Calcitonin Gene-Related Peptide Immunoreactive Nerves in Human and Rhesus Monkey Eyes

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Using immunohistochemical methods, calcitonin gene-related peptide localizes to peripheral nerve fibers of the human and rhesus monkey eye. Immunoreactive nerve fibers are found in the cornea, about limbal blood vessels and in the trabecular meshwork. Many immunoreactive iris nerve fibers are present, mostly within the stroma. A particularly dense network occurs just anterior to the iris sphincter muscle, but only a small number of immunoreactive nerve fibers are visualized within it. The ciliary muscle and ciliary processes also are innervated. Immunoreactive nerve fibers are associated with uveal blood vessels, most prominently in the choroid and ciliary body. Apposition of immunoreactive nerve fibers to uveal melanocytes is seen. In lower mammalian species, calcitonin gene-related peptide co-localizes with substance P in many ocular nerve fibers. The comparative distribution in human and monkey eyes of nerve fibers immunoreactive to these two peptides is discussed. Invest Ophthalmol Vis Sci 29:305–310, 1988

In addition to classical neurotransmitters, such as acetylcholine and norepinephrine, peripheral nerves to the mammalian eye contain many biologically active peptides. These neuropeptides are now known to have important neurotransmitter or neuromodulatory effects in peripheral tissues, including the eye. Of the three neuropeptides reported to date in ocular sensory nerves, substance P, calcitonin gene-related peptide (CGRP) and cholecystokinin, only the distribution of nerve fibers containing substance P has been described in the human and monkey eye. Using immunohistochemical techniques, we now have studied human and rhesus monkey eyes for the presence of CGRP, a 37 amino acid peptide recently found in ocular sensory nerves of rat and guinea pig.

Materials and Methods

Tissue Preparation

Human autopsy tissues were obtained shortly after death. Both eyes were obtained from a 15-month-old white male who died of multiple complications of prematurity, from a 16-year-old white female who died of postoperative cardiac arrest and from a 54-year-old white male who died suddenly of presumed myocardial infarction; one eye was obtained from a 54-year-old white female who died of lung carcinoma and cardiac arrest. The cornea and limbal region from each of these eyes had been excised previously as donor tissue for corneal transplantation. One intact anterior segment was obtained from an 11-month-old white male who died of cerebral hemorrhage; one, from a 40-year-old white male who died of brain carcinoma; and another, from a 68-year-old white female who died of a cerebral vascular accident. Both anterior segments were obtained from a 19-year-old white male and from a 25-year-old white male, each of whom died of accidental head trauma. Rhesus monkey eyes were obtained immediately after death from five animals killed under deep pentobarbital anesthesia as part of the polio vaccine testing program of the Bureau of Biologies of the Food and Drug Administration. This study was conducted in accordance with the ARVO Resolution on the Use of Animals in Research.

All tissues were fixed by immersion in Zamboni's solution for 48 hr at 4°C and then were washed overnight at 4°C in 0.1 M phosphate buffer, pH 7.4, containing 30% sucrose. Cryostat tissue sections, 16–20 μm thick were thaw-mounted on gelatin-coated slides, air dried, and stored at −20°C until stained.

Antisera

Three polyclonal antisera were used as immunohistochemical probes. All were generated in rabbits:

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Fig. 1. Calcitonin gene-related peptide-like immunoreactive nerve fibers supply the superficial limbus and cornea. (A) Immunoreactive nerve fibers (arrow) supply superficial limbal blood vessels (V) in the rhesus monkey. Some immunoreactive nerve fibers in this field are cut in cross-section (arrowheads). (B) An immunoreactive nerve fiber (arrow) lies in the anterior corneal stroma (STR) of the human. EP = corneal epithelium. (Fluorescence micrographs; magnification bars, 50 μm.)

one against the synthetic C-terminal sequence of CGRP \(^{11}\); one against a conjugate of synthetic rat CGRP \(^{2}\) and one against a gluteraldehyde conjugate of synthetic rat CGRP and bovine serum albumin (Cambridge Research Biochemicals, Cambridge, England).

Immunohistochemical Procedure

Immunofluorescence techniques were analogous to those employed previously. \(^{9}\) Tissues sections were incubated overnight at room temperature with one of the anti-CGRP sera diluted 1:400 in 0.05 M phosphate buffered saline containing 0.3% Triton X-100. After washing, the tissues sections were incubated for 30 min at room temperature with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (Cappel Laboratories, Malvern, PA) diluted 1:300 and also containing 0.3% Triton X-100. The tissue sections, mounted in a Tris-glycerin mixture, were examined for fluorescence with an epi-illumination system.

To assess immunohistochemical specificity, each of the primary antisera at 1:400 dilution was incubated overnight at 4°C with 1 μM CGRP (Peninsula Laboratories, Inc., San Carlos, CA). Each preabsorbed antiserum then was used instead of the primary antiserum in the same immunohistochemical procedure. As an additional control, tissue sections were incubated with the FITC-conjugated secondary antiserum alone.

Results

CGRP-like immunoreactive (-LI) nerve fibers were visualized in both human and rhesus monkey eyes and had the typical varicose appearance and meandering course of terminal nerve fibers. With only minor exceptions, the distribution of immunoreactive nerve fibers was identical for the human and for the monkey eye. Their distribution will be described together, with the few differences noted. Each of the three antisera stained ocular nerves in similar distribution. Control experiments confirmed the specificity of the immunohistochemical reaction: preabsorption of each primary antiserum or use of FITC-conjugated secondary antiserum alone failed to stain ocular nerve fibers.

Limbus and Cornea

Numerous immunoreactive nerve fibers surrounded the superficial limbal blood vessels in both human and monkey eyes (Fig. 1A). In part because of high autofluorescence, the cornea was more difficult to evaluate in many of the eyes. Nevertheless, a small number of immunoreactive corneal nerve fibers were seen in both species, more commonly in human than in monkey; they were visualized mostly within the anterior corneal stroma (Fig. 1B) and rarely within the epithelium.

Aqueous Outflow Apparatus

Immunoreactive nerve fibers were present in this region of both human and monkey eyes but were visualized more readily in the monkey. In both species, CGRP-LI nerve fibers occurred mostly in the posterior uveal portion of the trabecular meshwork. Sometimes they were seen in the anterior portions of
the trabecular meshwork, in the juxtacanalicular connective tissue near the inner wall of Schlemm's canal (Fig. 2) or at the outer wall of Schlemm's canal.

Iris

The iris of both species contained many CGRP-LI nerve fibers, primarily in the stroma. Most tended to lie in the middle portion of the stroma (Fig. 3A). Especially in the human, a small number of nerve fibers were seen in the anterior border layer; occasionally, CGRP-LI nerve fibers also were applied directly to melanocytes elsewhere in the iris stroma. Sometimes immunoreactive nerves were associated loosely with iris blood vessels, but many iris blood vessels had no CGRP-LI innervation.

An especially rich network of immunoreactive nerve fibers was present in the iris stroma either just in front of or directly applied to the anterior surface of the iris sphincter muscle (Fig. 3B). Occasionally a nerve fiber entered the sphincter, chiefly in its peripheral region (Fig. 3C). A small number of CGRP-LI nerve fibers were seen in the deep iris stroma, but only a few of these appeared to approach the iris dilator muscle (Fig. 3D).

Ciliary Body

A moderate number of CGRP-LI nerve fibers occurred within both longitudinal and oblique muscle fibers of the ciliary muscle (Fig. 4A) and within the lamina fusca. An association of CGRP-LI nerve fibers with arterioles was much more evident for ciliary than for iris blood vessels (Fig. 4B). Commonly, immunoreactive nerve fibers were applied closely to melanocytes in the ciliary body (Fig. 4C). More frequently in human, a modest number of immunoreactive nerves were present within the ciliary processes of both species (Fig. 5). Immunoreactive nerve fibers were more numerous in the anterior regions of ciliary processes but extended their entire length. CGRP-LI nerves were present in the pars plana stroma; but no clear nerve fibers were visualized in the capillary layer beneath the pars plana epithelium in either species, perhaps because of the high autofluorescence of this region.

Choroid

A moderate number of CGRP-LI nerve fibers was visualized in the choroid (Fig. 6), often surrounding the larger choroidal blood vessels. Rarely, individual nerve fibers were seen more superficially near but not within the choriocapillaris. Immunoreactive nerve fibers sometimes were seen in juxtaposition to choroidal melanocytes.

Discussion

Alternative processing of the calcitonin gene results in the expression of calcitonin in thyroid C cells and CGRP in nervous tissue; these two peptides contain no corresponding amino acid sequences.11 CGRP-LI nerves have been demonstrated in brain, spinal cord, sensory ganglia such as the trigeminal,6-8,11-15 and in many peripheral organs,7,16,17 including the eye.5-9 Recently, another similar peptide named β-CGRP has been discovered as the product of a gene different from that expressing CGRP; CGRP is now called α-CGRP to distinguish further these two peptides.18 The trigeminal ganglion contains both α-CGRP mRNA and β-CGRP mRNA. The primary antisera used here likely do not distinguish between these two closely related peptides, and the general term “CGRP-like immunoreactive” is appropriate for the present results, pending biochemical identification of the antigen in ocular nerves.

Despite unknown effects of age, premortem illness and systemic drug therapies on human autopsy specimens, there was remarkable similarity among tissues from different human eyes. Further, except for minor differences, the findings in the human were quite similar to those in the rhesus monkey. We therefore assume that the distribution of ocular nerves observed here is representative for both species.

In both rat and guinea pig, CGRP-LI ocular nerves derive from the trigeminal ganglion.5,8,9,13,19 By analogy, CGRP-LI ocular nerve fibers in human and monkey likely are sensory in origin. For rat and guinea pig, significantly more trigeminal neurons are immunoreactive for CGRP than for substance P; but essentially all substance P-LI cells also are immuno-
Fig. 3. Calcitonin gene-related peptide-like immunoreactive nerve fibers richly supply the iris. (A) In the iris root are seen both individual immunoreactive nerve fibers within a nerve trunk containing other nonreactive nerve fibers (arrowhead) and also free-running immunoreactive stromal nerve fibers (arrows). The tendency of immunoreactive nerve fibers to lie in the middle region of the iris stroma is demonstrated. (B) Immunoreactive nerve fibers (arrows) commonly are observed just anterior to the iris sphincter muscle (SPH). (C) Occasionally, a nerve fiber (arrow) is seen within the sphincter muscle itself. (D) Only rarely is an immunoreactive nerve fiber (arrow) seen in the deep iris stroma near the dilator muscle. AC = anterior chamber; CB = anterior ciliary body; PE = iris pigmented epithelium. (Fluorescence micrographs; rhesus monkey in A, B, C, and human in D; magnification bars, 50 μm.)

Fig. 4. Calcitonin gene-related peptide-like immunoreactive nerves are distributed throughout the ciliary body. (A) Immunoreactive nerve fibers (arrows) lie within the human ciliary muscle. (B) An immunoreactive nerve fiber (arrow) surrounds a blood vessel (V) in the human ciliary body. (C) An immunoreactive nerve fiber is directly apposed to one of the melanocytes (M) in this field from the rhesus monkey ciliary body. (Fluorescence micrographs; magnification bars, 50 μm.)
reactive for CGRP.\textsuperscript{5,13,14,19} Similarly, co-localization of CGRP and substance P occurs in ocular nerve fibers of both rat\textsuperscript{7} and guinea pig.\textsuperscript{7,18} We did not specifically study the co-localization of CGRP and substance P in the present study; but based on the findings in lower mammals and on the similarity in distribution of CGRP-LI ocular nerves to those containing substance P in both human\textsuperscript{6} and monkey,\textsuperscript{4} considerable co-localization of these two neuropeptides also is probable in the human and monkey eye.

Despite the likely co-localization, fewer CGRP-LI than substance P-LI nerve fibers are observed in three regions of human and monkey eyes: the cornea, the trabecular meshwork, and the ciliary process. Several reasons may account for the lower number of CGRP-LI nerve fibers visualized in these regions. First, unlike the findings in rat and guinea pig, not all substance P-LI trigeminal neurons of the primate may contain CGRP. Second, some trigeminal neurons may contain both peptides in their somata but express only one in their peripheral processes. Third, some ocular substance P-LI nerve fibers in human or monkey may not be of trigeminal origin, as recently observed in the guinea pig.\textsuperscript{19} Finally, technical considerations such as the high background autofluorescence of these areas may mask CGRP staining selectively. The current study does not distinguish among these alternatives.

An ocular role for CGRP is only beginning to be uncovered. CGRP is a potent vasodilator.\textsuperscript{20} In the rabbit eye, intracameral administration of CGRP causes disruption of the blood-aqueous barrier and an elevation of intraocular pressure.\textsuperscript{21} Unger and co-workers have proposed that CGRP and substance P act together in the neurogenic ocular injury response: CGRP mediating uveal vasodilation; substance P, miosis.\textsuperscript{21} In addition, however, the vascular effects of CGRP and substance P are synergistic, and CGRP potentiates the miotic effect of substance P,\textsuperscript{22} indicating even more direct interactions between these two peptides.

In addition to blood vessels, CGRP-LI nerve fibers innervate other intraocular tissues. For the cornea, CGRP-LI nerve fibers may serve a nociceptive function; but this hypothesis requires direct testing. The localization of CGRP-LI nerves in the aqueous humor outflow pathway of both human and monkey extends the list of biologically active substances found in this region.\textsuperscript{23} While the presence of nerves in the chamber angle has been long recognized, the functions of the innervation to this region remain to be established.

The juxtaposition of CGRP-LI nerve fibers to uveal melanocytes is analogous to observations for nerve fibers containing other neurotransmitters and neuropeptides.\textsuperscript{1} The manner and extent to which peripheral nerves interact with these cells require clarification beyond the already known sympathetic influence on uveal pigment synthesis.\textsuperscript{24}

CGRP is just one of many peptides found in ocular nerves. The large number of neuropeptides and the recognition of peptide co-localization in individual nerve fibers imply complex physiological interactions. Not only do individual neuropeptides need to be studied but the functional meaning of their co-lo-
calization specifically needs to be addressed. The recent studies with CGRP indicate that such interactions likely have great importance in the eye.

Key words: calcitonin gene-related peptide, innervation, sensory, eye, human, rhesus monkey, immunohistochemistry

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