Objective: To determine whether an association between keratoconjunctivitis sicca (KCS) and meibomian gland lipids exists in patients with chronic blepharitis.

Methods: Meibomian gland lipids were collected from normal patients and those with chronic blepharitis. Some of the chronic blepharitis patients had an ocular surface abnormality with apparent aqueous deficiency similar to KCS. Lipids were separated by thin-layer chromatography and polar lipids were further separated by high-pressure liquid chromatography with detection by UV absorbance. Lipids were identified by retention time with comparison with standards and by gas chromatography and mass spectroscopy.

Results: A strong association between specific lipids and KCS signs was observed only with the polar lipids. Low levels of 2 phospholipids, identified as phosphatidylethanolamine and sphingomyelin, were significantly (P < .05) associated with ocular surface abnormalities that were consistent with KCS.

Conclusions: Evaporative KCS syndrome (rather than tear insufficiency) in many individuals may be the result of polar lipid abnormalities. We believe that the 2 associated phospholipids identified in the patients with chronic blepharitis act as important structural components in the polar phase of the tear film lipid layer. We suggest that a deficiency in these lipids results in a poorly structured polar phase that in turn affects the nonpolar phase. Ultimately water transmission through the tear film lipid layer increases, thus resulting in evaporative KCS. These results should aid in development of tear film substitutes directed toward specific abnormalities.
PATIENTS AND METHODS

Informed consent was obtained from all patients and the study was conducted according to the tenets of the Declaration of Helsinki. Patients who had any form of CB in our classification system (thus all types were included) for at least 6 months were carefully evaluated. The patient population was 56% male (n = 61); of this male population, CB-KCS was present in 39% (n = 24) and CB-KCS was present in 30% (n = 23) of the female population. If the patients with a staphylococcal infection were omitted, the occurrence of CB-KCS in the female population was 38% (n = 11) and 41% (n = 23) in the male population. In a previous study, we published patient population's association with decreased levels of production of tear lysozyme in the CB-KCS patients as compared with the CB patients. Of these patients in the present study (n = 19), 7 had a clinical picture consistent with KCS that persisted after the CB had been controlled and treatment with ocular medications discontinued, ie, interpalpebral epithelial erosions with staining of conjunctiva and cornea with a vital stain (rose bengal), and decreased tear meniscus on slitlamp examination. None of these patients had a general meibum deficiency. Relatedly, it has been observed that lacrimal gland function is decreased in patients with KCS and increased evaporation. Meibomian gland secretions were collected as follows. After instillation of 1 drop of lidocaine onto the ocular surface, a lid conformer was placed into either the inferior or superior cul-de-sac. A sterile cotton swab was then passed over the lid margin to remove excessive debris and tears. The tarsal plate containing the meibomian glands was then squeezed between the swab and conformer. The secretions were harvested with a sterile platinum spatula. Samples were washed off the spatula with chloroform, collected in glass tubes, and evaporated to dryness with nitrogen. Samples were then squeezed (under nitrogen) and stored at −70°C.

Lipid classes were first separated by thin-layer chromatography as previously described. Samples were dissolved in chloroform and spotted on silica gel H plates, previously activated at 120°C. The plates were developed in hexane-diethyl ether-acetic acid (75:25:1), the region of origin containing the polar lipids was scraped off, and then it was placed in nitrogen-flushed screw-capped tubes.

The polar lipids were further separated by high-pressure liquid chromatography. The high-pressure liquid chromatography system (Waters Corporation, Milford, Mass) consisted of a universal liquid chromatograph injector (model U6K), automated gradient controller (model 680), high-pressure liquid chromatography pump (model 510), tunable absorbance detector (No. 484) set at a wavelength of 220 nm, and data module (model 740) to collect data. The mobile phase was a modification of a previously reported method and consisted of 73% acetonitrile, 2% methanol, 1.5% water, and 2.25 × 10−4% methylphosphonic acid (Aldrich, St Louis, Mo); pH was adjusted to 6.3 with ammonium hydroxide. The sample was dissolved in chloroform, evaporated to 4 to 6 µL, injected into the Waters system, and the polar lipids were separated on an aminopropyl silica column (Nucleosil-NH2, 5 µm, 250 × 4.6 mm (Sigma-Aldrich, St Louis) with an aminopropyl guard column. Column fractions were collected in screw-capped vials and evaporated under nitrogen at room temperature to reduce the volume.

Column fractions were transmethylated under nitrogen as follows. After transferring the samples to small screw-capped reaction vials (Pierce Reacti-Vial, Rockford, Ill) and adding sodium methoxide in methanol (Supelco, Bellefonte, Pa), they were heated in a heating block at 85°C for 15 minutes to transesterify the glycerolipids. The methyl esters were removed with heptane. After adding more sodium methoxide in methanol, the samples were heated for 18 hours at 85°C to transmethylate the sphingolipids.

Methyl esters were analyzed by a chemical ionization–gas chromatography–mass spectrometry (CI-GC-MS) system. This system (Hewlett Packard, Palo Alto, Calif) consisted of a 5890 series II GC and a 5971 mass selective detector with chemical ionization option. Samples were separated on a column (J & W Scientific 17HT, J & W Scientific, Folsom, Calif) (30-m, 0.25-mm inner diameter, 0.15-µm film thickness) with helium carrier gas and methane as the chemical ionization gas.

Lipids were identified using these 2 methods. Thus individual polar lipids were identified by retention time and comparison with lipid standards (sources: Sigma Chemical Co, St Louis; Avanti Polar Lipids, Alabaster, Ala; and Matreya, Pleasant Gap, Pa). Differential transmethylation and GC-MS identified the fatty acids and type of lipid (glycerolipid or sphingolipid). Data were analyzed statistically (t test) using the Statistica program (StatSoft, Tulsa, Okla).

We have presented elsewhere an analysis of the various lipid classes of the lipid layer of the tear film and proposed that the lipid layer is highly structured. The polar lipid layer may be only 1 to 3 molecules thick but serves a very critical function, ie, that of a surfactant creating a transition from a hydrophilic aqueous-mucin phase to a hydrophobic nonpolar lipid phase. Phosphatidylethanolamine and SM are most likely to be the key molecules in establishing the integrity and function of the polar layer. These 2 phospholipids are that the significance (P<.02) of low PE was much more important than low SM in male patients (Table). When the sum of PE and SM was compared for all patients, the sum for the CB-KCS group was significantly lower (P<.005) than that for the CB group (Figure 2).

RESULTS

Meibomian lipids are quite varied and more complex than previously understood. In our investigation of CB, we find that meibum polar lipids of normal individuals were not notably different from those of patients with CB without a KCS-like picture, composition of which is given in the Table. The most striking observation was in patients with CB with a KCS-like picture; phosphatidylethanolamine (PE) and sphingomyelin (SM) were significantly lower (P<.05) than in the patients with CB without associated KCS (Table, Figure 1). It was also observed

COMMENT

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prominent in lung surfactant phospholipids and are much more effective than phosphatidylcholine in maintaining in vitro lipid layer integrity and in lowering surface tension (surfactant properties).21 These polar lipids have also been observed to be dominant in rabbit meibomian glands.22 Although evidence has accumulated suggesting that meibomian disease and KCS syndrome may be related,1,6,9,23-26 this is the first evidence that specific lipids could be involved. Low levels of both PE and SM are significantly related to an ocular surface abnormality similar to KCS (Table), but PE appears to be more important, at least in men. Although we believe that it is the nonpolar lipid phase of the tear film that ultimately determines the tear film evaporation rate, the importance of the supporting structure of the polar lipid phase should not be ignored; as suggested by model systems27-28 both are important. We believe that it is both the structural and water transmission (activity) characteristics of the polar lipid phase that are compromised by low levels of PE and SM. The results presented in Figure 2 suggest that when PE and SM are at low levels, KCS is much more likely to occur.

The functionality of PE and SM in the polar lipid phase can be altered by associated conditions. The uniqueness of PE and its role in promoting a functional polar lipid phase can be understood in terms of the structure of PE. Thus, because of the molecular shape of the PE molecule, it has been proposed that the presence of PE reduces curvature stress,29 as would occur at the tear film surface. Also because of its molecular structure, and unlike phosphatidylcholine and SM, the amino (NH3+) group of PE which lacks the trimethylation (N\[CH3\]3+) of the choline group in phosphatidylcholine and SM is not stearically hindered. Therefore, PE is the only lipid present in meibum that contains an NH3+ group and thus can form ionic bonds and multiple hydrogen bonds (eg, to other lipids in the polar lipid phase).30 Finally, the amino group of PE (with a dissociation constant of about a pH of 7.5 in a membrane environment) can readily lose hydrogen-bonding ability at higher pHs, but especially

<table>
<thead>
<tr>
<th>Lipid†</th>
<th>Normal, Mean (SD) %</th>
<th>CB, Mean (SD) %</th>
<th>CB-KCS, Mean (SD) %</th>
<th>CB, Mean (SD) %</th>
<th>CB-KCS, Mean (SD) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>16.7 (4.0)</td>
<td>11.2 (6.9)</td>
<td>4.6 (3.2)†</td>
<td>12.8 (6.6)</td>
<td>3.9 (2.7)§</td>
</tr>
<tr>
<td>SM</td>
<td>14.0 (8.1)</td>
<td>12.6 (5.6)</td>
<td>7.2 (4.8)†</td>
<td>12.8 (6.4)</td>
<td>6.3 (5.0)</td>
</tr>
<tr>
<td>PC</td>
<td>21.6 (5.3)</td>
<td>26.8 (12.4)</td>
<td>33.7 (14.3)</td>
<td>26.8 (12.9)</td>
<td>34.0 (12.1)</td>
</tr>
<tr>
<td>CBS</td>
<td>23.5 (8.5)</td>
<td>26.9 (14.1)</td>
<td>26.2 (18.5)</td>
<td>26.7 (14.7)</td>
<td>29.4 (17.3)</td>
</tr>
<tr>
<td>P1</td>
<td>9.3 (8.1)</td>
<td>8.3 (5.8)</td>
<td>7.7 (6.6)</td>
<td>6.8 (5.4)</td>
<td>6.6 (6.3)</td>
</tr>
<tr>
<td>P2</td>
<td>3.2 (0.9)</td>
<td>2.7 (1.5)</td>
<td>2.8 (1.6)</td>
<td>2.6 (1.7)</td>
<td>3.1 (1.8)</td>
</tr>
<tr>
<td>P3</td>
<td>2.0 (0.5)</td>
<td>2.5 (1.7)</td>
<td>2.7 (2.0)</td>
<td>2.9 (1.8)</td>
<td>2.5 (2.4)</td>
</tr>
<tr>
<td>U1</td>
<td>6.6 (5.3)</td>
<td>4.1 (2.2)</td>
<td>6.2 (5.9)</td>
<td>3.3 (1.2)</td>
<td>6.6 (6.9)</td>
</tr>
<tr>
<td>U2</td>
<td>1.7 (0.4)</td>
<td>3.3 (3.8)</td>
<td>6.9 (6.0)</td>
<td>3.7 (4.3)</td>
<td>5.9 (5.4)</td>
</tr>
<tr>
<td>U3</td>
<td>1.2 (1.0)</td>
<td>1.5 (1.9)</td>
<td>2.0 (1.7)</td>
<td>1.6 (2.2)</td>
<td>2.5 (1.8)</td>
</tr>
</tbody>
</table>

*Number of individuals: normal, 4; with CB, 12 (9 male); with CB-KCS, 7 (5 male).
†PC indicates phosphatidylcholine; CBS, cerebroside; P, phospholipid unknown; and U, unknown.
‡Significantly different: P < .05, CB-KCS vs corresponding CB, as determined by the t test.
§Significantly different: P < .02; CB-KCS vs corresponding CB, as determined by the t test.
when calcium levels are high. These characteristics of PE enhance both its structure-forming and surfactant properties, but under abnormal conditions these properties can be diminished. Finally, the hydroxyl groups present in SM but not phosphatidycholine also increase its hydrogen-bonding ability and therefore its structure-forming properties. On the basis of these reports and our results, we believe that the functional polar lipid layer of the tear film can be compromised by abnormal conditions such as low levels of key polar lipids such as PE and SM.

Our results in no way suggest that all KCS conditions, whether due to high rates of aqueous evaporation, aqueous hyposcretion, or other lipid or mucin abnormalities, are the result of low levels of these 2 phospholipids. For example, we have reported an association of one type of CB, ie, rosacea, with meibomian keratoconjunctivitis33 and apparent nonpolar and polar lipid abnormalities34; more recently, there has been another report of an abnormal tear film associated with ocular rosacea. As a point of future interest, we would also like to point out that in Sjögren’s syndrome, with a sparsity of aqueous production, the presence of serum antiphospholipid antibodies (primarily IgA) are a commonly associated condition. In general high levels of IgA and lower levels of IgM are found in human tears37; in individuals with apparent IgA deficiency, IgM levels may be elevated. A recent study has determined that serum IgM but not IgG binds significantly to both PE and SM. Further studies will clarify the relevance of these points.

We, therefore, propose that in some KCS conditions, hyperevaporation of aqueous tears may result from low levels of PE and SM in meibum (as in the present investigation) and/or loss of PE and SM from the tear film because of antiphospholipid antibodies, lipases, or even reactive aldehydes. The resulting clinical picture would be that of a sicca syndrome (KCS) and would be caused by increased rates of aqueous evaporation. With these insights it may be possible to devise a tear replacement to correct the defect in many patients with aqueous deficiency and especially those with an evaporative KCS condition.

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