Gangliosides of Migrating and Nonmigrating Corneal Epithelium in Organ and Cell Culture

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**Purpose.** To identify major gangliosides—the sialated glycolipids—of corneal epithelium; to determine which specific gangliosides, if any, are synthesized in a higher amount or are downregulated during corneal epithelial cell migration; and to determine what role, if any, they play in the modulation of corneal epithelial cell proliferation.

**Methods.** [3H]-galactose-labeled and unlabeled glycolipids of migrating and nonmigrating rabbit corneal epithelium in cell and/or in organ culture were chromatographed on DEAE Sephadex to isolate gangliosides. The gangliosides eluted from the ion-exchange column were further characterized by thin-layer chromatography (TLC), glycosidase digestions, and TLC-immunostain analysis. A [3H]-thymidine incorporation assay was used to determine the effect of exogenous gangliosides on corneal epithelial cell proliferation.

**Results.** Upon TLC of the acidic fraction eluted from the DEAE column, only two radiolabeled glycolipids (GL1 and GL2), migrating as a doublet, were detected. Regardless of whether the epithelia were prepared by cell culture or organ culture, both GL1 and GL2 were present in a significantly higher amount in migrating compared to nonmigrating epithelia. Further characterization of GL1 and GL2 identified them as gangliosides known as GM3. TLC-immunostain analysis, as well as orcinol staining of thin-layer chromatograms of gangliosides of unlaabeled cells, revealed that GM3 also accumulates in a higher amount in migrating compared to nonmigrating corneal epithelial cell cultures. Exogenous addition of GM3, but not various other gangliosides, inhibited corneal epithelial cell proliferation in a dose-dependent manner.

**Conclusions.** GM3 is the major ganglioside present in corneal epithelium, and its levels are elevated during corneal epithelial cell migration. It is suggested that the ganglioside plays a role in events that modulate corneal epithelial cell proliferation. Invest Ophthalmol Vis Sci. 1996;37:501–510.

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After corneal epithelial injury, mitosis ceases at the wound periphery, and initial healing of the wound takes place by migration of the adjacent cells over the injured area.1,2 Once the coverage of the wound is complete, mitosis recommences and eventually results in a mature stratified corneal epithelium. Both cell migration and cell mitosis must occur at an appropriate rate if corneal epithelial wound healing is to occur without complications. Delayed reepithelialization or the failure of migrated epithelium to remain adhesive to the substratum are fundamental causes of recurrent or persistent epithelial defects.3,4 Similarly, an abnormality in the rate of cell proliferation after some forms of injury, such as excimer laser surgery, may lead to the development of corneal epithelial hyperplasia.5–9 The molecular mechanisms that induce cell migration and modulate cell proliferation during wound healing are under investigation. Early studies10–12 using plant lectins demonstrated that plasma membrane glycoconjugates play an important role in corneal epithelial wound healing by influencing cell migration. Cell surface glycoconjugates consist primarily of glycoproteins and glycolipids. Although various studies13,14 have focused on the role of corneal epithelial cell surface glycoproteins in corneal epithelial cell migration, relatively few studies have been reported on the role of glycolipids in corneal epithelial wound healing. In recent years, we have analyzed neutral glycolipids of corneal epithelium15,16 and have...
shown that specifically poly lactosamine glycolipids play a key role in corneal epithelial wound healing in a rabbit model.\textsuperscript{10} Little is known about the gangliosides—the sialated glycolipids—of corneal epithelium and the role they may play in events that mediate corneal epithelial cell migration and proliferation. Gangliosides, like neutral glycolipids, are constituted of a structurally varied class of complex carbohydrates. They are located predominantly in the external membrane of animal cells and have been shown to mediate cell–cell and cell–matrix adhesion and modulate cell proliferation by influencing signal transduction processes.\textsuperscript{17-20} The current study was designed to identify major gangliosides of corneal epithelium and to determine which specific gangliosides, if any, are upregulated or downregulated during corneal epithelial cell migration, as well as what role, if any, they play in the events that mediate corneal epithelial cell proliferation.

**MATERIALS AND METHODS**

**Synthesis of Glycosphingolipids by Migrating and Nonmigrating Rabbit Corneal Epithelium in Organ Culture**

To analyze gangliosides of migrating epithelia of corneas in organ culture, radiolabeled epithelia of partially healed corneas were collected by scraping with a blade.\textsuperscript{15,16} For this study, eyes from 2- to 3-month-old New Zealand rabbits (Pel-Freez Biologicals, Rogers, AK) were used. Before use, all eyes were examined for epithelial integrity by fluorescein staining, and approximately 30% of the eyes lacking an intact corneal epithelium were discarded. After washing the eyes in sterile saline, the epithelium from the central 8-mm diameter area was removed with a #15 Bard–Parker blade.\textsuperscript{16} Corneas were then excised with a 1- to 2-mm scleral rim and rinsed with a serum-free medium containing penicillin G (100 U/ml), streptomycin (100 \(\mu\)g/ml), and amphotericin (1 \(\mu\)g/ml). Epithelial fragments were peeled off with forceps and were cultured in 100-mm dishes in SHEM medium,\textsuperscript{23} which was constituted of EMEM:HAMS F-12 (1:1), calf serum (5%), dimethyl sulfoxide (5 \(\mu\)l/ml), gentamicin (1 \(\mu\)g/ml), epidermal growth factor (0.01 \(\mu\)g/ml), insulin (5 \(\mu\)g/ml), and cholera toxin (0.1 \(\mu\)g/ml). Epithelial fragments from three to four corneas were placed in 100-mm dishes. Within 3 days, approximately 30%–50% of each dish was populated with cells that were migrating away from explants. These cells were designated “migrating” corneal epithelial cells. Within 10 to 12 days, more than 90% of the dish area was covered with contact-inhibited polygonal cells designated “nonmigrating” corneal epithelial cells. Throughout this study, only primary cultures were used. The glycolipids of migrating and nonmigrating corneal epithelial cell cultures were radiolabeled by incubation with \(^{3}H\)-galactose (25 \(\mu\)Ci/ml) in 5 ml SHEM medium in 100-mm diameter dishes for 48 hours. Incorporation of \(^{3}H\)-galactose into glycolipids of migrating and nonmigrating cell cultures was 3.0 \(\times\) 10\(^{5}\) and 2.8 \(\times\) 10\(^{5}\) dpm/10\(^{6}\) cells, respectively.

**Isolation of Glycolipids and Thin-Layer Chromatography**

The lyophilized radiolabeled epithelia from fifteen 100-mm dishes were suspended in 0.5 ml distilled water. Ten volumes of chloroform:methanol (C:M, 2:1) were added, and samples were probe sonicated for 30 seconds (Branson Sonifier 25, constant output, setting 4). An equal volume of methanol was added to each tube, and the samples were further sonicated for an additional 30 seconds. The sonicated corneal epithelial cell extracts were centrifuged to remove protein. The harvested epithelia were processed for isolation of glycolipids. Unscraped corneas were processed concomitantly, as described above, to radiolabel glycolipids of nonmigrating corneal epithelium. Incorporation of \(^{3}H\)-galactose into glycolipids of migrating and nonmigrating corneal epithelium was 13.8 \(\times\) 10\(^{5}\) and 38.0 \(\times\) 10\(^{5}\) dpm/8 mm corneal button, respectively.
pellets, and the supernatants containing glycolipids, phospholipids, and neutral lipids were subjected to alkaline methanolysis (0.2 ml 1 N KOH in 1.2 ml C:M/1:2, 3 hours, 37°C) to eliminate diacylphosphoglycerides. The hydrolyzed samples were then neutralized with 1 N HCl, dried under a nitrogen stream, suspended in 3 ml C:M:0.1 M KCl (3:48:47), and passed through a reverse-phase Bond-Elut cartridge (Varian, Harbor City, CA) to remove salts. Radiolabeled glycolipids recovered from the Bond-Elut cartridge were separated on high-performance thin-layer chromatography plates (HPTLC; Alufolien, Kieselgel 60; EM Science, Cherry Hill, NJ) using a solvent system consisting of C:M:0.25% CaCl₂ (5:4:1). Various ganglioside standards including GM₃, GM₁, GD₃a, GD₃b, and GT₁b were obtained from Sigma Chemical (St. Louis, MO). Standards were chromatographed on the same plate as the radiolabeled samples. After TLC, the chromatograms were sprayed with EN' HANCE (DuPont NEN) and exposed to x-ray films (Kodak, Rochester, NY) at -70°C. After the fluorography, the plates were stained with an orcinol spray reagent to visualize glycolipids.

DEAE Sephadex Chromatography

To separate gangliosides from neutral glycolipids, the lipid extracts of radiolabeled corneal epithelium (0.5 - 4.0 × 10⁶ dpm in 1 ml methanol) were chromatographed on a DEAE Sephadex A-25 (Pharmacia LKB, Uppsala, Sweden) column (2 × 0.7 cm). Various glycolipid components were eluted from the column by a stepwise sequential elution using 5 ml each of methanol (fraction DE-1), methanol containing 0.02 M ammonium acetate (fraction DE-2), 0.08 M (fraction DE-3), and 0.5 M (fraction DE-4) ammonium acetate. Glycolipids eluted in fractions DE-1 to DE-4 were passed through the reverse-phase Bond-Elut cartridge again to remove salts before TLC analysis.²⁴

Neuraminidase Treatment

Radiolabeled samples eluted from the DEAE Sephadex column (30,000 dpm) and a GM₃ standard (5 µg) were treated with 0.02 units of neuraminidase (EC 3.2.1.18, Vibrio cholerae; Sigma) in 100 µl sodium acetate buffer (50 mM, pH 5.0) containing sodium taurocholate (1 mg/ml) and calcium acetate (0.1 mM) for 20 hours at 37°C. Negative controls involved incubation of the samples in the absence of the enzyme. After the enzyme treatment, the samples were dried under a nitrogen stream and suspended in 3 ml of C:M:0.1 M KCl (3:48:47) and passed through a reverse-phase Bond-Elut cartridge to remove salts. The products of the enzymatic hydrolysis were analyzed by TLC.

High-Performance Thin-Layer Chromatography Immunostaining

To identify GM₃ in the lipid extracts of migrating and nonmigrating corneal epithelial, an HPTLC-immuno-staining procedure,²⁵ coupled with the chemiluminescent detection system,²⁶ was used. Aliquots of the total lipid fractions derived from migrating and nonmigrating corneal epithelia (equivalent to 2 mg of original protein) and a GM₃ standard were chromatographed on aluminum-backed HPTLC plates using a solvent system consisting of C:M:0.25% CaCl₂ (5:4:1). After TLC, the chromatograms were treated with 0.05% polisobutylmethacrylate in n-hexane for 1 minute to prevent detachment of the silica gel during the subsequent procedures. The plates were then sequentially incubated with 1% bovine serum albumin (BSA) in PBS for 2 hours to block nonspecific binding, monoclonal antibody M-2590 (anti-NeuAc-GM₃, provided by Dr. Hirabayashi,²⁷ nonfunction blocking ²⁸) for 20 hours at 4°C and peroxidase-conjugated goat antimouse IgM (1:400 dilution in 1% BSA in PBS; Cappel Labs, Durham, NC) for 2 hours. The chromatograms were washed with PBS, and the antibody-reactive glycolipids were detected using a LumiGlo substrate kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for chemiluminescent detection of peroxidase-conjugated reagents. After the chemiluminescent detection, the same chromatogram was stained with an orcinol spray reagent to visualize glycolipids.²⁹

Cell Proliferation Assay

Primary cell cultures of rabbit corneal epithelium were dislodged by treatment with Dispase II (2 mg/ml, 37°C, 90 minutes). The cells were washed with Hanks' balanced salt solution by centrifugation, suspended in SHEM medium (2 × 10⁵ cells/ml), and replated in 12-well cell culture plates (COSTAR) at a density of 1 × 10⁶ cells/well. After overnight culture, the SHEM medium was replaced by MEM containing 0.4% BSA. [³H]-Thymidine (1 µCi; specific activity, 6.7 Ci/mmol; DuPont) was added to each well, and the cultures were incubated in the presence or absence of gangliosides (0.1 to 25 µg/ml) for 24 hours. At the end of the incubation period, the cells were sequentially washed with PBS and 5% cold trichloroacetic acid. Washed cells were solubilized in 1 N NaOH, neutralized by glacial acetic acid, and counted in a scintillation counter. To prepare media containing gangliosides, desired amounts of GM₃ or control gangliosides GM₁, GD₃a, and GT₁b (Sigma) in C:M (2:1) were aliquoted into screw cap tubes, and solvents were evaporated under a nitrogen stream. To the dried lipids, MEM (1 ml) was added and the samples were probe sonicated. To each sonicated sample, an equal volume of MEM containing 0.8% BSA was added. The stock
solutions of gangliosides thus prepared were then diluted with the serum-free medium supplemented with 0.4% BSA to achieve a final glycolipid concentration (0.1 to 25 μg/ml) and passed through a 0.22-μm sterile filter (Millipore, Bedford, MA) before use.

RESULTS

DEAE Sephadex Chromatography of Radiolabeled Glycolipids of Migrating and Nonmigrating Corneal Epithelia

The ion-exchange chromatography was effective in separating neutral glycolipids from gangliosides. Among various glycolipid standards chromatographed, neutral glycolipids (ceramidedihexoside, ceramidetrihexoside, and asialoGM1) were eluted in fraction DE1, monosialogangliosides (GM1 and GM2) were eluted in fraction DE2-DE3, and disialogangliosides and trisialogangliosides (GD1a and GT1b) were eluted in fraction DE4. Upon DEAE chromatography of the radiolabeled corneal epithelial lipid extracts, the bulk of the radioactivity (>75%) was eluted in the neutral fraction (DE1). Glycolipids eluted in the neutral fraction (Fig. 1, DE1) were not further characterized in this study. Most of the corneal epithelial gangliosides, representing 10% to 20% of the total radioactivity, were eluted in fraction DE3 (see legends to Figures 1 and 2). Little radioactivity (<5%) eluted in fractions DE2 and DE4. Upon TLC, two poorly resolved gangliosides, GL1 and GL2, were detected in fraction DE3 of migrating cell cultures (Fig. 1). The chromatographic mobility of the top band (GL1) of the doublet was similar to that of standard GM1. Both GL1 and GL2 were present only in trace amounts in the corresponding fraction obtained from the extracts of nonmigrating epithelia. Fraction DE2 of the migrating cell cultures contained small amounts of GL1 and GL2. No radiolabeled components were detected in fraction DE2 of nonmigrating cultures or in fraction DE4 of migrating and nonmigrating cell cultures. Similar results were obtained with [3H]-galactose-labeled extracts of migrating and nonmigrating epithelia prepared by organ culture (Fig. 2). Higher amounts of GL1 and GL2 were present in the extracts of migrating than in nonmigrating epithelia regardless of whether the epithelia were prepared by cell culture (Fig. 1) or organ culture (Fig. 2).

Chromatographic Patterns of Unlabeled Gangliosides of Migrating and Nonmigrating Epithelium Prepared by Cell Culture

To determine whether GL1 and GL2 are the major gangliosides present in corneal epithelium and whether migrating epithelium accumulates greater amounts of GL1 and GL2 than nonmigrating epithelium, we analyzed orcinol staining patterns of thin-layer chromatograms of gangliosides of unlabeled migrating and nonmigrating corneal epithelial cell cultures after DEAE chromatography. GL1 and GL2 were the only gangliosides detected in the lipid extracts of both migrating and nonmigrating cell cultures (Fig. 3). Both GL1 and GL2 were present in higher amounts in migrating than in nonmigrating cell cultures (Fig. 3). This experiment was not performed on epithelia prepared by organ culture because of the limited availability of the starting material.

Neuraminidase Treatment of GL1 and GL2

Because the chromatographic mobility of GL1 and GL2 on TLC plates and on the DEAE Sephadex column was similar to that of GM1, experiments were
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MNMNMNMN
CD-
CTH-
GM3-
aGM,-
GM,-
GD,-
GDlb-
GTlb-

FIGURE 2. Fluorograms of thin-layer chromatography showing radiolabeled glycolipids of migrating (M) and nonmigrating (N) corneal epithelium in organ culture after DEAE chromatography. Epithelia were radiolabeled with [3H]-galactose in serum-free medium for 24 hours and were then processed to isolate a lipid fraction as described in Materials and Methods. Aliquots of lipid fractions containing 5 x 10^4 dpm were chromatographed on DEAE Sephadex columns, as described in the legend to Figure 1, to obtain fractions DE-1 to DE-4. Total counts eluted in each fraction were chromatographed (DE-1, M 38,000, AT 57,500; DE-2, M 3,000, N 0; DE-3, M 7,500, N 4,000; DE-4, M 1,000, N 0). Because of limited availability of the starting material, unfractionated samples were not analyzed. Note that as observed with epithelia in cell culture, components GL1 and GL2 are present in a higher amount in the DE-3 fraction of migrating epithelia compared to that in the corresponding fraction of nonmigrating epithelia. Migration positions of various glycolipid standards are indicated in the left margin. CD = ceramidedihexoside; CTH = ceramidetrihexoside; aGM, = asialoGM1.

conducted to determine whether these two gangliosides are susceptible to neuraminidase treatment. Thin-layer chromatography pattern of the products of the enzymatic hydrolysis is shown in Figure 4. Neuraminidase treatment caused the migration position of GL1 and GL2 (left panel) as well as of standard GM3 (right panel) to shift from that of GM3 to that of ceramidedihexoside. Moreover, after neuraminidase treatment, the doublet GL1 and GL2 migrated as a single species. The chromatographic mobility of the components in the samples incubated in the enzyme buffer alone did not change (Fig. 4, lane C). This experiment confirmed that GL1 and GL2 are GM3.

HPTLC-Immunostaining of Glycolipids of Corneal Epithelium

Total lipid extracts of migrating and nonmigrating cell cultures of corneal epithelium were analyzed for reactivity with mAb M-2590 (anti-GM3). In the immuno

nostained chromatograms of the extracts of migrating epithelia, one component migrating a little slower than GM3 was detected (Fig. 5, arrow, right panel). In contrast, in the extracts of nonmigrating cultures, this component was not detected. Total lipid extracts of migrating and nonmigrating epithelia prepared by organ culture were not analyzed for reactivity with mAb M-2590 or for susceptibility to neuraminidase because of limited availability of the starting material.

FIGURE 3. Analysis of unlabeled gangliosides of migrating (M) and nonmigrating (N) corneal epithelial cells grown in tissue culture. Aliquots of lipid extracts derived from 20 mg of original cell protein were chromatographed on DEAE Sephadex columns, as described in the legend to Figure 1, to obtain fractions DE-1 to DE-4. Fraction DE-1, which is expected to contain neutral glycolipids, was discarded. One fourth of the total material eluted in fractions DE-2 to DE-4 was chromatographed on a TLC plate as described in Materials and Methods. Glycolipids were visualized by spraying the plate with orcinol. Glycolipid standards were detected in either fraction DE-2 or DE-4 (not shown). DE-3 contained two orcinol-positive components (GL1 and GL2) that were present in a higher amount in the fraction derived from migrating compared to that derived from nonmigrating epithelial cell cultures. Total lanes indicate orcinol staining pattern of the unfractionated material derived from 2 mg of original cell protein. CD = ceramidedihexoside; CTH = ceramidetrihexoside.

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Effect of Exogenous Addition of Gangliosides on Proliferation of Corneal Epithelial Cells

To determine the effect of various exogenous gangliosides on rabbit corneal epithelial cell proliferation, a \(^{3}H\)-thymidine incorporation assay was used. GM3 significantly inhibited the \(^{3}H\)-thymidine incorporation into corneal epithelial cells, whereas other gangliosides had little effect. Compared to cultures incubated in media alone, \(^{3}H\)-thymidine incorporation in the presence of GM3, GM1, and GD1b (25 \(\mu\)g/ml each) was 51% ± 6% (mean ± SEM) of 14 experiments (N = 42, \(P < 0.05\)), 99% ± 4% of four experiments (N = 12), and 98% ± 5% of three experiments (N = 9), respectively (Fig. 6). GM3 inhibited the \(^{3}H\)-thymidine incorporation in a dose-dependent manner in a concentration range of 0.1 to 25 \(\mu\)g/ml (four experiments, N = 12, Fig. 7). There was no significant effect on the \(^{3}H\)-thymidine incorporation at GM3 concentration below 0.1 \(\mu\)g/ml (not shown). From GM3 concentration 0.1 to 10 \(\mu\)g/ml there was a gradual decrease, and between 10 and 25 \(\mu\)g/ml there was a sharp decrease in the \(^{3}H\)-thymidine incorporation (Fig. 7). The inhibitory effect of GM3 on \(^{3}H\)-thymidine incorporation reached maximum at 25 \(\mu\)g/ml concentration. Use of a higher GM3 concentration—up to 100 \(\mu\)g/ml—did not further enhance the inhibitory effect on the nucleotide incorporation into corneal epithelial cells (data not shown).

DISCUSSION

In the current study, we demonstrate that GM3 is the major ganglioside synthesized by rabbit corneal epithelium in both cell and organ culture. Moreover, we demonstrate that migrating corneal epithelia synthesize markedly greater amounts of GM3 compared to nonmigrating epithelia. Although migrating epithelium in cell culture is also undergoing cell division, it appears likely that the elevated levels of GM3 detected...
with a serum-free medium containing 0.4% bovine serum. After overnight culture, the SHEM medium was replaced with a serum-free medium containing 0.4% bovine serum albumin, [\textsuperscript{3}H]-thymidine (1 \mu Ci), and various gangliosides (25 \mu g/ml). After 24 hours of incubation, the cells were rinsed with phosphate-buffered saline and solubilized, and the radioactivity was measured in a scintillation counter. A value of 1 was assigned to the proliferation rate ([\textsuperscript{3}H]-thymidine incorporation) of control cells incubated in a medium containing glycolipids and with respect to control cells. Mean values ± SEM (error bars) are shown. Note that GM\textsubscript{3} significantly inhibited [\textsuperscript{3}H]-thymidine incorporation (P < 0.05, N = 42, 14 experiments), whereas GM\textsubscript{1} (N = 12, four experiments) and GD\textsubscript{1a} (N = 9, three experiments) had little effect. Cultures incubated in media containing epidermal growth factor (0.1 \mu g/ml) served as a positive control.

FIGURE 6. Comparison of the effect of various gangliosides on [\textsuperscript{3}H]-thymidine uptake by corneal epithelial cells. Primary cultures of corneal epithelial cells were dislodged by dispase treatment and were seeded into each well (1 \times 10^5 cells/well in 0.5 ml SHEM medium) of 12-well cell culture plates. After overnight culture, the SHEM medium was replaced with a serum-free medium containing 0.4% bovine serum albumin, [\textsuperscript{3}H]-thymidine (1 \mu Ci), and various gangliosides (25 \mu g/ml). After 24 hours of incubation, the cells were rinsed with phosphate-buffered saline and solubilized, and the radioactivity was measured in a scintillation counter. A value of 1 was assigned to the proliferation rate ([\textsuperscript{3}H]-thymidine incorporation) of control cells incubated in a medium containing glycolipids and with respect to control cells. Mean values ± SEM (error bars) are shown. Note that GM\textsubscript{3} significantly inhibited [\textsuperscript{3}H]-thymidine incorporation (P < 0.05, N = 42, 14 experiments), whereas GM\textsubscript{1} (N = 12, four experiments) and GD\textsubscript{1a} (N = 9, three experiments) had little effect. Cultures incubated in media containing epidermal growth factor (0.1 \mu g/ml) served as a positive control.

FIGURE 7. Dose-dependent inhibitory effect of GM\textsubscript{3} on [\textsuperscript{3}H]-thymidine uptake by corneal epithelial cells. Results are expressed as described in the legend to Figure 6. Mean values ± SEM (error bars) of four experiments are shown. Note that the inhibitory effect of GM\textsubscript{3} on [\textsuperscript{3}H]-thymidine incorporation enhanced significantly (P < 0.05, N = 12) with an increasing GM\textsubscript{3} concentration.

in migrating epithelium were related, at least in part, to the process of cell migration because higher levels of GM\textsubscript{3} were detected in migrating epithelium in organ culture, which is not expected to undergo cell division during cell migration. Having established that migrating cell cultures synthesize greater amount of GM\textsubscript{3} than nonmigrating cell cultures, it was of interest to determine whether migrating cell cultures also accumulate greater amounts of GM\textsubscript{3}. Using unlabeled cell cultures—DEAE Sephadex chromatography—and orcinol staining of the thin-layer chromatograms clearly demonstrated that GM\textsubscript{3} is the major, if not the only, ganglioside present in corneal epithelium and that migrating cell cultures also accumulate greater amounts of GM\textsubscript{3} than nonmigrating cell cultures. On thin-layer chromatograms, GM\textsubscript{3} of corneal epithelium was seen as a doublet. GM\textsubscript{3} derived from a number of other tissues is known to migrate as a doublet because of heterogeneity either in the carbon chain of the fatty acid with respect to chain length, the extent of unsaturation and hydroxylation,\textsuperscript{30,31} or the sialic acid residue of GM\textsubscript{3} with respect to hydroxylation of the N-acetyl group, which results in the formation of the N-glycoly neuraminic acid.\textsuperscript{32,33} Our findings that neuraminidase treatment converted the GM\textsubscript{3} doublet into a single lactosylceramide species, and that anti-NeuAc-GM\textsubscript{3} mAb reacted with only one of the two bands of GM\textsubscript{3} doublet, suggest that perhaps the heterogeneity in the GM\textsubscript{3} species of corneal epithelium we detected may be related to the variation in the sialic acid residues. However, detailed structural studies should be carried out to establish conclusively the presence of NeuGa-GM\textsubscript{3} in corneal epithelium. Moreover, heterogeneity in the acyl moiety of the GM\textsubscript{3} species of corneal epithelium cannot be ruled out at this time.

GM\textsubscript{3} is a glycolipid found in a variety of cell types. In brain, it is present only in trace amounts, constituting less than 1% of the total gangliosides. In thymus and erythrocytes, GM\textsubscript{3} constitutes 17% and 37% of total gangliosides, respectively, and in a number of other tissues, including lung (64%), intestine (59%), and liver (69%), this glycolipid constitutes more than 50% of the total gangliosides.\textsuperscript{34} Paller et al\textsuperscript{35} measured the relative amounts of GM\textsubscript{3} in the epidermis of a variety of tissues and found that it constituted 51% (breast epithelium) to 74% (foreskin) of the total gangliosides. The current study indicated that GM\textsubscript{3} is the major, if not the only, ganglioside synthesized by and present in corneal epithelium, and its level is elevated during corneal epithelium cell migration.

GM\textsubscript{3} is known for its ability to regulate cell proliferation.\textsuperscript{36,37,38} It is well established that exogenous glycolipids, when added to media, are taken up by cells, become constituents of their plasma membranes, and function like those synthesized by cells.\textsuperscript{39-41} Various studies have tested the effect of exogenous
GM₃ on cell proliferation and have shown that the growth of various cell types, including BHK, KB, A431, 3T3 cells, and human keratinocytes, can be inhibited by the exogenous addition of GM₃ but not by most other gangliosides.²⁰,²⁸,³⁸,⁴² Similarly, in the current study, the exogenous addition of GM₃, but not of other gangliosides, was found to inhibit the proliferation of corneal epithelial cells measured by H-thymidine incorporation. Consistent with the observations that GM₃ inhibits cell mitosis, Paller et al.⁴³ have shown that the stratum corneum of human epidermis stains with anti-GM₃ antibody and that antibody binding in this region is diminished or absent in many disorders of hyperproliferation, including psoriasis, squamous cell carcinoma, and the hyperproliferative forms of ichthyosis. It would be of interest to determine whether GM₃ is involved in the pathogenesis of corneal epithelial hyperplasia known to occur after various forms of corneal insult, including excimer laser treatment for correction of myopia.²⁰,²⁸,⁴² Regarding the mechanism by which GM₃ inhibits cell mitosis, multiple studies have provided evidence that GM₃ functions by inhibiting activities of various protein kinases associated with growth factor receptors.²⁶,⁴⁰,⁴⁴,⁴⁶

Our finding that GM₃ levels are elevated during cell migration and that the ganglioside inhibits corneal epithelial cell proliferation in vitro leads us to speculate that elevated GM₃ levels may be responsible, at least in part, for the cessation of mitosis known to occur at the wound edge after corneal epithelial injury.¹ However, if GM₃ functions to inhibit cell proliferation, one might expect its level to be elevated on reaching confluence. In contrast, in the current study, confluent–contact-inhibited corneal epithelial cells were found to contain reduced levels of GM₃ compared to the sparse–dividing cell cultures. In various studies, depending on the cell type, GM₃ levels have been found to increase,⁴⁰–⁴⁶ as well as to decrease⁴⁶ upon cell cultures reaching confluence. The differential expression of GM₃ on contact inhibition in various cell types may be related to its role in the modulation of cell–cell interactions.⁵⁰,⁵¹ Perhaps in cell types in which GM₃ expression is elevated on reaching confluence, this ganglioside indirectly contributes to the cessation of mitosis by inducing cell contact inhibition, whereas in sparse cell cultures, GM₃ may inhibit cell mitosis by a more direct mechanism involving the inhibition of growth factor receptor kinases. In this respect, our data support the notion that GM₃ possibly helps prevent hyperproliferation of corneal epithelium but is not involved in events that mediate cell–cell interactions responsible for the induction of contact inhibition and cellular polarity.

Our findings that migrating corneal epithelium synthesize and contain an increased amount of GM₃ compared to nonmigrating corneal epithelia may have implications in events that mediate cell–substratum interactions during cell migration. In this regard, Zheng et al.²⁵,⁵⁵ have shown that liposomes containing integrin and a moderate amount of GM₃ adhere to fibronectin much more strongly than liposomes containing only integrin or integrin and other glycolipids, such as glucosylceramide or lactosylceramide. Our future studies will address whether GM₃ influences fibronectin–integrin interactions during corneal epithelial cell migration. Such studies should be greatly facilitated once function-blocking antibodies to the ganglioside are readily available.

Key Words
cell migration, cell proliferation, corneal epithelium, gangliosides, wound healing

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