devices (OVDs) were used, with 8 eyes operated with Viscoat® (dispersive) and 4 eyes operated with Healon V® (viscoadaptive) during viscodissection of DM.

Surgical Technique

Before surgery, pupils were constricted using 3 repeated drops of 3% pilocarpine. Following retrobulbar anesthesia using 2% Xylocaine, diluted (×100) povidone iodide was used for sterilization of the ocular surface, followed by sterilized disposable draping.

Recipient corneas were cut to approximately 2/3 thickness using vacuum trephines (Katena Products, Denville, NJ) with a diameter of 7.5 mm. The superficial stroma was removed by dividing the cornea into several quadrants, followed by lamellar dissections as described previously.[10] The anterior stroma is removed to offer a clear view of the deep stroma during the dissociation of DM described below. Lamellar dissection of the stroma was extended into the surrounding recipient rim, which will facilitate passing sutures into the recipient bed. A short side port was made in the temporal cornea, leaving as much distance from the donor bed as possible to avoid viscodissection extending to the side port wound.

After removal of aqueous fluid from the side port, the anterior chamber was replaced with Millipore-filtered room air. Similar to the method originally described by Melles et al,[8] the blunt end of a microsurgery knife (Ultrasharp 681.01, Grieshaber, Schaffhausen, Switzerland) was used to dissect the remaining stroma down to DM, using the reflection of the knife observed at the air-to-endothelium interface as a guide (Fig. 1A). A DLKP spatula (Maeda DLKP Spatula, Inami Inc, Tokyo, Japan) is bluntly inserted into the loose space between DM and the stromal fibers to create a pocket (Fig. 1B). After creation of a small DM detachment with BSS (Fig. 1C), viscodissection was performed to further extend DM detachment (Fig. 1D). Detachment of DM can be recognized by posterior "doming" of DM, which can be clearly distinguished from hydration of stromal tissue. Stromal hydration by BSS at this point indicates that the initial lamellar incision was not deep enough. If the pocket shown in Figure 1B is large enough to admit a 25-G cannula, then direct viscodissection is also an option. After complete dissection of DM, the overlying stroma is removed to expose the smooth surface of DM (Fig. 2). The area of DM detachment should extend outside the recipient bed because this will provide room for using scissors to remove the remaining deep stroma. DM detachment will extend to the limbus if a large number of OVD are injected; however, this is not necessary to complete the procedure.

Donor cornea was prepared with the Barron donor punch (Katena Products), with a diameter of 7.75 mm, and fine suturing forceps were used strip off DM. The donor button was then placed on the donor bed after residual viscoelastic material had been washed away. After 4 preplaced sutures, a single continuous running suture was performed with 10-0 nylon, with care taken not to perforate DM with the needle tip.

Air was left in the anterior chamber when DM was perforated during surgery. Air was injected until the area of the graft was covered by an air bubble in the supine position. Postoperative treatment included topical levofloxacin (Cravit, Santen Pharmaceuticals, Osaka, Japan) 5 times a day and topical dexamethasone (Sanbetasone, Santen Pharmaceuticals, Osaka, Japan) 5 times a day.

RESULTS

DLKP was successfully performed in 10 out of 12 eyes. Figure 3 shows a representative photograph of DLKP taken

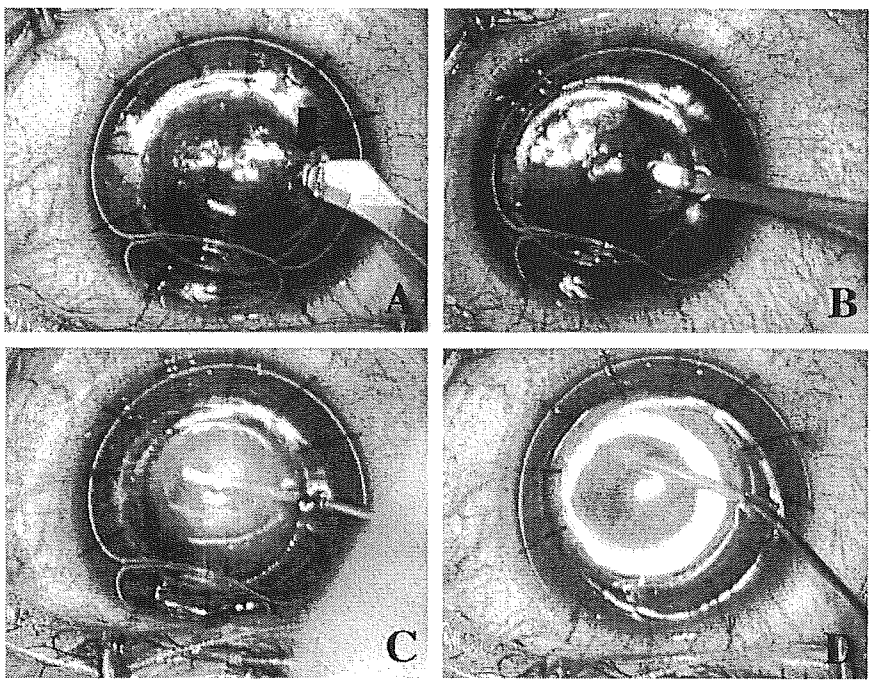

FIGURE 1. A, The blunt end of a microsurgery knife is directed toward DM using the mirror reflection (arrow) of an air bubble in the anterior chamber as a guide. B, A stromal pocket is created using a blunt spatula. C, BSS is injected into the pocket, creating a small DM detachment. D, OVD is injected into the pocket to further dissociate DM.

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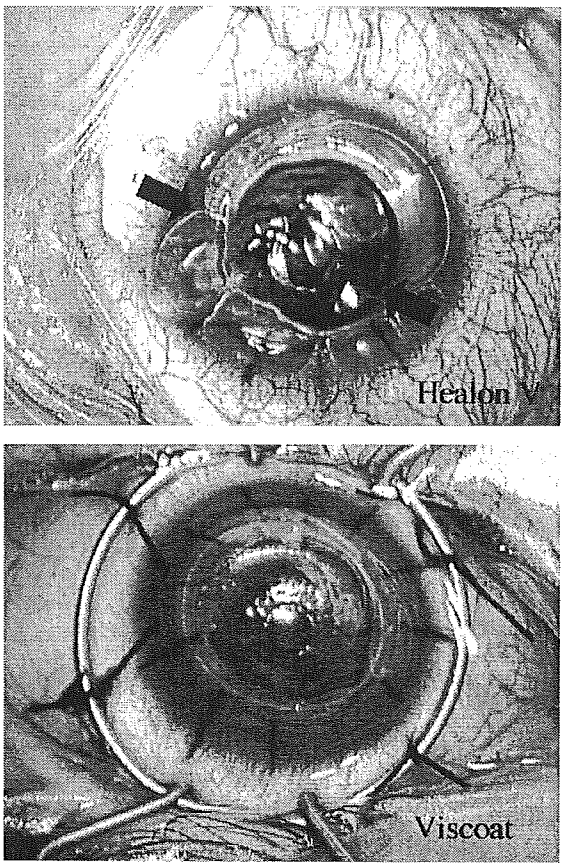

FIGURE 2. A thick layer of Healon V (between arrows) remains on the exposed host bed to protect DM, whereas Viscoat sloughs off to expose DM.

11 months postoperatively, with a clear interface between donor stroma and host DM. Both Viscoat and Healon V were effective during viscodissection. However, a viscoadaptive property of Healon V allows meticulous control of viscodissection, enabling the surgeon to insert the viscoelastic in the desired quadrant. Viscoat creates a concentric wave that extends detachment of DM in all directions. After complete removal of the remaining stroma, the different dynamics of the 2 viscoelastics is reflected in the amount of viscous material remaining on the recipient bed. As shown in Figure 2, Healon V covers DM, acting as a protective layer during handling of remaining tissue. Viscoat, on the other hand, inevitably sloughs off the surface to expose DM to room air.

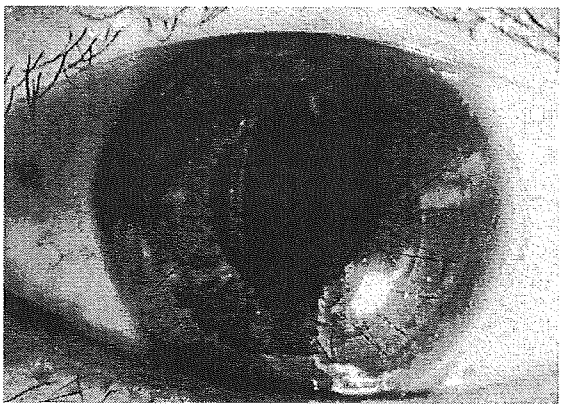

FIGURE 3. Postoperative photograph of DLKP in case 4. Minimal haze is observed in the stroma–DM interface, allowing for optical clarity comparable to that produced by PKP.

Rupture of DM was experienced in 6 cases, 5 of which had been operated using Viscoat. Of these 5 Viscoat cases, perforation occurred in 4 eyes during removal of residual stromal tissue, and in 1 case a large rupture occurred during viscodissection, presumably because of the weight of the bulk amount of Viscoat required to dissociate DM. The 1 case with perforation of the DM using Healon V occurred during suturing of the donor tissue. DLKP was completed in all rupture cases except for cases 3 and 12, where the patient wished to be converted to PKP during surgery. Pseudoanterior chambers were observed in cases 1 and 6, which were treated by additional injection of air into the anterior chamber.

Large tears in DM, even those that expand the entire diameter of the graft, can be managed by air and suturing of DM to the stroma. However, more than 1 procedure may be required to completely attach DM. Therefore, the authors inform patients before surgery as to the options available if a tear in DM occurs and decide whether to complete DLKP or convert to PKP according to what the patient wishes. Table 1 shows the surgical results in all patients.

Average endothelial cell density before surgery was 2995/mm^2, which decreased to 2310/mm^2 after 1 month following surgery. There was no statistical difference in endothelial cell loss between Viscoat and Healon V. None of the patients had episodes of increased ocular pressure following surgery, nor were there any other sequelae of note in any patient to date. Average corrected visual acuity at 1 month postop was 0.74 (approximately 14/20 Snellen), with an average astigmatism of −3.8 diopters. Refractive data were similar in both groups.

DISCUSSION

The surgical technique described in this paper is a modification of the methods reported by Melles et al.[8,9] One major difference is that a lamellar dissection of the diseased cornea is performed at the beginning of surgery. Exposure of DM is therefore performed within the lamellar dissection instead of from a limbal incision as introduced in the original method. The advantage of this approach is that a clearer view of DM is obtained when the stroma is opaque as a result of the original disease, and an additional incision is not required at the peripheral cornea. One drawback is that viscodissection is usually incomplete from the initial stromal pocket; however, this can be circumvented by creating a window in the stroma and adding OVD from any convenient location to dissociate segments of DM still attached to the stroma.

Complete exposure of DM is a fragile state, which may lead to higher incidence of DM rupture.[9] The overall rupture rate in the present study was 50%, which is slightly higher than those of previous studies.[7,14] However, comparing previous data can be confusing because of slightly different definitions

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TABLE 1. Summary of Data on Cases

Case	Surgery	OVD	DM Rupture	Reason for DM Rupture	BCVA	Endo (preop)	Endo (1 mo)	Astigamatism (1 mo) (Diopters)
1	DLKP	Viscoat	+	Host dissection	0.4	NA	906	−2.5
2	DLKP	Viscoat	−		1.0	3496	2380	−5
3	PKP	Viscoat	+	Host dissection	0.6	NA	NA	NA
4	DLKP	Viscoat	+	Host dissection	0.4	NA	3533	−2.5
5	DLKP	Viscoat	−		0.6	3355	3367	−4.5
6	DLKP	Viscoat	+	viscodissection	0.05	NA	713	−5.5
7	DLKP	Viscoat	−		0.8	3424	1795	−6
8	DLKP	Healon V	+	suture	0.9	2695	2358	−3
9	DLKP	Healon V	−		0.3	2785	2645	−1.5
10	DLKP	Healon V	−		0.8	2564	2463	−3
11	DLKP	Healon V	−		1.2	3105	2941	−4.5
12	PKP	Viscoat	+	Host dissection	0.8	NA	NA	NA

DLKP, deep lamellar keratoplasty; PKP, penetrating keratoplasty; OVD, ophthalmic viscosurgical device; Endo, endothelial density; NA, not available or does not apply (converted to PKP).

as to how "deep" DLKP was actually performed. Scarring of residual stromal tissue may leave a slight "haze" at the donor interface, which may affect visual acuity or contrast sensitivity.[13] All such concerns can be ignored if a "true" DLKP can be performed without the increased risk of DM rupture.

Although our study is too small for statistical analysis, none of the cases using Healon V experienced DM rupture during removal of host tissue. The 1 case that had a small perforation of DM was during suturing of host tissue. Suture complications can occur regardless of surgical technique but can be managed by air injection or direct suturing of DM at the area of perforation. The viscoadaptive quality of Healon V allows for injection with the minimum amount of OVD required to dissect the required area of DM. This should minimize the risk of a large tear observed in case 6, which could not bear the amount of OVD injected beneath the domed cornea of keratoconus. Furthermore, Healon V remains on the corneal surface after all of the overlying stroma has been removed, as shown in Figure 2. Lowering intraocular pressure by adjusting the amount of air within the anterior chamber will allow most of the exposed DM to be covered by Healon V. This is not the case with other OVDs because of the different viscous properties of these products. The presence of this thick layer of Healon V protects the DM from further damage during trimming maneuvers of the overlying stroma as well as during preparation of the donor. Only immediately before suturing is the DM completely exposed to open air. However, care must be taken to wash away all of the OVD from the DM surface to avoid trapping of OVD in the interface. This can be confirmed by observing a complete circular fluid meniscus along the circumference of the recipient bed after removal of excess fluid with a surgical sponge.

Endothelial loss varied among the cases, and the eyes with more cell loss seem to be those suffering from pseudoanterior chambers (cases 1 and 6). A large-scale retrospective study is currently under way to investigate the relationship between postoperative complications and endothelial cell loss. With the use of viscoadaptive OVDs, surgery time for DLKP can be reduced to what is required for PKP. If a fresh donor is available, surgery can be easily converted to PKP to avoid any delays in visual rehabilitation. Although the present study was not designed to evaluate refraction, the early postoperative data (1 month) seem to show that the rate of visual rehabilitation is promising. We are currently undertaking a prospective study to determine the safety and efficacy of DLKP, using viscoadaptive OVDs as standard protocol to dissociate Descemet membrane.

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Critical Role of the Fifth Domain of E-Cadherin for Heterophilic Adhesion with $\alpha_E\beta_7$, But Not for Homophilic Adhesion

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The integrin $\alpha_E\beta_7$ is expressed on intestinal intraepithelial T lymphocytes and CD8$^+$ T lymphocytes in inflammatory lesions near epithelial cells. Adhesion between $\alpha_E\beta_7{}^+$ T and epithelial cells is mediated by the adhesive interaction of $\alpha_E\beta_7$ and E-cadherin; this interaction plays a key role in the damage of target epithelia. To explore the structure-function relationship of the heterophilic adhesive interaction between E-cadherin and $\alpha_E\beta_7$, we performed cell aggregation assays using L cells transfected with an extracellular domain-deletion mutant of E-cadherin. In homophilic adhesion assays, L cells transfected with wild-type or a domain 5-deficient mutant formed aggregates, whereas transfectants with domain 1-, 2-, 3-, or 4-deficient mutants did not. These results indicate that not only domain 1, but domains 2, 3, and 4 are involved in homophilic adhesion. When $\alpha_E\beta_7{}^+$ K562 cells were incubated with L cells expressing the wild type, 23% of the resulting cell aggregates consisted of $\alpha_E\beta_7{}^+$ K562 cells. In contrast, the binding of $\alpha_E\beta_7{}^+$ K562 cells to L cells expressing a domain 5-deficient mutant was significantly decreased, with $\alpha_E\beta_7{}^+$ K562 cells accounting for only 4% of the cell aggregates, while homophilic adhesion was completely preserved. These results suggest that domain 5 is involved in heterophilic adhesion with $\alpha_E\beta_7$, but not in homophilic adhesion, leading to the hypothesis that the fifth domain of E-cadherin may play a critical role in the regulation of heterophilic adhesion to $\alpha_E\beta_7$ and may be a potential target for treatments altering the adhesion of $\alpha_E\beta_7{}^+$ T cells to epithelial cells in inflammatory epithelial diseases. *The Journal of Immunology,* **2005, 175: 1014–1021.**

E-cadherin, a classic member of the cadherin superfamily, is expressed on epithelial cells and mediates Ca^{2+}-dependent homophilic cell-cell adhesion (1–3). Classic cadherins contain five extracellular domains (ECs)[2] of ~110 aa each, a transmembrane domain, and a cytoplasmic domain. Structure-function analyses of the homophilic interactions of E-cadherin have largely focused on the NH_2-terminal EC domain (EC1), which contains a highly conserved His-Ala-Val motif (4–7). Indeed, protein fragments or peptides containing the His-Ala-Val sequence exerted limited effects on cell-cell adhesion (8, 9). Recently, crystallographic analysis has clearly demonstrated that conserved Trp in the EC1 domains of classical cadherins is critical for *trans*-interactions between E-cadherin molecules on different cells, serving as a strand dimer (10). However, several studies have suggested that ECs may be involved in cell adhesion in ways other than the role mediated by EC1. In human cancers, for example, E-cadherin gene mutations frequently occur in exons 7, 8, and/or 9 (corresponding to EC2 and EC3); these mutations are thought to result in the loss of the ability to undergo cell-cell adhesion (11–14). A study on the binding properties of the soluble C-cadherin ectodomain suggested that EC1 was not sufficient for complete homophilic binding (15). Furthermore, Corada et al. (16, 17) demonstrated that mAbs directed against EC3-EC4 affected VE-cadherin adhesion in endothelial cells.

The heterophilic interaction of E-cadherin and integrin $\alpha_E\beta_7$ has been previously documented (18–24). Integrin $\alpha_E\beta_7$ is expressed selectively on intestinal intraepithelial T lymphocytes under physiological conditions (25). Accumulating evidence indicates that $\alpha_E\beta_7{}^+$ is induced on T lymphocytes in the epithelia of skin, lung, salivary, and lacrimal glands and synovial membranes during inflammation (26–31), suggesting that this heterophilic interaction has a pathologic role. Since $\alpha_E\beta_7$ may have an important role in the selective localization or retention of a unique population of T cells in a specific tissue, the adhesion between $\alpha_E\beta_7$ and E-cadherin could be a potential target of therapeutic interventions for epithelial inflammation. Substitutions of a highly exposed and charged amino acid on mouse E-cadherin transfected into L cells demonstrated that Glu^{31} in EC1 is critical for binding with $\alpha_E\beta_7$ (32). Taraszka et al. (33) also elucidated that the substitution of the corresponding Glu in EC1 of human E-cadherin-Fc fusion proteins abrogated binding with $\alpha_E\beta_7$, confirming the previous observations. These studies clearly show that the specific heterophilic adhesion attributed to the exposed Glu^{31} in EC1 differs from homophilic adhesion. With the exception of EC1, however, the structures involved in heterophilic adhesion remain uncertain. To clarify the involvement of the EC domains in both heterophilic and homophilic adhesion, we performed cell aggregation assays using L cells transfected with specific domain-deleted E-cadherin mutations. The present report speculates on the characteristics of the E-cadherin domains involved in homophilic interactions and heterophilic interactions with $\alpha_E\beta_7$.

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Received for publication January 5, 2005. Accepted for publication May 13, 2005.

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[2] Abbreviations used in this paper: EC, extracellular domain; DiO, 3,3′-deoctadecyl-5, 5′-di(4-sulfophenyl)oxacarbocyanine sodium salt.

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0022-1767/05/$02.00

Materials and Methods

Cells

Mouse L-K (TK^-) fibroblasts were cultured in DMEM containing 10% FBS. K562 cells were cultured in RPMI 1640 containing 10% FBS. K562 cells double-transfected with α_E and β_7 were a gift from Dr. D. Erle (University of California, San Francisco, CA) and cultured in RPMI 1640 supplemented with 10% FBS, 500 μg/ml hygromycin B, and 500 μg/ml G418 (34).

Construction of E-cadherin deletion mutants

The expression vector containing human E-cadherin cDNA was a gift from Dr. Y. Shimoyama (National Okura Hospital, Tokyo, Japan). To examine the contribution of the different ECs to adhesion, the EC1, EC2, EC3, EC4, or EC5 domains or all of the EC domains were deleted using inverse PCR. Fig. 1 shows the domain deletion mutants (Δ) and the primers designed for the PCR experiments. The following primers were used: for Δ1, 5′-TCTCTTCTGTCTTCTGAGGCCAGGAGAGG-3′ (SIG-R) and 5′-ACCCAGGAGGTCTTTAAGGGGTCTG-3′ (2-F); for Δ2, 5′-GAATTCGGGCTTGTTGTCATTCTG-3′ (1-R) and 5′-AATCCCACCACGTACAGGGTCAG-3′ (3-F); for Δ3, 5′-GAAGATCGGAGGATTATCGTGGT-3′ (2-R) and 5′-GTGCCTCCTGAAAAGAGAGTGGAA-3′ (4-F); for Δ4, 5′-AGGCACAAAGATGGGGGCTTCATTCAC −3′ (3-R) and 5′-GAACCTCGAACTATTCTTCTGT-3′(5-F);forΔ5,5′-TGGTATGGGGGCGTTGTCATTCAC-3′ (4-R) and 5′-ATTCCTGCCATTCTGGGGATTCTTGGAG-3′ (TM-F); and for d0, SIG-R, and TM-F. Amplicons were ligated to form circular plasmids. The structures of the deletion mutants were confirmed by sequencing. Expression vectors bearing wild-type or mutated E-cadherin cDNAs were transfected into L cells using LipofectAMINE reagent (Invitrogen Life Technologies). The transfected cells were selected in DMEM supplemented with 10% FBS and 700 μg/ml G418, and colonies expressing high levels of E-cadherin were screened by Western blot analysis using the mAb 4A2C7 (Zymed).

RT-PCR

One microgram of total RNA from the L transfectants was used to generate single-stranded cDNA using AMV Reverse Transcriptase XL (Takara Bio). The cDNAs were amplified by PCR using Ex *Taq* polymerase (Takara Bio) and the following primers: for EC1, 5′-GACTGGGTTATTCCTCCCATCAGC-3′ (1-F) and 1-R; for EC2, 2-F, and 2-R; for EC3, 3-F, and 3-R; for EC4, 4-F, and 4-R; and for EC5, 5-F, and 5′-TTGCAATCCTGCTTCGACAGGCTGTGC-3′ (5-R).

Western blot

Cultured cells were rinsed in PBS and harvested in mammalian protein extraction reagent (Pierce). The total protein concentration was determined using a Micro BCA protein assay reagent kit (Pierce). GST fusion proteins containing the EC domains were produced in TOP10F′ cells carrying pGEX-4T-2 vectors and purified using GST purification modules (Amersham Biosciences). Cell lysates and the purified fusion proteins were subjected to SDS-PAGE and transferred to an Immobilon-P membrane (Millipore).

The membrane was blocked with 5% skim milk and incubated with the primary Abs. Goat anti-GST Ab was purchased from Amersham Pharmacia. Four different mouse mAbs against human E-cadherin were used. The binding epitopes recognized by SHE78-7 and HECD-1 (Takara Bio) were unknown. 4A2C7 was generated against a recombinant protein corresponding to the cytoplasmic domain, whereas G-10 (Santa Cruz Biotechnology) was raised against a recombinant protein corresponding to aa 600–707. The membrane was then incubated with the respective HRP-conjugated secondary Abs followed by an ECL system (Amersham Biosciences) and analyzed using LAS-1000 (Fuji film).

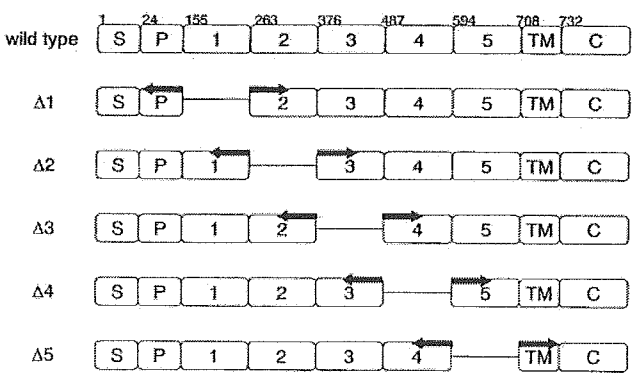

FIGURE 1. Schematic representation of the E-cadherin domain-deletion mutants and the PCR primers. Wild-type E-cadherin has five extracellular cadherin repeats, numbered 1–5 from the NH_2-terminal. The numbers above the columns indicate the amino acid number, counting from the start of the coding region. The arrows represent the primers used for inverse PCR. S, Signal peptide; P, propeptide; TM, transmembrane domain; C, cytoplasmic domain.

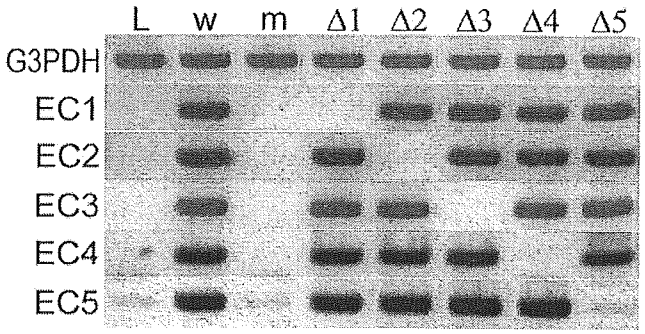

FIGURE 2. Expression of EC mRNA in L cells transfected with E-cadherin domain-deletion mutations. The primers were designed to amplify the various ECs. The mRNAs from parent L cells (L) and L transfectants with wild-type (w), mock (m), and mutated E-cadherin were analyzed using RT-PCR.

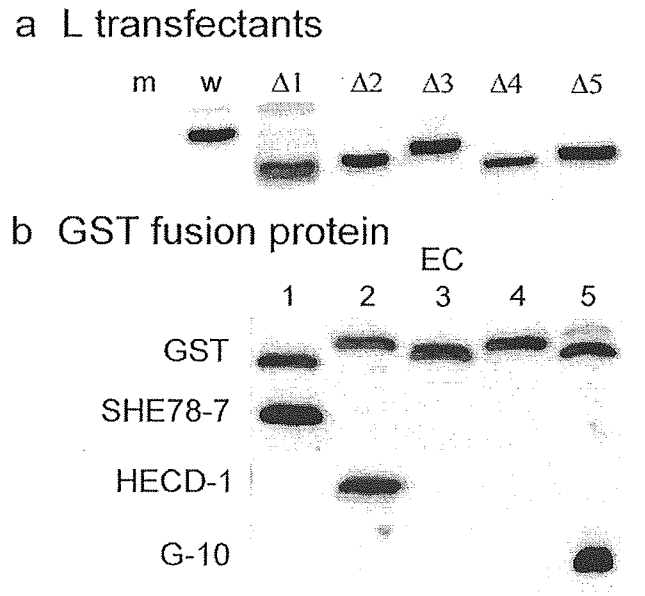

FIGURE 3. *a*, Immunoblot analysis of mutated E-cadherin expression in transfectants. Protein extracts of L cells transfected with mock (m), wild-type (w), and mutated E-cadherin were separated on a 7.5% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was then incubated with anti-E-cadherin Ab 4A2C7, which visualized the 120-kDa band of wild-type E-cadherin. *b*, Identification of the binding epitopes recognized by the mAbs. Purified GST fusion proteins containing the ECs were separated on a 7.5% polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The membranes were incubated with Abs against GST and E-cadherin (SHE78-7, HECD-1, and G-10).

Flow cytometry

The cell surface expression of E-cadherin in the transfectants was examined using flow cytometry. Adherent L transfectants were treated with 0.05% trypsin and 0.02% EDTA at 37°C for 10 min and then washed with DMEM containing 10% FBS. After washing with PBS, the cell suspension was incubated with anti-E-cadherin Ab or an isotype control on ice for 30 min, washed with PBS, and subjected to incubation with FITC-conjugated secondary Ab on ice for 30 min. Ten thousand stained cells were then analyzed using FACSCalibur and the CellQuest software (BD Biosciences).

Cell aggregation assays

The cell aggregation assays were performed according to a method described by Shimoyama et al. (35). Briefly, after the treatment of the adherent cells with 0.05% trypsin and 0.02% EDTA at 37°C for 10 min, the cells were washed and resuspended with DMEM containing 1.8 mM Ca^{2+} and 0.8 mM Mg^{2+} plus 10% FBS. The suspended cells were collected by centrifugation, washed with PBS, and resuspended with the above-mentioned medium.

To examine the homophilic interactions of E-cadherin, 10^5 L transfectants were added to each well of a 24-well plastic plate and incubated with rocking at 37°C overnight in a humidified atmosphere comprised of 7% CO_2 and 93% air. The resulting cell aggregates were examined using a phase-contrast microscope (Olympus).

To examine the heterophilic interactions between E-cadherin and $\alpha_E\beta_7$, L transfectants and K562 transfectants were used in a coaggregation assay. The former transfectants were labeled with 3,3′-dioctadecyl-5, 5′-di(4-sulfophenyl)oxacarbocyanine sodium salt (DiO; Molecular Probes), and the latter were labeled with CellTracker CM-DiI (Molecular Probes). The transfectants were then mixed (5×10^4 cells each), incubated as described above, and observed using a confocal laser-scanning microscope (TCS SP2; Leica). Mg^{2+} (1 mM) was added to the medium when indicated. In some experiments, the proportion of $\alpha_E\beta_7{}^+$ K562 cells in each aggregate was calculated. Micrographs of eight aggregates were taken in each assay. The numbers of K562 and L cells were counted, and the results were expressed as the percentage of K562 cells out of the total number of cells in the aggregate.

In the Ab-blocking experiments, the cells were incubated in the presence of 0.8 μg/ml of appropriate Ab.

Immunocytochemistry

Following the cell aggregation assays, the cell aggregates were collected and washed with PBS. For immunofluorescence staining, the aggregates were incubated with anti-E-cadherin Ab (HECD-1). Subsequently, the samples were incubated with Alexa Fluor 568-conjugated goat anti-mouse IgG Ab (Molecular Probes), followed by incubation with FITC-conjugated anti-$\alpha_E\beta_7$ Ab (Beckman Coulter). The specimens were observed using a confocal laser-scanning microscope.

Results

To confirm whether the transfectants expressed the desired region of E-cadherin, RT-PCR was performed for EC1, EC2, EC3, EC4, and EC5. Fig. 2 shows that the parent L cells did not exhibit any products amplified by human E-cadherin and that all five ECs were expressed in wild-type transfectants. All clones transfected with domain-deletion mutations of E-cadherin exhibited the expected expression patterns: Δ1 lacked EC1, Δ2 lacked EC2, Δ3 lacked EC3, Δ4 lacked EC4, and Δ5 lacked EC5.

The protein expression of mutated E-cadherin was examined by Western blot analysis using the appropriate Abs (Fig. 3*a*). 4A2C7, raised against a recombinant protein corresponding to the cytoplasmic domain, detected all of the deletion mutants. G-10, an alternative E-cadherin Ab that cross-reacts with mouse form (manufacturer's data), could not detect endogenous mouse E-cadherin in mock transfectants (data not shown). To clarify the binding epitopes recognized by the SHE78-7, HECD-1, and G-10 Abs, GST fusion proteins containing the EC domains were analyzed (Fig. 3*b*). SHE78-7 reacted with EC1, whereas HECD-1 reacted with EC2. G-10, raised against a recombinant protein corresponding to EC5, correctly detected EC5.

The expression of the mutated E-cadherin proteins on the cell surfaces of L transfectants was analyzed using flow cytometry (Fig. 4). Approximately 90% of the wild-type and Δ1, Δ3, Δ4, and Δ5 transfectants stained positive when incubated with HECD-1, but the Δ2 transfectants did not. In contrast, the Δ2 transfectants stained positive when incubated with SHE78-7 (Fig. 4); the wild-type, Δ3, Δ4, and Δ5 transfectants also stained positive when incubated with SHE78-7 (data not shown). These results show that the mutated E-cadherin proteins were expressed on the outer cell surface, indicating that protein transport to the plasma membrane was not impaired by the mutations.

To examine the homophilic interactions of the mutated E-cadherin proteins, L transfectants were subjected to a cell aggregation assay (Fig. 5). Mock transfectants were dispersed, whereas L cells expressing wild-type E-cadherin formed closely packed aggregates. Aggregation was strongly inhibited by the addition of SHE78-7 to the assay medium. HECD-1 also inhibited cell aggregation, but its inhibitory effect was not as strong as that of SHE78-7; G-10 did not have any effect on the aggregation. These results indicate that the adhesion of the L cells was mediated through E-cadherin, especially through the EC1 and EC2 domains, but not through the EC5 domain. Higher concentrations of Abs did not influence the results (data not shown).

Next, we examined the interaction of L cells transfected with domain-deletion mutants (Fig. 6). Cell aggregation was completely inhibited not only in the Δ1 transfectants, but also in the Δ2, Δ3, and Δ4 transfectants. In contrast, EC5-deficient transfectants formed cell aggregates. SHE78-7 and HECD-1 Abs also inhibited Δ5 aggregation (Fig. 6, *f* and *g*), indicating that Δ5 aggregates were similar to those produced by wild-type transfectants.

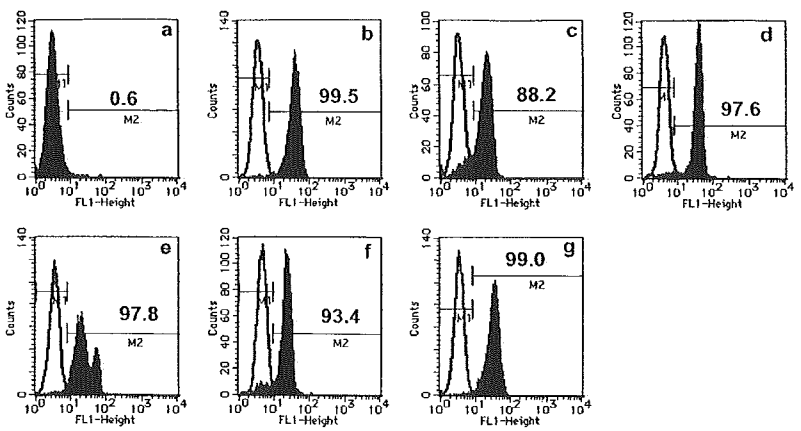

FIGURE 4. Flow cytometry analysis of E-cadherin expression on the cell surface of L transfectants. Mock (*a*), wild-type (*b*), Δ1 (*c*), Δ3 (*e*), Δ4 (*f*), and Δ5 (*g*) transfectants were stained with HECD-1, and Δ2 (*d*) transfectants were stained with SHE78-7. Open histograms indicate background labeling obtained with isotype controls, whereas filled histograms indicate labeling with the Ag-specific Abs.

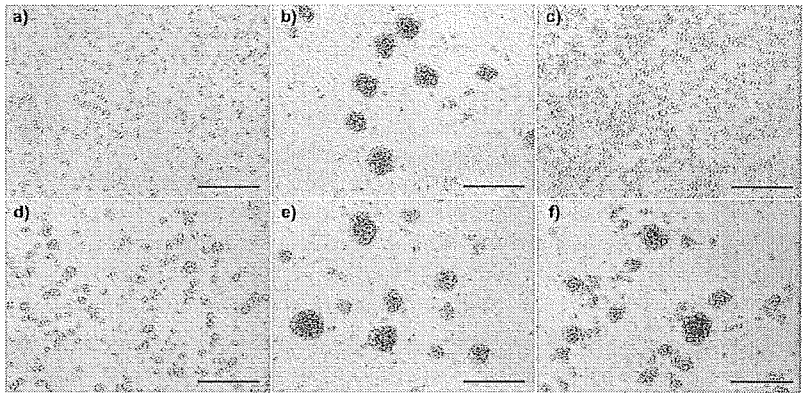

FIGURE 5. Homophilic interaction of wild-type E-cadherin. L cells transfected with mock (*a*) and wild-type (*b*) E-cadherin were used in cell aggregation assays. Wild-type transfectants were incubated in the presence of the anti-E cadherin mAbs SHE78-7 (*c*), HECD-1 (*d*), G-10 (*e*), or an isotype control (*f*). Bars, 300 μm.

To analyze heterophilic interactions, $\alpha_E\beta_7^+$ K562 cells and wild-type E-cadherin$^+$ L cells were mixed. Initially, the $\alpha_E\beta_7^+$ K562 cells did not adhere with the E-cadherin$^+$ L cells; in other words, only the E-cadherin$^+$ L cells adhered and formed cell aggregates (Fig. 7*a*). Thus, we modified the concentration of divalent cations in the assay medium, which originally contained Ca^{2+} and Mg^{2+}. Increasing the Ca^{2+} or Mg^{2+} concentrations had no effect on the aggregate constituents (data not shown). In contrast, Mn^{2+} supplementation led to a number of $\alpha_E\beta_7^+$ K562 cells being included in the cell aggregates (Fig. 7*a*). To confirm this observation, the numbers of the two cell types in each aggregate were counted. As shown in Fig. 8, the percentage of $\alpha_E\beta_7^+$ K562 cells in the cell aggregates significantly increased with Mn^{2+} supplementation (from 5.0 ± 0.8% without Mn^{2+} supplementation to 22.8 ± 0.9% with Mn^{2+} supplementation). Immunofluorescence staining of the cell aggregates clearly showed that E-cadherin and $\alpha_E\beta_7$ were localized on the cell membranes and in close contact (Fig. 7*b*). In subsequent coaggregation assays, Mn^{2+} was added to the assay medium.

The adhesion of $\alpha_E\beta_7^+$ K562 cells to E-cadherin$^+$ L cells was clearly inhibited by the presence of mAb against $\alpha_E\beta_7$, with the percentage of $\alpha_E\beta_7^+$ K562 cells in the cell aggregates decreasing to 2.9 ± 1.1% (Figs. 8 and 9*a*). This result shows that the $\alpha_E\beta_7^+$ K562 cells adhere to the E-cadherin$^+$ L cells via $\alpha_E\beta_7$ during cell aggregation. SHE78-7 inhibited aggregation in the heterophilic assay (Fig. 9*b*), whereas HECD-1 allowed the formation of some aggregates in which $\alpha_E\beta_7^+$ K562 cells were adhered to E-cadherin$^+$ L (23.0 ± 3.1%, Figs. 8 and 9*c*). G-10 did not inhibit the adhesion of the $\alpha_E\beta_7^+$ K562 cells to E-cadherin$^+$ L (25.9 ± 1.2%, Figs. 8 and 9*d*).

Next, the interactions of $\alpha_E\beta_7$ and the mutated E-cadherin proteins were examined. When $\alpha_E\beta_7^+$ K562 cells were mixed with the mock transfectants, no cell aggregates were formed (Fig. 10*a*). Similarly, the Δ1, Δ2, Δ3, or Δ4 transfectants and $\alpha_E\beta_7^+$ K562 cells formed no cell aggregates (data not shown). The mixture of $\alpha_E\beta_7^+$ K562 cells and Δ5 transfectants produced cell aggregates, but the percentage of $\alpha_E\beta_7^+$ K562 cells in the aggregates was significantly lower than that in aggregates formed from the mixture of $\alpha_E\beta_7^+$ K562 cells and wild-type transfectants (4.0 ± 0.9% vs 22.8 ± 0.9%; Figs. 8 and10*b*).

Discussion

In this study, we attempted to clarify the overall binding capability of E-cadherin for both homophilic adhesion and heterophilic adhesion to the integrin $\alpha_E\beta_7$ through the generation of domain-deletion E-cadherin mutants and the transfection of these mutants into L cells to determine the roles of the individual domains in cell adhesion. EC1, EC2, EC3, and EC4 domain-deletion mutants lost their homophilic binding ability, suggesting that these EC domains are indispensable for homophilic adhesion. Substantial evidence

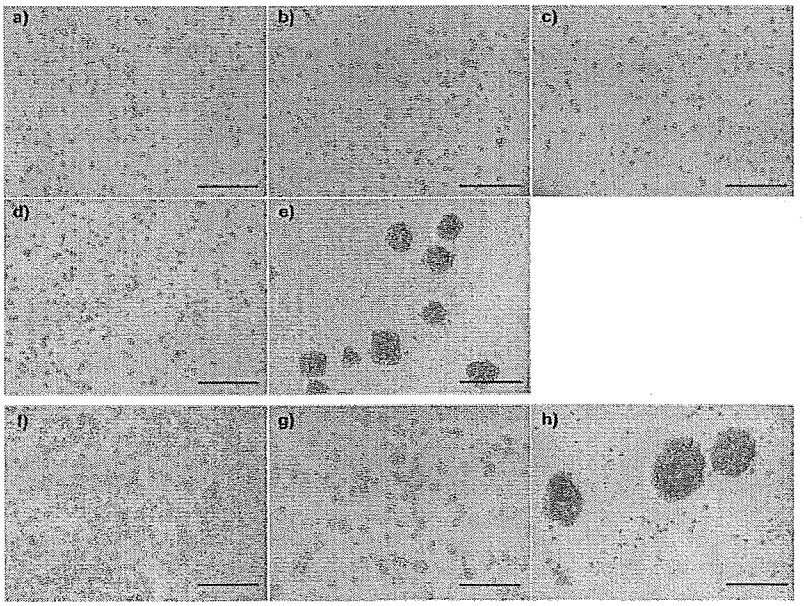

FIGURE 6. Homophilic interactions of domain-deletion mutants. L cells transfected with Δ1 (*a*), Δ2 (*b*), Δ3 (*c*), Δ4 (*d*), and Δ5 (*e*) were used in a cell aggregation assay. Δ5 transfectants were incubated with SHE78-7 (*f*), HECD-1 (*g*), or an isotype control (*h*). Bars, 300 μm.

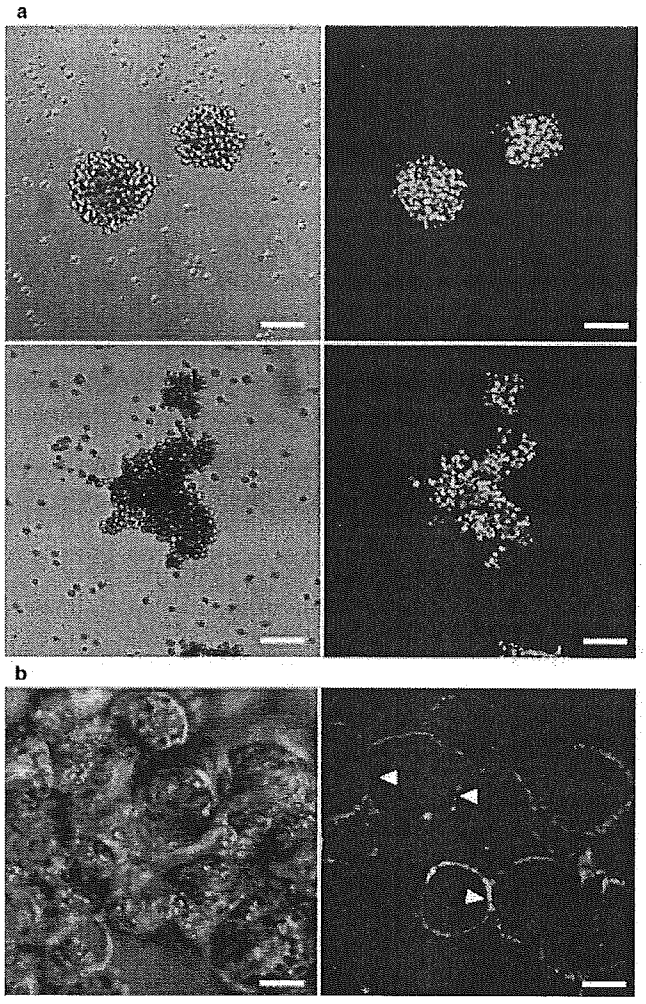

FIGURE 7. *a,* Heterophilic interaction of wild-type E-cadherin with $\alpha_E\beta_7$. Following the labeling of E-cadherin$^+$ L cells with DiO (green) and $\alpha_E\beta_7{}^+$ K562 cells with DiI (red), the cells were allowed to aggregate in assay medium with (*lower*) or without (*upper*) 1 mM Mn^{2+}. The dye localization into cytoplasmic vesicle made DiI-labeled K562 cells look smaller than they really are, while the plasma membrane of L cells was successfully labeled by DiO. Bars, 100 μm. *b,* Immunofluorescence detection of heterophilic aggregates. Cell aggregates were stained with anti-E-cadherin Ab, followed by an Alexa Fluor 568-conjugated secondary Ab, and with FITC-conjugated anti-$\alpha_E\beta_7$ Ab. E-cadherin (red) and $\alpha_E\beta_7$ (green) were localized on the cell membrane and in close contact with each other (arrowheads). Nomarski differential interference micrographs are shown to the *left* of each fluorescent photograph. Bars, 8 μm.

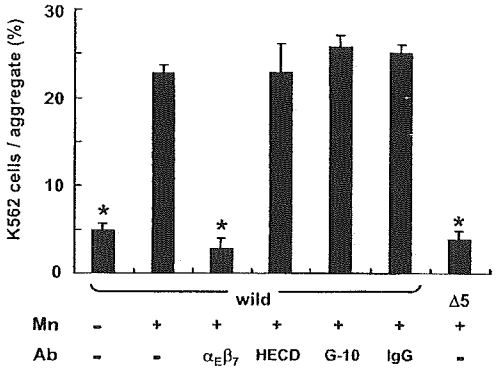

FIGURE 8. Effects of manganese, mAbs, and domain-deletions on heterophilic interactions. The numbers of K562 and L cells were counted, and the results were expressed as the percentage of K562 cells out of the total number of cells in the cell aggregate. Results are shown as the mean ± SEM (n = 8). Student's t test was used for the statistical analysis. The asterisks indicate a significant difference of $p < 0.001$, compared with wild-type transfectants cultured in the presence of Mn^{2+}.

suggests that the combination of *cis*-dimerization of two cadherin molecules on the same cell surface and *trans*-interactions between cadherin dimers on opposing cell surfaces maximizes homophilic adhesion. The widely accepted linear zipper model attributes the adhesive interfaces in the *cis*- and *trans*-interactions to EC1 (6, 7, 36). However, recent studies have introduced a new model for homophilic adhesion. Through the analysis of domain deletions in the *Xenopus* C-cadherin ectodomain using bead aggregation and cell adhesion assays, Clappuis-Flament et al. (15) demonstrated that the combination of at least three EC domains, such as EC1-EC2-EC3 or EC1-EC2-EC4, was required for *trans*-interaction and proposed an alternative model in which multiple ECs are required to achieve full adhesive capability. Several studies have provided data to support this model, suggesting the existence of multiple adhesive interfaces. For example, a mAb recognizing a potential epitope spanning the EC3–EC4 domains affected VE-cadherin adhesion in endothelial cells from umbilical veins (16). A mutant lacking the EC1 domain failed to exhibit homophilic adhesion, although the mutant could adhere to other mutants expressing wild-type E-cadherin (37). In contrast, a crystallographic analysis of C-cadherin supported the linear zipper model by showing that EC1 interacts with EC2 on other E-cadherin molecules on the same cell surface, leading to *cis*-dimerization (10). Our results showing that EC1- or EC2-deficient mutants failed to exhibit full homophilic adhesion are basically consistent with the above findings. However, mutants lacking either the EC3 or EC4 domains also failed to exhibit maximum adhesion. The fact that the present study used mammalian cadherin, whereas the previous study used *Xenopus* cadherin, may partially explain this discrepancy in findings. Another possible explanation may be that intracellular events were affected by the domain deletion in our aggregation assay, since the transmembrane and cytoplasmic domains were included in the domain-deletion human E-cadherin mutants. Compelling evidence suggests that the adhesive strength of cadherin is regulated by the transmembrane and cytoplasmic domains (3, 38–42). For example, the cytoplasmic tail of cadherin interacts with β-catenin and p120, which link cadherin to the actin cytoskeleton through α-catenin, and the regulation of the cadherin-catenin complex by diverse phosphorylation reactions influences the adhesive function of cadherin (43). In addition, the interaction of a motif in domain 4 of N-cadherin with the fibroblast growth factor receptor is required for neurite outgrowth (44), suggesting that EC4 may be involved in a cell signaling pathway in which fibroblast growth factor receptor controls the gene transcription and adhesive activity of cadherin via Snail, resulting in a loss of cell-cell adhesion (43). To the best of our knowledge, little information is available about the involvement of the membrane proximal domain EC5 in homophilic adhesion. EC5 might not participate in the adhesive bond, possibly explaining the preservation of homophilic adhesion in EC5-deficient mutants. Alternatively, conformational changes in E-cadherin resulting from the EC5 deletion may cause cell aggregation via a process different from that occurring with the native molecule. However, current data that the same mAbs inhibiting homotypic aggregates by wild-type transfectants (SHE78-7, HECD-1) also inhibited those by the Δ5 mutant would be evidence that the process of cell aggregation is similar.

Consistent with previous studies (21, 22, 45), we found that Mn^{2+} stimulated heterophilic interactions between $\alpha_E\beta_7$ and E-cadherin.

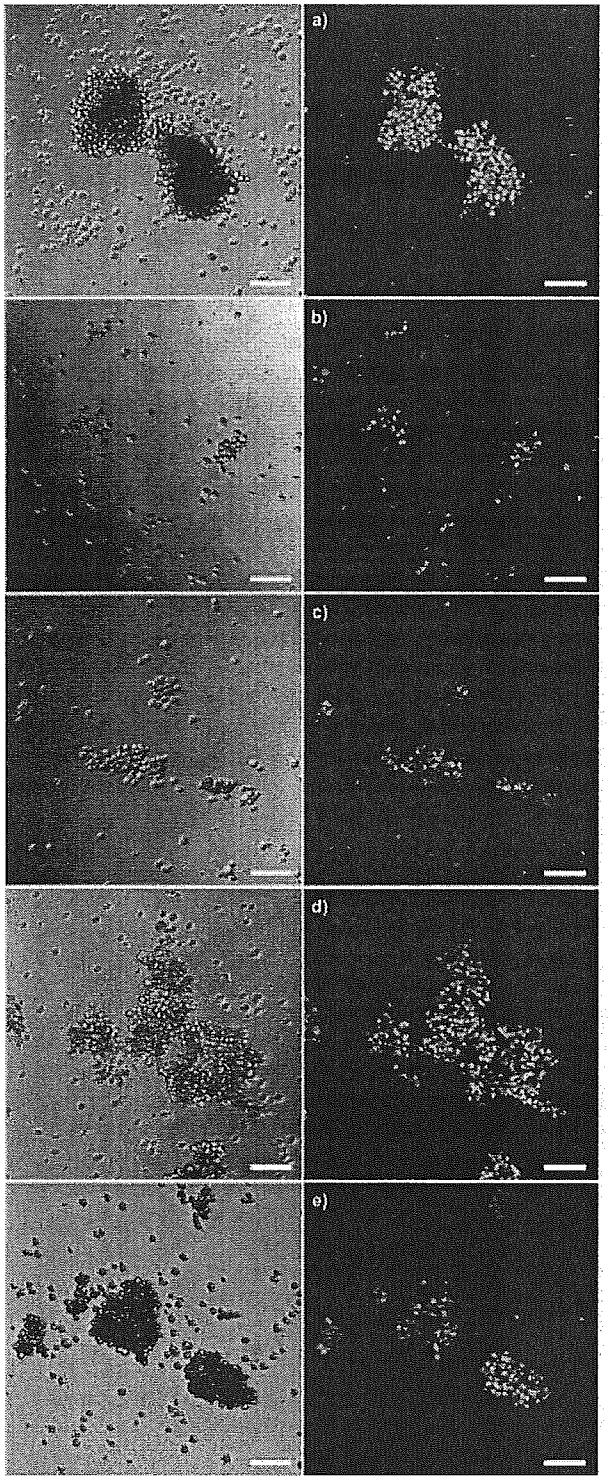

FIGURE 9. Heterophilic interaction of E-cadherin with $\alpha_E\beta_7$. Following the labeling of E-cadherin$^+$ L cells with DiO (green) and $\alpha_E\beta_7{}^+$ K562 cells with DiI (red), the cells were allowed to aggregate in assay medium containing 1 mM Mn^{2+} in the presence of anti-$\alpha_E\beta_7$ Ab (*a*) or one of the anti-E-cadherin Abs SHE78-7 (*b*), HECD-1 (*c*), or G-10 (*d*) or an isotype control (*e*). Nomarski differential interference micrographs are shown to the *left* of each fluorescent photograph. Bars, 100 μm.

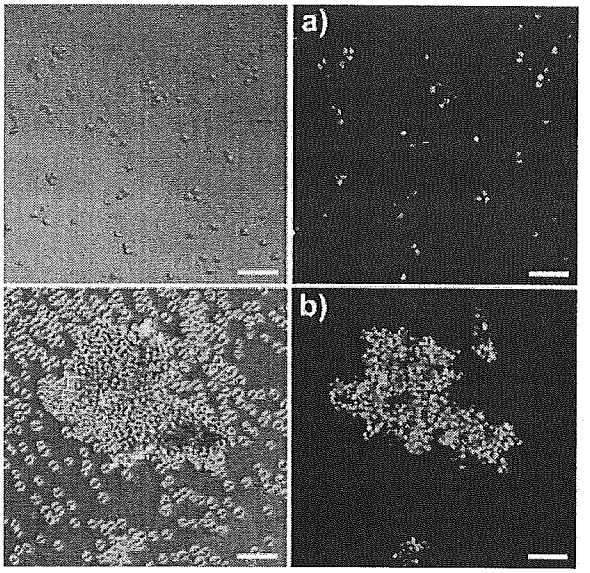

FIGURE 10. Heterophilic interactions of E-cadherin-deletion mutants with $\alpha_E\beta_7$. L cells transfected with mock (*a*) and Δ5 (*b*) were labeled with DiO (green), while the $\alpha_E\beta_7{}^+$ K562 cells were labeled with DiI (red). The cells were allowed to aggregate in assay medium containing 1 mM Mg^{2+}. Nomarski differential interference micrographs are shown to the *left* of each fluorescent photograph. Bars, 100 μm.

The functional activity of integrins is regulated through an inside-out signaling mechanism that quickly switches inactive forms to active forms. Divalent cations are also required for the acquisition of the active state. In particular, manganese has been shown to promote ligand binding by inducing a conformational change in the metal ion-dependent adhesion site (46–48).

To examine homophilic and heterophilic interactions, we adopted a cell aggregation assay that has been frequently used to evaluate cell-cell adhesion activity (4, 15, 35, 37). This assay method produced reproducible and clear results in the present study. When Δ1, Δ2, Δ3, or Δ4 transfectants were mixed with $\alpha_E\beta_7{}^+$ K562 cells, aggregation did not occur, indicating that all four domain-deletion mutants had lost their ability to undergo heterophilic adhesion with $\alpha_E\beta_7$. Alternatively, aggregation may have been prevented by a disruption in homophilic adhesion, although the ability to undergo heterophilic adhesion was retained. Heterophilic interactions have been suggested to be so weak that cell aggregation may not occur without homophilic adhesion; alternatively, $\alpha_E\beta_7$ adhesion may require an E-cadherin homophilic bond to be formed by *trans*-interactions among cells. The mAb HECD-1 against EC2 did not inhibit homophilic aggregation completely, allowing heterophilic adhesion and supporting the possibility that a homophilic scaffold may be necessary for heterophilic interactions. If so, the effect of the Δ1, Δ2, Δ3, and Δ4 mutants cannot be evaluated because these mutants prevent homophilic adhesion, and some degree of homophilic adhesion by the transfected L cells is a prerequisite to allowing a determination of the percentage of K562 cells included in the aggregates of E-cadherin-expressing cells. Therefore, the EC5 deletion mutant is the only mutant of the entire set used in this article that can be evaluated for loss of heterophilic adhesion in the K562 coaggregation assay.

We demonstrated that EC5 is critical for heterophilic adhesion with $\alpha_E\beta_7{}^+$ cells, but not for homophilic adhesion. This is the first evidence showing the involvement of ECs other than EC1 in heterophilic adhesion with $\alpha_E\beta_7$. Since the integrin $\alpha_E\beta_7$ plays a critical role in the selective localization of T cells in inflamed epithelia (26–31, 49), the adhesive interaction could be a potential target for therapeutic interventions. In contrast, mutational analyses of selected residues clearly revealed that the side chain of Glu^{31} is

important for integrin $\alpha_E\beta_7$ recognition, but not for homophilic adhesion, indicating that the E-cadherin residues critical for heterophilic adhesion to $\alpha_E\beta_7$ are distinct from those required for homophilic adhesion (32, 33). Higgins et al. (50) proposed a docking model involving the α_E A domain and EC1 in which the metal ion-dependent adhesion site cleft of α_E comes in contact with Glu^{31} of EC1 and the Phe^{298} projection of α_E coordinates with the hydrophobic pocket of EC1. Given a previous report showing that synthetic peptides encompassing Asn^{27}-Val^{34} in EC1 had very little inhibitory effect on the interaction with $\alpha_E\beta_7$ (32), however, full adhesive activity may require other binding sites or events, such as a conformational change in the cadherin or integrin molecules. Since domain 5 is located proximal to the membrane, it is less likely to serve as a direct binding site for the integrin, implying that the effect of the EC5 deletion on binding of the $\alpha_E\beta_7$ may be due to long-range effects that alter the conformation of more membrane distal domain of the molecules. However, the Δ5 mutants retained the ability to participate in homophilic adhesion and still bound the EC1-specific Ab SHE78-7 and the EC2-specific Ab HECD-1, indicating that a gross disruption of the conformation of the domain distal to EC5 is probably not occurring. The complexity of the EC5 conformational structure, containing asparagine residues of *N*-linked glycosylation and two intramolecular disulfide bonds, may favor the hypothesis that EC5 plays a role in the regulation of heterophilic adhesion via a change in conformation of the membrane proximal domain. Although the inside-out signaling mechanism that alters the conformational change in E-cadherin remains to be clarified, further functional and structural studies should provide an insight into our understanding of the role of EC5 in heterophilic adhesion. Identification of smaller deletions or point mutations in EC5 that also affected heterophilic adhesion by $\alpha_E\beta_7$ would be of considerable interest. The current data did not show that EC5-specific Ab G-10 block heterophilic adhesion of $\alpha_E\beta_7$ to E-cadherin. The adhesion was also not inhibited by the polyclonal Ab, which we newly generated in rabbits using a synthetic peptide corresponding to part of EC5 (our unpublished observations). If any anti-EC5 mAb blocks heterophilic adhesion to the same degree as anti-α_E, this would also be good supporting evidence for a role of EC5 in heterophilic adhesion.

Acknowledgments

We are grateful to Dr. D. Erle for providing K562-$\alpha_E\beta_7$ cells and Dr. Y. Shimoyama for providing the vector containing E-cadherin cDNA. We acknowledge the use of equipment belonging to the Saitama Medical School Research Center for Genomic Medicine

Disclosures

The authors have no financial conflict of interest.

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The Effect of Periocular Warming on Accommodation

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Purpose: To investigate the effect of periocular warming on accommodation amplitude and near vision after prolonged near work in visual display terminal (VDT) workers in an office setting.

Design: Prospective interventional observational case series.

Participants: Ten healthy Japanese male volunteers (mean age, 39.3±2 years [range, 36–43]) with a best distance corrected visual acuity (VA) of >20/20 who performed near work for 7 hours were included in the study.

Main Outcome Measures: The differences of subjective and objective accommodations, near acuity, and pupil diameters were compared and studied in 2 experimental settings with and without periocular warming.

Methods: Two experiments were performed on each subject on 2 different days. In one experiment, the subjects wore an eye mask and closed their eyelids for 10 minutes after 7 hours of VDT work (lid closure [LC] group). In the other experiment, the subjects applied warming sheets (WSs) to their eyelids and the periocular region and wore masks for 10 minutes again after 7 hours of VDT work (WS group). Visual display terminal work was performed under the same settings on both days. Subjective and objective accommodations, near vision, and pupil diameters were measured immediately before and immediately after the resting or warming procedure and again 90 minutes after the resting or warming procedures.

Results: Fifty percent of eyes had an increase in the accommodation amplitude of at least 0.5 diopters immediately after application of the WSs. Forty percent of eyes still maintained the accommodation amplitude level at 90 minutes. The mean near logarithm of the minimum angle of resolution VA immediately after application of WSs and at 90 minutes significantly improved relative to the near acuity values after simple eyelid closure.

Conclusion: Periocular warming was found to be associated with increases in subjective and objective accommodations, with a concomitant improvement in near VA. Periocular warming with disposable WSs is a simple and effective method of increasing near work–related ergonomy. *Ophthalmology 2005;112:1113–1118* © *2005 by the American Academy of Ophthalmology.*

Near work, particularly computer work, has been reported to cause accommodative dysfunction such as near blur, postwork distance blur, asthenopia, and headache.[1] The prevalence of eye symptoms after near work ranges from 25% to 93%, as reported by previous investigators.[1,2] Indeed, diffuse light reflections from computer screens seem to cause image degradation and decreased contrast, making it more difficult for the eyes to focus and maintain binocularity. Working under these conditions might increase the demand on the visual system and aggravate accommodative or refractive disorders.[1,3] Usually the first steps taken to overcome computer-related fatigue and accommodative disorders include simple workstation changes such as using blinds or drapes on windows, turning off a light fixture in the ceiling, swiveling the display position within acceptable ergonomic limits, or using hoods or baffles on the computer displays. If such simple changes do not provide a sufficient outcome, antireflection films can also be tried in conjunction.[1] Recently, it has been reported that periocular warming by an infrared warm compression device or disposable eyelid-warming devices effectively reduces fatigue symptoms after near work in office workers.[4,5] Therefore, we employed a disposable eyelid-warming device to investigate the effects of periocular warming on subjective and objective accommodative responses and

Originally received: September 11, 2004.
Accepted: December 6, 2004. Manuscript no. 2004-100.

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Presented in part at: Japanese Society of IOL and Refractive Surgery annual meeting, June, 2002; Tokyo, Japan. Also presented at: Association for Research in Vision and Ophthalmology meeting, April 27, 2004; Fort Lauderdale, Florida.

The authors have no proprietary interest in any of the products mentioned in the article.

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Published by Elsevier Inc.

ISSN 0161-6420/05/$–see front matter
doi:10.1016/j.ophtha.2004.12.040

near visual acuity (VA) in visual display terminal (VDT) workers.

Materials and Methods

Subject and Study Characteristics

Ten healthy male Japanese volunteers (mean age, 39.3±2 years [range, 36–43]) with a best distance corrected VA of >20/20 who performed VDT work for 7 hours were included in the study. None of the subjects had a history of any ocular or systemic diseases or received any particular diet or drug therapy that might have influenced the results of this study. Written informed consent was obtained from all subjects before the study. Ethical board review was not required.

Two experiments were performed on each subject on 2 different days. In one experiment, subjects wore an eye mask and closed their eyelids for 10 minutes after 7 hours of VDT work (lid closure [LC] group). In the other experiment, the subjects applied warming sheets (WSs; Eye Feel, Kao, Tokyo, Japan) to their eyelids and the periocular region and wore masks for 10 minutes again after 7 hours of VDT work, as shown in Figures 1 and 2 (WS group). Visual display terminal work was performed under the same settings on both days. The subjects were seated 50 cm from the computer displays, which all had a surface illumination of 80 candelas per square meter. Office room temperatures and humidity were kept constant on both experiment days, with the room temperature maintained at 25° C and relative humidity at 50%.

Testing

Subjective and objective accommodations, near vision, and pupil diameters were measured immediately before and immediately after the resting or warming procedure, and again 90 minutes after the resting or warming procedure. All examinations were performed after 7 hours of VDT work at 4 PM in all subjects.

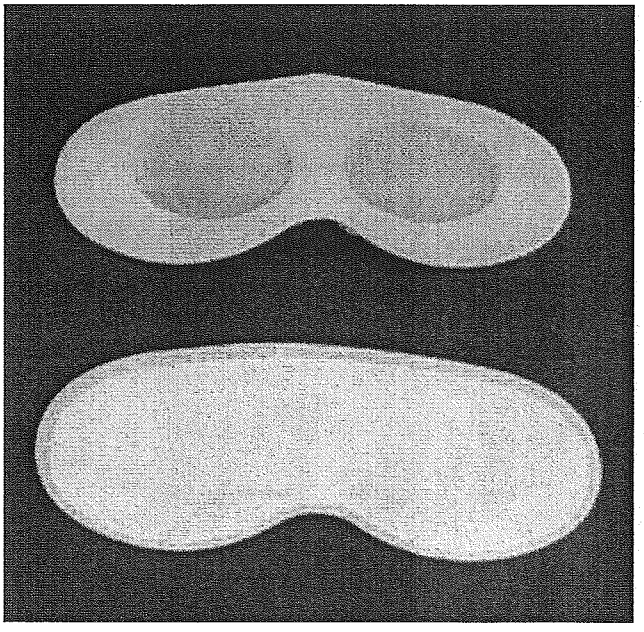

Figure 1. Disposable eyelid-warming device (Kao, Tokyo, Japan). The transparent sheet that is applied to the eyes is a synthetic paper made from polyethylene and polypropylene.

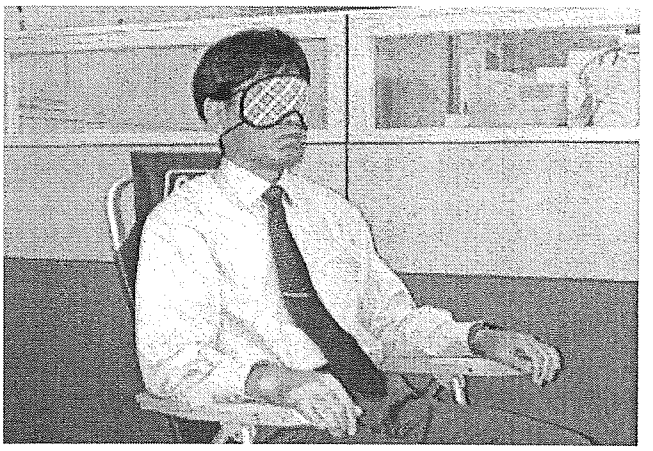

Figure 2. The heating sheet is applied to the periocular region, with an eye mask worn over it.

Subjective Accomodation

Near point measurements were carried out under best-corrected distance VA using an NP accommodometer system (KOWA Corp., Nagoya, Japan). The measurements were performed on each eye with the opposite eye occluded. A mean near point value was calculated from 10 near point measurements. Accommodative power for each eye was then calculated as A = 1/N (near point in meters) − 1/f (distance point in meters).

Objective Accommodation

The amplitude of accommodation was also evaluated objectively with the AA-2000 Accommodometer system (Nidek Inc., Aichi, Japan), with which a direct online recording of the accommodative stimulus versus accommodative response could be made by means of a modified infrared optometer of the Cornsweet type. The accommodometer employs a Badal optical system in which the stimulus position is controlled by a microcomputer. The optotypes of the system had a contrast of 100% with the background and were presented at a luminance of 10 candelas per square meter. The accommodative stimulus in the system can be moved from −12.5 diopters (D) to +12.5 D and then returned to −12.5 D with a constant velocity of 0.2 D/second while the infrared optometer is working continuously. The system feeds the analog signals of both the accommodative stimulus and the accommodative response into an x–y recorder that instantaneously provides a printout of a graph of accommodative stimulus (x-axis) versus accommodative response (y-axis).[6,7]

Pupillary Diameter

Pupil diameter was measured using a Corvard pupillometer (OASIS Medical, Inc., Glendora, CA) in a dark room immediately before, immediately after, and 90 minutes after the 10-minute resting or warming procedure.

Near Vision

Near VAs were measured with Landolt optotype near charts at 30 cm and a chart surface illumination of 600 lux in all subjects

Table 1. Changes in Accommodation

	D≤0.0		0.0<D<0.5		0.5≤D		
	N	%	*N*	%	*N*	%	Mann–Whitney *U* Test
Immediately after:							
Warming sheet	1	5	9	45	10	50	$P = 0.0027$
Lid closure	8	40	10	50	2	10	
90 min after:							
Warming sheet	2	10	10	50	8	40	$P = 0.0045$
Lid closure	9	45	10	50	1	5	

D = diopters.

immediately before, immediately after, and 90 minutes after the warming or resting procedure.

Eyelid-Warming Device

The eyelid-warming device (Kao) provided heat through a chemical reaction of the iron powders inside it upon exposure to air. The disposable device also provided moisture for approximately 20 minutes due to the presence of a specific transparent sheet made of synthetic paper that generated dampness with a chemical reaction. This wet-heat warming device was reported to be effective because it provided constant temperatures as well as a constant amount of generated steam with repeatability. It has also been reported that the temperature of the periocular skin remained at 40° C with the application of the warming device, which generated steam at a rate of 80 to 200 mg of H_2O/10 minutes for 20 minutes.[5]

Statistical Analyses

The changes in mean subjective and objective accommodation, near vision, and pupil diameters were analyzed with the Wilcoxon signed-ranks test. The differences in the number of eyes in different accommodation ranges in both experiment groups were analyzed with the Mann–Whitney *U* test. The differences in numbers of eyes in which the near vision improved by ≥2 lines in both experiment groups were analyzed with the chi-square test. Statcel software[8] was used for all statistical comparisons.

P values of <0.05 were considered statistically significant.

Results

All subjects completed the experiments successfully. The application of the WS was not associated with any side effects.

Changes in Accommodation

Fifty percent of the eyes had an increase in the accommodation amplitude of at least 0.5 D immediately after application of the WSs. Forty percent of eyes still maintained the accommodation amplitude level at 90 minutes (Table 1). On the other hand, 10% of the eyes with simple LC had an accommodation amplitude of at

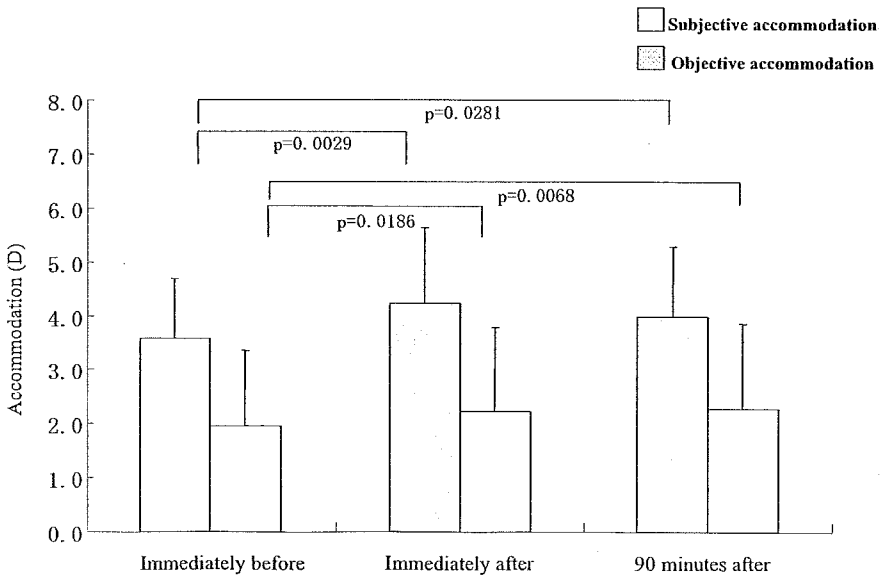

Figure 3. Changes in subjective and objective accommodation amplitudes with periocular warming. D = diopters.

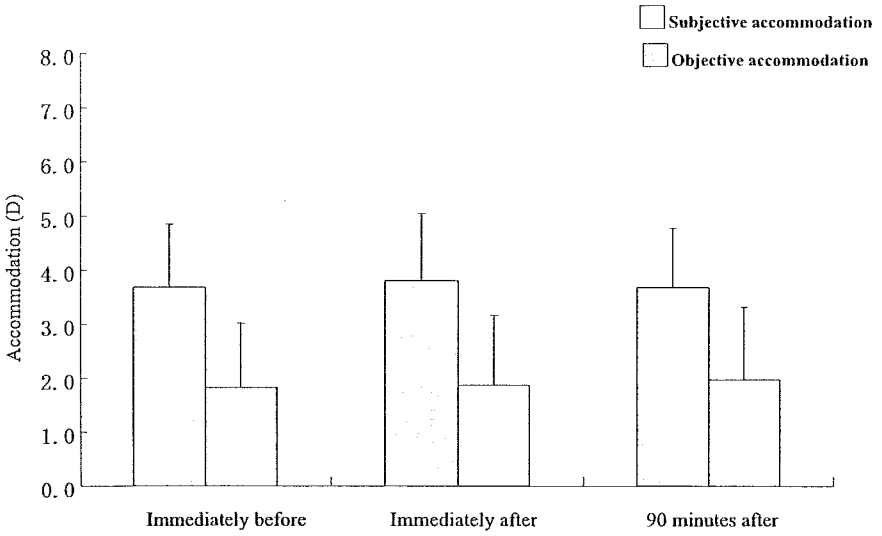

Figure 4. Changes in subjective and objective accommodation amplitudes with simple lid closure. D = diopters.

least 0.5 D immediately after LC, with a further decrease to 5% at 90 minutes. The percentage of eyes in different accommodation amplitude ranges in both experiment conditions is shown in Table 1.

The mean objective accommodation amplitude before the application of WSs was 3.58±1.11 D; it increased to a mean value of 4.23±1.43 D immediately after application of WSs and was recorded to be 3.98±1.31 D at 90 minutes. The difference was statistically significant ($P<0.05$).

The mean subjective accommodation amplitude before the application of WSs was 1.96±1.39 D; it showed a statistically significant increase to 2.23±1.57 D immediately after WS application, as shown in Figure 3. The mean subjective accommodation amplitude of 90 minutes after WS application was 2.27±1.60 D. Increases in accommodation amplitude immediately after WS application and at 90 minutes from the baseline value were statistically significant ($P<0.05$).

The mean objective and subjective accommodation amplitude values did not show significant changes immediately after eye LC and at 90 minutes compared with the baseline value in the LC group, as shown in Figure 4 ($P>0.05$).

Refractive Error and Accommodation Amplitude

The relationship between spherical equivalent (SE) and the changes in accommodation as well as the relationship between SE and the individual accommodation measurements taken before and after the procedures were also investigated. No correlation was observed in either the LC or the WS group.

Near Vision

The mean near logarithm of the minimum angle of resolution (logMAR) VAs before the experiments in the WS and LC groups were 0.03±0.01 and 0.03±0.01, respectively, as shown in Figure

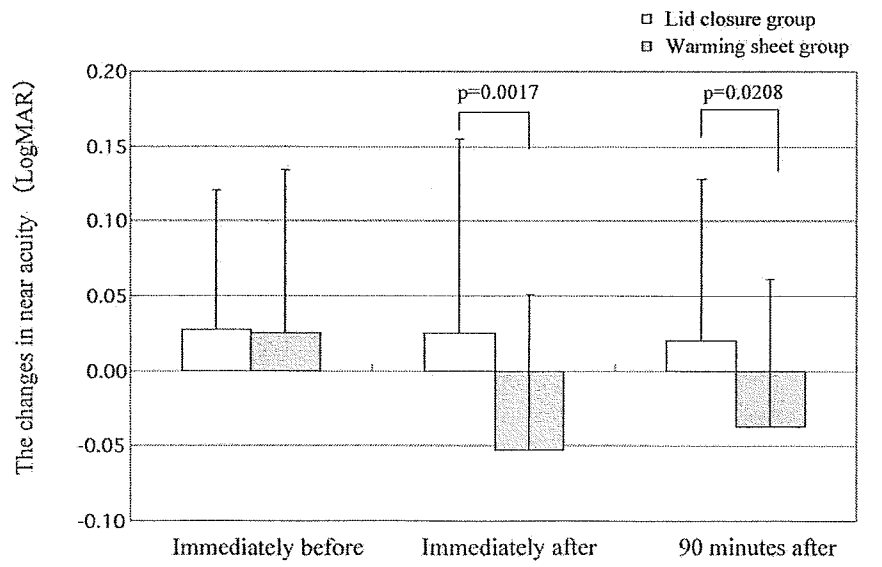

Figure 5. Changes in mean logarithm of the minimum angle of resolution (LogMAR) near acuity with periocular warming and simple lid closure.

Table 2. Numbers of Eyes with Near Vision Improvement of ≥2 Lines

Group	Immediately After	90 Minutes After	Chi-Square Test
Warming sheet	2	2	$P = 0.057$
Lid closure	0	0	

5. Mean near logMAR VAs immediately after application of WS and at 90 minutes were -0.05 ± 0.1 and -0.04 ± 0.1, respectively. Mean near logMAR VAs in the LC group immediately after eye LC and at 90 minutes were 0.03 ± 0.13 and 0.02 ± 0.11, respectively. There were no significant differences between mean near logMAR VA scores in the groups before the onset of experiments.

However, differences between mean near logMAR VA scores immediately after the onset of experiments and at 90 minutes were statistically significant ($P<0.05$). Ten percent of the eyes in the WS group had at least 2 lines of near vision improvement immediately after the experiment and had maintained that level of near vision at 90 minutes. None of the eyes in the LC group had a near vision improvement of ≥2 lines (Table 2).

Pupil Diameter

Figure 6 shows the changes in pupil diameter after the procedures. Pupil diameters immediately before, immediately after, and 90 minutes after resting the eyes in the LC group were 3.93 ± 0.58 mm, 3.34 ± 0.32 mm, and 3.94 ± 0.53 mm, respectively. Pupil diameters immediately before, immediately after, and 90 minutes after warming the eyes in the WS group were 4.18 ± 0.57 mm, 3.32 ± 0.30 mm, and 4.12 ± 0.53 mm, respectively. In both groups, there was a significant change in pupil diameter from immediately before to immediately after the procedures ($P = 1.9\times10^{-6}$ and $P = 5.7\times10^{-6}$, respectively). However, there was no significant change between the measurements taken before and the measurements taken 90 minutes after the procedure in either group.

Discussion

Accommodation is a dynamic optical change in the dioptric power of the eye providing the ability to change the point of focus of the eye from a distant subject to a near one.

The accommodation response in the eye can be blur, vergence, or pharmacologically driven and consists of pupillary constriction, convergence, and the act of accommodation. These responses are neuronally coupled through the preganglionic parasympathetic innervation extending from the Edinger–Westphal nucleus in the brain. The intraocular muscles are innervated by the postganglionic parasympathetic nerves.[9]

Recently it was reported that periocular warming by an infrared warm compression device or disposable eyelid-warming devices effectively reduces fatigue symptoms and improves tear stability after near work in office workers.[4,5] We employed a disposable eyelid-warming device to study further the effects of periocular warming on the accommodative response and near VA in VDT workers.

We found that periocular warming was associated with an increase of accommodation amplitude by at least 0.5 D immediately after warming in 50% of eyes, and that the effect was maintained in 40% of eyes 90 minutes after warming. We believe that the thermal stimulus from the periocular warming resulted in increased blood flow to the ocular region, enhancing the parasympathetic-driven responses in the ciliary muscle and the pupil, causing their constriction, thereby increasing the depth of focus of the eye. It has recently been confirmed that periocular warming

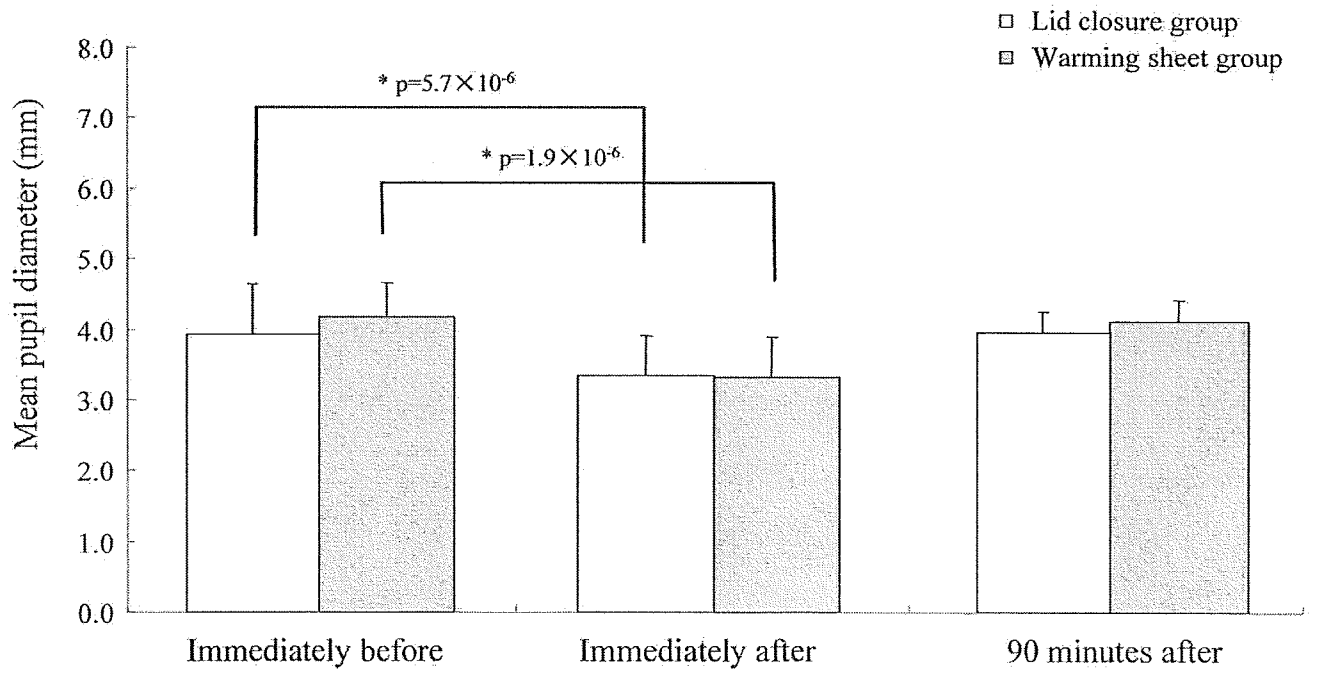

Figure 6. Changes in pupillary diameter with periocular warming and simple lid closure.

stimulates α brain wave activity and increases the high-frequency component of heart rate variability, suggesting an increase in parasympathetic activity caused by increased activity of the cardiac parasympathetic nerves through the trigeminal and facial nerves when the densely populated facial heat receptors are stimulated.[9]

We noted that the mean objective and subjective accommodative amplitudes increased significantly immediately after and 90 minutes after periocular warming. Interestingly, however, the mean subjective accommodation amplitudes were lower than the objective accommodation amplitudes at each experiment point. This might be explained by observations from previous reports that the accommodometer system measuring the subjective accommodative responses in this study by means of a modified infrared optometer of the Cornsweet type evaluated only the lens-related accommodative response, eliminating the effect of other factors on accommodation such as pupillary diameter, depth of focus, and patient-related psychological factors.[10–12]

To our surprise, we found that improvements in subjective and objective accommodation amplitudes by periocular warming were associated with simultaneous improvement in near VA values. Indeed, all subjects reported better display visibility and decreased ocular fatigue after periocular warming.

Indeed, prolonged near tasks have been shown to be associated with fatigue and difficulty in focusing in VDT workers.[12] Studies investigating the near acuity and accommodative changes with periocular warming in older individuals or presbyopic individuals would also provide interesting information.

In conclusion, periocular warming was found to be associated with increases in subjective and objective accommodations, with a concomitant improvement in near VA. We believe that periocular warming with disposable WSs is a simple and effective method of improving near work–related ergonomy.

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SCIENTIFIC REPORT

Decreased tear lipocalin concentration in patients with meibomian gland dysfunction

M Yamada, H Mochizuki, M Kawai, K Tsubota, T J Bryce

Br J Ophthalmol 2005;**89**:803–805. doi: 10.1136/bjo.2004.055822

Background/aim: Recent studies have demonstrated that tear lipocalin (TL) and phospholipids have a crucial role in maintaining tear film stability. The level of TL in patients with meibomian gland dysfunction (MGD) was examined and these data were correlated with the severity of their clinical disorder.

Methods: 12 patients with obstructive MGD, 12 patients with seborrhoeic MGD, and 12 age matched normal control subjects participated in this study. 3 µl of unstimulated tears were collected with a micropipette from the inferior tear meniscus in the right eye of all subjects. Tear samples were fractionated by high performance liquid chromatography, and TL concentrations were assayed with a bicinchoninic acid technique.

Results: The mean concentration of TL in patients with obstructive and seborrhoeic MGD was significantly lower than in normal controls. TL concentration correlated positively with tear film break up time and negatively with fluorescein staining scores.

Conclusion: These results suggest that TL deficiency may be a predisposing factor for the manifestation of symptoms in MGD.

Tear lipocalin (TL), formerly called tear specific prealbumin, comprises 15–33% of the mass of protein in tears.[1] TL is a member of the lipocalin superfamily that is produced in the lacrimal gland as well as in von Ebner's gland.[2–4] A number of lipophilic substances of different chemical classes, including fatty acids, fatty alcohols, phospholipids, glycolipids and cholesterol, are endogenous ligands of this protein.[5 6] Although the physiological roles of TL are not fully understood, recent studies have demonstrated that the low surface tension of tears is the result of a complex of TL with tear lipids.[7 8] Glasgow *et al* have stated that TL may be a key factor in maintaining tear film stability by acting as a lipid scavenger.[7] This protective function of TL may be compromised in dry eye syndrome.[9]

Meibomian gland dysfunction (MGD), a common, chronic condition of the posterior eyelids, comprises a significant part of dry eye syndrome.[10–12] The lipid layer, the outermost layer of the tear film produced by the meibomian glands, stabilises tear films by retarding evaporation and lowering surface tension. The compositional changes in lipids of meibum (that is, an increase in polar lipids) in MGD compromise tear film stability, which is clinically manifest as a shortened tear film break up time.[12]

The symptoms of MGD are non-specific and often similar to those of aqueous deficiency dry eye.[11 12] An important clinical feature of MGD is that findings on examination of the lid margin often do not correlate well with the severity of symptoms.[12] MGD is frequently associated with lacrimal insufficiency, and patients with both conditions are likely to be particularly symptomatic. These observations may suggest the presence of pathogenic factor(s) other than changes in the meibomian glands. In this study, we examined TL concentration in patients with MGD and correlated these results with the severity of their clinical disorder.

SUBJECTS AND METHODS

Twenty four patients with symptomatic MGD participated in this study. Diagnoses included obstructive MGD in 12 subjects (six female and six male, average age 67.8 (SD 11.5) years, and seborrhoeic MGD in 12 subjects (seven female and five male, age 62.4 (11.3) years). The diagnosis of obstructive MGD was made based on the presence of plugging of the meibomian gland orifices associated with thickened, erythematous eyelid margins. For the assessment of the meibomian gland orifices, digital pressure was applied on the upper tarsus. When cloudy meibum was expressed with more than moderate pressure, or when meibum could not be expressed even with the hard pressure, the diagnosis of obstructive MGD was confirmed. Seborrhoeic MGD was diagnosed based on the excessive expression of meibum, sometime associated with foam along the lateral lower eyelid margin. Twelve age matched normal subjects (six female and six male, age 61.0 (8.9) years) were used as normal controls.

Most patients had been treated with preservative free artificial tears and/or hyaluronic acid eye drops. Patients using antibiotic eye drops or steroidal eye drops at the time of study, or who had a history of ocular surgery, were excluded. The principles of the World Medical Association Declaration of Helsinki were followed. Each subject received a full explanation of the study, all procedures involved in the study, and provided written informed consent before enrolment. Approval for this investigation was granted by the committee for the protection of human subjects at the Keio University School of Medicine.

Non-masked observers performed a routine ocular examination on all subjects, followed by an examination of the ocular surface, including Schirmer testing, a cotton thread test, vital staining, and measurement of tear film break up time (BUT). For vital staining, 2 µl of a saline solution containing 1% fluorescein and 1% rose bengal was used.[13] Results were assessed semiquantitatively using a 0–9 grading scale for fluorescein staining in the cornea and for rose bengal staining in both the cornea and conjunctiva.[13 14] Tear film BUT was measured three times, and the measurements were averaged. Only the right eye of all subjects was used for analysis.

A volume of 3 µl of unstimulated tears were collected with a micropipette from the inferior tear meniscus in the right eye of all subjects on a separate day. Samples were stored at −80°C until assay. A volume of 1 µl of each tear sample was

Abbreviations: BCA, bicinchoninic acid; BUT, break up time; MGD, meibomian gland dysfunction; TL, tear lipocalin

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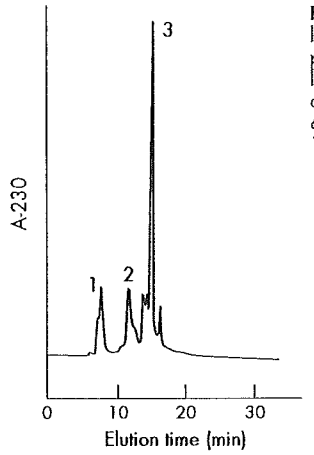

Figure 1 High performance liquid chromatography of tear proteins. The retention times of lactoferrin (1), tear lipocalin (2), and lysozyme (3) were 6.4 minutes, 10.8 minutes, and 14.1 minutes, respectively.

fractionated by high performance liquid chromatography (HPLC).[15] The determination of peaks in HPLC was carried out by using standards of lactoferrin, albumin, and lysozyme (Sigma, St Louis, MO, USA) based on the report of Baier *et al*.[15] The elution was performed using a TSK 3000SWxl column (Tosoh Inc, Tokyo, Japan) with a mobile phase of 0.5 M sodium chloride and 0.1 M sodium phosphate, pH 5.0. The flow rate was 0.7 ml/min and detection was performed using a ultraviolet detector set at 230 nm. The fractions containing TL were collected, and then concentrated by evaporation under nitrogen gas. The amount of TL was determined by a bicinchoninic acid (BCA) method. Total protein concentration was also determined by a BCA method using a 1 µl tear sample. The final results are expressed as mg/ml.

RESULTS

The chromatograms demonstrated excellent separation of TL from other proteins, and were free from interference by endogenous compounds. A typical chromatogram is shown in figure 1. The retention time of TL was 10.8 minutes.

TL concentration, total protein concentration, and the results of ocular surface examination are shown in table 1.

The mean concentration (SD) of TL in patients with obstructive MGD and seborrhoeic MGD was 0.89 (0.19) mg/ml, and 1.05 (0.22) mg/ml, respectively. In both groups, mean TL concentration was significantly lower than that of normal controls ($p = 0.00021$ and $p = 0.00042$, respectively, Mann-Whitney U test). There were no significant differences in total protein concentration among the three groups.

TL concentrations were not significantly correlated with the results of the Schirmer or cotton thread tests, or with the rose bengal staining score, but did correlate positively with tear film BUT ($r = 0.67$, $p = 7.8\times10^{-6}$; fig 2) and fluorescein staining scores ($r = -0.74$, $p = 1.8\times10^{-7}$; fig 3).

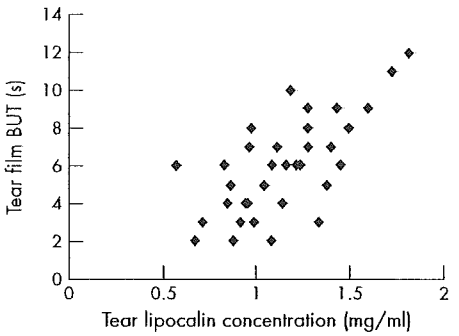

Figure 2 Tear lipocalin concentration was significantly correlated with tear film break up (BUT) time ($r=0.67$, $p=7.8\times10^{-6}$).

DISCUSSION

In this study, we have shown that mean TL concentrations in patients with MGD were significantly lower than concentrations in normal controls. This is not likely to be due to decreased tear secretion, because the same volume of tears was collected from all subjects, and the total tear protein concentration did not differ among patients with MGD and normal controls. Regression analyses revealed that TL concentrations were positively correlated with tear film BUT and negatively correlated with fluorescein staining scores. These results suggest that decreased TL concentration is associated with tear film instability and a more severe clinical manifestation of MGD.

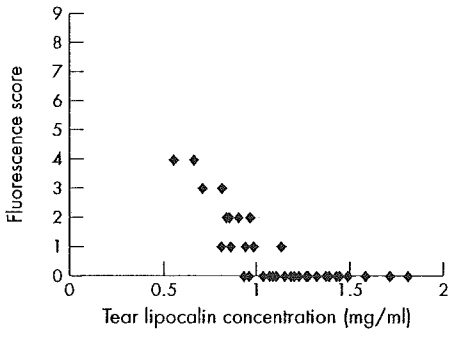

Figure 3 Tear lipocalin concentration was significantly negatively correlated with fluorescence score ($r=-0.74$, $p=1.8\times10^{-7}$).

Table 1 Tear lipocalin concentration, total protein concentration, and results of ocular surface examination (SD)

	Obstructive MGD	Seborrhoeic MGD	Normal controls
Schirmer test (mm)	5.4 (2.1)	6.4 (2.3)	8.5 (3.2)
Cotton thread test (mm)	10.4 (4.3)	12.7 (4.9)	13.7 (3.1)
Tear film BUT (s)	4.4 (1.7)	4.9 (2.1)	7.9 (1.9)
Fluorescein score	1.7 (1.5)	0.9 (1.1)	0
Rose bengal score	0.5 (0.7)	0.5 (0.6)	0.1 (0.3)
Tear lipocalin (mg/ml)	0.89 (0.19)*	1.05 (0.22)**	1.54 (0.23)
Total protein (mg/ml)	8.66 (1.50)	9.16 (1.76)	9.48 (1.72)

MGD, meibomian gland dysfunction.
The concentration of lipocalin in patients with obstructive MGD and seborrhoeic MGD was significantly lower than that of normal controls (*$p=0.00021$ and **$p=0.00042$, respectively).

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Recent studies have demonstrated that TL contributes to the high, non-Newtonian viscosity of tear film and its low surface tension.[7 8] These actions are thought to be exerted by the lipid binding properties of TL. In the lipocalin family, TL is unique because it binds most strongly the least soluble lipids.[5 6] In seborrhoeic MGD, excessive expression of meibum results in excessive concentrations of lipids in tear film. TL may contribute to tear film stability by acting as a lipid scavenger.[7] In obstructive MGD, low levels of two phospholipids, phosphatidylethanolamine, and sphingomyelin are associated with ocular surface abnormalities.[16] TL may adjust the lipid composition of tears by binding lipids from the tear fluid and/or releasing lipids into tear fluid. Intensive analysis of lipids compositions of tears and meibum from patients with MGD appears to be essential to test the hypothesis.

Lechner *et al* have recently reported that the expression of TL in tetracarcinoma derived NT2 precursor cells is enhanced by oxidative stress and that TL binds to several lipid peroxidation products.[17] These observations suggest that TL acts as a physiological epithelial protective factor by scavenging potentially harmful lipid peroxidation products. TL also has anti-inflammatory and antimicrobial activity.[18 19] It may exert a regulatory, dampening influence on the inflammatory cascade, thereby protecting against tissue damage from excessive inflammation. The negative correlation between TL concentration and fluorescein staining scores in the present study may be explained by the protective role of TL.

As mentioned in the introduction, findings on examination of the lid margin often do not correlate well with the severity of symptoms in patients with MGD.[12] MGD is frequently associated with lacrimal insufficiency, and patients with both conditions are likely to be particularly symptomatic. Our results suggest that decreased TL concentration in tears is one of the pathogenic factors other than changes in the meibomian glands for the development of clinical symptoms in MGD. Further investigations are required to determine the mechanism by which TL exerts protective effects on the ocular surface (that is, stabilising tear film by modulation of tear lipids, scavenging lipid peroxidation products, or regulating excessive inflammation).

To date, however, only a few investigators have reported pathophysiological changes in TL concentrations. The level of TL remains constant in non-stimulated and stimulated tears of normal subjects.[20] In soft contact lens wearers, the level of lipocalin in tears was significantly higher than that of normal controls.[21] Intolerant contact lens wearers had significantly higher amounts of lipocalin in their tears than tolerant contact lens wearers.[22] These observations appear to conflict with the proposed protective role of TL in maintaining tear film stability and epithelial integrity. However, Glasson *et al* have speculated that the greater concentration of TL observed in tears from intolerant contact lens wearers may be a response to increased lipid peroxidation products.[22] Further studies should be done to clarify this issue.

In summary, mean TL concentration was significantly lower in patients with MGD than in normal control subjects. TL concentrations correlated positively with tear film BUT and negatively with fluorescein staining scores. Our results suggest that a deficiency of TL may be a predisposing factor for the development of clinical symptoms in MGD.

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The authors have no proprietary interest in any materials in this manuscript.

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Accepted for publication 6 October 2004

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