Overall Distribution of Substance P Nerves in the Rat Cornea and Their Three-dimensional Profiles

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The overall distribution of substance P-like immunoreactive (SPI) fibers, examined by using whole-mounts of the rat cornea, was investigated by means of indirect immunofluorescence. SPI fibers entered the cornea from two levels; one from the middle layer of the sclera and the other from the episclera. From the sclera, a thick SPI fiber trunk, extending to the central part, subdivided into smaller SPI fiber bundles and approached the epithelium. The SPI fiber bundles from the episclera were smaller than those from the sclera. However, both fiber bundles formed a dense fiber network in the uppermost part of the stroma. This fiber plexus dissociated into SPI fibers extending to the superficial part of the epithelium where they formed an abundant arborization of fine SPI fibers. The results suggest that these fibers originate from SPI neurons in the trigeminal ganglion. Invest Ophthalmol Vis Sci 25:351–356, 1984

Immunohistochemical studies have demonstrated the presence of substance P (SP)-containing fibers in the cornea,1–10 although the role of this innervation is still equivocal. To elucidate the function of SPI in this area, it is important to explore the overall distribution of the SP fibers and their origins. However, the use of frozen sections1–7,9,10 provides only a limited picture of the overall distribution and three-dimensional profiles of SP in the cornea.

On the other hand, experiments using the whole-mount technique have succeeded in demonstrating SPI fibers in the cornea even though the density of the SPI fibers was reported to be rather low.8 However, we have demonstrated successfully a high density of SPI fiber network in the cornea on the whole-mounted tissue. In this article, we report the detailed profiles of SPI fibers in the cornea reconstructed from whole-mounts.

Tervo et al5,9 and Terenghi et al10 have insisted that corneal SP fibers originate from SP neurons located in the trigeminal ganglion, while Neuhuber et al11 have refuted this possibility. Therefore, we have reexamined, by experimental manipulation, whether or not SPI fibers in the rat cornea originates from SP neurons in the trigeminal ganglion.

Materials and Methods

Antiserum

The antiserum against synthetic SP was obtained after immunization of a rabbit with SP-bovine serum albumin conjugate. The specificity of this antiserum was tested by radioimmunoassay. The cross-reactivity of this antiserum with structurally similar peptides (eledoisin and physalaemin) was less than 0.02% and with other related peptides (somatostatin, glucagon, bradykinin, enkephalins, and endorphins) less than 0.01%. Specificity also was tested by the absorption test. Since the structures stained with SP antiserum were not seen in the section stained with the control serum (SP antiserum absorbed by an excessive SP, 1 µg/ml), the sections stained with SP antiserum were considered specific. Though the materials should be described correctly as showing SP-like immunoreactivity, we will use the simpler term, SPI.

Experimental Animals and Tissue Preparation

A total of 39 male albino rats weighing about 100 g were used. Twenty-five rats were used for observing the distribution of SPI in the cornea and the remaining 14 for elucidation of the origins of SPI fibers. In the latter group of animals, knife-cut or electrolytic coagulation of the ophthalmic nerve, immediately rostral to the trigeminal ganglion, were made stereotaxically. Sectioning was made with a fine knife. Electrolytic coagulation was made by passing an AC current for 20 min through a monopolar electrode. These operations were performed under sodium pentobarbital

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anesthesia (60 mg/kg, ip). After 5–7 days, the animals were anesthetized again and perfused via the ascending aorta with 50 ml ice-cold saline followed by 300 ml Zamboni's fluid.12 During the perfusion, the eyelid was pulled upward to make a reservoir for the Zamboni's fixative. After the perfusion, the cornea was removed completely, postfixed in the same fixative for 2 days at 4°C, and rinsed in 0.1 M phosphate buffer containing 30% sucrose for 1 day at 4°C.

Frozen sections were cut in a cryostat at a thickness of 10 μm and subjected to immunohistochemical analysis. The specimens for the whole-mounts were subjected directly to this analysis without separating the cornea into different layers or freezing after the rinsing with phosphate buffer.

Immunohistochemical Procedure

The materials for the whole-mounts were rinsed first in cold phosphate-buffer saline (PBS) for 10 min prior to processing for immunofluorescence.13 The materials were incubated overnight in a humid atmosphere at 15°C with SP antiserum, diluted to 1:1,000 with PBS containing 0.05% Triton X-100. Next, they were rinsed three times with PBS, and incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG goat serum 1:1,000 for 24 hr in the same atmosphere. Then, the cornea was rinsed three times (10 min each) with PBS; at this time, three razor cuts were made at the peripheral part to facilitate whole-mounting. The materials were mounted in a glycerine-PBS mixture (1:1), and a weight was put on the cover-glass to avoid wrinkling. The frozen sections also were processed as described above. The materials for the control experiments were incubated first with control serum (preabsorbed SP antiserum), after which they were processed as described above.

Results

Distribution of SPI Fibers in the Cornea

Frozen sections: As demonstrated in the previous studies, a large number of SPI fibers were observed in the cornea (Fig. 1). In the stroma, thick nonvaricose SPI fibers ran progressively superficial and entered the epithelium. At the upper part of the stroma, SPI fibers had a varicose appearance and formed a dense network especially in the uppermost part of the stroma. Some of the varicose fibers diverged from these fibers to the epithelial layer.

Whole-mounts: SPI fibers entered the cornea from two levels: one from the sclera and the other from the episclera. A thick fiber trunk, composed of numerous SPI fibers (Fig. 2A) entered the cornea and extended to the center, where it branched into several thinner segments. However, in the limbus, some of these thinner segments ran circularly (Fig. 2A). The centrally directed SPI fiber bundle further dissociated into thinner SPI fiber bundles and reached the uppermost part of the stroma, where they form a dense meshwork especially in the uppermost part of the stroma. Some of the varicose fibers diverged from these fibers to the epithelial layer.

Figure 3 shows SPI fiber bundles entering the cornea from the episclera. At the limbus, these fibers abruptly changed directions, ran circularly, and dissociated into thinner segments toward both the peripheral and central uppermost part of the stroma meshwork (Fig. 3). At this area, SPI fibers from the sclera and episclera formed a meshwork (Fig. 2B). The SPI fiber meshwork, located in the uppermost part of the stroma, often
Fig. 2. A. Fluorescent photomicrograph, focusing on the middle part of the stroma. Whole-mounts show a thick SPI fiber trunk (arrow) that entered the middle part of the stroma. Most of the bundles ran centrally, approached the epithelium, and subdivided (double arrowheads) while in the limbus, some ran circularly (single arrowhead) (×120). B. Fluorescent photomicrograph, focusing on the uppermost part of the stroma. Whole-mounts show a meshwork of SPI fibers in the uppermost part of the stroma. SPI fiber bundle from the stroma (arrow) form a dense meshwork in this layer (×120).

Fig. 3. Fluorescent photomicrograph focusing on the episclera. Whole-mounts show SPI fiber bundle (arrows) that enter the cornea at the limbus from the episclera. These fibers abruptly changed directions at the limbus (L) and ran circularly (arrowheads), dissociating into thinner SPI fibers (double arrowheads) that extended to the central region (C) (×200).
Fig. 4. Fluorescent photomicrograph, focusing on the epithelium. Whole-mounts show an abundant arborization of fine SPI terminals in the epithelium of the central part of the cornea (×200).

dissociated into thin SPI fibers that extended to the superficial part of the epithelium and terminated within the epithelium, where an abundant arborization of fine SPI fibers was seen (Fig. 4). In the epithelium, no regional differences in the density of the SPI fiber arborization was observed. The overall distribution of SPI fibers in the cornea of the rat is schematically shown in Fig. 5.

Fig. 5. Schematic representation showing the distribution of SPI fibers in the cornea, reconstructed from these photomontages of the cornea photographed at three different levels. Upper right shows the SPI fiber trunk that entered the stroma from the sclera. These fiber trunks extended to the central part, subdivided, and approached the epithelium. In the upper left, SPI fibers entered the cornea from the episclera, ran circularly at the limbus, often sending some fibers to the central part. In addition, an SPI fiber network was located in the uppermost part of the stroma. In the lower half, an abundant arborization of fine SPI terminals is seen within the epithelium.
Fig. 6. Fluorescent photomicrograph of a frozen section through the cornea. Seven days after complete neurotomy of the ophthalmic branch of the trigeminal nerve. Note a disappearance of SPI fibers in the cornea. Abbreviations are the same as in Figure 1. (×100).

Origins of SPI Fibers in the Rat Cornea

Partial transection of the ophthalmic branch of the trigeminal nerve caused an ipsilateral decrease in SPI fibers in the cornea. Moreover, the decrease was proportionate to the extent of the lesion. Complete transection of the ophthalmic nerve resulted in the almost complete disappearance of SPI fibers in the cornea (Fig. 6).

Discussion

SPI fibers have been reported to be present in the cornea of various mammals by the frozen section technique.1-7,9,10 Recently, Miller et al succeeded in demonstrating SPI fibers in the cornea on whole-mounted preparations, and they reported that although a network of SPI fibers could be seen in the cornea, the density was rather diffuse.8 In contrast, the present study, which also uses whole-mounted tissue, revealed a markedly dense network of SPI fibers. This finding provides evidence that these fibers enter the cornea at two levels: one from the middle layer of the sclera and the other from the episclera with circumferential distribution of SPI fibers in the limbal region.

Though the previous authors separated the cornea into different layers for improving the penetration of the antiserum into the cornea, we used the entire cornea for the whole-mounted preparation. The other major differences in the experimental design are that in our study: (1) the tissues were kept overnight in the hypertonic 30% sucrose-phosphate buffer; (2) the incubation time for the second antiserum was longer; and (3) the titer of the antiserum was higher.

It remains to be confirmed whether or not the rat corneal SPI fibers originate from SPI neurons located in the trigeminal ganglion. Neuhuber et al11 reported that rat cornea was not innervated by SPI neurons in this ganglion and suggested that the corneal SPI may be extraneuronal. However, several investigators using experimental immunohistochemical manipulations reported that SPI fibers in the cornea of the rat and rabbit originate from SPI neurons in the trigeminal ganglion.5,8,14 Our present findings support the latter hypothesis because trigeminal neurotomy resulted in a disappearance of SPI fibers in the cornea.

On the other hand, radioimmunoassay study of SP in the cornea of the mouse showed that trigeminal neurotomy reduced the SP level by only 42% while neonatal capsaicin treatment caused an 80% loss.15 This finding suggests that SPI fibers in the cornea of the mouse are innervated by sources other than the trigeminal ganglion. The discrepancy of the results between this radioimmunoassay study15 and the present and previous studies5,8,14 may be due to differences in the species or antisera used. However, it also is important to confirm the exact extent of the transection, because, as shown in the present study, the degree of the decrease of SPI fibers in the cornea depends upon the extent of the lesion.

It has been considered that nerve fibers located in the cornea are composed of sensory components, which are terminals in the cornea that receive sensory information from the cornea related to the central nervous system; therefore, the terminals located in the cornea might not contain neurotransmitter or neuromodulators. However, in the last 10 years, the pres-
ence of acetylcholinesterase, catecholamine, and serotonin-containing fibers as well as SPI fibers in the cornea has been reported.16–19 Thus, further studies are needed to explore the function of the neurotransmitters or neuromodulators in the cornea.

Key words: substance P, cornea, whole-mounts, overall distribution

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