Changes in Chick Corneal Lipids during Development

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Chick corneas from days 9 through 18 of embryonic development were analyzed for phospholipid and glycolipid content, and for phospholipid and ganglioside classes using chromatographic techniques. Little change in phospholipid headgroup classes was detected during this time span, with the exception of a steady increase in the content of phosphatidylserine from 12 to 18% of the total. Changes in glycolipid content also were observed, with a maximum in the sphingolipid/phospholipid ratio at day 14. This increase was correlated with the appearance of highly sialylated gangliosides at days 14 and 15; these ganglioside species then declined in abundance until they were no longer detectable at day 18. These results indicate that gangliosides with high sialic acid content undergo stage-specific developmental cycles in the chick cornea. Furthermore, the data suggest that phosphatidylserine content may be a sensitive biochemical measure of corneal innervation. Invest Ophthalmol Vis Sci 25:1151-1155, 1984

Gangliosides are a group of amphiphilic glycosphingolipids that are preferentially located at the cell surface of eucaryotic cells. The lipid portion of these molecules is embedded in the membrane bilayer, and the oligosaccharide chain, containing one or more sialic acid groups, extends into the aqueous environment. This surface location permits gangliosides to modulate effects of extracellular ligands on cell functions, and the large number of possible oligosaccharide chains allows for a wide variety of specific interactions. Gangliosides have been implicated as receptors or modulators of receptors for hormones, interferon, opiates, toxins, and growth-regulatory substances. Additionally, developmentally important functions such as cell adhesion, growth factor responses, and formation of neuromuscular junctions also are thought to be influenced by the glycolipid composition of the cell plasma membrane. Inhibition of degradation of gangliosides, as in the various sphingolipidoses such as Tay-Sachs disease, is correlated always with mental retardation, further strengthening the link between gangliosides and normal development, particularly in the nervous system. Recent reports also have linked the appearance of stage-specific antigens with particular gangliosides; the reappearance of these ganglioside antigens in transformed cells also has attracted considerable interest. These observations indicate the possibility of an important role for gangliosides in development of various adult structures, and provided the impetus for us to examine the ganglioside content and classes during development of chick corneas.

The chick corneal system is suited ideally for this type of study, as it is a readily accessible tissue, with defined patterns of glycosaminoglycan and collagen deposition, innervation, and clearing to give the transparent adult structure. Because these processes are well studied and occur on a very reproducible time schedule, it should be possible to correlate changes in the ganglioside content or glycolipid classes with developmental changes. This report details our initial observations on ganglioside pattern changes during corneal development. The data suggest that lipid changes occur concommitantly with such processes as nerve migration, interactions between corneal cells and the nervous tissue, and corneal clearing.

Materials and Methods

Corneal Dissection

All corneas used in these experiments were derived from White Leghorn (Gallus domesticus) eggs, which were obtained from local suppliers. The eggs were incubated in a forced draft incubator at a temperature of 38°C. The embryos were staged according to the normal developmental tables of Hamburger and Hamilton. Anterior halves of eyes were dissected free from extraembryonic membranes in cold saline-G solution. Following dissection, the corneas were placed in a fresh cold solution of saline-G. A cornea

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punch was used as a trephine (1.8, 2.1, 2.1, 2.7, 2.7, and 2.7 mm for days 9, 13, 14, 15, 16, 17, 18, respectively) to ensure that no scleral ring remained attached to the corneas. Corneas were collected and placed on ice in a 13 × 100 mm glass screw cap tube until 52 corneas were gathered from each age group. After collection, each group of corneas was stored at −20°C prior to lipid extraction. Embryos and eggs were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

**Lipid Extraction and Analysis**

One milliliter of chloroform/methanol (2/1, v/v) was added to the tubes containing the corneas. After heating to 60°C for 5 min, the samples were cooled, and were then homogenized in a 5.0-ml glass homogenizer with a tight-fitting teflon pestle. After homogenization, samples were again heated to 60°C for 5 min and applied to a dry Unisil (Clarkson) silicic acid column according to the method (A) of Irwin and Irwin.18 Neutral lipids and phospholipids were eluted with 2.0 ml chloroform/methanol (2/1). Gangliosides were eluted with 5.0 ml of chloroform/methanol/water (50/50/15, v/v/v). Both samples were then dried under nitrogen at 40°C. Samples were resuspended in 0.5 ml solvent (chloroform for the neutral lipids and phospholipids, and chloroform/methanol/water (50/50/15) for the gangliosides).

**Thin Layer Chromatography**

Gangliosides were spotted on 250-μm Silica Gel G TLC plates (Analtech), dried under a stream of air, and developed in chloroform/methanol/15 mM MgCl₂/NH₄OH (60/35/7.5/3) (19). Gangliosides were detected by spraying with resorcinol reagent,19 and all lipids were visualized by spraying the plates with 50% H₂SO₄, and charring overnight at 130°C.

The neutral lipid and phospholipid fractions were spotted at the lower left corner of a 20 × 20 cm Silica Gel G TLC plate (Analtech) and developed in the two-dimensional solvent system previously described.20 Lipids were visualized by spraying the plate with 50% H₂SO₄ and charring as described above.

**Analytical Methods**

Phosphate was assayed by the method of Ames,21 using the charred spots from the TLC plates. Sphingosine was assayed by a modification of the method of Naoi et al.22 Sulfatide (Supelco) standards (0–100 nmol) and unknowns were added to 13 × 100 mm tubes, and 0.5 ml 1 N HCl (in methanol/water, 82/18, v/v) was added to each tube. The tubes were capped with teflon-lined screw caps, and heated at 90°C for 2.0 hr. After cooling, 0.25 ml 2 N NaOH, and 0.75 ml sodium borate buffer (0.2 M, pH 8.0) were added and mixed. Fluorescamine (0.5 ml, 0.15 mg/ml in acetone, freshly prepared) was added and mixed immediately. Three milliliters of chloroform then was added to all tubes, the tubes were capped, and mixed well using a vortex mixer for 30 sec each. Tubes were centrifuged for 5 min at 1000 rpm in a table-top clinical centrifuge. The upper (aqueous) phase was discarded. The lower (chloroform) phase was used for analysis by fluorescence emission at 480 nm, excitation at 390 nm, to quantitate long chain sphingosine bases, using a Spex Fluorolog spectro-fluorimeter.

**Materials**

All solvents were analytic grade and were obtained from Mallinckrodt (St. Louis, MO). Lipid standards were obtained from Supelco, TLC plates from Analtech, and other reagents were from Sigma (St. Louis, MO).

**Results**

Preliminary experiments using corneal lipid extracts from alternate days during chick development from day 9 to day 18, indicated that a significant increase in complex gangliosides could be detected on day 15 (data not shown). Further experiments then were performed on corneal lipid extracts from days 13, 14, 15, 16, and 17. These extracts were analyzed for glycolipid content, phospholipid content and glycolipid and phospholipid classes by thin layer chromatography.

Phospholipid and glycolipid content were measured on total lipid extracts before silicic acid column chromatography. These results are shown in Figure 1. Phospholipid content increased steadily with developmental age; this increase was seen in two separate experiments shown. In contrast, glycolipid content, as measured by sphingosine and corrected for sphingomyelin content (see below), was seen to undergo a significant increase from day 13 to day 14, then decrease during the next 2 days. The lower panel of Figure 1 shows the glycolipid/phospholipid ratio, which also peaked at day 14 of development.

Phospholipid head group classes were measured after thin layer chromatography on Silica Gel G. Spots charred by H₂SO₄ at 130°C were scraped from the plates and assayed as described in Materials and Methods. These data are shown in Table 1. No large and reproducible changes were seen in any of the phospholipid head group classes, with the exception of phosphatidylserine. This aminophospholipid was seen to increase from 12% of the total phosphate at
Fig. 1. Glycosphingolipid and phospholipid content of chick corneas during embryonic development. Corneas were dissected, extracted, and analyzed for phosphate and sphingosine as described in the text. Five percent of the phospholipid content was subtracted from the sphingolipid content in order to correct for sphingomyelin. Results shown are from two separate experiments. Upper panel: □ = phospholipids, experiment 1; ■ = phospholipids, experiment 2; ○ = glycolipids, experiment 1; and • = glycolipids, experiment 2. Lower panel: Δ = glycolipid/phospholipid; ratio (mol/mol) calculated from average values in experiments 1 and 2.

Table 1. Phospholipid composition of total lipid extracts from embryonic chick corneas

<table>
<thead>
<tr>
<th>Days</th>
<th>%PC*</th>
<th>%PE†</th>
<th>%SM‡</th>
<th>%PS§</th>
<th>%Other PL‖</th>
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<tbody>
<tr>
<td>9</td>
<td>43</td>
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<td>5</td>
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<td>16</td>
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<td>18</td>
<td>10</td>
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</tbody>
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*PC = phosphatidylcholine.
†PE = phosphatidylethanolamine.
‡SM = sphingomyelin.
§PS = phosphatidylserine.
‖Other PL includes cardiolipin, phosphatidylinositol, phosphatic acid and phosphatidylglycerol.

Discussion

The changes observed in phospholipid and glycolipid, specifically ganglioside, content could potentially

day 9 to 18% of the total on day 18. An apparent increase in the other phospholipid classes at day 9 is merely due to high levels of color development from the silica gel itself, relative to the low actual phosphate levels. The low level of sphingomyelin (5–8%) was subtracted from the molar content of glycolipid sphingosine to give the results shown in Figure 1.

Ganglioside classes were qualitatively examined after silicic acid chromatography fractionation, using thin layer chromatographic techniques. These results are shown in Figure 2. The solvent used to develop these TLC plates separates gangliosides into groups based on the number of sialic acids in the oligosaccharide chain. It is apparent that all samples from all days examined had detectable levels of mono- or disialo-gangliosides, and that there was no change in this amount over developmental time. However, the content and types of more highly sialylated gangliosides seemed to undergo a stage-specific change. Specifically, the number of ganglioside species with two or more sialic acid groups was increased on days 14–16 and decreased thereafter. No other change in ganglioside species was detected reproducibly in these preparations.

Fig. 2. Thin layer chromatograph of gangliosides from chick corneas. Corneas were dissected, lipids were extracted and fractionated as described in the text. The glycosphingolipid fractions were spotted on 250 µm Silica Gel G TLC plates, and developed as described in the text. Lipids were visualized by spraying the plate with sulfuric acid/water (50/50, v/v), and charring overnight at 130°C. PL = Phospholipid (not positive in resorcinol sprayed plates), A = day 12, B = day 13, C = day 14, D = day 15, E = GM1 standard, F = day 16, G = day 17, H = day 18, I = day 19. Arrows indicate gangliosides with more than one sialic acid per molecule.
be reflecting one or more of several changes known to be occurring in the chick cornea during development. It is known that corneal nerves encircle the chick cornea during days 5–10, begin to migrate into the cornea per se on day 11 or 12, and complete the innervation of the cornea by day 18. It is possible that the increase in corneal phosphatidylserine (Table 1) arises from membranes of the nerve cells, which are known to be enriched for this particular phospholipid. Our data certainly are consistent with that interpretation; further experiments are planned in order to address this question and to determine if, in fact, phosphatidylserine can be used as a biochemical monitor of corneal innervation.

It is possible that the changes in ganglioside content also are due to nerve migration into the cornea, for gangliosides are common components of nervous, particularly synaptic, tissue. This possibility is rather unlikely to be the entire explanation, however, for two reasons. First, the basic ganglioside pattern is found as early as day 9, when no silver-staining nerves are seen in the chick cornea. Secondly, the number of nerves in the cornea increases with developmental age, while the ganglioside content increases until day 15 and then decreases.

A further observation that remains to be explained is the appearance and disappearance of highly sialylated ganglioside species on days 14–16. Again, it is probably not due entirely to neurite migration into the cornea, as one then would expect to see a constant or increasing ganglioside composition. It is certainly possible that these gangliosides are of nervous origin, and their appearance and disappearance merely reflect a change in the ganglioside composition of the nerves perhaps related to their function, superimposed on the background of gangliosides derived from the epithelial cells and keratocytes. Further experiments will be required to assess accurately the contribution of each of these corneal cell types to the ganglioside compositions reported here.

A final possibility that deserves mention is based on previous observations that ganglioside compositions can influence both nerve migration and neuromuscular junction formation. It is possible that the gangliosides in corneal tissue are specific modulators of neurite migration or synapse formation (if such connections form in cornea) or both. Obviously, proof of such an hypothesis awaits the precise chemical identification and cellular localization of these interesting and developmentally important lipids.

The changes in corneal glycolipids described above occur during the same period of time as several other morphogenetic events in the cornea: corneal innervation per se begins on day 11 or 12 and reaches the corneal center by day 18 (15); the increase in thickness of the corneal epithelium, coinciding with an increase in the number of cells, begins on day 10 or 11 and continues until day 20 (24–25); the degree of sulfation of corneal glycosaminoglycan (12) and thyroxine-induced increases in synthesis of 3′-phosphoadenosine-5′-phosphosulfate (PAPS) both increase significantly during the period while corneal innervation is occurring (26–29), transparency of the cornea begins to increase on day 14 (16), under the influence of thyroxine (27–29) and is complete by day 19 or 20 (10). The extent to which any of these developmental events is dependent upon corneal innervation or upon changes in lipid composition in any corneal cell layer must await further experiments. The identity of the highly sialylated gangliosides also remains to be determined. Due to the small amounts of lipid available, complete characterization of these lipids may not be possible. It should be possible to obtain carbohydrate compositional data for these corneal lipids from the amounts presently available. Such experiments are presently underway.

Key words: cornea, development, gangliosides, phospholipids, corneal nerves

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