the asymptomatic side was infused with 100 μL of 0.5% timolol maleate, and dacryocystography was performed to determine the width of the lumen of the nasolacrimal drainage system.**

Results: **Timolol reduced the width of the lumen significantly, and the changes were more marked in the nasolacrimal duct (NLD), especially the middle and lower regions. The width of the lacrimal sac was not significantly changed.**

Conclusions: **The decrease in the width of the nasolacrimal drainage system lumen, especially the NLD, by a β-adrenergic antagonist suggests that the drainage of tears through the NLD may be influenced, in part at least, by the autonomic nervous system.**

INTRODUCTION

OUR EARLIER STUDIES showed that 5% phenylephrine hydrochloride, an α-1 adrenergic agonist, significantly increased the width of the lumen of the nasolacrimal drainage system, especially the width of the nasolacrimal duct (NLD).[1] On the other hand, 2% pilocarpine hydrochloride, a muscarinic cholinergic agonist, reduced its width.[1] These changes probably resulted from a change in the thickness of the wall of the NLD,[2–10] (i.e., a change in the blood flow and volume of the thick cavernous wall of the NLD probably altered its thickness, and consequently, the width of the lumen.[1,4–10] This cavernous body is also well developed in the nasal mucosa[11,12] and is associated with nasal patency.[13] The mucosa of the membranous NLD gradually attains the morphologic and physiologic characteristics of the nasal mucosa as it approaches the nasal cavity.[1,14]

The rate of blood flow and volume in the nasal mucosa are known to be under autonomic control,[15,16] and there are anastomosis between the dense plexus of the nasal mucosa, the lacrimal sac, and the NLD.[17] Therefore, it is highly likely that the width of the lumen of the NLD is also partly under autonomic control, and α-1 adrenergic and muscarinic cholinergic receptors probably control the width.[1]

The role of β-adrenergic receptors on the nasolacrimal drainage system has not been investi-

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