Autonomic Innervation of Preretinal Blood Vessels of the Rabbit

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The preretinal blood vessels, that is, blood vessels lying on the inner surface of the retina, were observed by SEM examination using digestion methods, TEM examination and fluorescence histochemical examination using the Falck-Hillarp method. The nerve endings on the preretinal arterioles were distributed from the optic disc to the periphery. The longest nerve terminals from the optic disc to peripheral arterioles were about 9 mm. There were also a few nerve endings on the preretinal veins. These nerve endings had a series of axonal varicosities with diameters between 0.5 and 1.5 μm, which contained empty synaptic vesicles and cored synaptic vesicles. The number of nerve endings on these arterioles decreased with the shortening of the diameter of the retinal arterioles. Fluorescent nerve fibers with axonal varicosities were distributed on the wall of the preretinal blood vessels in the fluorescence histochemical study. These fluorescent nerve fibers were numerous near the optic disc, but there were only a few fluorescent nerve fibers on the peripheral blood vessels. The nerve endings on the preretinal blood vessels disappeared following superior cervical ganglionectomy. The present study shows that the preretinal blood vessels in rabbit eyes are innervated by the sympathetic nerve originating from the superior cervical ganglion. Invest Ophthalmol Vis Sci 28:1752-1760, 1987

Up to the present, many have concluded that the rabbit preretinal blood vessels, that is, the blood vessels lying on the surface of the retina, had no autonomic nerve innervation. Laties, using the fluorescence histochemical method, reported that the central retinal artery of the rabbit lost its autonomic nerve supply when it entered the eyeball beyond the lamina cribrosa. Similar results were reported regarding the retinal arterioles of the New World monkey, the Old World monkey and the cat. Light microscopic examination of the human retina using the silver impregnating method demonstrated that nerve fibers were present along the retinal blood vessels of humans. However, as the sympathetic nerve endings of the retinal blood vessels have not been demonstrated by transmission electron microscopic examination, it is generally thought that the retinal blood vessels of mammals do not have sympathetic nerve fibers. Recently, though, some authors reported that under transmission electron microscopic and fluorescent examination, adrenergic nerve fibers were seen in the wall of human retinal blood vessels on the disc beyond the lamina cribrosa and sometimes even a few millimeters onto the retinal surface from the disk. These facts show that there is a possibility that sympathetic nerves enter the retina beyond the lamina cribrosa in mammals. Seitz reported that the vasodilation of the preretinal vessels of the rabbit occurred after bilateral superior cervical ganglionectomy. Therefore, it is possible that rabbit preretinal blood vessels are innervated.

The present study reveals the autonomic nerve innervation of the rabbit preretinal blood vessels from the disc to periphery and the origin of the autonomic nerve supply of the preretinal blood vessels.

Materials and Methods

Eighty eyes of 40 adult Japanese white rabbits (Oryctolagus cuniculus var domestica) weighing 2–3 kg were used. Either a unilateral superior cervical ganglionectomy or a nervectomy between the superior cervical ganglion and the middle cervical ganglion was performed on 25 rabbits to study the origin of the nerves which were distributed on the retinal vessels. The contralateral eyes served as control. The rabbits that were operated on which sacrificed 1–30 days after surgical treatment by an intravenous overdose of sodium pentobarbital. Fifteen rabbits were used for the control study of the normal retinal blood vessels. Immediately after enucleation, the anterior parts of the eyeballs were removed. Specimens for scanning electron microscopy (SEM), transmission electron...
Fig. 1. SEM micrograph of the preretinal blood vessels of the rabbit. The peripheral retina is to the left and the optic disc is to the right. Preretinal arterioles and veins travel horizontally on the surface of the retina. A: preretinal arteriole, V: preretinal vein. The inset is an enlargement of the rectangle. The rectangle position is on about a 6 mm periphery from the optic disc. There are nerve endings on the preretinal arteriole, and capillaries enter the retina, a: arteriole, v: vein, c: capillary, arrow: nerve fibers.

microscopy (TEM) and fluorescent histochemistry were processed by the methods discussed below. Retinae of both the left and right eyes of the rabbits that were operated on were treated together in a single vial under the same conditions. These investigations conformed to the ARVO Resolution on the Use of Animals in Research.

SEM Specimens

Three kinds of digestion methods were used:
1. Using Uehara's method (1980), unfixed tissues were digested in Eagle's solution containing collagenase and hyaluronidase at 37°C for 20–60 min. After enzymatic digestion, they were fixed with 2% glutaraldehyde-0.1M cacodylate buffer and postfixed by 2% OsO₄-0.1M cacodylate buffer (pH 7.4), and then treated in 8N HCl at 60°C for 15–40 min.
2. Retinae with attached choroid and sclera were immersed in 2% glutaraldehyde-0.1M cacodylate buffer (pH 7.4) for 2 hr and then postfixed in 2% OsO₄-0.1M cacodylate buffer (pH 7.4) for 3 hr. After fixation, these specimens were treated in 8N HCl at 60°C for 10 min. As this recipe has produced constant results, we could easily compare the superior cervical ganglionectomy specimens with control specimens.
3. After prefixation with 2% glutaraldehyde-0.1M cacodylate buffer (pH 7.4) for 2 hr, the specimens were postfixed in 2% OsO₄-0.1M cacodylate buffer (pH 7.4) for 3 hr and then, in order to remove the adventitia, they were treated in 0.1M cacodylate buffer (pH 7.4) with Branson (Shelton, CT) B-32 ultrasonic cleaner for about 30 min.

Specimens obtained by these three methods were dehydrated in a graded series of alcohol and dried in a Hitachi (Tokyo, Japan) HCP-2 critical point dryer. They were spattered with platinum and palladium, and then examined with a Hitachi HS-700 scanning electron microscope.

TEM Specimens

The retinae which were removed from the enucleated eyes were immediately immersed in 2% glutaraldehyde-0.1M cacodylate buffer (pH 7.4) for 2 hr and postfixed in 2% OsO₄-0.1M cacodylate buffer (pH 7.4) for 2 hr. Specimens were dehydrated in an alcohol series and embedded in EPON 812. SEM specimens which had been photographed by SEM were then embedded in EPON 812. Specimens were cut into thin sections using a Porter-Blum (Newtown, CT) MT-2 ultramicrotome and stained doubly with both uranyl acetate and lead acetate. They were then examined under a JEM-100CX (Tokyo, Japan) transmission electron microscope.

Fluorescent Specimens

The Falck-Hillarp method was used. Immediately after the eyeballs were enucleated, the retinae
were separated from the choroid and sclera. The whole retina was mounted on a glass slide and dried in hot air for 15 min. It was then placed in a freezer-dryer for 3 hr. The specimen was incubated in paraformaldehyde gas at 80°C and thereafter whole retina was observed by a Nikon (Tokyo, Japan) Fluophot using a DM 445 barrier filter and an IF 385-420 excited filter.

Results

Preretinal Blood Vessels

The preretinal blood vessels obtained through the digestion methods were directly visualized under SEM (Fig. 1). The preretinal arterioles and veins traveled on the retina horizontally in a parallel manner. The diameter of the trunk arterioles and veins were over 60 μ near the optic disc; the minimum diameter was about 8 μ in the peripheral arterioles (Fig. 1). The preretinal arterioles with a diameter of over 60 μ were surrounded with three or four sheets of smooth muscle layers, but the arterioles with a diameter of about 10 μ were encircled with a smooth muscle layer. On the other hand, veins were encircled with cells such as pericytes which had many cell processes. Capillaries then entered into the retina (Fig. 1, inset).

Nerve Innervation of Preretinal Blood Vessels

The nerve innervation along the vascular tree occurred in both directions from the optic disc. The nerve endings mainly existed on the wall of the preretinal arterioles from optic disc to periphery and...
Fig. 3. SEM micrographs of the nerve density of the nerve plexuses on the preretinal arteriole. These figures show that the number of nerve fibers depends on the diameter of the preretinal arteriole. (A) Various sized nerve fibers (N) travel on the surface of the media of the preretinal arteriole with diameter of about 50 μm near the optic disc. The nerve density is the highest in this area. An arteriole (Ar) which branches from the trunk arteriole (A) has few nerve fibers. Schwann cell body (S) is in the shape of triangle. (B) SEM micrograph of the nerve plexus on the preretinal arteriole with diameter of about 30 μm. The number of nerve fibers is decreased in comparison with that of the large preretinal arteriole with diameter of about 50 μm, but their arrangement is like that of the large preretinal arteriole. An arteriole (Ar) which branches from the trunk arteriole (A) has one or two nerve fibers. N: nerve fibers, S: Schwann cell body.
sometimes on the retinal surface between the retina and the vitreous membrane. Usually the preretinal veins and capillaries had no nerve fibers (Fig. 2). However, sometimes a few thin nerve fibers were observed on the vein within about 2 mm from the optic disc and on the capillaries (Fig. 1, inset).

The number of axons in the nerve fibers was between one and six. Through SEM observation it was seen that the longest distance of the nerve endings from the optic disc was about 6 mm. Therefore, the original distance from the optic disc was about 9 mm because SEM specimens contract to about two-thirds of their fresh size owing to digestion, dehydration and drying.

The number of nerve endings on the wall of preretinal blood vessels depended on the diameter of the blood vessels. The arrangement of the nerve ending plexuses of the preretinal arterioles with a diameter of over 40 μ near the optic disc was as follows. Many nerve fibers existed on the media of preretinal arterioles. The diameters of the nerve fibers were between 0.2–2 μ (Fig. 3A). Several large nerve fibers traveled on the preretinal arterioles parallel to the longitudinal axis of the preretinal arterioles, and they branched and anastomosed (Fig. 3A). Fine nerve fibers with axonal varicosities ran in variable directions on the wall of the arterioles. The triangle-shaped point of the branch or the anastomosis was the soma of the Schwann cell (Fig. 3A). The number of nerve fibers decreased as the diameter of the preretinal arterioles decreased. Therefore, arterioles which branched from the trunk arteriole near the optic disc had one or two nerve fibers (Fig. 3A). In about a 4 mm periphery around the optic disc, there were about three to four nerve fibers on arterioles with a diameter of between 20 and 40 μ. These nerve fibers were smaller than those of the preretinal arterioles near the optic disc. Some thin nerve fibers with axonal varicosities traveled spirally on the arterioles with a diameter of between 20 and 40 μ, and sometimes the nerve endings formed loops. Arterioles which branched from the trunk arteriole had one or two nerve fibers (Fig. 3B). In about a 9 mm periphery around the optic disc, almost all preretinal arterioles became precapillary arterioles. Some thin nerve fibers with axonal varicosities spiraled around the precapillary arterioles with a diameter of about 10 μ (Fig. 3C). Figure 4 shows a schematic diagram of the distribution of the adrenergic nerve endings on the wall of blood vessels from the optic disc to the periphery.

Axonal varicosities contained empty and cored synaptic vesicles (Fig. 5A, B). The distance between the axonal varicosity and the smooth muscular surface was variable. For instance, near the optic disc, it
Fig. 4. Schematic diagram of the nerve distribution on the wall of the arterioles from the optic disc (Od) to the periphery. The density of nerve fibers decreases with the decreasing diameter of the preretinal arteriole. The nerve endings extend to precapillary arterioles. a: arteriole, v: vein, S: Schwann cell body.

Fig. 5. The interrelationship between axonal varicosities and blood vessels. (A) TEM micrograph of the axonal varicosity on a preretinal arteriole with diameter of about 50 μ. The media contains three layers of smooth muscle cells (Sm). The distance between the axonal varicosity and the outer smooth muscle cell is about 500 nm. The axonal varicosity (Av) contains cored synaptic vesicles. It is not sheathed in a Schwann cell process. (B) TEM micrograph of axonal varicosity on a preretinal arteriole with diameter of about 10 μ. Large and small cored vesicles and empty vesicles are visible in the axonal varicosity (Av). The axonal varicosity is in contact with the basement membrane of the smooth muscle cell of the preretinal arteriole. The axonal varicosity, except for the contact side to the smooth muscle cell (Sm), is sheathed in a Schwann cell process (S). The axonal varicosity includes cored synaptic vesicles.
Fig. 6. Fluorescent nerve fibers (arrow) on the preretinal blood vessels.

was over 500 nm (Fig. 5A). In the precapillary arterioles, the axonal varicosities directly contacted the basement membrane of the smooth muscle of the preretinal arterioles (Fig. 5B).

Fluorescence Histochemical Examination

Fluorescent nerve fibers were observable on walls of the preretinal blood vessels (Fig. 6). These nerve fibers had axonal varicosities. Large preretinal blood vessels near the optic disc had heavy fluorescent staining. However, only one or two fluorescent nerve fibers were observed on the preretinal arterioles with a diameter of about 10 μm.

Denervation Experiments

Seven days after ganglionectomy, nerve fibers on the preretinal blood vessels could not be observed by SEM (Fig. 7), TEM and fluorescence histochemical examination. However, the nerve distribution around the preretinal blood vessels was the same as that of the control eyes after the nervectomy between the superior cervical ganglion and the middle cervical ganglion.

Discussion

It has been concluded that the retinal blood vessels of mammals have no noradrenergic innervation. With respect to the rabbit, a previous physiological study by Seitz demonstrated the vasodilation of the preretinal vessels of the rabbit after bilateral superior cervical ganglionectomy. His report suggested sympathetic nerve innervation of the preretinal blood vessels of the rabbit. However, Laties et al denied the sympathetic nerve innervation of rabbit preretinal blood vessels using the Falck-Hillarp method. Therefore, many have believed that there was no nerve innervation of rabbit preretinal blood vessels. Nevertheless, our present study using SEM and the Falck-Hillarp method proved that the preretinal blood vessels of the rabbit eye were supplied with sympathetic nerve fibers from the optic disc to periphery.

The Falck-Hillarp method makes noradrenergic nerves fluorescent, and TEM examination usually shows that small cored synaptic vesicles store noradrenaline. In our results, nerve fibers on the preretinal arterioles were fluoresced by the Falck-Hillarp method, and axonal varicosities included cored synaptic vesicles. After the superior cervical ganglionectomy, it was shown by SEM, TEM and fluorescent histochemical examination that nerve fibers around the preretinal arterioles had disappeared. Judging from these results, nerve fibers around the preretinal arterioles must be sympathetic nerve endings originating from the superior cervical ganglion.

Why was there a difference between Laties' and our results? Laties used rabbits as experimental animals, the same as we did (Japanese white, Oryctolagus cuniculus var domestica); there was therefore no species difference between Laties' and our experimental animals. We think that the difference between his and our results is due to the difference in the observing and the drying method. Since, except for cutting, we observed the whole retina, we could search for fluorescent nerve fibers on the complete wall of the blood vessels. Laties also used a freeze vacuum drying method, while we used hot air to dry the specimens. Thus our techniques may be the main reason that we were able to observe noradrenergic nerves on the wall of preretinal blood vessels.

There is little doubt that this heavy sympathetic nerve innervation of preretinal blood vessels controls the blood flow of the preretinal blood vessel system. With respect to the nerve innervation of capillaries of the brain, Rennels et al reported that noradrenergic terminals were found close to the pericytes of the capillaries; they suggested the influence of nerve terminals on cerebral microcirculation. However, with regard to the preretinal blood vessels of the rabbit,
most of the nerve fibers were observable on arterioles, and sometimes a small number of thin nerve fibers were found on veins and capillaries. Therefore, the nerve innervation of veins or capillaries may have no influence on the blood flow of the preretinal blood vessels.

Asymmetric disposition of nerve endings on the preretinal blood vessels is found on other animal vessels, and Burnstock also described that adrenergic nerves generally spread into the adventitia of arterioles with a diameter within 200 μ. Our results also showed that nerve endings had asymmetric disposition and spread into the adventitia. Therefore, the influence of synaptic vesicles may be different according to the region of the smooth muscle cell layers.

**Key words:** autonomic innervation, superior cervical ganglion, preretinal arterioles, rabbit, scanning electron microscope

**Acknowledgments**

The author thanks Miss Rumiko Itoh for her assistance and Professor Shinji Kurimoto and Dr. Tsunetami Nomura for valuable advice.

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