Peptidergic Innervation of the Primate Meibomian Gland

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Purpose. To localize and characterize nerves in primate meibomian glands using immunohistochemical staining for neuropeptides and neuronal enzymes.

Methods. Upper eyelids were obtained from seven rhesus monkeys (Macaca mulatta) and one cynomolgus monkey (Macaca fascicularis). The tissues were fixed either by immersion in Zamboni’s fixative or by transcardiac perfusion with paraformaldehyde and glutaraldehyde and were then postfixed. Cryostat tissue sections of the lids were stained by immunohistochemistry using rabbit antisera to neuron-specific enolase (NSE), tyrosine hydroxylase (TH), neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP), and substance P (SP), followed by a fluorescence visualization system.

Results. Used as a marker for the overall nerve distribution, NSE antibodies revealed abundant smooth and varicose nerve fibers closely apposed to the basement membranes of acini of the meibomian glands. Numerous nerve fibers near the meibomian gland acini were immunoreactive for NPY and VIP, but nerve fibers containing TH, CGRP, and SP were more sparse in the meibomian glands. Nerve fibers also were visualized in other eyelid structures, including conjunctiva, epidermis, hair follicles, and subconjunctival lymphoid follicles.

Conclusions. The meibomian glands of rhesus and cynomolgous monkeys are richly innervated by diverse nerve fiber types. The immunohistochemical staining suggests a largely parasympathetic origin for this innervation, with relatively smaller contributions from sympathetic and sensory sources. These findings also suggest that meibomian gland secretion is under the control of diverse neurotransmitter–neuromodulator mechanisms. Invest Ophthalmol Vis Sci. 1996; 37:238-245.
tion, the vasculature of meibomian glands in monkey, humans, and a number of nonprimate species appears to contain nerve fibers immunoreactive for neuropeptides, VIP, and SP.6

In the current study, we sought to characterize more extensively the innervation of the primate meibomian gland using immunohistochemical techniques in eyelid specimens from the rhesus monkey (Macaca mulatta) and the concomitant monkey (Macaca fascicularis).

METHODS

Antisera and Peptides

The primary antibodies used in this study included antisera to neuropeptide Y (NPY), VIP, and SP, each purchased from INCSTAR Corporation (Stillwater, MN). Other primary antibodies included antiserum to neuron-specific enolase (NSE) (Polysciences, Warrington, PA), antisera to TH (Eugene Tech International, Ridgefield Park, NJ), and antiserum to CGRP (Cambridge Research Biochemicals, Wilmington, DE). To assess the specificity of the immune reactions, the synthetic peptides NPY, VIP, SP, and CGRP were obtained from Peninsula Laboratories (San Carlos, CA).

Tissue Preparation

Eyelids were obtained from seven rhesus monkeys and one concomitant monkey killed for unrelated experiments at the Yerkes Regional Primate Research Center (Atlanta, GA). The animals included males and females and ranged in age from 8.5 months to 27 years. The investigation adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The tissue was fixed in most cases by transcardiac perfusion with paraformaldehyde (3% to 4%) and glutaraldehyde (0 to 0.1%), with or without post-fixation. In two cases, the tissue was fixed by immersion in Zamboni’s fixative. After fixation, the tissues were cryoprotected in 30% sucrose in 0.1 M phosphate-buffered saline (PBS) overnight. Cryostat sections of the upper eyelids, 16 μm thick, were thaw-mounted on gelatin-coated slides, dried at room temperature, and stored at −20°C until stained.

Immunohistochemical Procedure

In the immunohistochemical staining for NSE, TH, NPY, and VIP, all rinses were made with 0.02 M PBS, pH 7.4, and all dilutions were made using the same buffer with 0.3% Triton X-100. After washing in PBS, the tissue sections were incubated at room temperature for 15 minutes with 10% normal donkey serum. Subsequently, they were incubated overnight at room temperature with antiserum diluted 1:250 (TH), 1:500 (NPY and VIP), or 1:800 (NSE). To visualize the immunoreaction, the washed tissue sections were incubated for 1 hour at room temperature with biotinylated donkey anti-rabbit immunoglobulin G (Amersham, Arlington Heights, IL), diluted 1:200, and subsequently incubated for 30 minutes at 37°C with rhodamine-labeled streptavidin (Amersham) diluted 1:200.

The staining procedure for CGRP and SP was identical except that all washes were made with 0.02 M PBS containing 4.0% NaCl and 0.2% Triton X-100, pH 7.4; the primary antisera were diluted with 0.02 M PBS with 0.3% Triton X-100 and 0.1% bovine serum albumin at least 1 hour before use. These modifications decreased nonspecific staining. The CGRP antisera was used at a dilution of 1:1000, and the SP antisera was used at a dilution of 1:250 or 1:400.

As a control for immunohistochemical specificity, the peptide antisera (NPY, VIP, CGRP, and SP) were incubated overnight at 4°C with 1 μM concentrations of the corresponding synthetic peptide before use in immunohistochemical staining. As an additional control, the primary antisera were omitted in the staining procedure.

RESULTS

Each of the antisera stained structures in the lids having the typical meandering appearance of peripheral nerve fibers, with both varicose and smooth fibers visualized. There was minor variation between specimens from different animals in the intensity of staining, perhaps related to differences in fixation. Among tissue sections from the same animal, however, the results generally were consistent. Despite the small variations, definable nerve fiber distribution patterns emerged from the various specimens. Table 1 summarizes the results, with the density of the nerve fiber types graded on an arbitrary scale of 0 to ++++, 0 signifying no nerve fibers visualized and ++++ signifying the maximum density visualized.

Neuron-Specific Enolase

Many nerve fibers were visualized in the meibomian glands of all specimens (Fig. 1). They encircled the acini and duct structures, usually in bundles of fine smooth and varicose fibers, sometimes forming networks between the acini. To the level of resolution attainable by light microscopy, nerve fibers appeared to be apposed to the basement membrane of the acini. No nerve fibers were visualized between individual acinar cells.

Nerve fiber bundles coursed in the substantia propria of the conjunctiva, and individual varicose nerve fibers occurred just under the epithelium, running generally parallel to the conjunctival surface. There
TABLE 1. Nerve Fiber Distributions

<table>
<thead>
<tr>
<th>Meibomian Gland</th>
<th>Conjunctiva</th>
<th>Epidermis</th>
<th>Hair/Cilia follicles, Glands of Zeis</th>
<th>Blood Vessels</th>
<th>Lymphoid Follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSE</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TH</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>NPY</td>
<td>++</td>
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<td>0</td>
<td>+</td>
<td>+</td>
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<tr>
<td>VIP</td>
<td>++</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>CGRP</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>SP</td>
<td>+</td>
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<td>+</td>
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</tbody>
</table>

The distribution of histochemically defined nerve fiber types in the primate eyelid is summarized, with a scale of 0 to +++ indicating approximate density. Within the meibomian gland, there was no evident difference in location of the various peptidergic nerve fibers visualized by the methods used. Where their identity was unambiguous, the innervated blood vessels generally were arterioles.

NSE = neuron-specific enolase; TH = tyrosine hydroxylase; NPY = neuropeptide Y; VIP = vasoactive intestinal polypeptide; CGRP = calcitonin gene-related peptide; SP = substance P.

were fine varicose nerve fibers running between epithelial cells, sometimes reaching to the conjunctival surface. The tarsal conjunctiva generally contained more nerve fibers than the marginal region.

Subconjunctival lymphoid follicles were seen in several specimens. A few nerve fibers were visualized in these follicles—sometimes, but not always, in association with structures resembling blood vessels. The nerve fibers traveled both at the surface and in the parenchyma of the lymphoid follicles.

Nerve fiber bundles also were seen at the base of the epidermis, with individual fine varicose nerve fibers running in the epithelium. Nerve fibers in the epidermis were more numerous near the lid margin than in other regions. In a few specimens, the basal epithelium contained isolated clusters of NSE-immunoreactive cells resembling Merkel cells.14

Hair and cilia follicles were surrounded by smooth and varicose nerve fibers, encircling the follicles or radiating from them in sometimes complex patterns. Nerve fibers were seen running between the follicles and associated glands of Zeis, sometimes looping partially around the glands. Nerve fiber bundles also coursed near and around blood vessels and between fascicles of the orbicularis oculi.

Tyrosine Hydroxylase

Direct innervation to the meibomian glands by TH-immunoreactive nerve fibers was scant. A few faint varicose nerve fibers were seen near glands or running between acini (Fig. 2A). Some fibers were apposed to the basement membrane of acini, but others ran in the interstitium near acini. In contrast to the sparse glandular innervation, the blood vessels were richly supplied by TH-immunoreactive nerve fibers (Fig. 2A, insert).

Some fine varicose TH-immunoreactive nerve fibers appeared in the substantia propria of the conjunctiva, sometimes associated with small blood vessels. Subconjunctival lymphoid follicles in a few of the sections contained TH-immunoreactive nerve fibers. These nerve fibers tended to cluster near the edges of the lymphoid follicles, sometimes along vessel-like structures.

Neuropeptide Y

Many fine smooth and varicose NPY-immunoreactive nerve fibers invested the meibomian glands: Most of them encircled the acini and central duct, with some forming networks between acini (Fig. 2B). The nerve fibers tended to distribute evenly throughout the glands in relatively high density. In several specimens, some fine varicose nerve fibers were seen in networks in the substantia propria of the upper tarsal conjunctiva, but none were visualized in subconjunctival lymphoid follicles. A few fine varicose nerve fibers were seen encircling several hair or cilia follicles. Immunoreactive nerve fibers surrounded blood vessels in most specimens, varying in density among specimens.

Vasoactive Intestinal Polypeptide

Many smooth and varicose nerve fibers immunoreactive for VIP surrounded the meibomian gland acini and ducts, sometimes forming networks around them. In general, they were distributed evenly within the glands, occurring in density comparable to that of NPY-immunoreactive nerve fibers (Fig. 2C).

Some fine varicose nerve fibers were observed in the substantia propria and just below the epithelium of the conjunctiva, running mostly parallel to the conjunctival surface but sometimes oriented perpendicularly to it. In several specimens, fine varicose nerve fibers were visualized near blood vessels. No nerve fibers were seen in the epithelium of the conjunctiva nor in lymphoid follicles.
FIGURE 1. Sagittal section of an upper eyelid from a rhesus monkey immunostained for NSE, illustrating numerous nerve fibers (arrowheads) encircling meibomian gland acini (asterisks). In addition, nerve fibers are seen in the conjunctiva (C), epidermis (E), and near blood vessels (V) and hair follicles (F). Nerve fiber bundles (arrow) course between fascicles of the orbicularis oculi muscle (M). Bar = 300 μm.

Calcitonin Gene-Related Peptide

In most specimens, the meibomian glands contained only a few faint varicose CGRP-immunoreactive nerve fibers in a somewhat patchy distribution (Fig. 3A). They were either apposed to the acini or located in the interstitium near them. Clear innervation was evident surrounding nearby blood vessels (Fig. 3A, insert).

In contrast to the meibomian glands, there were numerous fine varicose nerve fibers in the substantia propria of the conjunctiva, some associated with small blood vessels. In addition, many CGRP-immunoreactive nerve fibers coursed just under the conjunctival epithelium and between epithelial cells, mostly in the upper tarsal conjunctiva. Some of these intraepithelial nerve fibers extended to the conjunctival surface. CGRP-immunoreactive nerve fibers were seen in a few subconjunctival lymphoid follicles, both at the surface and in the parenchyma, sometimes associated with structures resembling blood vessels.

The epidermis contained fine varicose nerve fibers just below or sometimes within the epithelium. Most of the intraepithelial nerve fibers were seen in
FIGURE 2. (A) Only a few tyrosine hydroxylase (TH)-immunoreactive nerve fibers (arrowheads) are seen in apposition to meibomian gland acini (asterisks), in contrast to their abundance near a small arteriole of the lid (insert). In addition to their location near acini, as seen here, TH-immunoreactive nerve fibers also traveled in the interstitium between acini. (B) Numerous neuropeptide (NPY)-immunoreactive nerve fibers (arrowheads) are apposed closely to meibomian gland acini (asterisks). (C) Vasoactive intestinal polypeptide-immunoreactive nerve fibers (arrowheads) in the meibomian gland (asterisks) occur in distribution and density similar to that of NPY-immunoreactive fibers. Bars = 75 μm.

the marginal portion of the lid. Varicose nerve fibers encircled hair and cilia follicles, sometimes forming dense and intricate patterns around them. A few nerve fibers also were seen running between some cilia follicles and their associated glands of Zeis.

Substance P

Nerve fibers immunoreactive for SP were generally more sparse than those for CGRP but had a similar distribution (Fig. 3B). As with CGRP, SP-immunoreactive nerve fibers were seen in subconjunctival lymphoid follicles, sometimes associated with vessel-like structures (Fig. 4).

DISCUSSION

Meibomian glands from both M. mulatta and M. fascicularis contain abundant nerve fibers as visualized using immunohistochemical staining for NSE, a useful marker for most types of neurons and many neuroendocrine cells. The localization of NSE probably provides an approximation of the entire population and distribution of nerve fibers in the lid. The presence of nerve fibers in the meibomian glands distinguishes them from other sebaceous glands, which generally lack innervation.

Large subsets of these nerve fibers were immunoreactive for NPY or VIP, many apposing the basement membrane of meibomian gland acini or surrounding duct structures. Vasoactive intestinal polypeptide serves as a marker for parasympathetic nerves in the eyelid originating, at least in part, in the pterygopalatine ganglion. The origin of NPY-containing nerves is more complex: NPY is found both in sympathetic nerves and in parasympathetic nerves from the pterygopalatine ganglion and perhaps other sources. Tyrosine hydroxylase is the rate-limiting enzyme in catecholamine biosynthesis, and it is a useful marker for sympathetic nerves. Although NPY-containing nerve fibers in the meibomian glands were numerous, nerve fibers containing TH were relatively scarce, suggesting that many of the NPY-containing nerve fibers in the meibomian gland may not be sympathetic but instead are parasympathetic in origin. Denervation or anterograde tracing studies are necessary for definitive characterization of these NPY-containing nerve fibers. The presence of nerve fibers in the meibomian gland of parasympathetic origin is consistent with earlier reports of cholinergic nerve fibers in rat and primate meibomian glands. In addition to nerve fibers near meibomian gland acini, others have reported VIP-containing nerve fibers in smaller quantities in the intrinsic vasculature of the gland in rat. We cannot exclude that some of the VIP- or NPY-containing nerve fibers we observed were innervating blood vessels.

Unlike the VIP- and NPY-containing nerve fibers,
FIGURE 3. (A) Few calcitonin gene-related peptide (CGRP)-immunoreactive nerve fibers (arrowheads) are seen in the meibomian gland compared to a small arteriole of the lid (insert). The CGRP-immunoreactive nerve fibers illustrated here lie in close apposition to acini (asterisks), but they also were seen in the interstitium between acini or near the gland, as in Figure 3B. (B) As with CGRP, substance P (SP)-immunoreactive nerve fibers (arrowheads) are sparse in the meibomian gland (asterisks) relative to their density around an arteriole (insert). SP-immunoreactive nerve fibers generally had the same distribution as CGRP-immunoreactive nerve fibers. Here, a few weakly stained nerve fibers are seen in the glandular tissue, whereas short segments of more intensely stained nerve fiber trunks (arrows) travel in the adjacent connective tissue. Bars = 75 μm.

the TH-immunoreactive nerve fibers in our primate meibomian gland specimens were not regularly apposed to the acini, but they also were supporting a nonsympathetic origin for the bulk of the acinar peptidergic nerve fibers. The vascular innervation by TH-containing nerve fibers was prominent. These findings concur with reported observations of sympathetic nerves in the rat meibomian gland, innervating mostly blood vessels and, to a lesser extent, meibomian gland acini. In reports on rat, however, the density of sympathetic innervation appears to be greater than in the primate specimens studied here.

Sparse nerve fibers immunoreactive for CGRP and SP were present in the meibomian glands. In the eyelid, CGRP serves as a marker for what is likely a subset of sensory nerves originating in the trigeminal ganglion. Substance P-immunoreactive neurons similarly are present in the trigeminal ganglion. Both CGRP and SP are found in many sensory perivascular nerve fibers. In the meibomian gland, the CGRP- and SP-immunoreactive nerve fibers appeared to be terminal nerve fibers apposed to acini in some cases, but possibly innervating small blood vessels not readily visualized by the current methods. Similarly in rat, CGRP- and SP-immunoreactive nerve fibers are present between meibomian gland acini, where the CGRP-immunoreactive nerve fibers appear to innervate blood vessels more than acini.

Our observations suggest that meibomian gland function may be under neural control through direct effects on the synthesis or extrusion of meibomian lipids by the acini or indirectly through vascular effects, or both. There is little understanding of the control of meibomian gland secretion. Unlike most sebaceous glands, the meibomian gland does not appear to be under hormonal control. Evidence for neural control of secretion includes an observation in...
rabbit of meibomian secretion stimulated by physostigmine,\(^\text{22}\) a finding consistent with the histochemical evidence of numerous cholinergic and presumed parasympathetic nerves in the meibomian gland.\(^\text{10,18}\)

The role of sympathetic and sensory nerves in the meibomian gland is less clear. Long-term administration of epinephrine in rabbits results in clinical and histologic meibomian gland dysfunction, but this could be a direct toxic effect of epinephrine rather than a neural mechanism.\(^\text{1,23}\) The rat preputial gland, one of the few innervated sebaceous glands, responds to \(\alpha\)-adrenergic stimulation with immediate extrusion of preformed secretions.\(^\text{24}\) Whether meibomian glands have a similar response is unknown. The action, if any, of sensory nerves in the meibomian gland could be related to the effector role of CGRP in some glandular tissue or to the vasodilatory effects of SP and CGRP.\(^\text{25,26}\)

In addition to nerves in the meibomian glands, we observed nerves in or near many other eyelid structures, such as conjunctiva, cilia and other hair follicles, and glands of Zeis. Our findings generally concur with published descriptions of conjunctival and subconjunctival vascular innervation\(^\text{7–9}\) and of cilia and hair follicles.\(^\text{27}\) One study\(^\text{12}\) of human eyelids reported cholinesterase-reactive nerves near glands of Zeis, but we observed mostly sensory nerves and, in a few instances, nerves containing NPY near the glands of Zeis.

An additional finding is the presence of nerve fibers in subconjunctival lymphoid follicles. Previous studies have demonstrated innervation to lymph organs, both primary (e.g., thymus) and secondary (e.g., spleen, lymph nodes, and gut-associated lymphatic tissue). Results in these studies have included noradrenergic nerve fibers containing NPY and nerve fibers containing SP, CGRP, and VIP located in the lymphoid parenchyma or in association with blood vessels. Various immune cells possess receptors to neuropeptides (including VIP, SP, somatostatin, and opiates) that appear to have an immunomodulatory effect.\(^\text{28}\) Similar to observations in primary and secondary lymph organs, we visualized presumed sympathetic and sensory nerve fibers in the parenchyma and along blood vessels of subconjunctival lymphoid follicles, findings that concur with a previous report\(^\text{5}\) of CGRP-immunoreactive nerve fibers in the lymphoid layer of rat conjunctiva. Because we had only a few samples of these follicles to study, we cannot exclude the presence of other nerve fiber types. The function of these nerves in subconjunctival lymphoid follicles remains to be elucidated.

In conclusion, the meibomian glands of the primate species \(M.\) \textit{mulatta} and \(M.\) \textit{fascicularis} have a rich innervation, with nerve fibers predominantly associated with acinar structures. Our immunohistochemical observations support the hypothesis that this innervation is largely parasympathetic, but further experiments are needed to confirm this. Sparse sympathetic and sensory innervations appear to supply both acini and blood vessels in the meibomian glands. In addition, subconjunctival lymphoid follicles contain apparently sympathetic and sensory nerve fibers in their parenchyma and associated blood vessels. Although the presence of nerves in these structures suggests modulation by neuropeptides or neurotransmitters, definition of their function awaits direct physiologic study.

**Key Words**

dry eye, immunocytochemistry, meibomian gland, neuropeptide, primate

**References**


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