The skeletal muscle vascular supply closely correlates with the muscle fiber surface area in the rat*

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Summary. The skeletal muscle capillary supply (capillarity) dynamically changes in response to muscle conditions such as growth, atrophy, and hypertrophy. The capillary number-to-fiber ratio is reported to correlate closely with the muscle fiber cross sectional area. However, little information is available regarding the capillarity of neonatal and very young skeletal muscles. In this study, the vascular endothelium was reliably stained with an anti-PECAM-1 antibody, and relationships between the capillarity and muscle fiber parameters were analyzed. For assessment of the capillarity, we used the capillary length-to-fiber ratio, due to the presence of transversely running vessels. In young and adult rats, the capillary length-to-fiber ratio was proportional to both the muscle fiber cross sectional area and muscle fiber radius. However, when these data were analyzed together with data from neonatal and very young rats, the capillary length-to-fiber ratio correlated more closely with the muscle fiber radius than the muscle fiber cross sectional area in the tibialis anterior muscle. The capillary number-to-fiber ratio demonstrated results very similar to the capillary length-to-fiber ratio.

During muscle atrophy after denervation, the number of capillaries was decreased in a non-apoptotic manner as revealed by electron microscopy, maintaining the close relationship between the parameters described above. In conclusion, capillarity was closely correlated with the muscle fiber radius (which represents the perimeter) during growth and atrophy. This indicates that the capillarity is linked to the muscle fiber surface area (which is determined by perimeter and section thickness), in agreement with the essential role of the cell membrane in the transport of materials by simple diffusion or active transport.

Introduction

Skeletal muscle is mainly involved in action and movement, which requires large amounts of glucose, fatty acids, and oxygen. These materials are supplied by blood vessels and incorporated into the muscle fiber through the cell membrane. Conversely, metabolic wastes are discarded outside the cell membrane and removed by blood vessels. It is well known that the capillarity of skeletal muscle adapts to various physiological and pathological conditions such as normal development, ageing, atrophy, and hypertrophy. To evaluate changes in the capillarity and elucidate the remodeling mechanism of the vascular network, numerous morphometric parameters have been created and implemented such as the capillary number-to-fiber ratio (the number of capillaries / muscle fibers; Loats et al., 1977; Sillau and Banchero, 1977), the capillary density (the number of capillaries / muscle cross sectional area; Ripoll et al., 1979), the capillary perimeter / fiber perimeter (Mathieu-Costello et al., 1991, 1996; Kano et al., 2000), the capillary surface area / fiber surface area, the capillary length / adjacent fiber surface area (Kubínová et al., 2001), and the capillary-
to-fiber perimeter exchange index (Hepple, 1997). Still, the skeletal muscle capillarity of various animals and different muscles was difficult to compare due to heterogeneity of the parameters. Among these, the most widely used parameters are the capillary number-to-fiber ratio and the capillary density. It has been repeatedly reported that the capillary number-to-fiber ratio is proportional to the muscle fiber cross sectional area, and that this index is increased through normal development (Loats et al., 1977; Sillau and Banchero, 1978; Ripoll et al., 1979; Carpenter and Karpatic, 1982) and hypertrophy (Plyley et al., 1998) but decreases during atrophy due to denervation (Borsoi et al., 2000) or hind-limb suspension (Kano et al., 2000). On the other hand, capillary density has been reported to decrease proportionally to increases in the muscle fiber cross sectional area (Sillau and Banchero, 1977; Ripoll et al., 1979). Capillarity is also claimed to correlate to mitochondrial volume in muscle fibers (Mathieu-Costello et al., 1996; Hepple et al., 1998).

However, it is unclear what factors play the major roles in determining the number and distribution of blood vessels in the skeletal muscle and which parameter is most appropriate to represent capillarity. Furthermore, information is lacking concerning the capillarity of neonatal and very young muscles due to the technical reasons mentioned below.

To detect capillaries in skeletal muscle, gelatin injection (Krogh, 1919), ATPase staining (Sillau and Banchero, 1978; Ripoll et al., 1979; Hansen-Smith et al., 1992a), lectin histochemistry (Hansen-Smith et al., 1989, 1992a; Ahmed et al., 1997), alkaline phosphatase reaction (Hansen-Smith et al., 1989; 1992b), and direct measurement of blood vessels in semithin sections (Carpenter and Karpati, 1982; Hansen-Smith et al., 1989; Kano et al., 1997) have been conventionally used. However, the gelatin injection was incomplete (Krogh, 1919), ATPase staining for capillaries gave unreliable results (Hansen-Smith et al., 1992a), and the alkaline phosphatase reaction did not visualize all capillaries (Hansen-Smith et al., 1992b; Ushiki and Abe, 1998), especially in neonatal and very young animals (Hansen-Smith et al., 1989), due to low enzyme activity. Counting blood vessels in semithin sections is laborious and inappropriate for the examination of a large number of samples. Thus, the lack of a reliable and appropriate method for the identification of all blood vessels has hindered a precise assessment of capillarity in neonatal and very young animals. However, an antibody against PECAM-1 (platelet endothelial cell adhesion molecule, identical to CD31) is known to visualize blood vessels with excellent reliability and has been applied to the evaluation of capillarity (Brey et al., 2002; Charifi et al., 2003; Lyon et al., 2007). This antibody was therefore used in the present study to examine whether or not the capillary number-to-fiber ratio is proportional to muscle fiber cross sectional area in neonates and very young rats. However, transversely running blood vessels were often encountered in early postnatal muscles. Thus, it was necessary to use the capillary length-to-fiber ratio as well as the capillary number-to-fiber ratio. We attempted to clarify which factor is most closely correlated with capillarity, reasoning that when the most correlated parameters are found and employed, the skeletal muscle capillarity can be easily and reliably compared among various animals and different conditions. We also examined the process of capillary degeneration using a model of muscle atrophy after denervation.

**Materials and Methods**

Rats were reared at 22–25°C with a constant humidity of 55 ± 5% and a 12 h light-dark cycle (light 8:00–20:00). Food and water were supplied *ad libitum*. The present experimental procedures were approved by the Committee of Research Facilities for Laboratory Animal Science, Natural Science Center for Basic Research and Development, Hiroshima University.

**Normal muscle development**

Wistar rats aged 0, 3 days, 1, 2, 3, 4, 8 weeks (3 legs removed from 3 animals in each group), and 24 weeks (4 legs removed from 2 animals), and weighing 4 to 458 g were used. Newborn to 4-week-old rats were both males and females since identification of sex is difficult in neonates, while rats aged 8 weeks or older were males. With respect to normal development, rats were sacrificed by an overinhalation of diethyl ether, and the tibialis anterior muscles were removed due to their easy identification and large size. The soleus muscles of 8-week-old animals were also used for comparison; however, the soleus muscles of neonatal animals were difficult to prepare and too small to measure.

**Denervated group**

Six 8-week-old male rats were used. The animals were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg, body weight) assisted by inhalation of diethyl ether when necessary. The skin over the lateral left thigh was incised and the sciatic nerve was exposed. The sciatic nerve was isolated from the surrounding connective tissue and cut at the mid-thigh.
The proximal cut end of the sciatic nerve was reflected proximally and sutured to the nearby fascia, and the skin was closed by suturing. This procedure resulted in a permanent denervation of the leg muscles. The right hindlimb was untreated and used as a control. At 3 and 6 weeks after surgery, rats were sacrificed by an overinhalation of diethyl ether and the tibialis anterior and plantaris muscles (3 legs from 3 animals in each group) were removed.

**Hypertrophy group**

Eight-week-old male rats were used. Under pentobarbital anesthesia, the left Achilles tendon was cut, and the gastrocnemius and soleus muscles were reflected upward together to the extent that about 2 cm of the distal portion of the plantaris muscle was exposed. The reflected Achilles tendon was then sutured to the nearby fascia, with the right hindlimb left untreated and used as a control. Two weeks after tenotomy, the rats were sacrificed and the plantaris muscles were removed and weighed using an electric balance. The plantaris muscles in some rats were not sufficiently hypertrophied in terms of muscle wet weight when compared with the control sides. Thus, those 3 specimens hypertrophied more than 20% compared with their control side were selected for further examination.

**Preparation of specimens for light microscopy**

Sample muscles were removed and cut at their mid-belly portion transversely to their long axis and supported by 6% aqueous tragacanth gum jelly on cork disks (about 20 mm in diameter and several mm thick). Muscles and cork disks were then immersed and frozen together in isopentane cooled with liquid nitrogen. Cryosections (10 μm in thickness) were cut in a cryostat and used for the detection of blood vessels by anti-PECAM-1 immunohistochemistry. Attention was given to cutting the specimens as perpendicularly as possible to the long axis of the muscle.

**Immunostaining**

Sections were air-dried, fixed in acetone for 10 min, and rehydrated in 0.01 M phosphate-buffered saline (PBS; pH 7.4) for 5 min. Endogenous peroxidase was inactivated by incubation of the sections in methanol containing 0.3% H2O2 for 20 min. After 2 rinses with PBS for 5 min each, nonspecific binding sites were blocked by treating sections with PBS containing 1% normal horse serum for 30 min. After blotting away the blocking solution, sections were incubated with an anti-PECAM-1 monoclonal antibody (clone TLD 3A12, Becton Dickinson Bioscience, San Jose, CA, USA; 1: 250 dilution) for 2 h at room temperature. Sections were rinsed with PBS 3 times for 5 min each and incubated with the secondary antibody (horse biotinylated anti-mouse IgG, 1: 250 dilution; BA-2001, Vector Laboratories, Burlingame, CA, USA) for 1 h. Then the sections were rinsed with PBS twice for 5 min each and incubated with a streptavidin-biotin complex (1: 50 dilution; Elite ABC, Vector Laboratories) for 30 min. After rinsing, immunoreactivity was visualized with 0.05% 3, 3'-diaminobenzidine tetrahydrochloride in a 0.05 M Tris-HCl buffer (pH 7.2) and 0.01% hydrogen peroxide, followed by staining with eosin. Sections were then dehydrated, cleared with xylene, and mounted. Negative controls incubated with PBS instead of the primary antibody were all negative for PECAM-1 staining.

**Electron microscopy**

For electron microscopy, some specimens from denervated plantaris muscles were dissected in 3% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) and immersion fixed in the same fixative for 2 h at 4°C. Then specimens were postfixed in a 1% OsO4 in a 0.1 M phosphate buffer (pH 7.4) for 4 h at 4°C, rinsed with 10% saccharose 3 times for 10 min each, and stained en bloc with 3% uranyl acetate for 1 h at room temperature. Specimens were dehydrated with an ascending series of ethanol and embedded in epoxy resin. Ultrathin sections (0.1 μm in thickness) were cut, stained with uranyl acetate and lead citrate, and observed with a JEM-1200EX electron microscope (JEOL, Tokyo).

**Counting of the number of capillaries**

Each central area of transverse sections cut from the midbelly portion of the muscles was used for measurements, since the neonatal rats specimens were small. This protocol was also helpful for avoiding regional differences within each specimen. One central field of each section of the tibialis anterior muscle was photographed using a DP70 digital camera (Olympus, Tokyo) at a magnification of 20× in 0- and 3-days-old rats and at a magnification of 10× for rats older than 1 week. Each field was photographed twice: first with a light blue filter (daylight filter) to reveal muscle fibers, and second with an orange filter to reveal blood vessels for the easier recognition of muscle fibers and blood vessels, respectively. Each field contained more than 190 muscle fibers. Digital images were displayed on a computer, and the number of muscle fibers were manually counted using Image J 1.32j software (National Institutes of Health, Bethesda, MD, USA).
USA). Muscle fibers showing their entire cell boundary within the microscopic field were counted as 1, and fibers for which the entire cell boundary was not included in the field were counted as 0.5. Similarly, blood vessels showing their entire cross section within the microscopic field were counted as 1, and vessels not showing their entire cross section were counted as 0.5 (Plyley et al., 1998; Charif et al., 2003).

The capillary number-to-fiber ratio is a parameter often and widely used to represent capillarity; however, in the present study, a considerable number of capillaries ran transversely to the long axis of the skeletal muscle, particularly in neonatal and very young rats. Sullivan and Pittman (1987) mentioned that capillary-fiber contact length is a better indicator of capillarity than the number of capillaries around a fiber. We therefore mainly used the capillary length-to-fiber ratio in the present study. First, the number of blood vessels was counted. Transversely cut blood vessels were presumed to be 10 \( \mu m \) in length, which equaled the thickness of the sections. The length of longitudinally cut blood vessels was measured on the order of \( \mu m \). Then total capillary length was calculated as follows: total capillary length (\( \mu m \)) = 1 \times \text{number of capillaries showing their entire cross section} \times 10 + 1/2 \times \text{number of capillaries not showing their entire cross section} + \text{sum of lengths of longitudinally cut blood vessels (Fig. 1).}

Next, using digital images, representative portions of muscle fibers stained with eosin were chosen. Areas with the same color were automatically selected and changed to black by Photoshop CS software (Adobe, San Jose, CA, USA). The total black area in each field was computed using Image J 1.32j. This total muscle cross sectional area was divided by the number of muscle fibers counted manually to calculate the mean cross sectional area of the muscle fiber. Based on the above methods, we calculated the following parameters: the mean muscle fiber cross sectional area (total muscle cross sectional area / number of muscle fibers), the mean muscle fiber radius

\[
\frac{\left( \frac{1}{\pi} \text{the mean muscle fiber cross sectional area} \right)}{1},
\]

mean capillary length-to-fiber ratio (total length of blood vessels / total number of muscle fibers). All data represent mean values ± SD (standard deviation).

**Statistical analysis**

The muscle wet weight, the muscle fiber cross sectional area, and the capillary length-to-fiber ratio were found to distribute normally using StatView J 5.0 (SAS Institute Japan, Tokyo). Using Excel (Microsoft, Seattle, WA, USA), data were then analyzed with Student’s t test in cases with isodispersion or Welch’s t test in cases with anisodispersion. Statistical significance was set at P<0.05. Regression analysis was used to investigate relationships between capillary length-to-fiber ratio and muscle fiber cross sectional area, between capillary length-to-fiber ratio and mean muscle fiber radius, between capillary number-to-fiber ratio and muscle fiber cross sectional area, and between capillary number-to-fiber ratio and mean muscle fiber radius.

**Results**

**Capillarity in the normal developing tibialis anterior muscle**

At 0 days to 1 week of age, muscle fibers were thin and most fibers were not provided with blood vessels. At 0 days to 2 weeks of age, blood vessels were found to run in longitudinal and often transverse directions in muscles (Fig. 2). With development, an increasing number of muscle fibers accompanied blood vessels in their peripheral distributions, and transversely running blood vessels became less frequent. The muscle cross sectional area apparently increased after 1 week of age. In adult rats, most blood vessels ran longitudinally along muscle fibers (Fig. 2).
Fig. 2. Light micrographs of transverse sections of the tibialis anterior muscle during normal development. Staining with an anti-PECAM-1 antibody and eosin. Bars=50 μm. a: At 0 days after birth (0 d). Muscle fibers are thin and most have no capillaries in their peripheries. Capillaries are small in number and frequently run transversely. b: At 3 days (3 d). Muscle fibers and capillaries are similar to those at 0 days. c: At 1 week (1 w). Capillaries are sparse and have contact with a small number of muscle fibers. d: At 2 weeks (2 w). Capillaries are seen in close vicinity to most muscle fibers. e: At 4 weeks (4 w). Capillaries run along the long axis of the muscle. f: At 8 weeks (8 w). Cross sections of several capillaries are observable around each muscle fiber.
The capillary length-to-fiber ratio increased in parallel with the muscle fiber cross sectional area from 0 days to 4 weeks of age (y = 0.002x + 0.0015, where y = capillary length-to-fiber ratio and x = muscle fiber cross sectional area, $R^2 = 0.9765$). After 4 weeks of age, the increase in the capillary length-to-fiber ratio was attenuated (y = 0.0005x + 1.2436, x and y are the same as above; $R^2 = 0.7287$). When all data ranging from 0 days to 24 weeks

Fig. 3. The relationship between capillary length-to-fiber ratio and muscle fiber cross sectional area of the normal tibialis anterior muscle. The lines indicate the results of regression analysis.

Fig. 4. The relationship between capillary length-to-fiber ratio and muscle fiber radius of the normal tibialis anterior muscle. The correlation is stronger than in Figure 3.

Fig. 5. The relationship between capillary number-to-fiber ratio and muscle fiber cross sectional area of the normal tibialis anterior muscle. The lines indicate the results of regression analysis.

Fig. 6. The relationship between capillary number-to-fiber ratio and muscle fiber radius of the normal tibialis anterior muscle. The correlation is stronger than in Figure 5, but slightly weaker than in Figure 4.
of age were combined and analyzed together, the capillary length-to-fiber ratio correlated with the muscle fiber cross sectional area \( (y = 0.001x + 0.3257, \text{x and y are the same as above}; R^2 = 0.8799) \) (Fig. 3). On the other hand, the increases in capillary length-to-fiber ratio actually paralleled the increases in muscle fiber radius \( (y = 0.1106x - 0.4024, \text{where y = the capillary length-to-fiber ratio and x = the muscle fiber radius, } R^2 = 0.9580) \) (Fig. 4). Thus, the capillary length-to-fiber ratio was more closely correlated with the muscle fiber radius than the muscle fiber cross sectional area. The ratio of the capillary length-to-fiber ratio to the radius of the soleus muscle at 8 weeks of age was comparable to that of the tibialis anterior muscle (Fig. 4).

Furthermore, the results using the capillary number-to-fiber ratio showed very similar results assessed by the capillary length-to-fiber ratio. The capillary number-to-fiber ratio correlated with the muscle fiber cross sectional area \( (y = 0.0009x - 0.2423, \text{where y = the capillary number-to-fiber ratio and x = the muscle fiber cross sectional area, } R^2 = 0.8643)(\text{Fig. 5}) \). Also, the capillary number-to-fiber ratio again correlated more closely with the muscle fiber radius \( (y = 0.1024x + 0.4342, \text{where y = the capillary number-to-fiber ratio and x = the muscle fiber radius, } R^2 = 0.9481; \text{Fig. 6}) \) than the muscle fiber cross sectional area.

**Distribution of blood vessels in denervated muscles**

At 3 and 6 weeks after denervation, the values of muscle wet weight / body weight of denervated tibialis anterior and plantaris muscles were significantly decreased compared with those of the control side. The muscle fiber cross sectional area and the capillary length-to-fiber ratio were apparently decreased (Fig. 7, Table 1).

With atrophy-induced decreases in muscle fiber radius, the capillary length-to-fiber ratio also decreased (Fig. 8). The muscle fiber radius and capillary length-to-fiber ratio of atrophied muscle were reduced in reverse to the increases observed during normal development (Fig. 8). It was noteworthy that the combined data of normal (control) and denervated tibialis anterior muscles again demonstrated a good linear correlation with the muscle fiber radius \( (y = 0.1081x - 0.4441, \text{where y = the capillary length-to-fiber ratio and x = the muscle fiber radius, } R^2 = 0.9282)(\text{Fig. 8}) \). The normal and denervated plantaris muscles also demonstrated a good linear correlation with the muscle fiber radius \( (y = 0.1156x - 0.7691, \text{x and y are the same as above, } R^2 = 0.9004)(\text{Fig. 9}) \).

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**Fig. 7.** Light micrographs of transverse sections of the plantaris muscle after denervation. Staining with an anti-PECAM-1 antibody and eosin. Bars=100 µm. a: Control side (Con). b: Denervated side at 3 weeks (3 w DN) after operation. Muscle fibers are thinner than in the control (panel a). c: Denervated side at 6 weeks (6 w DN) after denervation. Atrophy is advanced compared with panel b.
Table 1. Atrophy after denervation of the tibialis anterior and plantaris muscles.

<table>
<thead>
<tr>
<th></th>
<th>Muscle wet weight / body weight (mg/g)</th>
<th>Muscle fiber cross sectional area (μm²)</th>
<th>Capillary length-to-fiber ratio (μm/fiber)</th>
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</thead>
<tbody>
<tr>
<td>Tibialis anterior muscle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denervation 3 weeks</td>
<td>Control 2.36 ± 0.10</td>
<td>1988 ± 336</td>
<td>21.1 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>Denervated 0.80 ± 0.06*</td>
<td>1062 ± 186*</td>
<td>15.6 ± 3.6</td>
</tr>
<tr>
<td>Denervation 6 weeks</td>
<td>Control 2.34 ± 0.14</td>
<td>2421 ± 151</td>
<td>28.1 ± 8.0</td>
</tr>
<tr>
<td></td>
<td>Denervated 0.60 ± 0.04*</td>
<td>672 ± 196*</td>
<td>10.9 ± 2.2*</td>
</tr>
<tr>
<td>Plantaris muscle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denervation 3 weeks</td>
<td>Control 1.21 ± 0.08</td>
<td>2183 ± 322</td>
<td>22.4 ± 5.7</td>
</tr>
<tr>
<td></td>
<td>Denervated 0.40 ± 0.03*</td>
<td>563 ± 37*</td>
<td>8.9 ± 0.9*</td>
</tr>
<tr>
<td>Denervation 6 weeks</td>
<td>Control 1.44 ± 0.10</td>
<td>2124 ± 202</td>
<td>22.6 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>Denervated 0.46 ± 0.06*</td>
<td>587 ± 212*</td>
<td>6.9 ± 1.2*</td>
</tr>
</tbody>
</table>

*P<0.05 versus control group.

Fig. 8. The relationship between capillary length-to-fiber ratio and muscle fiber radius of the normal and denervated tibialis anterior muscles. The line indicates the relationship in the normal developing tibialis anterior muscle shown in Figure 4.

Fig. 9. The relationship between capillary length-to-fiber ratio and muscle fiber radius of the normal and denervated plantaris muscles. The line is the same as shown in Figures 4 and 8.
Fig. 10. Transverse electron micrographs of capillaries in the denervated plantaris muscle. Bars=1 μm. a: Normal capillary. Endothelial cells (E) and their basement membranes continuously encircle the capillary. b: Cytoplasmic processes (C) of endothelial cells are discontinuous. c: Endothelial cells almost disappear and a red blood cell (R) directly contacts the basement membrane. d: Endothelial cells and the capillary lumen have disappeared. Only a collapsed irregularly shaped basement membrane (B) is recognizable.
Electron microscopy of capillaries in denervated muscle

Fine structural changes in skeletal muscle after short-term denervation have been reported elsewhere (Sakakima et al., 2000) and were not the main topic of the present study, so we focused our observations on blood vessels. In denervated muscles, capillaries with various degenerating images were observed. Endothelial cells were interrupted to various extents or even disappeared so that red blood cells directly faced basement membranes (Fig. 10). Sometimes, only the basement membrane remained, showing a collapsed irregular circle without endothelial cells. However, no characteristic markers of apoptosis were observed.

Distribution of blood vessels in the hypertrophied plantaris muscle

In the hypertrophied plantaris muscle, the muscle cross sectional area increased by 28% and the muscle fiber radius increased by 13% (Fig. 11). On the other hand, the capillary length-to-fiber ratio remained constant and did not increase in parallel with the muscle fiber radius (Table 2).

Discussion

The main finding of the present study was the reliable measurement of skeletal muscle capillarity in neonatal and very young rats. Antibodies against PECAM-1 allow clear and reliable identification of microvessels (Charifi et al., 2003). This antibody is known to stain both blood vessels (Scholz and Schaper, 1997) and

Table 2. Hypertrophy after tenotomy of the plantaris muscle.

<table>
<thead>
<tr>
<th>Plantaris muscle</th>
<th>Muscle wet weight / body weight (mg/g)</th>
<th>Muscle fiber cross sectional area (μm²)</th>
<th>Radius of muscle fiber (μm)</th>
<th>Capillary length-to-fiber ratio (μm/fiber)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.08 ± 0.12</td>
<td>1754 ± 311</td>
<td>23.6 ± 9.9</td>
<td>21.3 ± 2.6</td>
</tr>
<tr>
<td>Hypertrophy 2 weeks</td>
<td>1.37 ± 0.12*</td>
<td>2242 ± 273</td>
<td>26.7 ± 9.3</td>
<td>20.5 ± 1.7</td>
</tr>
</tbody>
</table>

*P<0.05 versus control group.
the lymphatic endothelium, though lymphatic vessels are stained less intensely than blood vessels (Sauter et al., 1998; Ebata et al., 2001; Schacht et al., 2004). In the endomysium, few or no lymphatic vessels were detected, in contrast to blood vessels (Kato, 2000; Kivelä et al., 2007). These findings are in agreement with our preliminary experiments and suggest that lymphatic vessels are unlikely to interfere with the quantification of blood vessels.

Capillary number-to-fiber ratio versus capillary length-to-fiber ratio

The capillary-to-fiber ratio (the capillary number-to-fiber ratio in the present study) has been widely used to evaluate capillarity. The capillary number-to-fiber ratio is a two-dimensional concept, while the capillary length-to-fiber ratio is a three-dimensional concept that takes into account transversely running, tortuous, and branching capillaries. These two parameters would give identical values if all blood vessels ran straight and longitudinally. Sullivan and Pittman (1987) reported a high incidence of transversely running capillaries in the soleus muscle of adult hamsters using electron microscopy. Transversely running blood vessels were also observed in the present study, particularly in neonatal and very young rats. Although the capillary length-to-fiber ratio may not precisely measure obliquely running blood vessels, it appears to be a better index than capillary number-to-fiber ratio for the assessment of capillarity. However, the capillary number-to-fiber ratio also correlated well with the muscle fiber radius in the present study. Capillary number is easy to measure and can be used to represent the skeletal muscle capillarity, albeit slightly less accurately than when using the capillary length-to-fiber ratio. At any rate, both capillary length-to-fiber and capillary number-to-fiber ratios correlated more closely with the muscle fiber radius than the muscle fiber cross sectional area.

Muscle fiber cross sectional area versus muscle fiber radius

The capillary number-to-fiber ratio has been reported to increase in proportion to the muscle fiber cross sectional area (Sillau and Banchero, 1978; Ripoll et al., 1979). However, to our knowledge, the capillarity of neonatal and very young rats has not been examined thus far. Our data for young and adult rats demonstrated a close relationship between the capillary length-to-fiber ratio and muscle fiber cross sectional area in agreement with Ripoll et al. (1979). In addition, our data for newborn to adult rats clearly demonstrated that the capillary length-to-fiber ratio increased linearly with a higher correlation to radius ($R^2 = 0.9580$) than muscle fiber cross sectional area ($R^2 = 0.8799$); they refute the opinion that muscle fiber cross sectional area is the major factor affecting skeletal muscle capillarity (Ripoll et al., 1979). Furthermore, during muscle atrophy, the capillary length-to-fiber ratio decreased in parallel with the reduction of the muscle fiber radius in the tibialis anterior muscle. Thus, it is plausible that the capillarity of the rat tibialis anterior muscle is more closely related to the muscle fiber radius rather than to the muscle fiber cross sectional area.

Muscle fiber surface area versus muscle fiber volume

The finding that capillarity is more proportional to radius than to muscle fiber cross sectional area implies that capillarity correlates more closely to muscle fiber surface area than to muscle fiber volume. If a muscle fiber is considered a cylinder, its perimeter ($2\pi r$) is derived from the radius ($r$). The muscle fiber surface area ($2\pi rh$) is therefore the product of the perimeter ($2\pi r$) and the length ($h$; thickness of muscle section). The muscle fiber volume ($\pi r^2 h$) is the product of the cross sectional area ($\pi r^2$) and the length ($h$).

In the present study, the mean radius ($r$) of the muscle fiber was computed from the mean muscle fiber cross sectional area under the assumption that muscle fiber cross sections are roughly circular (Mathieu-Costello et al., 1991; Hepple, 1997). This assumption appears incorrect as shown in Fig. 2. However, when numerous muscle fibers are averaged, the ratio between the real cross sectional area and the computed cross sectional area may fluctuate within a narrow range regardless of rat age. Hepple et al. (1998) defined a "fiber shape factor" as fiber shape factor $= 4\pi \times$ muscle fiber cross sectional area / (muscle fiber perimeter)$^2$. Charif et al. (2003) termed this factor the "form factor" and used it to assess obliquity in fiber sections. These papers reported that the above-mentioned factor was not significantly different between groups. Therefore, the following equation probably stands: the real muscle fiber cross sectional area $= \pi r^2 \times C$, where $C$ is the mean quotient of the real cross sectional area and the calculated cross sectional area ($\pi r^2$). Similarly, the real muscle fiber perimeter $= 2\pi r \times R$, where $R$ is the mean ratio of the real perimeter to the calculated perimeter. When $R$ fluctuates within a narrow range and can be considered almost constant, the calculated muscle fiber radius is likely proportional to the muscle fiber perimeter even when the shapes of muscle fiber cross sections are not circular.
**Muscle hypertrophy**

The capillary number-to-fiber ratio has been studied in the hypertrophied plantaris muscle (Kano et al., 1997; Plyley et al., 1998; Egginton et al., 1998) and reported to increase at 2 weeks (Egginton et al., 1998) and 3 weeks (Plyley et al., 1998) after the induction of hypertrophy. The weight of the plantaris muscle in the present study significantly increased when compared to controls, whereas the capillary length-to-fiber ratio did not. The duration of 2 weeks after the induction of hypertrophy may have been insufficient to observe any effect, since capillary proliferation is reported to lag behind muscle hypertrophy (Plyley et al., 1998).

**Fine structural changes in the capillaries of denervated muscle**

During muscle atrophy, muscle fibers become thin and their radii become smaller. To maintain the close correlation between the capillary length-to-fiber ratio and the muscle fiber radius, capillaries should decrease in number and length with the advancement of atrophy (Fig. 8, 9). As might be expected, degenerating capillaries have been observed during muscle atrophy after denervation (Carpenter and Karpati, 1982; Borisov et al., 2000). Degenerated capillaries have also been reported in aged rats (Desaki and Ezaki, 2002). In these studies, necrotic or damaged endothelial cells have been observed by electron microscopy. Fujino et al. (2005) also observed an apoptotic endothelium in unweighted rat hind-limbs by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL). In our study, electron microscopy did not reveal characteristic apoptotic markers such as chromatin condensation, nuclear fragmentation, or the formation of apoptotic bodies (Kerr et al., 1994). Therefore, it is unlikely that capillaries degenerate by apoptosis after denervation. The mechanism of vascular degeneration during atrophy is unknown and needs to be clarified.

**The relationship between capillarity and mitochondria**

It has been stated that capillarity is proportional to mitochondrial volume (Mathieu-Costello et al., 1996; Hepple et al., 1998). Although the soleus muscle is a well-known slow muscle containing more mitochondria than fast muscles, the soleus (slow) and tibialis anterior (fast) muscles showed very similar plots in Figure 4 in the present study. These findings suggest that capillarity is regulated by muscle fiber area rather than mitochondrial content. It is also necessary to take into account the transportation of various nutrients such as glucose and amino acids that are transported and mainly metabolized in the cytosol of muscle fibers. Oxygen and lipid-soluble substances cross the lipid bilayer of the plasma membrane by simple diffusion, while some substances need specific carrier proteins for transport (Guyton and Hall, 1996). Carrier proteins in the cell membrane play an essential role in the transportation of materials. In conclusion, capillarity is probably determined on the balance of various factors including muscle fiber surface area and mitochondrial content. Among these factors, muscle fiber surface area appears to be most correlated with capillarity.

**References**


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