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Characterization of ocular gland morphology and tear composition of pinnipeds

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Abstract

Objective—The importance of tear film integrity to ocular health in terrestrial mammals is well established, however, in marine mammals, the role of the tear film in protection of the ocular surface is not known. In an effort to better understand the function of tears in maintaining health of the marine mammal eye surface, we examined ocular glands of the California sea lion, and began to characterize the biochemical nature of the tear film of pinnipeds.

Procedures—Glands dissected from California sea lion eyelids and adnexa were examined for gross morphology, sectioned for microscopic analysis, and stained with haematoxylin and eosin.

The tear film was examined using interferometry. Tears were collected from humans and pinnipeds for analysis of protein and carbohydrate content.

Results—The sea lion has sebaceous glands in the lid, but these glands are different in size and orientation compared to typical meibomian glands of terrestrial mammals. Two other accessory ocular glands located dorso-temporally and medially appeared to be identical in morphology, with tubulo-acinar morphology. An outer lipid layer on the ocular surface of the sea lion was not detected using interferometry, consistent with the absence of typical meibomian glands. Similar to human tears, the tears of pinnipeds contain several proteins but the ratio of carbohydrate to protein was greater than that in human tears.

Conclusions—Our findings indicate that the ocular gland architecture and biochemical nature of the tear film of pinnipeds have evolved to adapt to the challenges of an aquatic environment.

Keywords

ocular surface; tear film; pinnipeds; meibomian glands

INTRODUCTION

The importance of tear film integrity to ocular health in terrestrial mammals is well established [1]. In contrast, in the case of marine mammals, land dwellers that evolved back to the sea in several clades between 30 and 60 million years ago [2], the role of the tear film in protection of the ocular surface is not known. In the process of their evolution from land back to the sea, the ocular apparatus, which marine mammals had inherited from their terrestrial ancestors, had to adapt to the challenges of an aqueous environment [3].

In terrestrial mammals, contributions from mucin-producing goblet cells, aqueous-secreting lacrimal glands, and lipid-producing meibomian glands are the major constituents of the precorneal tear film [1]. The mucosal, aqueous, and lipid components of the tear film are essential to maintaining a healthy ocular surface. The mucosal and aqueous constituents work in a complex integrated fashion to bathe, lubricate, and protect the cornea from particulate and pathogenic invasion [4, 5]. The outer lipid layer provides stability and retards evaporation of the inner aqueous-mucosal phase of the tear film [6, 7].

Compared to the understanding of the terrestrial mammalian tear film, the current state of knowledge regarding the role of marine mammal tears in protection of the ocular surface is in its infancy. In the class Mammalia, there are three orders that comprise only marine mammals: pinnipeds (seals, sea lions, walruses), cetaceans (whales, dolphins, porpoises), and sirenians (dugongs, manatees); and little is known of how the composition of ocular secretions is correlated with eye surface disorders in these animals. However, manifestations of ocular surface disease in marine mammals, both in the wild and in rehabilitative facilities, have been well documented [8–12]. Keen eyesight is essential for foraging for food and avoidance of predators, and therefore key to survival, and this may help to explain the unique degree of scotopic development of the visual system in certain marine mammals [3, 13, 14].

In light of the well-established protective role of tears, and the prevalence of eye disease in marine mammals, it is important to gain more knowledge with respect to the ocular glands that produce the marine mammal tear film, as well as the nature of the tear film itself. In the California sea lion, there has recently been a comprehensive report on the anatomy of the globe [15], but little is known of the nature of ocular adnexal tissues. The purpose of the present study was to examine the ocular gland structure of the California sea lion, and to begin to characterize the biochemical nature of the tear film of different species of

pinnipeds, with the goal of advancing our understanding of the role of tear film in marine mammals.

MATERIALS AND METHODS

Morphology

Analyses were performed on ocular tissues collected from four necropsied sea lions (*Zalophus californianus*), animals that had been euthanized for reasons unrelated to this study. This group comprised two adults (5+ years old, male and female), a juvenile (2–4 years old, male), and a pup (less than 2 years old, male). All tissues were fixed in 10% commercial formalin at the time of necropsy, and stored for several days prior to being submitted for analysis. Fixed tissues from sea lions consisted of the eyeball together with the adherent tissues of the upper and lower lids and the skin (Fig. 1A). For lid examination prior to embedding, the globe and lids were halved in sagittal direction, and visualized with a stereo magnifier (surgical microscope, Wild-Leitz, Wetzlar, Germany). For photo-documentation, a stand-alone digital camera (Nikon Coolpix 995) in macro-mode was used with a ruler as size indicator. Ocular glands were dissected from the extra-orbital adnexa. For histochemical analyses of lids and glands, tissues were washed and then embedded in paraffin. Sections of 5 micron (μm) thickness were prepared with a rotary microtome and stained with haematoxylin and eosin (H&E).

Interferometry

A hand-held mobile tear film interferometer (custom designed and built by Marshall Doane) was used to evaluate Harbor seal (*Phoca vitulina*) and California sea lion (*Zalophus californianus*) tear film according to previously described procedures [16, 17]. For this study, marine mammals were called out of the water onto a platform, by their handlers, and allowed to rest for 3–5 minutes before the optical scan was performed. Optical interferometry is a non-invasive technique that can be employed to visualize the structure of the tear film *in vivo*. In essence, the light beam from the mobile interferometer is aimed at the ocular surface at a distance of about 35 mm, and the images of the tear film are recorded to a high-resolution camera in real time. In the presence of an outer lipid layer, such as exists on the ocular surface of terrestrial mammals, the beam is specularly reflected from the air-lipid and lipid-inner aqueous layer of the tear film, effectively splitting the original beam into two reflected rays that can be optically recombined and focused, thereby permitting determination of the distance between the two interfaces. In the presence of an outer lipid layer, interference fringes can be seen that represent the thickness distribution of that layer, with a limit of detectability of approximately 0.05 μm in visible light [17].

Tear Collection

Tears were collected, using non-invasive methods, from 4 California sea lions (*Zalophus californianus*), 6 Harbor seals (*Phoca vitulina*), 5 Northern Fur seals (*Callorhinus ursinus*), and 5 human volunteers (*Homo sapiens*). For marine mammals, all tear collection procedures were conducted in accordance with Animal Care and Use Committee approvals at the respective institutions. For human tear collection, this study was performed in accordance with the Declaration of Helsinki and approval from the Schepens Eye Research Institute Institutional Review Board. All human subjects completed an institutional review board-approved questionnaire and consent form prior to participating in the study.

For marine mammal tear collection, animals were called out of the water by their handlers, who waited for 2–3 minutes to allow tank water to drain from the ocular area, and then collected tear secretions. A cellulose Weck-cel (Beaver-Visitec International Inc., Waltham, MA) sponge was put to the ocular surface at the junction of the lower lid and sclera for 30–

60 seconds to allow for sufficient absorption of the tear fluid. Tears from humans were collected either by aspiration with a plastic micropipette or through absorption with Weck-cel cellulose sponges. To elute tears from Weck-cel spears, sponges were placed into the top section of a Spin-X filter (22 μ m) tube (Corning, Lowell, MA) and centrifuged at 12K rpm in a mini-centrifuge. The filter and sponge were then removed and the eluted tear sample was stored in the Spin-X tube at -80°C .

Biochemical Analyses

Assays were performed to determine overall carbohydrate and protein content of tears. Carbohydrate content of tears was measured using a slightly modified version of a phenol-sulfuric acid microplate method [18]. The modified protocol was as follows: 150 microliters (μ l) of chilled sulfuric acid was added to 30 μ l of sample in a well of a 96-well microplate, followed by addition of 20 μ l of 5% phenol in water. The plate was then incubated for 5 minutes in an oven heated to 100°C . After chilling the plate on ice, and then warming to room temperature, absorbance was measured at 490 nm by microplate reader in a spectrophotometer. Analysis of tear protein concentration was performed using a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL). Tear samples were subjected to gel electrophoresis to separate proteins. Proteins were then detected using Pierce Silver (Thermo-Scientific, Waltham, MA) or SYPRO Ruby (Sigma-Aldrich, St. Louis, MO) gel staining kits to determine molecular weight distribution. A protein standard ladder (Novex® Sharp, Invitrogen, Carlsbad, CA) was used to estimate molecular weights of electrophoresed proteins.

RESULTS

Morphology of sea lion eyelid

Observation of the fixed tissues, sectioned prior to embedding, revealed that the eyelids consisted mainly of the muscular layer of the orbicularis muscle and were lacking a distinct tarsus. The lid margin attenuated within a short distance to a sharp tip. Towards the outer aspect, underneath the epidermis, the dermis was readily visible as a narrow pale layer that extended along the entire external surface of both lids (Fig. 1B).

In paraffin sections through the lids, the dermal layer consisted of many hair follicles and bundles of small associated sebaceous glands, with pale staining by H&E, that together formed a hair-gland layer of 1 mm thickness (Fig. 2A). At the lid margin solitary sebaceous glands occurred as individual structures without association to hair follicles (Fig. 2B). These individual glands had a length of 1–2 mm. They had a distinct central duct around which several holocrine acini were arranged. The acini had intensely stained small basal and larger pale central cells. The cells in the middle of the acini and at the start of emerging ductules showed nuclear pyknosis. Nuclear pyknosis, a characteristic finding of the central acinar cells in sebaceous glands, was specific to these cells and not observed in other cells in the glands or elsewhere in the tissue. The terminal part of the central duct opened to the surface of the eyelid and was characterized by an ingrowth of the keratinized epidermis (Fig. 2C).

Morphology of temporal and medial ocular glands

Two larger glands were identified (Fig. 3) that appeared to be lacrimal in nature, and therefore are likely to be involved in the principal formation of the aqueous component of the tear. A typical main lacrimal-like gland (LG, Fig. 3A & B) was located dorsotemporally beneath the superior eyelid. Another gland was located medially, associated with the inner aspect of third eyelid, or nictitating membrane, herein referred to as the nictitans gland (NG, Fig. 3C & D). In terrestrial mammals, glands associated with the nictitating membrane have been characterized as lacrimal in nature [19–21], although they are sometimes anatomically

localized in proximity to Harderian glands [22]. The LG (Fig. 4A) and NG (Fig. 4B) of the sea lion were very similar histologically, with tubulo-acinar arrangements, and potentially secrete an aqueous component of the tear.

In vivo tear film interferometry

To determine whether pinnipeds have a lipid layer on the outer surface of the tear film, sea lions and seals were evaluated by interferometry. The surface of the eye was scanned using a mobile interferometer, and the scans were recorded on video. Analyses of video recordings indicate that the lipid layer of the tear film typically observed in terrestrial mammals is undetectable in pinnipeds, at least over the time course of these recordings, which for the sea lion lasted several minutes. Interferometric scans of human tear film (Fig. 5A) reveal a smooth, thin lipid, which is not evident on the surface the sea lion (Fig. 5B) or seal eye (not shown).

Biochemical analyses of pinniped tears

Protein—Two standard methods were used to detect proteins after gel electrophoresis of tear samples, silver (Fig. 6A) and luminescent (Fig. 6B) staining. Both methods revealed similar banding profiles, with detection of multiple protein bands in the range of 30 to 120 kilodaltons, similar to human tears in number of bands and intensity of staining, if not in exact migration patterns.

Protein and carbohydrate concentration of tears—Protein and carbohydrate concentrations were measured in tear samples from humans and pinnipeds (harbor seal, fur seal, sea lion). Overall, ratios of carbohydrate to protein were greater in pinnipeds as compared to humans (Table I), however there was variation among individuals and between species of pinnipeds (Table II).

DISCUSSION

In this study, we examined the ocular anatomy of the sea lion to determine the types of glands present in the lids and adnexa, with the goal of beginning to establish the source of the tear film. We also evaluated the structure of the tear film to determine whether we could detect an outer lipid layer as is found in terrestrial mammals, and we analyzed basic biochemical composition, with respect to protein and carbohydrate, of human and pinniped tears.

We found substantial differences in the glandular architecture of sea lions as compared to that reported for humans, as well as other types of marine mammals as described below. In view of the divergent evolutionary paths of marine mammals and humans, and their respective aquatic and atmospheric environments, the finding that the ocular gland architecture is substantially different is not surprising. However, it is of interest that there are differences between sea lions and reported ocular anatomic structures for other marine mammals. We speculate that the reason for these differences may lie in the varying degrees of adaptation to the aquatic environment across marine mammal species. For example, pinnipeds spend a significant amount of time hauled out on land, whereas cetaceans are almost entirely aquatic.

With respect to glands of the eyelid, the sea lion has numerous sebaceous glands at the lid margin, but these glands are different in size and orientation compared to typical meibomian glands of terrestrial mammals [23]. Of particular note, the lid glands of the sea lion do not impinge on the ocular surface, but rather are on the external lid oriented away from the eye. This is consistent with the lack of a detectable lipid layer on the surface of the sea lion eye.

The typical lipid layer on the surface of the human eye is estimated to be 0.015–0.160 microns over a mucin-aqueous phase of 3–40 microns in thickness [24], so a lipid layer of this thickness or greater should be detectable by the interferometer that was used to examine the ocular surface of the sea lion. Interference colors will not be present if the reflecting lipid layer is less than 0.04 – 0.05 microns, therefore a very thin tear film layer could escape detection. It is also possible that after an extended time of exposure to air, lipids produced by the sebaceous glands located in the sea lion eyelid could flow to the ocular surface, however there is currently no evidence to support this conjecture. With respect to lid glands, sea lions may be similar to other marine mammals. For example, manatees do not have classical eyelids, but rather muscular lids that close by a sphincter action [25] and lack meibomian glands [26]. With regard to cetaceans, it has been stated in a review that whales do not have meibomian glands, whereas dolphins do [27]. However closer examination of the literature reveals that the reference regarding whales has a cursory statement that meibomian glands were not present, but there is no anatomical data to support the claim [28]; and the dolphin study is a treatise on biochemical composition of ocular secretions, and does not investigate eyelid glands [29]. To our knowledge there are no scientific reports of the presence of meibomian glands in cetaceans.

Two other accessory ocular glands were located dorsolaterally and medially. These glands were very similar morphologically, with tubulo-acinar bundles, and may be analogous to the aqueous-producing nictitating and lacrimal glands of terrestrial mammals. The sea lion lacks a large belt-like gland wrapping around the globe, which has been described in cetaceans, including the Atlantic bottlenose dolphin [30] and baleen whales [31], and has been proposed to be analogous to the Harderian gland in structure and function [31]. However, the analogy of the cetacean ocular gland to the Harderian is controversial. Whereas size and location may be similar, there are many differences, including the lack of lipid staining in the gland [31]. Also, the ocular gland of the bottlenose dolphin is more developed rostrally, and is laterally oriented on the corneal plane, rather than extending posteriorly to the back of the globe [30].

The orbital gland documented for cetaceans is much greater in size and area when compared to the two adnexal ocular glands of the sea lion. It has been noted that Atlantic bottlenose dolphins produce copious amounts of tears [30]. However, in our experience with pinnipeds, there was not enough volume to collect tears with syringes, and instead, ocular secretions were absorbed onto cellulose sponges. Again, these differences in tear volume may reflect the varying degrees of adaptation of marine mammalian species to an aquatic environment.

With respect to biochemical composition, the pinniped tear film has both similarities and dissimilarities as compared to that of humans. Of particular note, an outer lipid layer, ubiquitous in terrestrial mammals, was not detected on the ocular surface of the sea lion. This may be a common feature across marine mammal species. At one time it was thought that the marine mammal tear film must have an oily, hydrophobic nature to repel water and protect the eye, but as the understanding of tear film has become more sophisticated, it has become accepted that the tear film of marine mammals may be more hydrophilic to retain hydration at the eye surface [30]. These revelations are born of the realization that the tear film of those cetaceans and sirenians that have been studied is viscous and mucous-like, not oily in nature [26, 30, 32, 33]. In consideration of the prominent sebaceous lid margin glands, it is possible that after an extended time out of water, a tear film lipid layer develops in pinnipeds. However, verification of the presence or absence of a tear film lipid layer after longer periods of time in an air atmosphere has yet to be established. Our finding that the sea lion eye lacks an outer tear film lipid layer, under the conditions of these experiments, is consistent with findings in other marine mammals.

Similar to human tears, the tears of pinnipeds contain several proteins from 30 to 120 kilodaltons in size. This supports the concept that the marine mammal has a substantial aqueous component in the tear film because smaller proteins would reside in the hydrophilic component of ocular secretions [1]. However, the ratio of carbohydrate to protein in pinniped tears was greater than that in human tears. This may reflect a greater prevalence of large glycosylated proteins, and possibly mucins, in pinniped tears. The possible presence of mucins would be consistent with the reported highly viscous, or mucous-like, quality of tears from a variety of marine mammal species [26, 30, 32].

In view of the findings presented in this study, we hypothesize that the ocular gland architecture and the tear film of marine mammals have both evolved to adapt to the challenges of varying exposures to an aquatic environment. Furthermore, we postulate that mucins are likely to be present in the aqueous phase of the marine mammal tear film, and that mucins may play a crucial role in protection of the ocular surface. This hypothesis is supported by our finding that the ratio of carbohydrate to protein was greater in tears from pinnipeds as compared to human tears. However, further study is needed to detect and identify mucins at the marine mammal ocular surface.

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Figure 1.

Gross morphology of California sea lion eyelids: (A) Fixed tissues from a sea lion consisted of the left eyeball together with lids and the skin. (B) A sagittal cross section shows the internal composition of the eyeball (including the cornea, sclera, and iris) and the eyelids. The eyelid consisted mainly of pale red tissue with a narrow pale layer (arrow) underneath the epidermis. A centimeter ruler is shown as size marker.

Figure 2.

Histology of California sea lion upper eyelid: (A) Histology showed that the eyelid has an outermost hair-gland layer consisting of hair follicles (arrowheads), and their associated sebaceous glands, on top of the mass of the orbicularis muscle. Occasional sections of hair shafts (arrow) are seen also above the level of the epidermis. To the inner aspect is the conjunctiva (conj). (B) At the lid margin, individual sebaceous glands (one indicated by an arrow) without association with hair follicles occurred. (C) Histological micrograph of these glands at higher magnification showed a straight central duct (cd) around which pale sebaceous acini (a) were arranged and connected to the duct via smaller ductules (de) into the central duct. The terminal part of the central duct has an ingrowth (arrow) of the epidermis (epi), and opens to the anterior surface of the eyelid (large arrow). Paraffin sections, H&E, size marker is 1000 µm in (A) & (B), and 100 µm in (C).

Figure 3.

(A) Photograph of the right eye of a normal California Sea lion showing the location of the main lacrimal-like gland (LG). (B) Photograph (courtesy of Sarah Corner, Resident, Zoological Pathology Program, Univ. of Illinois; taken at the Marine Mammal Center, Sausalito, CA.) of a gross dissection during a necropsy of California Sea lion eye with an arrow marking the location of the LG. (C) Gross morphology of California sea lion ocular glands: Eyeball is shown with lid structures removed from the globe and photographed from behind to expose the bulbar surface of the nictitans and the palpebral surface of the conjunctiva. The position of the nictitans gland (NG) is marked. (D) Micrograph of nictitating membrane (third eyelid) showing nictitans gland.

Figure 4. Histological sections of California sea lion ocular glands: Temporal main lacrimal gland (A) and medial nictitans gland (B). H&E, magnification is 20×, size marker is 50 µm.

Figure 5.

Interferometric scans of the ocular surface: Scan of a normal human eye (A) (image courtesy of M. Doane), showing fringe patterns indicating a lipid layer of uniform thickness over the corneal surface, and a close-up of a scan of a sea lion eye (B) without any fringe pattern, therefore lacking evidence of a lipid layer. The image in panel B was captured as a freeze frame from a film of a continuous scan of the eye taken over a period of time in excess of 4 minutes.

Figure 6. Protein gels: Left panel (A), silver-stained 12% polyacrylamide gel with 36 μ g of protein loaded into sample lanes. Right panel (B), Sypro-stained 3–8% gradient pre-cast polyacrylamide gel with 8 μ g of protein loaded into sample lanes. For both panels, individual lanes are from a single gel run. Samples were protein molecular weight markers (lanes A1, B1); human tear (lanes A2, B 2&3); sea lion tear (lanes A3, B4); and seal tear (lanes A4, B5).

Table 1

Comparison of protein and carbohydrate tear concentrations in humans and pinnipeds: Values are expressed $\pm$ standard deviations. Ranges of values and numbers of animals sampled are indicated in parentheses. Concentrations are expressed in milligrams/milliliter.*

Tear Source	Protein (*mg/ml)	Carbohydrate (CHO) (*mg/ml)	Ratio CHO/Protein	Ratio normalized to human
Human	3.82 $\pm$ 1.85 (2.36–5.90, n=3)	0.53 $\pm$ 0.47 (0.40–1.15, n=5)	0.14	1
Pinniped	0.92 $\pm$.52 (0.26–2.10, n=13)	0.79 $\pm$ 0.42 (0.20–1.35, n=10)	0.86	6.14

Table 2

Protein and carbohydrate concentrations of tears of pinnipeds: Values are expressed $\pm$ standard deviations. Ranges of values and numbers of animals sampled are indicated in parentheses. Concentrations are expressed in milligrams per milliliter.*

Tear Source	Protein (*mg/ml)	Carbohydrate (CHO) (*mg/ml)	Ratio CHO/Protein	Ratio normalized to human
Harbor Seal	0.43 $\pm$ 0.19 (0.26–0.69, n=4)	0.83 $\pm$ 0.41 (0.36–1.35, n=4)	1.93	13.78
Fur Seal	1.25 $\pm$ 0.54 (0.77–2.10, n=6)	0.88 $\pm$ 0.42 (0.40–1.30, n=5)	0.70	5.00
Sea Lion	0.89 $\pm$ 0.09 (0.81–0.98, n=3)	0.20 (n=1)	0.22	1.57
Total Pinniped:	0.92 $\pm$.52 (0.26–2.10, n=13)	0.79 $\pm$ 0.42 (0.20–1.35, n=10)	0.86	6.14