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The Morphological Changes in the Capillary Architecture of the Tibial Nerve Associated with Spontaneous Aging and Aerobic Exercise Intervention during Aging in Rats

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Abstract. [Purpose] Peripheral nerve degradation associated with aging is linked to failure of interactions in capillary metabolism. The aim of this study was to morphologically investigate the age-related changes in the capillary architecture of the tibial nerve in spontaneous aging and with aerobic exercise intervention in rats. [Subjects] Male Sprague-Dawley rats (n=15) were used in the present study. [Methods] The rats were divided into control (Cont, n=5), elderly (Elder, n=5), and elderly with aerobic exercise (Elder+Ex, n=5) groups. Aerobic training of low intensity was performed for 10 weeks using a treadmill starting at 96 weeks of age by the Elder+Ex group. The capillary diameter, cross-sectional area and number of microvascular ramifications in the tibial nerve were compared among the Cont (20-week-old), Elder (106-week-old) and Elder+Ex groups using three-dimensional images gained from confocal laser scanning microscopy. [Results] The capillary diameter, cross-sectional area and number of microvascular ramifications in the Elder group were significantly smaller than those observed in the Cont and Elder+Ex groups. [Conclusion] These findings suggest that the capillaries in the peripheral nerve degrade with spontaneous aging and that aerobic exercise of low intensity promotes angiogenesis, and protects the capillary from oxidative stress.

Key words: Capillary, Tibial nerve, Three-dimensional image

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INTRODUCTION

Dysfunction of the peripheral nerves in the lower extremities induces a decline in the ability to balance in the elderly[1, 2)]. Previous studies have shown that functional deficits in the peripheral nerves are related to the occurrence of falls by the elderly[3, 4)]. Morphologic and morphometric studies have clearly demonstrated that the number and density of both myelinated and unmyelinated fibers in peripheral nerves are progressively reduce with age in mice[5)]. An electrophysiological study showed that the nerve conduction velocity of the peripheral neurons in the lower extremities and the action potentials of the soleus in the elderly are significantly decreased in comparison with those observed during youth[6)]. These investigations demonstrate that the peripheral nerves gradually degenerate with age. In cerebral microvascular pathology, microvascular abnormalities complicate or precede neurodegeneration[7, 8)]. The energy metabolism of peripheral neurons is primarily dependent on intracytoplasmic (axon and myelin) glycolysis and intramitochondrial oxidative phosphorylation processes. In particular, most ATP and creatine phosphate molecules are formed by these processes. The supply of oxygen and glucose required for the endoneurial oxidative glycolysis process decreases due to a progressive reduction in the peripheral nerve blood flow and an increase in nerve vascular resistance in elderly rats[9)]. This finding suggests that a decrease in the nerve blood flow is generated by a reduction in microvascular diameter.

Angiogenesis is promoted by VEGF (Vascular Endothelial Growth Factor). VEGFs are growth factors of endothelial cells that are secreted for capillary sprouting and arterial differentiation[10, 11)]. VEGFs are elicited depending under hypoxic conditions by hypoxia inducible factor-1 (HIF-1), a transcription factor[12)]. Capillaries are induced to grow in the direction of apoptosis when VEGF expression is downregulated[13)]. Regarding cerebral microvessels, VEGF expression decreases following a decrease in the reactivity of HIF-1 to hypoxia with age[14, 15)]. In the cerebral cortex of elderly rats, HIF-1 is stored in the neuronal cytoplasm, and this accumulation can lead to the inhibition of HIF-1 transcriptional activity[16, 17)]. Moreover, a decline in the

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level of HIF-1 transcriptional activity with age is related to neuronal death via VEGF downregulation. The failure of angiogenesis induced by hypoxia due to endogenous oxidative stress may be caused by a reduction in the number of interactions between the nerve and capillary. A previous study found that long-term aerobic exercise decreases lipid peroxidation and Schwann cell apoptosis in aged rats[18]. The authors concluded that aerobic exercise training protects peripheral nerves by attenuating oxidative reactions and protecting Schwann cells and myelin sheaths from the pathologic changes that occur during normal aging. However, it remains morphologically unknown whether degeneration of the capillary occurs with spontaneous aging or whether long-term aerobic exercise retards the capillary degeneration associated with aging.

Our objective was to morphologically investigate the influence of changes in the capillary architecture associated with spontaneous aging and long-term aerobic exercise training during aging in the tibial nerves of rats.

MATERIALS AND METHODS

Animals

The experiments were approved by the Animal Care and Use Committee of Himeji Dokkyo University and were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (National Research Council, 1996). Male Sprague-Dawley (SD) rats (10 weeks old, n=15) were used in the present study. The rats were randomly divided into control (Cont, n=5), elderly (Elder, n=5) and elderly with aerobic exercise (Elder+Ex, n=5) groups at 20 weeks of age. Each rat in the Cont, Elder (spontaneous aging) and Elder+Ex groups were housed for 10 (Cont group) or 96 (Elder and Elder+Ex groups) weeks in a room with light and dark changes every 12 hours controlled at a temperature of 22 ± 2 °C with 40–60% humidity. Each rat was housed in a cage of the same size and provided ad libitum food and water. The rats in the Elder+Ex group ran on a treadmill starting at 96 weeks of age for ten weeks at the following intensity and frequency: 15 m/min, 0° gradient of running surface, 0.5 hour once a week. The blood lactate levels of the Elder+Ex group were measured in blood samples extracted from the tail vein before and after exercise using a blood lactate test meter (Lactate Pro2 LT-1730; Arkray, Shigma, Japan). As there were no differences in the blood lactate concentrations (<2 mmol/L) between the pre- and postexercise measurements, the training was assumed to fall within the category of aerobic exercise.

Methods

The body weights of the rats under all three conditions were measured at 20 and 106 weeks of age. At 20 weeks of age in the Cont group and 106 weeks of age in both the Elder and Elder+Ex groups, each rat was anaesthetized with pentobarbital sodium (50 mg/kg; i.p.). Then, the abdominal cavity was opened and both the left common iliac artery and vein were ligated, followed by insertion of a catheter to maintain perfusion of the abdominal aorta with contrast medium to the right hind limb and extraction of the testicular fat. A series of nerves from the sciatic to the distal end of the tibia in the right hind limb were perfused for three minutes to wash out the intravascular blood with a 0.9% physiological saline solution containing 10,000 IU/L of heparin at 37 °C, and 10% glucose. Then, contrast medium, consisting of 1% fluorescent material (PUSR80; Mitsubishi Pencil, Tokyo, Japan), 8% gelatin (Nakalai Tesque, Kyoto, Japan) and distilled water, was administered into the circulation of a series of nerves from the sciatic to the distal end of the tibia. The whole body of each rat was immediately immersed in cold saline for 10 minutes following perfusion with contrast medium that completely filled the entire microvasculature under a physiologically similar perfusion pressure[19]. A series of nerves from the sciatic to the distal end of the tibia in the right hind limb were excised and frozen in isopentane precooled in liquid nitrogen. The nerves were stored at −80 °C until the capillary in the tibial nerve was observed using a confocal laser microscope that could scan the capillary to determine the three-dimensional (3-D) architecture[20].

To observe the 3-D architecture in the capillary, confocal laser scanning microscopy (CLSM) (TCS-SP; Leica Instruments, Mannheim, Germany) was used in fluorescence mode with an argon laser (488 nm)[19, 21]. The nerve sample block was cut at a distance 5 mm from the distal end of the nerve in a cryostat microtome (CM3050; Leica Microsystems, Mannheim, Germany) at −20 °C, then the section was fixed with 4% paraformaldehyde (pH 7.4) and thawed to room temperature for five minutes. Images of the CLSM were obtained using a ×20 objective lens. Each nerve section that was perfused with contrast medium was scanned and visualized to a depth of 50 μm at 1 μm/slice, and the CLSM images were drawn as 3-D images with a depth of 50 μm. Using the 3-D images created by the CLSM, the capillary diameter and number of microvascular ramifications per nerve section in 40–60 capillaries were measured on several images measuring 800 × 800 μm^2 in area utilizing the ImageJ software program. Next, each capillary cross-sectional area was calculated according to $\pi \times radius^2$ (the square of half of the capillary diameter measured on the 3-D CLSM image).

The Kruskal-Wallis test was performed under three groups to compare the body mass, and adipose weight (the testicular fat weight) of the three groups. One-way analysis of variance (ANOVA) was performed to compare the capillary diameter, cross-sectional area and number of microvascular ramifications of the three groups. The Scheffé post hoc test was employed when the results of the Kruskal-Wallis test and ANOVA were significant. Differences with p-values of less than 0.05 were considered to be statistically significant.

RESULTS

The Kruskal-Wallis test revealed significant differences in body mass among the three groups ($p < 0.01$) (Table 1). The post hoc test showed the mean values of the body masses of the Elder and Elder+Ex groups were significantly

Table 1. The capillary diameter, capillary cross-sectional area and number of microvascular ramifications in the tibial nerve

	Cont (20 weeks old)	Elder (106 weeks old)	Elder+Ex (106 weeks old)
Body mass (g)	604.0 ±56.0*	888.0±87.5	774.5±177.0$^{\#\S}$
Adipose weight (mg)	4938.3±1456.3*	9998.2±2352.5	7072.7±2650.8*
Capillary diameter (μm)	7.63±0.10*	4.98±0.11	7.85±0.11*
Capillary cross-sectional area ($10^{-3}\times mm^2$)	52.12±1.33*	22.14±0.85	51.40±1.34*
The number of microvascular ramifications	44.42±2.08*	31.00±3.78	46.08±3.05*

The Kruskal-Wallis test showed that the body mass ($p < 0.01$) and adipose weight ($p < 0.05$) were different among the three groups. ANOVA showed that the capillary diameter, cross-sectional area and number of microvascular ramifications were different among the three groups ($p<0.0001$). The values of the body mass and adipose weight are presented as median ± quartile deviation. The values of the capillary diameter, cross-sectional area and number of microvascular ramifications are presented as the mean ± SEM. *: significant difference between the Cont and Elder groups and between the Elder and Elder+Ex. groups using the post hoc test ($p<0.05$); $^{\#}$: significant difference between the Cont and Elde+Ex. groups using the post hoc test ($p< 0.05$); §: difference between the Elder and Elder+Ex groups ($p< 0.1$).

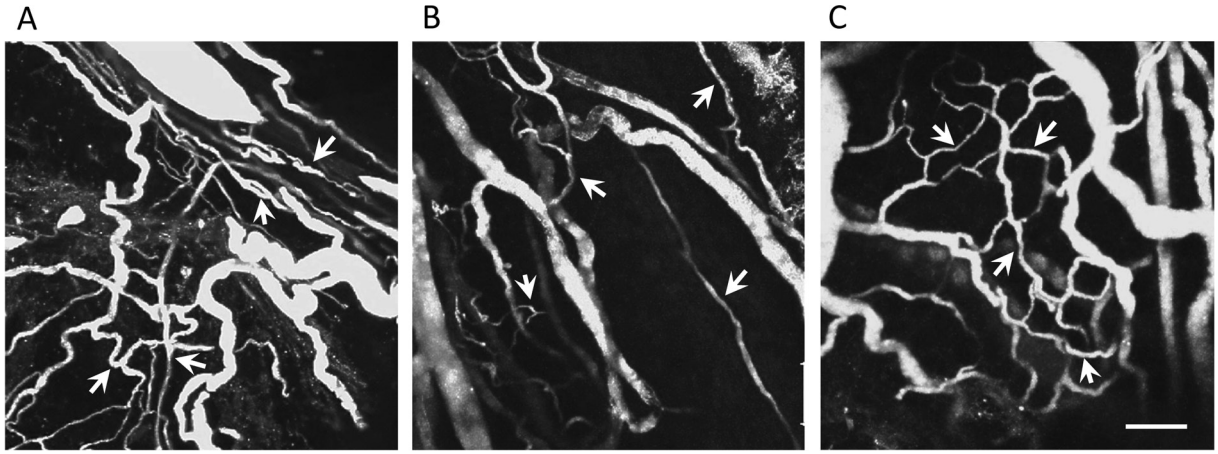

Fig. 1. Confocal laser scanning microscopic images of the capillaries in tibial nerve (A: Control; B: Elderly ; C: Elderly with aerobic exercise). In each image, the tibial nerve run obliquely from the upper left to the lower right. The capillaries in Cont (image A) and Elder+Ex (image C) seemed to be more compact than Elderly (image B). The arrows indicate the capillaries. The scale of a bar is 100 μm.

greater than that of the Cont group ($p< 0.05$), and the body mass value of the Elder group tended to be greater than that of the Elder+Ex group ($p< 0.1$). The Kruskal-Wallis test also revealed significant differences in adipose weight among the three groups ($p < 0.05$). The adipose weight of the Elder group was significantly greater than those of the Cont group ($p< 0.05$) and the Elder+Ex group ($p < 0.05$).

The mean values of the capillary diameter, capillary cross-sectional area and number of microvascular ramifications of the three groups were significantly different ($p< 0.0001$) according to ANOVA (Table 1). Subsequently, the post hoc test showed that the values of the Elder group were significantly smaller than those of the Cont ($p< 0.05$) and Elder+Ex groups ($p< 0.05$).

The capillaries observed in the three groups were presented as 3-D images constructed using CLSM (Fig. 1). The capillaries in the tibial nerves of the rats in the Cont and Elder+Ex groups were observed to be concentrated in close proximity to one another and ramifications (or anastomoses) were frequently observed. Conversely, the 3-D images of the Elder group rats showed spacious fields due to capillary loss and degeneration.

DISCUSSION

In the present study, we morphologically investigated age-related changes in capillaries and the preventive effects of aerobic training on capillary degeneration in the tibial nerves of rats. No previous studies have analyzed the capillaries of the tibial nerve using 3-D images captured with the CLSM. We demonstrated that the capillaries of the tibial nerve degenerate with spontaneous aging, and that aerobic exercise of low intensity prevented capillary degeneration and prompted angiogenesis.

The mean body mass value was highest in the Elder group, and lowest in the Cont group. Similarly, the mean adipose weight of the Elder group was greater than that of the Cont group, while the value of the Elder+Ex group was lower than that of the Elder group. These findings indicate that there is a relationship between age-related increases in body mass and the levels of lipids and that aerobic training decreases the levels of lipids[18].

Furthermore, the capillary diameter, cross-sectional area and number of microvascular ramifications of the Elder group were smaller than those of the Cont group. These

declines indicate degradation and apoptosis of the capillaries in the tibial nerve with age. Recent investigations have demonstrated that the onset of cell death is associated with elevated intraneuronal levels of reactive oxygen species (ROS)[22–24] and free radicals[24], and that the reactivity of HIF-1 is a transcriptional factor of angiogenesis that decreases with age[16, 17]. HIF-1 induces VEGF expression, which promotes angiogenesis. Therefore, a decline in VEGF expression may have led to the decreases seen in capillary diameter, cross-sectional area and number of microvascular ramifications in the Elder group.

In contrast, the capillary diameter, cross-sectional area and number of microvascular ramifications by the Elder+Ex group were greater than those of the Elder group. Aerobic exercise was performed by the Elder+Ex rats from 96 to 106 weeks of age. Degradation of the capillaries in the tibial nerve was expected at 96 weeks of age, similar to that observed in the Elder group, but aerobic exercise restored capillary sprouting. It has been shown that aging is associated with an increased level of nerve malondialdehyde (a marker of lipid peroxidation) and a higher degree of Schwann cell apoptosis in sedentary rats[18]. However, an exercise training group exhibited diminished nerve lipid peroxidation and Schwann cell apoptosis. Lipid peroxidation of both Schwann cells and capillaries can produce free radicals, and nerve tissue and endothelial cells can be injured by free radicals[25, 26]. In the skeletal muscle of rats, it has been shown that aerobic training increases the levels of succinate dehydrogenase (SDH) activity, an indicator of the mitochondrial oxidative enzyme activity, which leads to capillary sprouting and prevents microangiopathy[20, 27]. The mitochondrial oxidative enzyme activity in capillaries and neurons also promotes lipid metabolism and decreases lipid peroxidation. There are likely associations between the mitochondrial oxidative enzyme activity, a decrease in free radicals and improvements in the responsiveness of HIF-1 to hypoxia. Therefore, it seems likely that VEGF expression is upregulated and capillary sprouting is reactivated during aerobic exercise. In humans, aerobic exercise lasting approximately 30 minutes, in which the lactate concentration in the blood remains below 2 mmol/L, is equivalent to moderate running[28] by healthy adults and walking by the elderly[29, 30]. Therefore, remodeling of peripheral nerve capillaries of elderly patients can be promoted at the intensity and frequency of exercise performed by the SD rats in the present study.

The present CLMS study morphologically demonstrated that spontaneous aging induces capillary degradation in the tibial nerve, and that aerobic exercise promotes angiogenesis. In the present experiment, it was not shown whether ROS and free radicals increase, or HIF-1 and VEGF expressions decline with spontaneous aging, or whether aerobic exercise restores these factors. Therefore, future research should histochemically and biochemically investigate the influence of the expressions of these factors on peripheral nerves with aging.

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