Adrenergic and cholinergic innervation of the anterior segment of the normal and glaucomatous dog

Robert M. Gwin,* Kirk N. Gelatt, and Chung Y. Chiou

Adrenergic and cholinergic innervation of the anterior segment was examined in the normal and glaucomatous dog. Adrenergic innervation was demonstrated by the fluorescence histochemical technique of Falck-Hillarp. Corneal adrenergic fluorescence consisted of mild stromal fibers running parallel to the corneal epithelium. Fine fluorescent fibers occurred in the trabecular meshwork and the anterior cilioretinal sinus. Dense adrenergic innervation was present in the subepithelial portions of the ciliary body with extensions into the stroma of the ciliary body processes. Fibers were prominent in scleral outflow channels (venous sinus plexus) and iris dilator musculature. Less extensive adrenergic innervation was present in iris sphincter and ciliary body musculature. Cholinergic activity was evaluated by the thiocholine method for cholinesterase staining and a modified method of Mindel and Mittag for choline acetyltransferase activity. Cholinergic activity in the cornea was limited with the majority of activity in the corneal epithelium. Low choline acetyltransferase activity and little appreciable cholinesterase staining were present in the trabecular meshwork and associated outflow channels. The ciliary body and ciliary processes demonstrated intense cholinesterase staining and high choline acetyltransferase activity. Cholinesterase staining revealed activity in ciliary musculature and epithelial cells. Moderate cholinesterase staining and choline acetyltransferase activity were present in iris tissue. Beagles with advanced glaucoma tended to exhibit less intense fluorescence of adrenergic fibers. Choline acetyltransferase levels were also lower in glaucomatous specimens. The apparent decrease in cholinergic and adrenergic activity may be secondary.

Key words: dog, glaucoma, autonomic innervation, adrenergic, cholinergic, anterior segment

The autonomic nervous system participation in the regulation of ocular physiology, specifically that dealing with aqueous production and outflow, has long been recognized. Development of histochemical techniques that identify adrenergic and cholinergic nerves has permitted evaluation of their relationship to ocular structures.

A sensitive histochemical method for the identification of adrenergic nerves is based on the principle that adrenergic neurotransmitters (i.e., norepinephrine) are transformed into intensely fluorescent isoquinoline derivatives by condensation with formaldehyde.1, 2 By this method the ocular sympathetic innervation in the monkey, cat,
rabbit, mouse, rat, and guinea pig has been described.3-6

Variations of the original histochemical technique for the localization of cholinesterase activity by Koelle and Friedenwald7 have been developed8-10 and applied to localization of ocular cholinergic activity in several species.3, 4, 11-14 Although this type of histochemical localization reveals enzyme activity on an anatomical basis, these cholinesterases (true and nonspecific) are not solely concerned with the hydrolysis of acetylcholine; hence the sites of enzyme reactivity do not always represent cholinergic nerve fibers. A sensitive measurement of cholinergic nerve fiber activity may be achieved by quantitative analysis of the enzyme choline acetyltransferase, which is responsible for the synthesis of acetylcholine. Choline acetyltransferase activity has been estimated in ocular tissues of several species.15-21 Although this enzyme is quite specific for cholinergic innervation, the required homogenization of tissues precludes the localization of anatomical relationship achieved with conventional cholinesterase localization method.

A strain of beagle dogs with spontaneous open-angle glaucoma associated with a decreasing facility of outflow has been described.22-24 These animals are responsive to topically applied adrenergic and cholinergic agents.25, 26

Since comparative studies of the autonomic innervation of the canine eye are lacking, the purpose of this study is to describe the adrenergic and cholinergic innervation of the anterior segment in normotensive and glaucomatous dogs.

Materials and methods

Adrenergic innervation. A total of 20 beagle eyes were used (15 normal, five glaucomatous). All animals were anesthetized with intravenous sodium pentobarbital. Within 10 sec following enucleation, each globe was submerged in Freon 22, supercooled with surrounding liquid nitrogen, for 2 min. The globe was then placed on Dry Ice and bisected in the anterior-posterior direction with a frozen razor blade. The sectioned globes were then sealed in plastic bags and stored in an ultra-low temperature freezer. The sectioned globes were freeze-dried (Lab Conco, 75050 freeze-drying unit; Lab Conco, Kansas City, Mo.) in a vacuum at -20° C (sample temperature) for 16 to 24 hr, removed, and placed separately in a covered beaker containing paraformaldehyde crystals (5 gm). The beakers were then placed in an oven at 80° C for 1 hr according to a previously described technique,2 after which the tissues were immediately embedded in paraffin. Tissue sections (10/um) were cut, placed directly on glass slides, covered with xylene and cover slips, and heated on a slide warmer. It is important to avoid exposure of the sections to water and humidity. The mounted sections were examined by a fluorescence microscope with a BG12 exciter filter and an Olympus 530 barrier filter. Photographs were taken with GAF 500 film (GAF Corp., New York, N. Y.). As a control, two dogs were given reserpine (25 mg/kg) intraperitoneally 26 hr prior to enucleation and again 2 hr before enucleation, and the eyes were processed as above for catecholamine fluorescence. Reserpine exhausts the catecholamine supplies of the adrenergic terminals.

Cholinesterase studies. A total of 12 eyes were used (eight normal, four glaucomatous beagles) for the cholinesterase localization. Determination of cholinesterase activity was performed following Gerebtzoff's variation of the thiocholine method of cholinesterase localization.10 Eyes were frozen in Freon 22 or Dry Ice immediately after removal from the anesthetized dogs, sectioned (10/um) in a cryostat at -20° C and placed on cover slips. The sections were incubated in a solution containing acetylthiocholine at 37° C for 3 hr at pH 6. Control sections were placed in a preincubation solution which contained 3.5 X 10^-6M diisopropyl fluorophosphate for 30 min. All slides were counterstained with eosin.

Table I. Choline acetyltransferase activities in various ocular structures

<table>
<thead>
<tr>
<th>Choline acetyltransferase activity (pmol Ach synthesized/min/mg tissue)</th>
<th>Normal dog</th>
<th>Glaucomatous dog</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corneal epithelium</td>
<td>4 0.94 ± 0.12</td>
<td>4 0.95 ± 0.16</td>
</tr>
<tr>
<td>Corneal stroma</td>
<td>4 0.11 ± 0.03</td>
<td>4 0.03 ± 0.02</td>
</tr>
<tr>
<td>Iris</td>
<td>9 2.33 ± 0.71</td>
<td>6 0.80 ± 0.12</td>
</tr>
<tr>
<td>Corneoscleral angle</td>
<td>9 0.08 ± 0.03</td>
<td>6 0.05 ± 0.02</td>
</tr>
<tr>
<td>Ciliary body</td>
<td>9 5.46 ± 0.48</td>
<td>6 1.94 ± 0.30*</td>
</tr>
<tr>
<td>Retina</td>
<td>9 0.92 ± 0.14</td>
<td>6 0.89 ± 0.10</td>
</tr>
</tbody>
</table>

*Significantly different from normal dog at p < 0.05.
Fig. 1. Fluorescing adrenergic fibers in the corneoscleral trabecular meshwork (arrows) located between the anterior chamber (AC) and the sclera (S). (Freeze-dried; ×400.)

**Choline acetyltransferase studies.** For determination of choline acetyltransferase activity, 15 globes (nine normal, six glaucomatous beagles) were removed from anesthetized dogs and immediately cooled in liquid nitrogen for 4 to 6 sec, then placed on Dry Ice for tissue dissection under an operating microscope. The corneal epithelium was stripped off in eight globes prior to corneal removal for separate analysis. Following removal of the cornea, the iris was excised. The globe was then sectioned longitudinally, and the ciliary body and outflow channels were dissected from other ocular structures. All tissues were immediately frozen in liquid nitrogen and stored in an ultralow temperature freezer. Separate sections were fixed in 10% buffered formaldehyde, processed, and stained with hematoxylin and eosin for verification of dissected structures. Choline acetyltransferase activity was determined with a modified method of Mindel and Mittag.21

**Results**

**Cornea.** Catecholamine fluorescence was sparse in the canine cornea. Adrenergic nerves were identified as short, fluorescent segments parallel to the corneal epithelium.
throughout the corneal stroma. Branching was not prominent, and innervation of the corneal epithelium was not demonstrated. Adrenergic innervation was similar in glaucomatous and normotensive dogs.

Cholinergic innervation of the cornea was limited. The acetylthiocholine method for staining cholinesterase demonstrated no activity. The choline acetyltransferase assay revealed limited activity in the cornea, with most activity in the corneal epithelium (Table I).

Iris. Adrenergic fluorescence of the iris was most prominent beneath the posterior pigmented epithelium in the iris dilator muscle which extends almost the entire length of the iris. The iris sphincter muscle also displayed mild fluorescence. Fluorescence was also intense around most of the major blood vessels of the iris. Both glaucomatous and normotensive dogs had similar adrenergic patterns, although these patterns were less intense in advanced glaucomatous specimens.

Cholinesterase activity in the iris was moderate with the acetylthiocholine method but difficult to evaluate due to heavy pigmentation. The choline acetyltransferase assay revealed fairly high levels of activity in iris tissue (Table I). Although statistically insignificant, there was a tendency for the glaucomatous dog to have lower acetyltransferase activity in the iris than the normal dog.

Filtration mechanism. The filtration mechanism in the dog includes the corneoscleral trabecular meshwork, associated venous sinus plexus, and the ciliociliary cleft (equivalent of uveal meshwork).

Fine adrenergic nerve fibers were distributed throughout the canine filtration

Fig. 2. Adrenergic fibers surrounding aqueous draining channels of the venous sinus plexus (large arrows) and associated corneoscleral meshwork (small arrows) reveal adrenergic innervation. The anterior chamber (AC) is at the top of the photo while the sclera (S) is at the bottom. (Freeze-dried; x400.)
mechanism. In the corneoscleral trabecular meshwork, abundant fibers ran parallel with the trabecular tissue and exhibited a finer quality than those present in the iris, ciliary body, and cornea (Fig. 1). Adrenergic innervation of the draining venous sinus plexus was also evident (Fig. 2). In beagles with advanced glaucoma the fluorescence was less intense than in normotensive dogs.

The ciliochoroidal cleft contained very fine, delicate uveal meshwork which was difficult to maintain during processing and sectioning. However, in several instances the anatomical relationships were preserved, and adrenergic innervation was demonstrated. Similar to the corneoscleral trabecular meshwork, the innervation pattern consisted of very fine fibers in moderate to abundant numbers.

The acetylthiocholine method for cholinesterase indicated a lack of cholinesterase activity in the corneoscleral meshwork and in the ciliochoroidal cleft. Assays for choline acetyltransferase in the area of corneoscleral meshwork indicated minimal activity in both normal and glaucomatous dogs (Table I).

Ciliary body and ciliary processes. Adrenergic innervation in the ciliary body was quite extensive beneath the ciliary body epithelium in both the pars plicata and pars plana (Fig. 3). However, the ciliary body muscle was almost totally devoid of adrenergic fibers. The stroma of the ciliary processes was richly innervated with adrenergic fibers; these fibers did not extend into the epithelial layers (Fig. 4). Findings were similar in glaucomatous and normotensive dogs.

Cholinesterase activity contrasted sharply to the adrenergic innervation. The immediate subepithelial portion of the ciliary body demonstrated no cholinesterase activity; however, the ciliary musculature exhibited high amounts of cholinesterase (Fig. 5). The nonpigmented epithelial cells in the ciliary
Fig. 4. Adrenergic innervation (long arrows) of a canine ciliary body process. The nonpigmented ciliary body epithelium has autofluorescence (short arrows). (Freeze-dried; X200.)

body processes contained considerable cholinesterase activity (Fig. 6). The amount of cholinesterase activity in the pigmented epithelial cells of the ciliary body processes could not be estimated because of the large amounts of pigment. The stroma of the ciliary body processes retained minimal cholinesterase stain. Cholinesterase activity in glaucomatous dogs followed the same pattern as dogs with normal intraocular pressure but appeared quantitatively less intense. The ciliary body and processes in the glaucomatous dogs were moderately atrophic.

Choline acetyltransferase activity in the ciliary body correlated with the cholinesterase staining procedure and was the highest of the tissues studied (Table I). The choline acetyltransferase activity in the glaucomatous ciliary body was significantly lower than in ciliary body tissue from a normotensive dog (p < 0.05). Considerable levels of choline acetyltransferase activity occurred in the retina of both normotensive and glaucomatous dogs.

Discussion

Corneal fluorescence of adrenergic nerves in the dog is similar to that in the cat and rabbit.3 4 No pattern of innervation could be ascertained from the corneal stromal adrenergic fibers which ran parallel to the epithelium in all layers of the stroma. Fibers approaching either the epithelium or endothelium were not detected. The functional significance of this innervation is unclear.

The lack of cholinesterase activity in the cornea was unexpected, since cholinergic fibers have been demonstrated in the cornea of several other species.3 4 The majority of choline acetyltransferase activity was in the corneal epithelium. High corneal choline acetyltransferase activity occurs in the rabbit, bovine, and human corneal epithelium, whereas little has been found in the cat.19 21
Fig. 5. Composite of two photomicrographs of the ciliary body and associated structures. The ciliary body musculature beneath the pars plana (A) and the pars plicata (B) stains heavily for cholinesterase except in the immediate subepithelial regions (arrows). The cilioconal sinus (C), a pectinate ligament (D), and the corneoscleral trabecular meshwork (E) contain no cholinesterase activity. (Acetylthiocholine stain and eosin; light microscopy; ×100.)

Fig. 6. Heavy cholinesterase staining in the nonpigmented epithelium of the canine ciliary body and its processes (arrows). (Acetylthiocholine stain and eosin; light microscopy; ×200.)
Adrenergic innervation of the filtration mechanism in the dog occurred in the uveal as well as the corneoscleral components. The corneoscleral innervation consisted of fibers to both the trabecular meshwork and the venous sinus plexus. Adrenergic innervation of these areas along with those fibers in the ciliary cleft may be responsible for increasing the facility of outflow following topical application of adrenergic agents, although the exact mechanisms by which this occurs are not known.

The sparse cholinesterase and choline acetyltransferase activity in the filtration mechanism would seem to indicate that the mechanism of action for cholinergic pressure reducing activity in the dog lies in other areas. The major sites of cholinesterase activity found in the anterior segment of the dog are the iris, the ciliary muscle, and the epithelium of the ciliary body processes. Cholinergic activity in the ciliary body musculature may result in an alteration of tension on the filtration mechanism with a resultant increase in facility of outflow. Such a mechanism of action has been demonstrated in the cynomolgus monkey. Although the dog does not have a scleral spur, it does have similar ligamentous attachments that may function in the same manner. Alteration of cholinergic activity within the epithelium of ciliary processes may be responsible for a decrease in aqueous humor production.

The glaucomatous beagles exhibited decreased choline acetyltransferase activity in the majority of tissues examined; the low cholinergic activity was significant in the ciliary body tissues. Since they also had advanced chronic glaucoma and a generalized atrophy of all structures in the anterior segment, the reduction in choline acetyltransferase could either be an effect or part of the genesis of the glaucoma. There is no way to tell from the present study. In the future, examination of young beagles prior to the onset of advanced glaucoma may answer this question.

REFERENCES
20. Williams, J.D., and Cooper, J.R.: Acetylcholine in


