Architecture of blood vessels in the mouse infraorbital nerve
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Abstract

We examined the normal intraneural vascularisation of the infraorbital nerve from the infraorbital foramen to the peripheral vibrissae to know a normal intraneural vascularisation in a peripheral pure sensory nerve. Indian ink was injected into the heart of the mouse for observation of vascular architecture in the infraorbital nerve. Three-dimensional images of blood vessels in the infraorbital nerve were then reconstructed using 3D visualization software. Optical microscopic observation of the peripheral section of the normal mouse infraorbital nerve (near mouse vibrissae) revealed fascicles of nerve fibers (10-60 μm in diameter) covered with perineurium, with one blood vessel normally present within the fascicle. Optical microscopic observation of the cross-section of normal mouse infraorbital nerve near the infraorbital foramen revealed nerve fiber fascicles (about 150 μm in diameter) covered with epineurium extending from proximal to peripheral areas, increasing in number as they branched. In the nerve fascicle surrounded by the perineurium near the infraorbital foramen, a few blood vessels were distributed. As the nerve fascicle branched and extended, the blood vessels also branched, providing one blood vessel in each nerve fascicle. There were multiple blood vessels in between the epineurium and perineurium. The blood vessels in nerve fascicles consisted of thin branches communicating with blood vessels outside of the perineurium. Nerve fascicles were surrounded by networks of blood vessels communicating with capillaries, arteriolaris and venulae. Some blood vessels showed chain-like distribution in nerve fascicles. In the peripheral part of the infraorbital nerve, the blood vessel in the nerve fascicle exited before the nerve entered a vibrissa. The blood vessel goes out of the nerve to this point that enters the vibrissa.

Key words: blood capillary, architecture, peripheral nerve, infraorbital nerve, mouse
Introduction

Trigeminal neuralgia is a neuropathic pain sensation in the maxillofacial area that is characterized by sharp paroxysmal electric-like spasms. Because of the expectation of long-term effects, this condition is frequently treated with nerve block therapy. The purpose of nerve block therapy is to obtain analgesic effects for a certain period using destructive agents or a high concentration of local anesthetic. Kashima et al. investigated the histological effects on nerve tissue when high concentrations of local anesthetic (5% lidocaine) and absolute (99%) alcohol were used for nerve block therapy. They found that the process of nerve regeneration was similar for both solutions, although there was some difference in the duration of regeneration and growth of the denatured nerves.

In terms of nerve regeneration after nerve injury, the promotion of nerve regeneration is important. However, for relief of pain in pain clinics, the prolongation of the effects of a nerve block is essential. In addition to various factors that contribute to nerve growth such as nerve growth factor (NGF) and fibroblast growth factor (FGF), nutrition is important for the regeneration and growth of nerves in wound healing. In peripheral nerves, blood vessels around the nerve tissue supply nutrition through the axonal transport system, including slow anterograde axonal transport related to nerve regeneration through the blood-nerve barrier (BNB). Therefore, to delay nerve regeneration after a nerve block, regeneration of blood vessels should be suppressed.

Previous research into regeneration of nerve and blood vessel morphology in mammals and amphibians has investigated mixed nerves (motor and sensory nerves) such as spinal nerves, median nerves, sciatic nerves, tibial nerves, and femoral nerves, and motor nerves such as facial nerves. However, these studies did not describe the region or thickness of the nerves. One earlier study reported that motor and sensory nerves grew at different rates and that sensory nerves recovered function faster than motor nerves.

According to Goran et al., the morphology of blood vessels around human peripheral median nerve fascicles takes a reticular form with numerous anastomoses around the epineurium and perineurium; although the number is few, capillaries exist in the endoneurium, occasionally form the epineurium and perineurium; although the number is few, capillaries exist in the endoneurium and sometimes form the epineurium and perineurium; although the number is few, capillaries exist in the endoneurium and perineurium. Therefore, to delay nerve regeneration after a nerve block, regeneration of blood vessels should be suppressed.

Materials and Methods

Experimental animal: A total of seven male mice (C57BL/6, body weight 20-30 g, 7 weeks old) (Clea Japan, Osaka, Japan) were used in the present study. After the purchase of the mice, they were acclimated for one week prior to the experiment at the Institute for Experimental Animal Sciences Iwate Medical University (air temperature: 23±1°C, humidity: 50±10%). We observed no oral or systemic abnormalities in the mice. During the experimental period, the mice were provided with free access to solid feed (Oriental Yeast, Tokyo, Japan) and tap water. This research was approved by the Committee on Animal Experiments of Iwate Medical University (Approval number 20-011) and was performed in accordance with the Guidelines for Animal Experiments at Iwate Medical University.

Experimental methods:

1) Light-microscopic histological images

Two mice were euthanized with an intraperitoneal injection of pentobarbital sodium. The heads were removed and fixed in 4% formaldehyde, then demineralized using a Plank-Rychlo’s solution After dehydration with an ascending series of alcohols and penetration with 2-propanol, resin-embedded tissue samples were fabricated using glycol methacrylate (GMA) semimer and monomer. Using a microscope (Jung Autocut, Leica, Milton Keynes, UK), a series of 3-μm frontal plane sections were fabricated and stained with toluidine blue (pH 7.4). Then, both the left and right sides of the head were observed using an optical microscope (E800, Nikon, Tokyo, Japan) with a chilled 3CCD camera (Hamamatsu Photonics, Hamamatsu, Japan).

2) Vascular reconstruction

To observe vascular reconstruction in the infraorbital nerve, four mice were euthanized with an intraperitoneal injection of pentobarbital sodium. Indian ink was then injected into the heart of the mouse to be used to fabricate the models, and all of the heads were removed and fixed in 4% formaldehyde. After decalcification in Plank-Rychlo’s solution and dehydration with an ascending alcohol series, the samples were embedded in paraﬃn. Eight paraﬃn-embedded samples were fabricated by cutting the right and left sides of the heads into a series of 10-μm frontal plane sections using a microtome (Sledge Microtome VS-400®, Sakura, Tokyo, Japan). Two of these samples were used to create 331 three-dimensional images of reconstructed blood vessels to a length of 3310 μm. In addition, a series of 3-μm frontal sections were prepared from resin-embedded samples from the mouse that had been injected with Indian ink for observation of nerve fascicles near the vibrissae.

The two-dimensional images of a series of sections were input to a computer (G4-450, Macintosh, St Louis, MO) from a light microscope (E1000M®, Nikon) with a chilled 3CCD camera (C5810®, Hamamatsu Photonics). Using Photoshop® CS3 Extended ver.10.0 (Adobe, San Jose, USA), we selected blood vessels in nerve fascicles with branches that were clearly observable on the Windows Vista monitor. The axis was then precisely aligned by extracting blood vessels and nerve fascicles from the two-dimensional images, and the blood vessels and nerve fascicles were color-coded for image processing including threshold processing (dichotomizing). Three-dimensional images of blood vessels in the infraorbital nerve were then reconstructed using 3D visualization software (ZedView®DB, Ver.5.0, Atsumi Ishizuka et al.: Architecture of blood vessels in nerve
These images were used to create rotating three-dimensional images to examine the architecture of the blood vessels in the infraorbital nerve from all aspects.

**Results**

*Histological observation of resin embedded sections (overview Fig. 1):* The resin embedded sections are sufficiently thin (3μm) to allow for histological observation from low to high magnification. In the present study, we examined both the inside and outside of nerve fascicles stained with toluidine blue.

Microscopic observation of cross-sections of the infraorbital nerve as it emerged from the infraorbital foramen in normal mice revealed a bundle of approximately 20 nerve fascicles 2-50 μm in diameter. The nerve fiber fascicles were surrounded by the perineurium, and the epineurium surrounded the bundle of fascicles, forming the infraorbital nerve. In the infraorbital canal, we observed approximately 10 perineurium fascicles with a diameter of 400 μm covered by the epineurium. More than 20 nerve fiber fascicles 400 μm in diameter were present in the infraorbital canal near the infraorbital foramen (Fig. 2). From the infraorbital foramen, the nerve bundles extended peripherally along the piri-form aperture (Fig. 3 and 4).

Blood vessels of varying diameter (10-50 μm) were observed in the epineurium. The perineurium contained mainly blood vessels of about 10 μm diameter, but a small number of vessels about 30 μm in diameter were also found.

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**Fig. 1.** (a) Mouse resin-embedded section, near vibrissa. Tongue, T; Bone, B; Nerve, N; Muscle, M; Vibrissa, Vi; Incisor, I; Molar1, M1. (b) Near the infraorbital foramen. infraorbital nerve (red dotted line). (c) In the infraorbital canal.
In the nerve fiber fascicles, one capillary about 2-10 μm in diameter was normally contained within the endoneurium (Fig. 5). Peripheral mouse infraorbital nerves branch into the deep vibrissal nerves, whose nerve fibers enter the follicle of the vibrissa. In our observation the nerve fascicles were approximately 60 μm in diameter. The blood vessels distributed peripherally in the nerve fascicles merged into the neighboring blood vessels without entering the follicle of the vibrissa (Fig. 6).

Three-dimensional reconstruction of the blood vessels in the nerve fascicles of the infraorbital nerve: Observation of blood vessels in the paraffin sections was facilitated by the thickness of the sections (10 μm) and the injection of Indian ink (Fig. 7). These samples were used for observation at low magnification or for three-dimensional image reconstruction, but they were not appropriate for observation at high magnification due to their relative thickness.

In the present study, two three-dimensional reconstruction images were developed: one of multiple nerve fascicles (Fig. 8, 3310 μm) and one of a branched nerve fascicle (Fig. 9, 3310 μm). The location of the images was the halfway point between the infraorbital foramen and vibrissa with numerous collections and branches of nerve fascicles. In the images with multiple nerve fascicles, blood vessels of various diameters were observed all around the nerve fascicles, communicating with each other and forming networks. A proximal thick nerve fascicle including several blood vessels branched into thinner nerve fascicles as it proceeded peripherally (Fig. 8). The blood vessels running in a thin nerve fascicle became almost one vessel along a branch of the nerve and the blood vessels in the nerve fascicle branched.
Fig. 5. Resin-embedded section Vessel in perineurium. Epineurium, Ep; Perineurium, Pe. Toluidin blue, scale bar: 50 µm.

Fig. 6. Resin-embedded section. A blood vessel can be seen entering the vibrissa with the nerve fiber fascicle. Toluidin blue, scale bar: 100 µm.
into thin lateral vessels, penetrating the perineurium. These thin vessels communicated with surrounding capillaries or arterioles and venules and formed vascular plexuses (Figs. 8 and 9).

As shown in figure 9, there are three thin blood vessels which connect the vessels in the nerve with the vessels in the perineurium. However, we were unable to speculate about the direction of blood flow since the blood vessel in the nerve fascicle was continuously distributed longitudinally, and differentiation between very thin arteries and veins
was difficult. In the stem of the nerve fascicle, there was frequently only one blood vessel, but occasional chain type branching was found. In the nerve fascicle on the proximal side, several branches of intraneural blood vessels were observed; after extending for approximately 10-30 μm, the nerve was observed to branch, and one blood vessel entered each nerve fascicle (Fig. 9).

We created a three-dimensional reconstruction of the area where the infraorbital nerve enters a vibrissa using 71 3-μm resin-embedded sections (total 213 μm) with India ink injection. The infraorbital nerve extended to the tip of nose following the nasal bone after emerging from the infraorbital foramen. In this way, the nerve went towards the skin and changed its direction about 90° to head toward a vibrissa at a diameter of approximately 60 μm. In the follicle of the vibrissa, the tip of the nerve branched like a bamboo rake and spread thinly. The only blood vessel present in the nerve fascicle exited the fascicle approximately 400 μm before it entered a vibrissa, so no blood vessels were present in the nerve fascicle after that point (Fig. 10). The follicle of the vibrissa was filled with blood and a blood vessel approximately 30 μm in diameter entered the follicle along with the nerve. The blood vessels in the follicle of the vibrissa do not connect to the blood vessels in the nerve. The blood vessels in the nerve connect to the blood vessels surrounding the nerve near the follicle of the vibrissa.

**Discussion**

According to previous investigations of the circulation around the spinal nerve, blood is supplied by segmental arteries such as the vertebral artery, the intercostal artery and the lumbar artery, forming a rete venosum in the veins. One of the characteristics of blood vessels in the spinal nerve is that the arteries are distributed more sparsely in the white
matter than the gray matter\cite{28, 29}. Lundborg observed blood vessel morphology in peripheral nerves by examining sectioned human median and ulnar nerves. These nerves are mixed nerves (including both motor and sensory nerves); none of the studies have investigated the morphology of blood vessels in pure sensory nerves or peripheral nerves close to receptors. In the present study, we examined the morphology of blood vessels in the infraorbital nerve, a branch of the maxillary nerve, itself a branch of the trigeminal nerve.

Rauber/Kopsch shows the appearance of two or more blood vessels in the ischiadic nerve fascicle. But, this research is an observation of the pure sensory peripheral nerve, and the kind and the thickness of the nerve are different.\cite{43}

In the microcirculation of the human median nerve, Lundborg found that the blood vessels of the epineurium form dense anastomoses with the vascular plexus of the perineurium consisting of blood vessels existing in between all layers of the perineurium. These blood vessels in the perineurium are distributed longitudinally in general, and run long distances throughout the perineurium, entering the endoneurium obliquely through the deep perineurium. Occasionally, capillary systems in nerve fiber fascicles are connected in chains showing a V-loop structure. The study reported that these structures indicate anastomoses between capillaries neighboring each other in the endoneurium\cite{11}.

However, according to Nakao et al.\cite{19} and Shibata\cite{17}, the capillaries distributed inside and outside the nerve fascicle of the normal Oryctolagus cuniculus sciatic nerve exhibit a different structure. They observed that the extraneural vascular system consists of fine networks surrounding the nerve fascicle, whereas the intraneural vascular system is distributed longitudinally in spirals along the direction of the nerve fibers.

In the present study, we observed the microcirculation structure in the mouse infraorbital nerve immediately after emerging from the infraorbital foramen, where it forms networks of anastomoses between blood vessels in the nerve fascicle and around the perineurium and epineurium. We also found that in the fiber fascicle surrounded by the perineurium, one blood vessel was usually distributed longitudinally in the endoneurium, branching in accordance with the nerve branches. In addition, one blood vessel generally ran

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Fig. 10. Three dimensional reconstruction of the nerve entering the vibrissa. The blood vessel in the nerve fascicle exits before entering a vibrissa.
longitudinally along the branched nerve. The existence of chainlike capillaries in the nerve fascicle was also observed (Fig. 9). Relative to the human median nerve and the Oryctolagus cuniculus sciatic nerve, the mouse infraorbital nerve emerged from the infraorbital foramen with a smaller number of thinner diameter nerve fiber fascicles that were closer to receptors. We suggest that these differences may be attributed to differences in the morphology of blood vessels between the species, even though the studies all relate to blood vessels in peripheral nerves.

It has been shown that the cut end of nerves or peripheral nerves from affected areas produce not only nerve growth factor (NGF) from Schwann cells, but also substances that may be involved with the control of axonal growth. In addition, some studies have suggested that macrophages may secrete substances promoting axonal regeneration (FGF, PDGF and interleukin-1 etc.). There are factors that affect nerve growth, including cell adhesion substances, trophic and growth promoting factors, and a neurotrophic factor. NGF functions as both a growth and neurotropic factor. As described above, Schwann cells and macrophages in the peripheral nerves from affected areas cooperate in developing the optimal microenvironment for axonal regeneration and growth. A regeneration blastema is considered to regenerate from Ranvier's node in the proximal side of affected areas, and regenerated axons extend closely along the surface layer of the basal membrane tube in Schwann cells. Previous reports have shown that the substances needed for axonal regeneration are considered to be transported by slow anterograde axonal transport for the function of growth and maintenance of axons. However, these studies did not describe the presence of blood vessels in nerve fascicles; the relationship between axonal regeneration and substances transferred by capillaries in nerves has not been revealed.

In the present study, we examined only pure sensory nerves. Previous studies have reported that sprouting occurs at the cut end of nerves in sensory nerve fibers, but this phenomenon does not occur in motor nerve fibers. However, previously no differences were found between sensory and motor nerves in the growth of regenerating axons. In effector organs, the cut end of nerves with Wallerian degeneration undergo sprouting and nerve growth utilizing the inside of Schwann cells' basement membrane tube as a scaffold for adhesion. In contrast, in motor nerve terminals, growth starts using the skeletal muscle basement membrane as a scaffold, but according to one study, the number of fibers sprouting from nerve fibers was low, suggesting that this scaffold is not suitable for adhesion relative to Schwann cells.

The present study revealed that one blood vessel enters each nerve fascicle emerging and branching from the infraorbital foramen. Blood vessels never enter nerves after neural regeneration. Extension of blood vessels always occurs earlier than in nerves or simultaneously, sprouting and growing from the cut end of nerves. Blood vessels play a major role in transporting nutrients and essential substances for organs and tissues and removing waste products. Therefore, it is reasonable to assume that blood vessels exist abundantly around nerves, but vascular construction around nerves and nerve fascicles has not been extensively examined. Previous studies have suggested that administration of vasodilatory drugs such as PG and ATP groups and stellate ganglion blocks (SGB) promote restoration and regeneration of nerve tissues. This fact indicates the existence of abundant blood supply around nerves, and a close involvement with neuronal regeneration. During neural grafting, it is thought that the existence of both preexisting blood vessels in grafts and the surrounding blood vessels in the host tissues promotes early blood flow resumption and extension of new blood vessels, contributing to the recovery of neural function. The present study demonstrated the significance of the presence of one blood vessel in a nerve fascicle as well as blood vessels surrounding the fascicle. Blood flow in the surrounding vascular systems is important for axonal regeneration and extension by slow anterograde axonal transport and supply of essential substances and energy. A factor for newly formed vessels called Ang 2 interacts with an endothelial cell receptor, Tie 2, and acts as a guide to avoid contacts between endothelial cells and the external cellular matrix. As a result, vascular regeneration stops due to termination of endothelial cell growth or endothelial cell death. Ang 2 is receiving attention as a target of cancer treatment due to their role in new blood vessel formation in tumors. By using factors that delay vascular regeneration or methods that partially block blood flow, neuronal regeneration will be delayed and the effects of a block will persist for a long duration. These results report about capillaries around nerves; therefore, it is necessary to examine whether the same type of results can be obtained in blood vessels existing in nerve fascicles. We also need to examine whether the BNB exists or not.

Trigeminal neuralgia is characterized by sudden and intense pain in the face and commonly affects middle-aged women. Although several treatment options including medications are available, a nerve block is often performed in difficult cases. Three months after a nerve block, regeneration and growth of myelinated nerves with nearly normal morphology can be observed, although different timing and images of neural destruction are evident when comparing cases involving the application of a high concentration of local anesthetic with the application of absolute alcohol. Since clinically many cases require a second nerve block three to six months after treatment, an extension of the nerve block's effects will be beneficial for patients in terms of reduction of stress and risk after treatment.

We are planning to observe differences in the regeneration of the blood vessels distributed in and around nerve fascicles over time in further projects.

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