Neutral Glycolipids of Migrating and Nonmigrating Rabbit Corneal Epithelium in Organ and Cell Culture

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It is generally believed that plasma membrane glycoconjugates influence corneal epithelial cell migration after wounding. Previous studies have focused on the role of glycoproteins in this event. The present study was designed to determine whether migration-specific glycolipids are synthesized by epithelium of healing rabbit corneas. Migrating and nonmigrating rabbit corneal epithelia were incubated with [3H]-galactose in an organ culture system for 48 hr. At the end of the labeling period, a neutral glycosphingolipid (NGSL) fraction was isolated from each radiolabeled epithelium and was analyzed by thin-layer chromatography. Three radiolabeled NGSL components, M1, M2 and M3 (M1–M3), were present in significantly higher amounts in the extracts of migrating as compared to nonmigrating epithelium. Chromatographic mobility of M3 was similar to that of a standard glucosylceramide; M1 and M2 migrated more slowly than M3. For characterization of the migration-related NGSL, a large amount of the starting material is required. Experiments, therefore, were conducted using cell cultures of rabbit corneal epithelium. Confluent (nonmigrating) cell cultures of rabbit corneal epithelium were found to synthesize either minimal or undetectable amounts of NGSL M1–M3. In contrast, we found that the NGSL M1–M3 are synthesized as major components by sparse (migrating) corneal epithelial cell cultures. Components M1–M3 were synthesized as major components by sparse cultures even in the absence of cell mitosis. This suggests that the increased synthesis of components M1–M3 by sparse cell cultures may be related to cell migration rather than cell mitosis. It was shown further by orcinol staining of thin-layer chromatograms of NGSL of unlabeled cells that components M1 and M3 are present in higher amounts in migrating as compared to nonmigrating epithelial cells in culture. Invest Ophthalmol Vis Sci 31:689–695, 1990
signed to determine whether: (1) migration-specific neutral glycosphingolipids (NGSL) are synthesized by healing rabbit corneal epithelium in organ culture; and (2) whether NGSL synthesized by corneal epithelium in tissue and organ culture are similar.

Materials and Methods

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

All materials for organ and cell culture were purchased from Flow Laboratories (McLean, VA) unless stated otherwise. SHEM media was prepared as described by Jumblatt and Neufeld. For this study, we used rabbit eyes from Pel-Freez Farms, Inc. (Rogers, AR), and processed approximately 80 to 100 eyes at one time. The eyes from 2- to 3-month-old New Zealand rabbits reached our laboratory on wet ice within 24 hr after slaughter. After washing the eyes in sterile saline, the central corneas were demarcated by an 8 mm trephine, and the epithelium within the trephined area was removed with a #15 Bard-Parker blade. The corneas were then excised with a 1 to 3 mm scleral rim, rinsed, and were then incubated in the modified SHEM media containing 25 \( \mu \)g/ml of streptomycin, (70 \( \mu \)g/ml) and amphotericin B (450 \( \mu \)g/ml) and were then immersed in a neomycin-gramacidin-polyoxymyxin B solution (Burroughs Wellcome, Research Triangle Park, NC) containing penicillin G (1.8 mg/ml), streptomycin (4.2 mg/ml) and amphotericin B (0.09 mg/ml) for 5 to 7 min. The corneas were rinsed, and were then incubated in the modified SHEM media at 37°C in a CO2 incubator. The reepithelialization was monitored by staining the corneas periodically with Richardson stain. To radiolabel the glycolipids of the migrating corneal epithelium, the corneas were allowed to heal partially for 48 hr and were then incubated for an additional 48 hr in the modified SHEM media containing 25 \( \mu \)Ci/ml of 6-[\(^3\)H]-galactose (20 Ci/mM, American Radiolabeled Chemicals, St. Louis, MO). At the end of the labeling period, the corneas were rinsed extensively with cold PBS, central 8 mm buttons were cut out with a trephine and the migrating epithelium was collected by scraping with a #15 Bard-Parker blade under a dissecting microscope. At the time of harvesting the migrating epithelia from healing corneas, a central area of approximately 4 mm (12.6 mm²) remained deepithelialized. Care was taken not to let the corneas fully heal prior to collection of migrating epithelium. This ensured minimal contamination with nonmigrating epithelium. To prepare radiolabeled nonmigrating epithelium, unscraped corneas were processed concomitantly as described above.

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

The corneal epithelial cells were grown in tissue culture using Pel-Freeze eyes. Briefly, after the eyes were washed in sterile saline, the corneas were excised and rinsed in HEPES-buffered Eagles Minimum Essential Media (EMEM-HEPES):HAMS F-12 (1:1). After the endothelium and posterior stroma were removed with forceps, the anterior stromas with intact epithelium were incubated at 37°C for 1 hr with 1 unit/ml of Dispase II (Boehringer Mannheim, Indianapolis, IN) in calcium- and magnesium-free Hank’s buffer. Epithelial fragments were peeled off with forceps and cultured in 100 mm dishes using SHEM media. Epithelial fragments from three corneas were placed in a 100 mm dish. The medium was changed two or three times a week. Within 3 days, approximately 50% to 70% of the dish was populated with rapidly dividing cells, designated as migrating corneal epithelial cells. Within 10 to 12 days, 90% to 95% of the dish was populated by tightly packed polygonal cells, designated as nonmigrating corneal epithelial cells. Epithelial cell cultures obtained from the Pel-Freeze eyes were morphologically indistinguishable by phase contract microscopy from those prepared from rabbits killed in our laboratory.

To radiolabel the GSL of migrating and nonmigrating corneal epithelial cells in tissue culture, approximately 0.5 to 2.0 \( \times 10^6 \) cells in culture in 100 mm dishes were incubated with 5.0 ml of SHEM media containing 5 \( \mu \)Ci/ml of 6-[\(^3\)H]-galactose (20 Ci/mM) for 48 hr. At the end of the labeling period, the medium was removed and the cells were washed extensively with phosphate-buffered saline, pH 7.4 (PBS). Using a cell scraper, the cells in each dish were dislodged in 0.5 ml of cold PBS and were then placed into screw-cap tubes on ice and lyophilized. In two experiments, cell cultures were treated with 5-fluorouracil (5-FU, 10 \( \mu \)g/ml, LyphoMed Inc., Rosemont, IL) for 48 hr, to inhibit the cell mitosis, prior to incubation with [\(^3\)H]-galactose. To ensure that treatment with 5-FU effectively inhibited cell mitosis, incorporation of [\(^3\)H]-thymidine was measured in the 5-FU-treated cells as well as the control cells, not treated with the drug. For this, cell cultures were incubated with [\(^3\)H]-thymidine (1 \( \mu \)Ci/ml, 15 Ci/mM) for 24 hr, washed extensively and subjected to trichloroacetic acid (TCA) precipitation. The radioactivity was then measured in the washed TCA precipitate of the radiolabeled cells.
Isolation of Neutral Glycosphingolipids From Radiolabeled Epithelia

Radiolabeled epithelia were subjected to a series of extraction procedures to prepare a lower phase containing NGSL, phospholipids and neutral lipids and an upper phase containing gangliosides. Investigations on gangliosides will be reported in a separate publication. The lower phase was subjected to methanolation with C:M:1M KOH (0.8:0.4:0.2) for 5 hr at 37°C to eliminate diacylphosphoglycerides. After methanolation, the samples were neutralized with HCl, dried at 40°C with a stream of N2, suspended in 1.0 ml distilled water, dialyzed against cold distilled water over a period of 24 hr with eight changes of 10 liters each, lyophilized, dissolved in CHCl3:methanol (2:1) and analyzed by thin-layer chromatography (TLC). Cholesterol was not removed from the lower phase prior to analysis because it migrated with the solvent front during TLC and, therefore, did not interfere with the analysis of NGSL.

Thin-Layer Chromatography of Neutral Glycosphingolipids

NGSL fractions containing 30,000 to 50,000 cpm were spotted on 10 x 10 cm silica gel, 60 plates (HPTLC, EM Science, Cherry Hill, NJ). Separation of NGSL was performed using a solvent containing chloroform:methanol:H2O (65:25:4). NGSL standards used in this study consisted of glucosylceramide, galactosylceramide, lactosylceramide, ceramidetrihexoside and globoside. Glucosyl-, galactosyl- and lactosylceramides were obtained from Sigma Chemical Co. (St. Louis, MO), whereas ceramidetrihexoside and globoside were kindly provided by Dr. F. Jungalwala of the Eunice Kennedy Shriver Center (Waltham, MA). The standards were chromatographed on the same plate as the radiolabeled NGSL. After the separation of the NGSL, the TLC plate was allowed to dry and was then cut with a glass cutter to separate the section of the plate consisting of standards. The rest of the plate was sprayed with En3Hance (New England Nuclear, Boston, MA) and subjected to fluorography using X-omat AR film (Kodak, Rochester, NY). The standards were visualized by spraying the plate with an orcinol spray reagent.

We compared fluorographic patterns of thin-layer chromatograms of migrating and nonmigrating corneal epithelium. The components that were present in the migrating epithelium in higher concentrations were designated "M" components. To determine whether M components also accumulate in greater amounts in the migrating epithelium compared to the nonmigrating epithelium, NGSL were isolated from unlabeled corneal epithelial cells in culture and were chromatographed on a thin layer plate as described above. Various NGSL components in the chromatogram were then visualized by staining with orcinol.

Results

Healing of Deepithelialized Corneas

Figure 1 shows the rate of healing of corneal epithelium in organ culture. At 48 hr after injury, when radiolabeled galactose was added, an approximate 6 mm (28.3 mm²) area of central cornea remained deepithelialized. Similarly, when migrating epithelium was collected at 96 hr after injury, an area of central cornea of about 4 mm (12.6 mm²) remained deepithelialized. Reepithelialization of 8 mm wounds was complete in 5 to 6 days (data not shown). We initially observed that after 6–7 days in organ culture, corneal epithelium tends to deteriorate. We, therefore, performed no experiments to determine whether NGSL synthesized by fully healed corneas were similar to NGSL synthesized by normal corneas.
Yield of Radiolabeled NGSL

Incorporation of \(^{3}H\)-galactose into NGSL of migrating corneal epithelium was approximately 1.5 to 2 times greater than in that of nonmigrating epithelium (Table 1). Yield of migrating and nonmigrating epithelium, expressed as micrograms of protein/cornea, was 12 and 6 times greater, respectively, from epithelium grown in cell culture compared with epithelium prepared by organ culture (Table 1).

Chromatographic Patterns of Radiolabeled Neutral Glycosphingolipids of Migrating and Nonmigrating Corneal Epithelia

TLC patterns of radiolabeled NGSL from four preparations of epithelia prepared by organ culture and three preparations of epithelia prepared by cell culture were analyzed. To ensure the reproducibility of the data, NGSL from different preparations were not pooled but were analyzed individually. Representative TLC patterns of radiochemically detected NGSL of migrating and nonmigrating corneal epithelial cells derived by organ as well as tissue culture techniques are shown in Figure 2. TLC patterns of the epithelia derived by tissue and organ culture were remarkably similar. Regardless of whether epithelia were grown in tissue or organ culture, three NGSL components, M1, M2 and M3, were present in significantly higher amounts in migrating as compared with nonmigrating corneal epithelial cells (Fig. 2). Greater amounts of NGSL components, M1, M2 and M3, were detected in all seven chromatograms of NGSL of migrating epithelia than in those of seven nonmigrating epithelia. However, in some chromatograms, components M1 and M2 were not resolved (Fig. 3). Chromatographic mobility of M3 was similar to that of a standard glucosylceramide, and components M1 and M2 migrated a little more slowly than M3 (Fig. 2). The relative intensity of the M components was greater in the chromatograms of all three preparations of migrating epithelia prepared by cell culture than in the chromatograms of the four preparations of migrating epithelia prepared by organ culture.

Several other differences (Fig. 2) between the TLC patterns of migrating and nonmigrating epithelium (eg, the presence of two components migrating slightly faster than CM in the chromatogram of only nonmigrating cell cultures) are not discussed here because they were not found consistently in all chromatograms analyzed in this study.

<table>
<thead>
<tr>
<th>Culture technique</th>
<th>Epithelium</th>
<th>Incorporation of radioactivity (cpm x 10^-2)/mg of cell protein</th>
<th>Protein yield (μg/cornea)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organ culture</td>
<td>Nonmigrating</td>
<td>0.86 ± 0.14</td>
<td>49 ± 0.700</td>
</tr>
<tr>
<td>Organ culture</td>
<td>Migrating</td>
<td>1.85 ± 0.24</td>
<td>12 ± 1.470</td>
</tr>
<tr>
<td>Tissue culture</td>
<td>Nonmigrating</td>
<td>0.93 ± 0.24</td>
<td>290 ± 2.300</td>
</tr>
<tr>
<td>Tissue culture</td>
<td>Migrating</td>
<td>1.43 ± 0.23</td>
<td>140 ± 1.500</td>
</tr>
</tbody>
</table>

* ± SEM of three preparations.
migrating rabbit corneal epithelial cells continue to synthesize M components in the absence of cell mitosis (Fig. 3).

Chromatographic Patterns of Unlabeled Neutral Glycosphingolipids of Migrating and Nonmigrating Epithelia Prepared by Cell Culture

To determine which, if any, of the migration-related component M1, M2 and M3 also accumulated in greater amounts in migrating epithelium compared with nonmigrating epithelium, we analyzed thin-layer chromatograms of NGSL of three unlabeled preparations of migrating and nonmigrating epithelial cell cultures. Greater amounts of at least two NGSL components, M1 and M3, were detected in all three preparations of migrating epithelial cell

Effect of 5-Fluorouracil Treatment on the Synthesis of M Components

That the treatment of the cell cultures with 5-FU almost completely inhibited cell mitosis was shown by the marked reduction of [3H]-thymidine incorporation in the 5-FU-treated cells (75 cpm/mg cell protein, a mean of two preparations) compared to control cells, not treated with the drug (25,076 cpm/mg cell protein, a mean of three preparations). Comparison of the fluorography patterns of NGSL of 5-FU-treated cells with that of control cells revealed that the
cultures compared with those of respective nonmigrating epithelial cell cultures (see Fig. 4 for a representative orcinol staining pattern). This experiment was not performed on epithelia prepared by organ culture due to lack of sufficient quantities of the starting material.

**Discussion**

Our results demonstrate that the TLC patterns of NGSL synthesized by corneal epithelia prepared by tissue and organ culture are remarkably similar. Our data also demonstrate that three glycolipids, M1, M2 and M3, are synthesized in greater amounts by migrating epithelial cells when compared with nonmigrating epithelial cells grown in tissue as well as in organ culture. Having established that three NGSL, M1, M2 and M3, are synthesized in increased amounts by migrating epithelium compared with nonmigrating epithelium, it was of interest to determine if any of the M components also accumulate in greater amounts in migrating epithelia. The method employed using unlabeled cell cultures and orcinol staining of the thin-layer chromatograms clearly demonstrated that two of the three M components, M1 and M2, also accumulate in greater amounts in migrating compared to nonmigrating epithelium.

During the initial phase of organ and tissue culture of corneal epithelium, cell migration is a major event; after the initial phase, cell migration and cell mitosis occur concomitantly. The M components identified in this study were synthesized even in the absence of cell mitosis and are therefore likely to be related to cell migration rather than to cell mitosis.

The migration-related glycolipids identified in this study are likely to be some form of ceramidemonohexosides since they migrated with a relative mobility similar to that of a standard glucosyl/galactosylceramide. Greater synthesis of components M1, M2 and M3 during corneal epithelial cell migration may have been induced by activation or increased synthesis of enzymes involved in the synthesis of the M components. A detailed characterization of components M1, M2 and M3 would be prerequisite to testing our hypothesis. Since similar data were obtained from epithelial cells prepared in tissue and organ culture, we suggest that these epithelial cells grown in tissue culture are suitable for preparing large amounts of M1, M2 and M3 needed for the detailed characterization of these components. Since 80% of the cellular glycolipids usually are located on the cell surface, the majority of the NGSL identified in this study are likely to be associated with corneal epithelial plasma membranes. In the present study, however, experiments were not performed to determine whether migration-related NGSL components M1, M2 and M3 are located on corneal epithelial cell membranes. The biological significance of components M1, M2 and M3 in corneal epithelial cell migration and wound healing remains to be established. Studies have shown that in a murine model, P. aeruginosa binds preferentially to injured corneas rather than to normal corneas. It is reasonable, therefore, to suggest that NGSL components M1, M2 and M3 may serve as bacterial receptors and play a role in the development of corneal infection. We have recently determined using a thin-layer chromatogram overlay procedure that radiolabeled P. aeruginosa bind to NGSL component M3.

**Key words:** glycolipids, corneal epithelium, epithelial cell migration, cell culture, organ culture

**Acknowledgments**

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