

# Remodeling of the Vascular Bed and Progressive Loss of Capillaries in Denervated Skeletal Muscle

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## ABSTRACT

Very little is known regarding structural and functional responses of the vascular bed of skeletal muscle to denervation and about the role of microcirculatory changes in the pathogenesis of post-denervation muscle atrophy. The purpose of the present study was to investigate the changes of the anatomical pattern of vascularization of the extensor digitorum longus muscle in WI/HicksCar rats 1, 2, 4, 7, 12, and 18 months following denervation of the limb. We found that the number of capillaries related to the number of muscle fibers, i.e. the capillary-to-fiber ratio (CFR), decreased by 88%, from  $1.55 \pm 0.35$  to  $0.19 \pm 0.04$ , during the first 7 months after denervation and then slightly declined at a much lower rate during the next 11 months of observation to 10% of the CFR in normal muscle. Between months 2 and 4 after denervation, the CFR decreased by 2.4 times, from 58% to 24% of the control value. The loss of capillaries during the first 4 months following nerve transection was nearly linear and progressed with an average decrement of 4.16% per week. Electron microscopy demonstrated progressive degeneration of capillaries following nerve transection. In muscle cells close to degenerating capillaries, the loss of subsarcolemmal and intermyofibrillar mitochondria, local disassembly of myofibrils and other manifestations of progressive atrophy were frequently observed. The levels of devascularization and the degree of degenerative changes varied greatly within different topographical areas, resulting in significant heterogeneity of intercapillary distances and local capillary densities within each sample of denervated muscle. Perivascular and interstitial fibrosis that rapidly developed after denervation resulted in the spatial separation of blood vessels from muscle cells and their embedment in a dense lattice of collagen. As a result of this process, diffusion distances between capillaries and the surfaces of muscle fibers increased 10–400 times. Eighteen months after denervation most of the capillaries were heavily cushioned with collagen, and on the average 40% of the muscle cells were completely avascular. Devascularization of the tissue was accompanied by degeneration and death of muscle cells that had become embedded in a dense lattice of collagen. Immunofluorescent staining for the vascular isoform of  $\alpha$ -actin revealed preservation of major blood vessels and a greater variability in thickness of their medial layer. Hyperplastic growth of the medial layer in some blood vessels resulted in narrowing of their lumens. By the end of month 7 after denervation, large deposits of collagen around arterioles often exceeded their diameters. Identification of oxidative muscle fibers after immunostaining for slow-twitch myosin, as well as using ultrastructural criteria, has shown that after 2 months of denervation oxidative muscle fibers were less susceptible to atrophy than glycolytic fibers. The lower rate of atrophy of type I muscle fibers at early stages of denervation may be explained by their initially better vascularization in normal muscle and their higher capacity to retain capillaries shortly after denervation. Thus, degeneration and loss of capillaries after denervation occurs more rapidly than the loss of muscle fibers, which results in progressive

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Grant sponsor: NIH; Grant number: PO1-Ag-10821; Grant number: T32 AG-00114.

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Received 5 December 1998; Accepted 30 November 1999

decrease of the CFR in denervated muscle. The change of capillary number in denervated muscle is biphasic: the phase of a rapid decrease of the CFR during the first 7 months after nerve transection is followed by the phase of stabilization. The presence of areas completely devoid of capillaries in denervated muscle and the virtual absence of such areas in normal muscle indicate the development of foci of regional hypoxia during long-term denervation. The anatomical pattern of muscle microvascularization changes dramatically after nerve transection. Each muscle fiber in normal muscle directly contacts on average 3–5 capillaries. In contrast, in denervated muscle, groups of muscle fibers are served by single capillaries spatially separated from them by dense collagen insulation. Taken together, these results suggest that the remodeling of the vascular bed in the direction of a less oxygen-dependent metabolism and impairment of microcirculation are integral components in the pathogenesis of post-denervation muscle atrophy. Insufficient vascularization and a vast accumulation of collagen in denervated muscle appear to be among the factors that block regeneration of long-term denervated muscle after experimental or clinical reinnervation. *Anat Rec* 258: 292–304, 2000. © 2000 Wiley-Liss, Inc.

**Key words:** capillaries; arterioles; vascularization; skeletal muscle; denervation; muscle atrophy

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Morphofunctional changes in macro- and microcirculation are important factors controlling trophic environment in organs and tissues. Striated muscle of mammals, both skeletal and cardiac, is richly vascularized. Contractile activity depends on an active consumption of energy substrates and replenishment of their pools, and requires continuous removal of end products of energetic metabolism. The rate of oxygen consumption by working skeletal muscle attains the second highest absolute value of all tissues (for literature see Groebe, 1994). These functional characteristics explain the intimate anatomical interrelations between muscle cells and the microvascular bed. Each muscle fiber is encircled by a network of anastomosing capillaries located close to its surface (reviewed by Ellis et al., 1983; Hoppeler et al., 1997). Even a short-term block of the normal blood supply to a skeletal muscle, regardless of whether or not it is followed by reperfusion, causes significant injury to muscle cells (e.g. Menger et al., 1992; Menger and Messmer, 1993; Hvaal et al., 1996). Damage to blood vessels and the temporary interruption of the circulation followed by ischemia causes massive cell death after the transplantation of whole skeletal muscles (reviewed by Faulkner et al., 1994).

Historically, the microscopic anatomy of capillaries in muscle tissue has been investigated in relation to their functional role in oxygen transport. One of the first comprehensive publications concerning structural aspects of the microcirculation in muscle is the classical work of Krogh (1919), who considered capillaries as nearly straight and regularly spaced tube-like structures that provide homogeneous free diffusion of oxygen in the muscle tissue. However, later studies showed that the Krogh model significantly oversimplified morphofunctional interrelations of capillaries and muscle cells, and that, in some respects, it was incorrect (for discussion see Ellis et al., 1983; Loats et al., 1978; Groebe, 1996; Hoppeler et al., 1997). To quantify muscle capillarity, Krogh suggested using their numerical density, i.e. the number of capillaries per unit area of a cross-section. Evaluation of skeletal muscle capillarity using average capillary densities has significant drawbacks (discussed in detail by Ellis et al., 1983; Hoppeler et al., 1997). This approach assumes an evenness of the distributions of capillaries in muscle cross

sections and does not reflect the heterogeneity in capillary spacing and oxygen diffusion distances from individual capillaries. Capillary counts per unit area also strongly depend on the shrinkage of the tissue samples and the planes of sectioning. Summarizing data concerning the capillarity in skeletal muscle of different mammalian species, Plyley and Groom (1975) found a great variability of capillary counts in the same muscles reported by different investigators. For example, in the gastrocnemius muscle of cat, the range of numerical capillary densities varied between 379 (Schmidt-Nielsen and Pennycuik, 1961) and 2,341 per 1 mm<sup>2</sup> (Paff, 1930). Similar significant contradictions in publications regarding this issue and enormous variability of the number of capillaries per unit cross-sectional area even for the same muscle of the same species were reviewed by Schmalbruch (1985). These differences in the capillary counts appear to be accounted for by different types of fixation, processing, embedding, and spatial orientation of tissue samples as well as by the specificity and sensitivity of techniques used for capillary staining. At the same time little is known as yet concerning the difference in capillarization between different strains of experimental animals and variations between individual animals within the same experimental group.

Another traditional criterion of evaluation of muscle microvascularization is the number of capillaries per number of muscle fibers, or capillary-to-fiber ratio (Loats et al., 1978; Hoppeler et al., 1997). Unlike capillary density, this characteristic does not depend on the distortions of normal tissue geometry during preparation of the sample. However, like capillary density, capillary-to-fiber ratio is an averaging characteristic that assumes the evenness of capillary distribution and free diffusion of oxygen in the tissue. Thus, both capillary density and capillary-to-fiber ratio do not reflect the changes in the topographical interrelations of capillaries and muscle fibers, the heterogeneity of intercapillary distances and the changes in diffusion distances between capillaries and surfaces of muscle cells. It is becoming increasingly apparent that the application of these criteria alone, as it has been usually performed in older literature and in numerous recent publications, represents a significantly oversimplified approach to the analysis of the state and functional activity

of the microvascular bed. This is especially relevant to studies of developmental and pathological processes that are frequently accompanied by quantitative shifts of the stromal and parenchymal components of a tissue, changes in cell sizes, and alterations of structure and protein composition that directly affect the rate of oxygen diffusion. A number of electron microscopic studies of normal skeletal muscle tissue demonstrated a close topographical association of capillaries with subsarcolemmal accumulations of mitochondria (for discussion see Mathieu-Costello et al., 1992; Hoppeler et al., 1997). Such a microvascular design facilitates direct and rapid oxygen transport from the blood to mitochondria of muscle fibers. Recent data concerning oxygen transport, diffusion and consumption in skeletal muscle indicate that the dynamics of oxygen transport in muscle tissue are much more complex than was assumed earlier and that the heterogeneity of intercapillary spacing is a very important factor controlling oxygenation of the tissue (Hoofd and Turek, 1996; Groebe, 1994, 1996).

Denervation of skeletal muscle results in increasing impairment of its functional capacity, atrophy of muscle fibers and a loss of up to 70–85% of the mass of the tissue (Gutmann and Zelena, 1962; Hnik, 1962; McComas, 1996). Despite a number of early and recent descriptive publications concerning the gross, microscopic and ultrastructural morphology of denervated muscle (for literature see Lu et al., 1997; Viguie et al., 1997), the cellular mechanisms and molecular basis of post-denervation muscle atrophy are still poorly understood. It also remains unclear why post-denervation muscle atrophy is fully reversible only if re-innervation occurs no later than during the first few months after nerve injury. Changes in trophic characteristics of denervated skeletal muscle are also poorly studied as yet, and little is known regarding the remodeling of the vascular bed following nerve transection. For these reasons, in the present paper we tried to answer to the following questions: To what degree is the microanatomy of the vascular bed in skeletal muscle affected by denervation? Does denervation alter the spatial relationship between blood vessels and muscle fibers? What is the time-course of vascular changes following nerve transection? Does hypervascularization or hypovascularization of muscle fibers occur at any stage after the loss of neural control? Thus, the purpose of this study was to investigate the process of remodeling of the vascular bed in long-term denervated rat skeletal muscle with special reference to structural aspects of interactions of muscle fibers and capillaries.

## MATERIALS AND METHODS

### Muscle Denervation

The experiments were conducted on adult (4-month-old) male WI/HicksCar rats maintained at the animal facility at the Department of Biology, University of Michigan. In all experiments the animals were anesthetized with ether. The right legs of the rats were first denervated by sectioning the sciatic nerve high in the thigh, suturing the proximal and distal stumps, and implanting the proximal stump into a hip muscle and the distal stump into the popliteal space. This procedure allows a permanent and complete denervation of the lower leg (Carlson and Faulkner, 1988). The animals were sacrificed under ether anesthesia 1, 2, 4, 7, 12, and 18 months after denervation.

### Experimental Design

Tissue samples of the extensor digitorum longus (EDL) muscle were taken from 35 animals. For quantitative electron microscopic study of microvascularization, the number of capillaries per cross-sectioned muscle fiber was counted in samples taken from 28 animals. These samples were distributed in the following groups: normal muscle of 8-month-old animals (6 samples), normal muscle of 16-month-old animals (control for aging, 3 samples) and denervated tissue: 1 month after denervation (3 samples), 2 months (3 samples), 4 months (4 samples), 7 months (5 samples) 12 months (3 samples) and 18 months after denervation (2 samples). Capillaries topographically associated with 1,100–1,350 muscle fibers were counted at each time point. For light microscopy, samples of the extensor digitorum longus and tibialis anterior (TA) were taken 0, 2, 4 and 7 months after denervation from 3 animals in each group. Cross-sections were prepared through the equatorial part of each muscle and mounted in groups of four on histological slides. Indirect immunofluorescent staining for alpha-smooth muscle actin was used as a marker of macrovascularization. Three slides per each time point were studied from each animal. To determine the capillary-to-fiber ratio we divided the number of counted capillaries by the number of muscle fibers topographically associated with them at each time point. An average weekly rate of capillary loss was calculated by dividing the value of the total linear decrease of the capillary number by the number of weeks of observations.

### Electron Microscopy

The samples of muscle tissue were prefixed immediately after excision from the limb in ice-cold solution of 2.5% glutaraldehyde and 4% formaldehyde in 0.1 M isoosmotic phosphate buffer, pH 7.4 for 1 hr. Then the tissue was cut into several smaller pieces and fixed for an additional 4 hr at 3°C in fresh aliquots of fixative. After removal of the fixative, the samples were washed in 3 changes for 15 min each in 0.1 M phosphate buffer. The tissue was postfixated with 1% OsO<sub>4</sub> in 0.1 M phosphate buffer for 1.5 hr at 3°C, washed again in the buffer solution 3 times (15 min each wash) and dehydrated through a graded ethanol series to absolute ethanol. After infiltration with epoxy resin the samples were embedded in Spurr medium (Ted Pella, Inc). After polymerization of the medium in blocks, sections were prepared using a Reichert-Young Ultracut E ultramicrotome and mounted on grids. After staining with uranium acetate and lead citrate, the sections were examined with a Philips CM 10 electron microscope at an accelerating voltage of 60 kV.

### Immunocytochemistry and Light Microscopy

The middle part of each muscle was fixed in freshly prepared ice-cold 4% solution of paraformaldehyde in isotonic phosphate buffer, pH 7.2–7.4 for 6–8 hr. After washing out the fixative, the samples were dehydrated, embedded in paraffin using standard techniques, sectioned 8 µm and immunostained using the technique of indirect immunofluorescence. Monoclonal antibodies to alpha-isoform of vascular actin (Sigma), von Willebrandt factor (Dako), laminin (Chemicon) and slow myosin (Developmental Hybridoma Bank, University of Iowa) were diluted according to the recommendation of the producers. Fluorescein-conjugated rabbit anti-mouse IgG (Cappel) and goat anti-

rabbit IgG (Sigma) were used as secondary antibodies. The immunofluorescent reaction was observed with Nikon Optiphot fluorescence microscope using an excitation filter at 470–490 nm and an emission barrier filter at 510–560 nm wavelengths.

## RESULTS

Histological and immunocytochemical staining clearly revealed the localization of capillaries in muscle tissue in normal control samples (Fig. 1) and up to 2 months after denervation. (Fig. 8a,b). Light microscopic study indicated the presence of vascular pathology in denervated muscle that included a decrease in the number of capillaries, changes of their structure and the collapse of their lumens. However, neither routine hematoxylin-eosin staining or immunocytochemical labeling of capillaries with anti-laminin and anti-von Willebrandt factor antibodies provided clear contours of capillaries at more advanced stages of muscle atrophy (not shown). Thus, light microscopy did not allow us to perform a reliable quantitative analysis due to significantly increased frequencies of faint patterns of staining for the capillary markers. Similar conclusions concerning the difficulties of reliable identification of total capillary population even in normal muscle tissue at the light microscopic level were made by other authors (Hammersen, 1968; Hammersen and Appel, 1976; Schmalbruch 1985). For this reason, we used ultrastructural identification of capillaries in our study of the time-course and dynamics of vascular changes in denervated muscle. Electron microscopy has shown that in normal EDL muscle capillaries were localized in close proximity to the basal laminae of muscle fibers, and each muscle fiber usually made contact with 3–4 capillaries (Figs. 2a, 3a,b). Depending on their location, two types of capillaries can be distinguished in normal tissue: those contacting several (usually 3–4) muscle fibers (Fig. 3a) and capillaries localized between the lateral surfaces of two neighboring fibers (Fig. 3b). The topographical association of capillaries with subsarcolemmal accumulations of mitochondria was frequently observed (Figs. 2a, 3a, 9a). Less often, capillaries were located near myonuclei (Fig. 3b) and satellite cells (Fig. 5a). As shown in Figures 2a and 3a,b, the basal laminae of neighboring muscle fibers in normal control muscle were located close to one another. Collagen was occasionally detected between myocytes as single fibrils or small fibrillar clusters, and its presence between myocytes and capillaries was found rarely.

The scheme of our experiments and the results of capillary counts in normal and denervated muscle are presented in Table 1. To check on the degree to which the capillarity in normal skeletal muscle changes during the course of experiments as a result of aging, we used the tissue of 16-month-old animals as the second control for possible age-related changes in the tissue. As one can see from Table 1, the changes of capillarity between the two groups of control animals were not significant. The data presented in Table 1 show that the number of capillaries in denervated muscle tissue dramatically decreases from month 1 to month 18 after nerve transection. We found that several weeks after denervation of the muscle its microvascular bed underwent significant remodeling. The number of capillaries counted per 100 muscle fibers decreased from  $155 \pm 35$  in normal muscle to  $117 \pm 5$  and  $90 \pm 4$  after 1 month and 2 months after denervation, respectively (Table 1). This comprises 24% and 42% de-

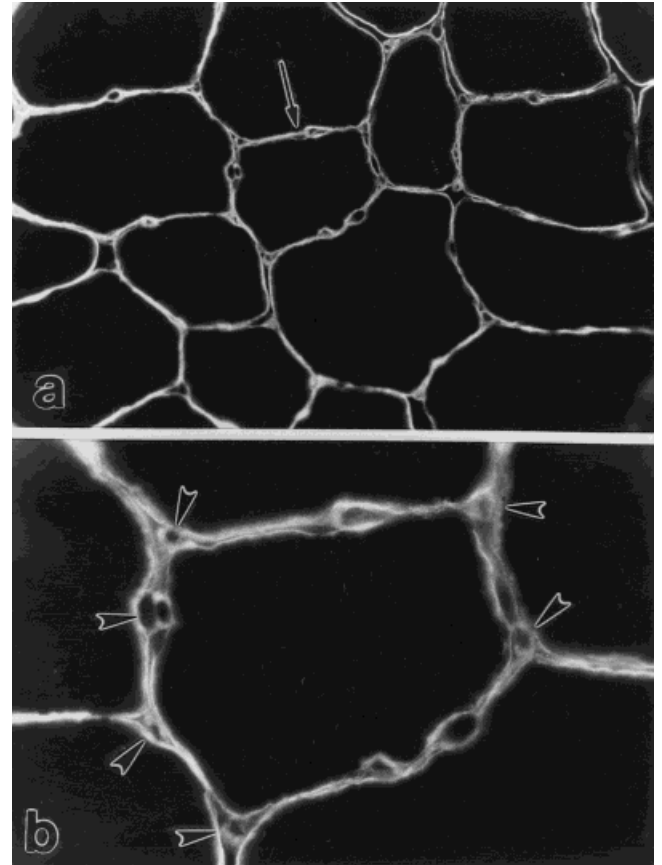


Fig. 1. Immunofluorescent staining for laminin reveals localization of capillaries (arrows) in cross-cryosectioned control EDL muscle. Arrow in (a) indicates the fiber shown in detail in (b). Arrowheads in (b) indicate the localization of capillaries. Note good preservation and minimal shrinkage of the tissue after rapid freezing. Magnification  $\times 254$  (a) and  $\times 634$  (b).

creases from the control value (Fig. 4). Noticeable changes in muscle structure occurred already 1 month after nerve transection, and these changes progressed during the second month after denervation. The most discernible manifestation of this remodeling was the beginning of a progressive spatial separation of capillaries from muscle cells as well as a separation of muscle cells from each other by deposits of fibrillar collagen (Figs. 2b, 3c,d). We did not observe intense proliferation and/or intercalation of fibroblasts between muscle fibers or muscle fibers and capillaries. As a result of advancing interstitial fibrosis, capillaries that contacted several muscle cells in normal muscle (as shown in Fig. 3a) were usually located closer to one of them and were separated by much greater distances from the others (Fig. 3c). Capillaries contacting lateral surfaces of two muscle fibers in normal muscle (Fig. 3b) 2 months after denervation were located at approximately equal distances from each of the muscle fibers (Fig. 3d) or remained closer to one of the fibers. Structural changes of the capillary endothelium indicate alterations of its functional properties. Unlike normal muscle, endothelial cells in denervated muscle more frequently formed processes and ruffles inside capillary lumens, and exhibited some

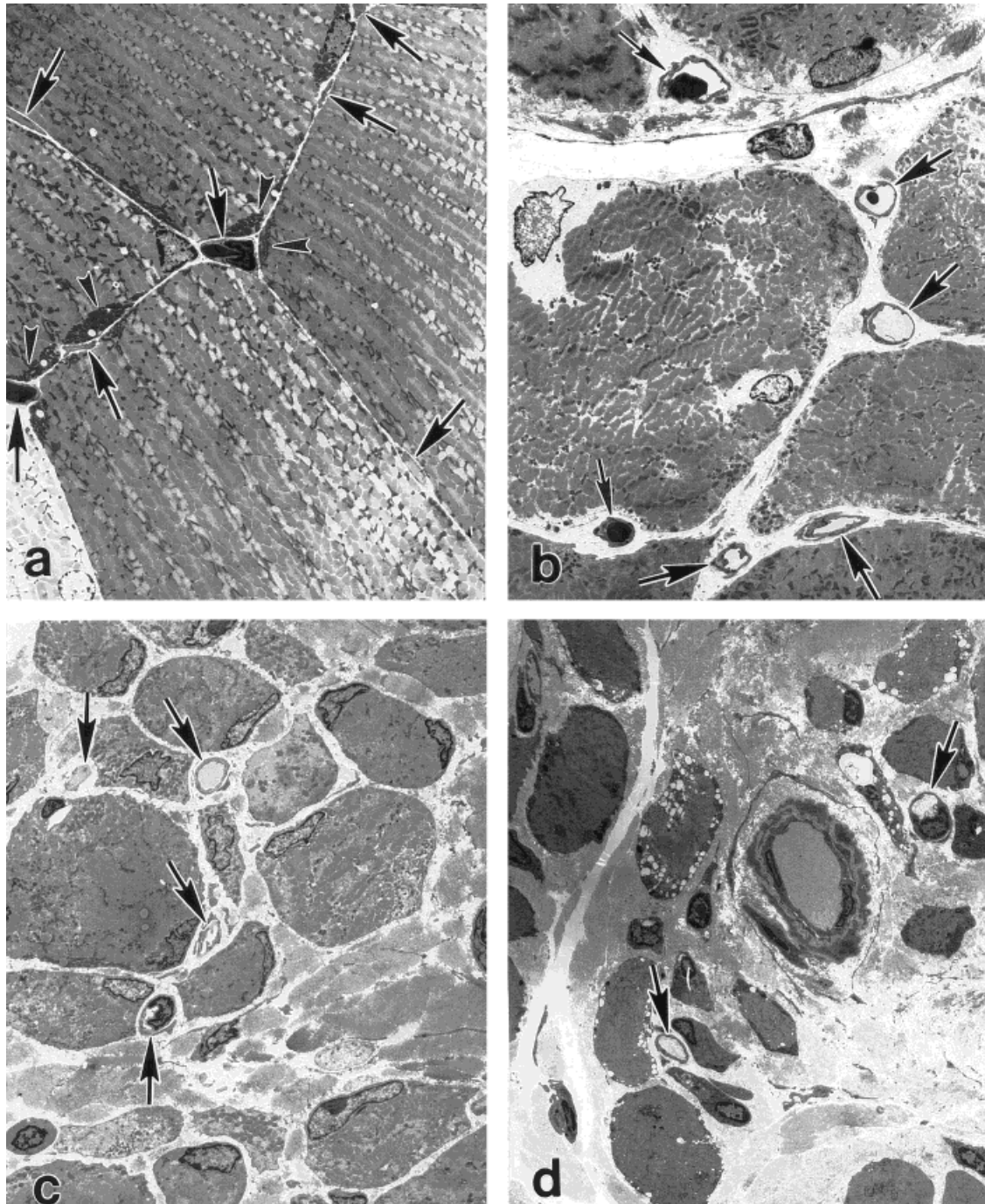


Fig. 2. Localization of capillaries in normal and denervated EDL muscle. **a**: Normal muscle. **b-f**: Denervated muscle; 2 months after denervation (**b**), 4 months (**c**); 7 months (**d**), 12 months (**e**) and 18 months (**f**) after denervation. Arrows indicate capillaries, arrowheads in (**a**) show

subsarcolemmal clusters of mitochondria. Note the spatial separation of capillaries from muscle fibers resulting from interstitial fibrosis and the decrease of their number per field of view. **a-e**: Magnification  $\times 1,650$ .

degree of constriction which resulted in decrease of capillary diameters (compare Fig. 3a,b and Fig. 3c,d). Pericytes also formed thin cytoplasmic processes sometimes extending outside the folded and thickened basal lamina (Fig. 3c). Electron microscopy has shown that the number of capillaries during the first weeks of denervation decreased

as a result of their degeneration and atrophy. One and two months after denervation, when the loss of capillaries still did not reach its advanced stages, regions of relatively unchanged capillary number (Fig. 2b) and areas of local relative hypervascularization were observed. We found numerous degenerating and dead capillaries in the muscle

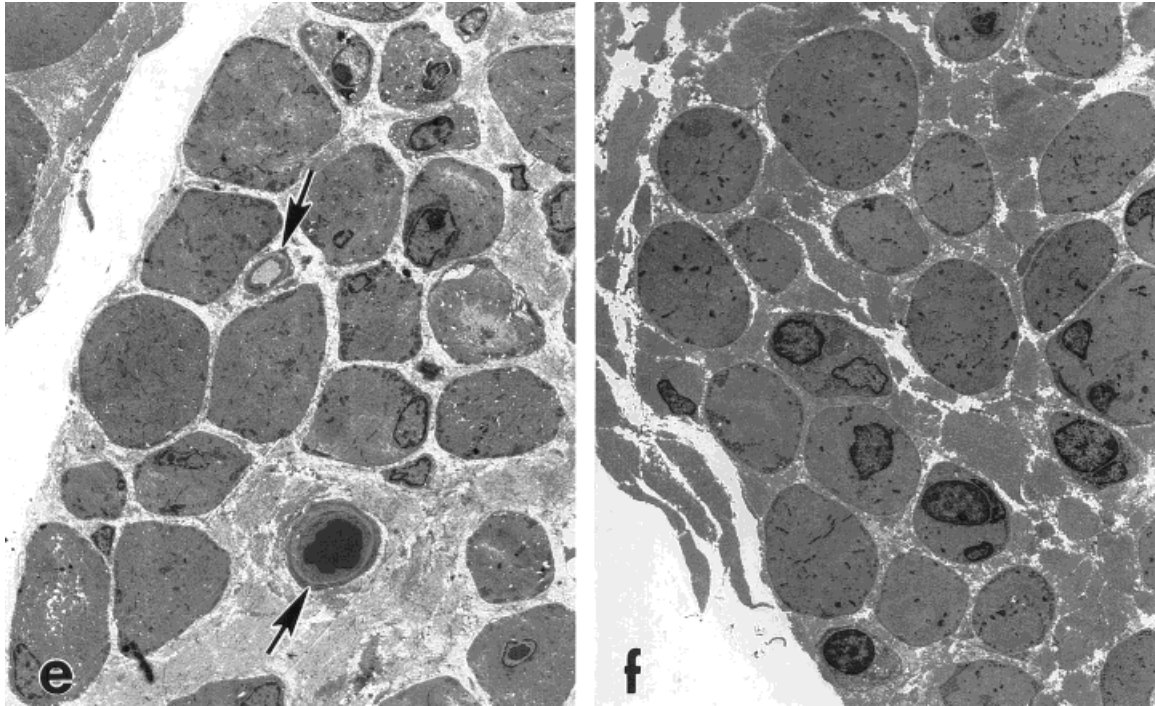


Figure 2. (continued)

tissue denervated for 1 and 2 months (Fig. 5a). A significant loss of capillaries after denervation temporally preceded the development of morphologically detectable manifestations of degeneration of muscle fibers. It is important that at all stages after denervation capillaries degenerated non-uniformly in different areas of the tissue. This heterogeneous pattern of capillary loss was especially evident 2 and 4 months after denervation. Similarly, the degree of development of degenerative changes of muscle cells varied greatly in different areas of the same muscle and even among individual muscle cells in the same topographical area.

In muscle tissue denervated for 2 and 4 months, subsarcolemmal mitochondria in oxidative fibers were less abundant than in controls (Fig. 2a and Fig. 2c). Degenerating mitochondria, lipofuscin granules and residual bodies were seen mostly in subsarcolemmal areas (Figs. 3d, 5a) and to a lesser extent between myofibrils. Formation of electron-lucent areas free of organelles around some of myonuclei (Fig. 2b), early stages of disalignment of myofibrils and disorganization of the contractile system (Figs. 3c,d, 5b) represent other manifestations of the degenerative changes in denervated muscle fibers. During the course of long-term denervation, large areas of the muscle underwent significant devascularization. The local numerical densities of capillaries per unit area were very heterogeneous in denervated muscle. Figure 2 shows the fields of view of the same size in normal innervated muscle (Fig. 2a) and at different stages following denervation (Fig. 2b–f). In control samples, we usually found the presence 5–12 capillaries per field of view. Unlike normal control muscle, areas containing a low capillary number (Fig. 2d,e) and avascular zones (Fig. 2f) have been fre-

quently observed in the tissue denervated for 7, 12 and 18 months. However, the heterogeneous pattern of devascularization was evident even at late stages of denervation. Focal areas completely devoid of capillaries were intermixed with regions of slightly increased or nearly normal intercapillary distances. Figure 2 also demonstrates that the anatomical pattern of microvascularization changes significantly after nerve transection. Each muscle fiber in normal muscle directly contacts on average 3–5 capillaries (Fig. 2a), whereas whole groups of muscle fibers in long-term denervated muscle are served by single capillaries separated from them by dense layers of collagen deposits (Fig. 2d–f).

Our results show that the capillary-to-fiber ratio diminished most dramatically during the first 4 months of denervation (Fig. 4). During this period, it decreased nearly linearly 4 times from the control level, with an average rate of 4.16% a week. Figure 4 also shows that the capillary to fiber ratio decreased most dramatically between months 2 and 4 after denervation (from  $0.9 \pm 0.04$  to  $0.37 \pm 0.04$ ), which constituted a 2.4-fold drop during this period. By this time the number of capillaries had dropped by 75% from the level observed in normal innervated muscle (Fig. 4). Interstitial fibrosis progressed dramatically between 2 and 4 months after denervation (Figs. 2c, 7a,b). As a result of the interstitial deposition of collagen fibers, the linear distances between muscle cells and capillaries greatly increased (Fig. 2c). In tissue denervated for 4 months most of the blood vessels were separated from muscle cells by deposits of collagen. At this stage, we also observed degenerating and dead capillaries and their remnants (Fig. 5b). The loss of capillaries did not depend on the levels of atrophy of individual muscle cells: this pro-

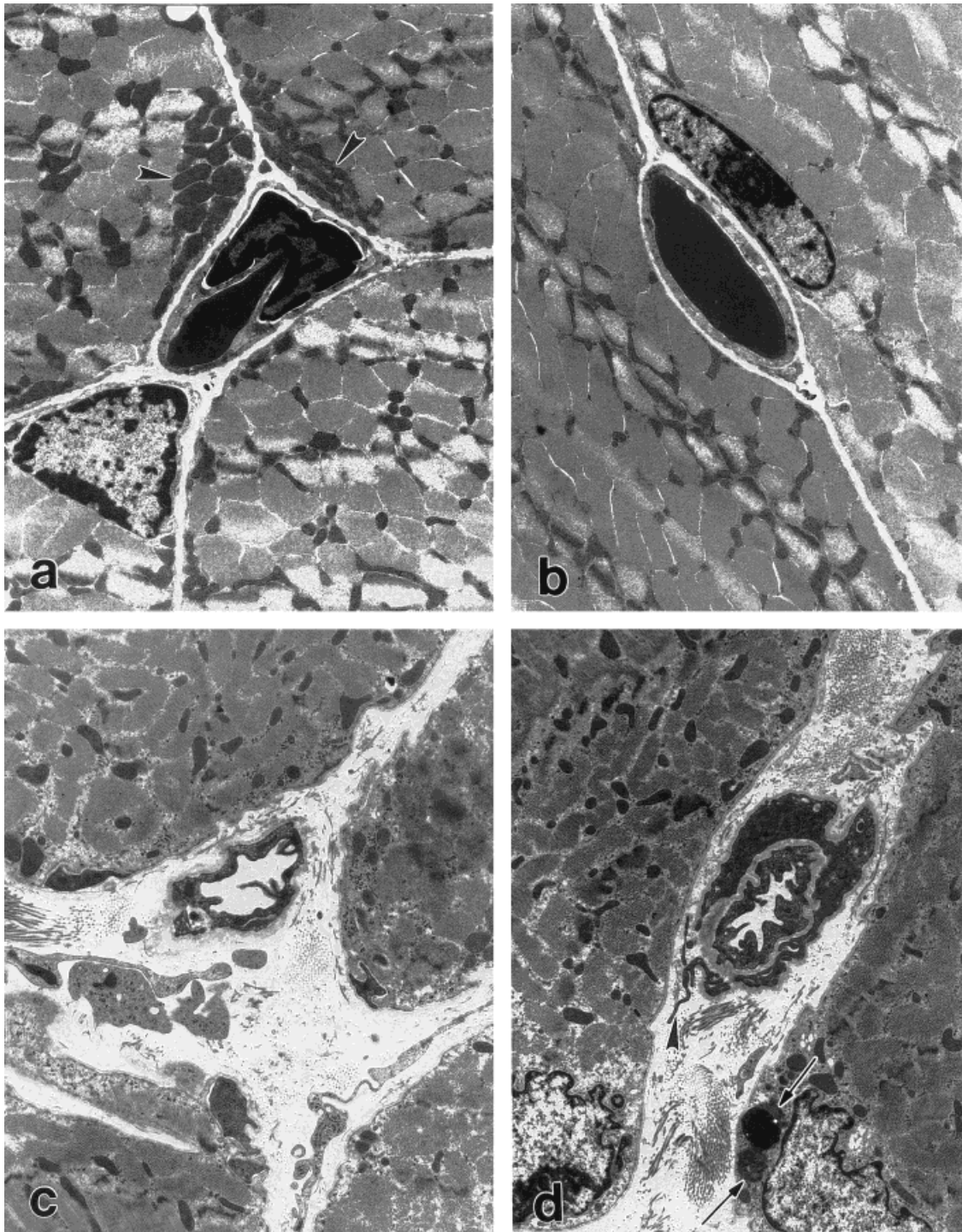


Fig. 3. Two types of localization of capillaries in skeletal muscle: a capillary contacting several muscle fibers **(a)**; a capillary located between the lateral surfaces of two neighboring fibers **(b)**. These two types of capillaries in muscle denervated for 2 months are shown in **(c)** and **(d)**, respectively). Early stages of progressive spatial separation of capillaries from muscle fibers and their insulation with collagen are already clearly

visible. Note processes of endothelial cells in capillary lumens (c and d), cytoplasmic processes of a pericyte (arrowhead in d) and some constriction of capillaries in denervated muscle. Arrowhead in (a) indicates a subsarcolemmal accumulation of mitochondria; small arrows in (d) indicate degeneration of subsarcolemmal organelles. Magnification  $\times 6,610$ .

**TABLE 1. Number of capillaries per 100 muscle fibers in normal and denervated EDL muscle\***

	Number of capillaries/ 100 muscle fibers	Counted capillaries/ counted muscle fibers	Number of animals/group
Normal muscle			
8-month-old rats	155 ± 35	1,571/1,013	n=6
16-month-old rats	141 ± 16	1,458/1,034	n=3
Denervated muscle			
1 <sup>a</sup>	117 ± 5	1,198/1,024	n=3
2	90 ± 4**	949/1,057	n=4
4	37 ± 4**	394/1,065	n=5
7	19 ± 4**	226/1,332	n=6
12	16 ± 3**	196/1,251	n=3
18	16 ± 3**	214/1,362	n=2

\*Values are the means ± SD.

\*\* Statistically significant differences from the value observed in the EDL of control 8-month-old animals, *P* < 0.05.

<sup>a</sup> Months after denervation.

**CAPILLARY / MUSCLE CELL RATIO**

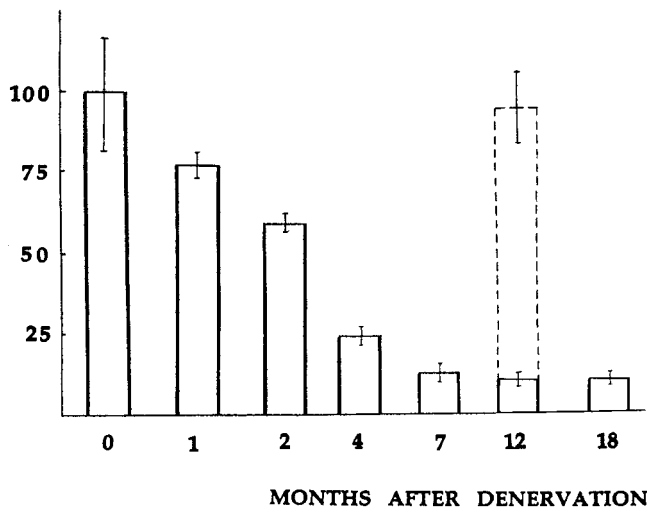


Fig. 4. Progressive loss of capillaries during the course of long-term denervation. Ordinate, capillary / muscle cell ratio, percentage of the control value. Abscissa, time after denervation, months. Dashed column, control for aging (16-month-old animals). Values are the means ± SD. Differences between control and the groups denervated for 2, 4, 7, 12 and 18 months are statistically significant at *P* < 0.05.

cess occurred around muscle fibers of both larger and smaller sizes. A noticeable feature of muscle tissue denervated for 4 months is the presence of degenerating and dying muscle cells embedded in a dense collagen lattice that was often completely devoid of blood vessels (Fig. 7). Degenerative changes in mitochondria and the formation of lucent areas near myonuclei visible in some cells after 2 months of denervation further progressed in 4-month denervated muscle.

Seven months after nerve transection the number of capillaries related to the number of muscle fibers in denervated muscle was 8.3 times (88%) lower than in the control, and by 18 months of denervation it was 10 times lower than in normal muscle, with capillary-to-fiber ratios of  $0.19 \pm 0.04$  and  $0.16 \pm 0.03$ , respectively (Fig. 4). Thus the number of capillaries after 7 months of denervation decreased nearly 2 times from the level observed after 4

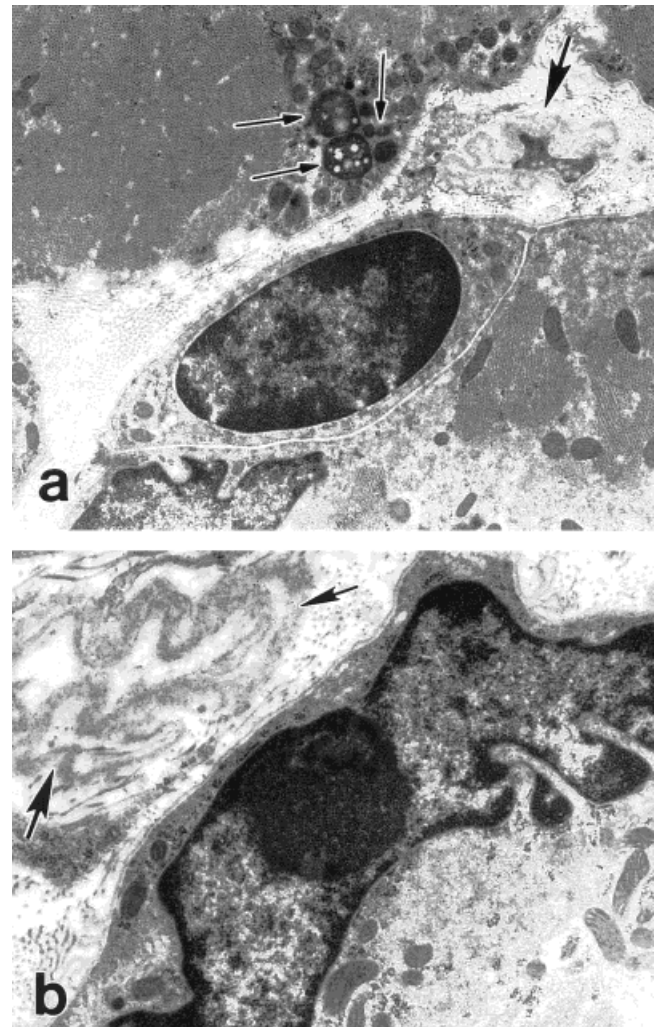


Fig. 5. Degeneration and death of capillaries in denervated EDL muscle. **a:** Two months after denervation. **b:** Four months after denervation. Large arrows indicate capillaries, small arrows indicate degenerating subsarcolemmal mitochondria. Note swollen empty basal lamina of the dead capillary near a satellite cell. Magnification ×11,500 (a), ×15,500 (b).



### NUMBER OF AVASCULAR MUSCLE CELLS, %

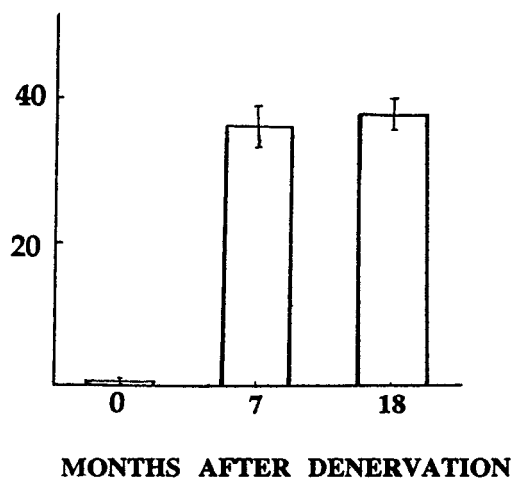


Fig. 6. Number of avascular muscle fibers in the denervated EDL muscle. Ordinate, number of avascular muscle fibers, %; Abscissa, time after denervation, months. Differences between control and denervated groups are statistically significant at  $P < 0.05$ .

months of denervation. In muscle denervated for 7 months most of the myocytes were densely insulated with collagen (Fig. 2d). With increasing time of denervation, progressive interstitial fibrosis further separated capillaries and muscle fibers at longer distances from each other. By 7 months after denervation and later, practically all capillaries were separated from muscle cells by more or less developed layers of dense collagen fibrils (Fig. 2d-f). Distances between capillaries and the surfaces of the muscle fibers increased 10–400 times by 12 and 18 months after denervation. Completely avascular muscle fibers were observed beginning from month 4 of denervation, and their number increased from virtually 0% in control muscle to 33–48% by 18 months after denervation (Fig. 6). In avascular fibers evidence of degenerative processes was frequently observed (Fig. 2d,e). Degenerating and moribund muscle cells observed beyond months 3 and 4 of denervation were usually embedded in a dense lattice of fibrillar collagen (similarly to those shown in Fig. 7). A distinctive feature of myocytes not subjected to intense degenerative processes after long-term denervation is much smaller numbers of subsarcolemmal mitochondria (compare Fig. 2a and Fig. 2e,f). During 7, 12 and 18 months after denervation, the separation of muscle fibers from each other and from capillaries continued as a result of further accumulation of fibrillar collagen (Fig. 2d-f). After 7 months of denervation, the capillary-to-fiber ratio stabilized at 12%, 11% and 10% of the levels seen in control innervated muscle after 7, 12 and 18 months of denervation, respectively (Fig. 4). Thus, quantitative electron microscopic analysis has shown that the capillary-to-fiber ratio progressively decreased nearly 10 times during 18 months of denervation.

Indirect immunofluorescent staining for slow-twitch myosin, a marker of oxidative type I muscle fibers has shown that during the first few weeks of denervation slow-type muscle fibers were less susceptible to atrophy than fast muscle fibers (Fig. 8). At later stages type I fibers also demonstrated significant atrophy (not shown). Elec-

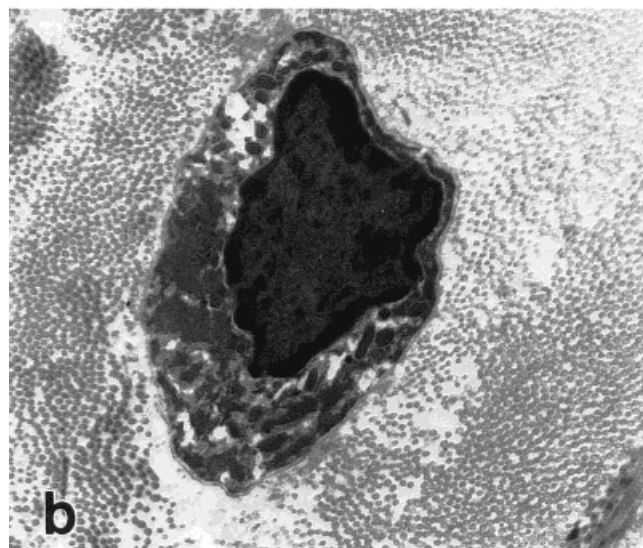
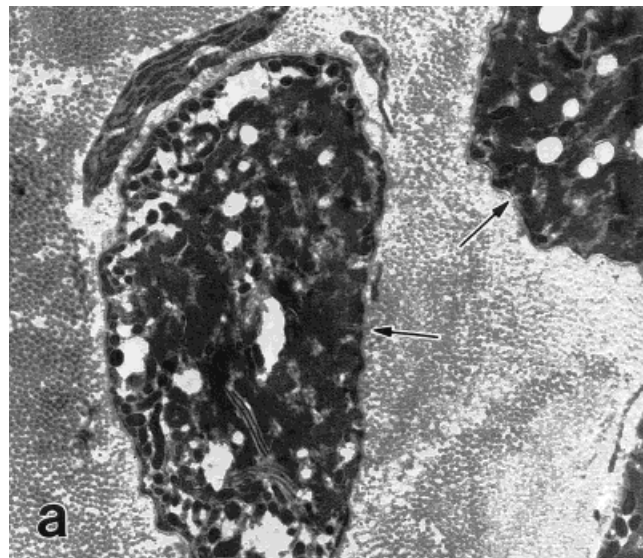


Fig. 7. Severe atrophy and degenerative changes in avascular fibers in long-term denervated muscle: degenerating muscle fiber surrounded by dense collagen deposits. Arrows in (a) indicate avascular degenerating muscle cells. Panel (b) shows a severely atrophied moribund muscle fiber embedded in collagen lattice. Magnification  $\times 8,900$  (a),  $\times 11,500$  (b).

tron microscopy confirmed this observation and has shown that many slow oxidative fibers not only underwent slower atrophy but also retained their capillaries longer than fast glycolytic fibers (Fig. 9).

Immunostaining for the alpha-isoform of vascular actin revealed preservation of major blood vessels in the denervated muscle. Fibrosis in perivascular regions resulted in heavy insulation of arterioles in a collagen lattice (Fig. 10a,b). After 7 months of denervation collagen deposits around small blood vessels frequently equaled or even exceeded their diameters (Fig. 10b).

### DISCUSSION

Our results show that the vascular bed and microcirculation undergo dramatic remodeling in denervated mus-

cle. We found that progressive impairment of the normal anatomical pattern of capillary distribution occurs in skeletal muscle with increasing time of denervation. Mass degeneration and death of capillaries during the first 7 months after denervation causes a significant decrease in their number, and leads to devascularization of significant numbers of muscle fibers. This indicates that alterations of the circulation is an integral component of the pathogenesis of post-denervation muscle atrophy. Of considerable interest is evident topographical heterogeneity of capillary distribution observed at all stages after denervation. Large heterogeneity of capillary spacing and the development of zones with zero capillary counts leads to the formation of areas of local chronic or acute hypoxia. The heterogeneous pattern of capillary degeneration could result from non-uniform loss of vasculotropic factors in different regions of denervated muscle or/and from different sensitivity of different domains of the microvascular bed to denervation. Progressive spatial separation of capillaries from muscle cells that occurs in muscle during the course of long-term denervation appears to be another important factor contributing to the development of post-denervation muscle atrophy. The rare occurrence of fibroblasts between muscle cells indicates that great amounts of collagen can be synthesized by muscle cells themselves. This interpretation is supported by recent findings concerning the capacity of skeletal muscle fibers to produce collagen (for literature see Kivirikko et al., 1995). The conclusion that interstitial fibrosis can lead to hypoxia of the muscle cells is supported by recent studies on cardiac muscle (Shimoyama et al., 1994; Sabbah et al., 1995). Earlier studies of Rakusan (1971) have shown that a 70% increase in oxygen diffusion distance can decrease  $PO_2$  in myocardial cells to zero and result in hypoxia, even provided that the remaining parameters of oxygen delivery were normal. In our experiments, we observed that the increase in diffusion distances between muscle cells and capillaries frequently exceeded 70%. It is interesting that progressive interstitial fibrosis has been described in the heart under a number of conditions associated with restriction of blood flow and insufficient delivery of oxygen to myocardial cells (Bishop, 1984). Similar to our data, multifocal areas of fibrosis surrounding small arteries and arterioles have been observed in the chronically hypoxic myocardium (reviewed by Ferrans, 1984). Little is known regarding similar changes in skeletal muscle. However, thickening of the medial layer of the vessel wall and decreased diameters of their lumens observed in some arterioles after long-term denervation (Fig. 9) is also consistent with the data obtained in studies of ischemia-related cardiovascular pathology.

To our knowledge, this work is the first study concerning the quantitative dynamics and the time-course of structural changes of the vascular bed during the course of long-term denervation. Microangiographic studies of blood flow in skeletal muscle have been concentrated on the first hours and days of denervation (Chen et al., 1992a, 1992b). An important indication of the possibility of similar processes occurring in human muscle pathology is the data of Carpenter and Karpati (1982) who found necrotic capillaries in muscle biopsies obtained from patients suffering from amyotrophic lateral sclerosis and axonal and demyelinating neuropathies. Interestingly, some ultrastructural changes in capillaries, such as formation of processes and ruffles by pericytes

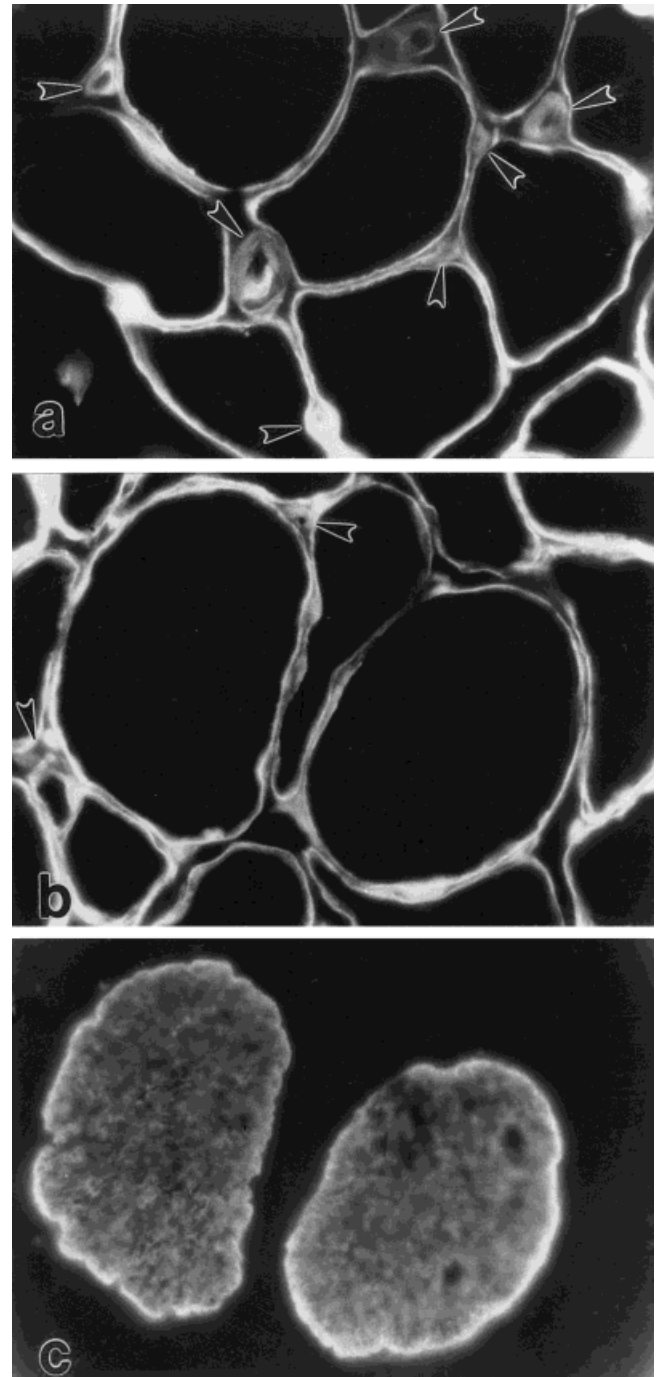


Fig. 8. Different susceptibility to post-denervation atrophy of fast and slow types of muscle fibers as revealed by double immunolabeling for slow myosin and laminin. Laminin staining of a highly vascularized area in 2-month denervated muscle (a); immunostaining of normal muscle for slow myosin (c); the same field immunostained for laminin (b). Note the difference in the degree of atrophy of slow and fast muscle fibers. Arrows in (a) and (b) indicate the localization of capillaries. Magnification  $\times 634$ .

and endothelial cells, that we observed in acutely denervated muscle are typical of human muscle in clinical cases of these neurodegenerative disorders. However,

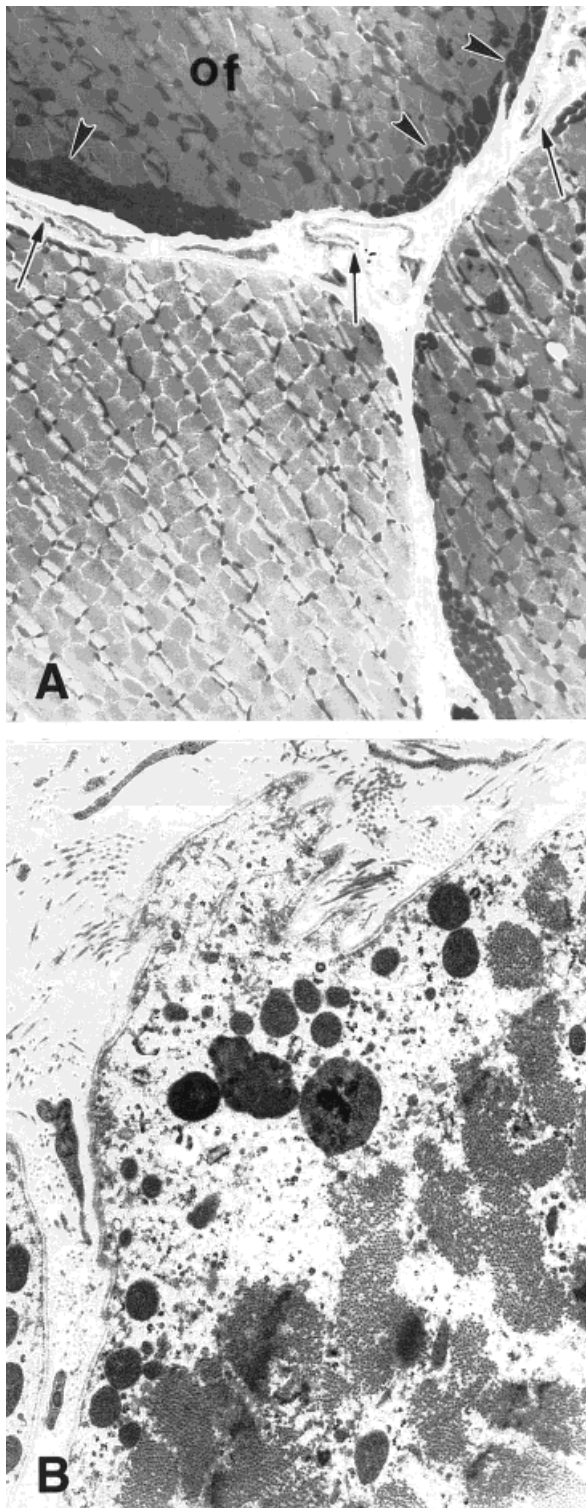


Fig. 9. Capillaries associated with oxidative muscle fibers. Note the topographical association of capillaries (arrows) with large amounts of subsarcolemmal and intermyofibrillar mitochondria (arrowheads) 1 month after muscle denervation (a) and the loss of subsarcolemmal mitochondria associated with the loss of capillaries 2 months after denervation (b). Also note the change from dense mitochondrial clusters (a) to sparse groups (b) of oxidative fiber. Magnification:  $\times 3,420$  (a),  $\times 17,000$  (b).

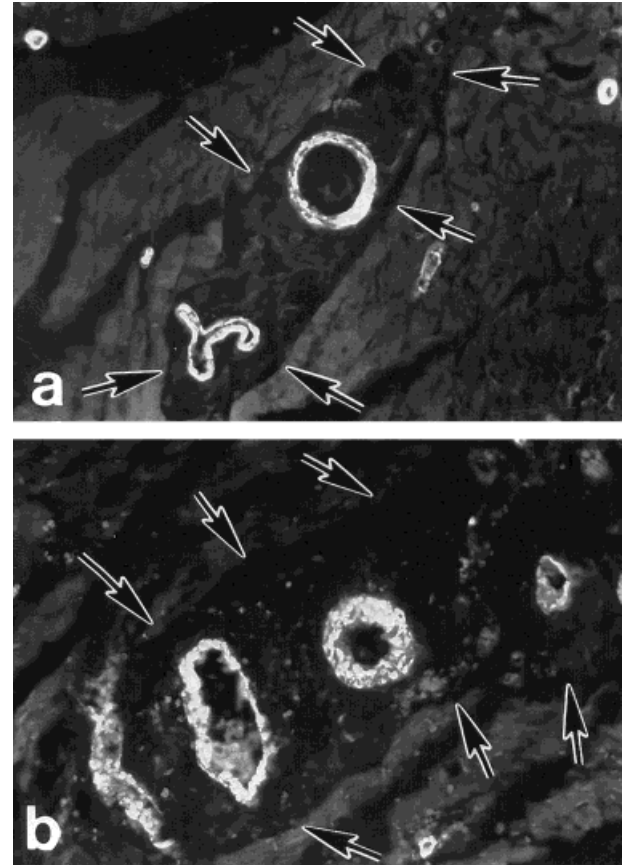


Fig. 10. Progressive fibrosis in perivascular regions of denervated muscle. Note the insulation of arterioles in a collagen lattice 2 months after denervation (a). By 7 months after denervation (b) collagen deposits significantly increased in size and the medial layer of some blood vessels is larger than at early stages after denervation. Indirect immunofluorescent staining for vascular  $\alpha$ -actin shows the location of blood vessels in the same anatomical region 2 and 7 months after denervation. Arrowheads indicate the boundaries of muscle fibers and the connective tissue surrounding blood vessels. Magnification  $\times 120$ .

clinical material does not permit one to study the kinetics of this process. Thus, our data show that as a result of both focal reduction in capillary densities and a spatial separation of capillaries from muscle cells by deposits of collagen, the linear diffusion distances for oxygen between capillaries and muscle cells are significantly increased. Both decrease of the capillary-to-fiber ratio and an increase of the distance between capillaries and fiber mitochondria are considered among the leading factors contributing to the development of hypoxia in skeletal muscle (Sieck and Johnson, 1996). This supports the conclusion that ischemia developing in denervated skeletal muscle may contribute to the atrophy of the tissue occurring from month 1 to month 7 after denervation.

Mitochondria are the major sites of cellular oxygen consumption, and their distribution in normal muscle cells is topographically associated with capillaries (for review see Hoppeler et al., 1991). The greater part of mitochondria in muscle cells (especially in oxidative fibers) is extensively clustered under the sarcolemma. Localization of capillar-

ies in close proximity to the basal laminae of muscle fibers at short distances from subsarcolemmal accumulations of mitochondria directly facilitates the transport of oxygen into the cells (for literature see Kreuzer et al., 1997; Hoppeler et al., 1991). This localization explains the increased local oxygen consumption in subsarcolemmal areas and creates intracellular gradients of oxygen distribution inside muscle cells (Kreuzer et al., 1997; Groebe, 1994). The oxidative capacity of myocytes can be adequately characterized by their mitochondrial volume and density. In a variety of muscles, mitochondria have been demonstrated to be distributed most densely near capillaries, and their number decreased with distance toward the center of the fiber (for literature see Hoppeler et al., 1997). Such observations led to the appealing conclusion that mitochondrial location in muscle fibers is related to mitochondrial function. Loss of mitochondria is an important indicator reflecting metabolic remodeling of long-term denervated muscle. Our data show that the densities of mitochondria in muscle cells dramatically decrease during the course of long-term denervation. This indicates that myocytes in denervated muscle should undergo adaptation to a less oxygen-dependent metabolism. This conclusion is supported by the data concerning changes of the energetic metabolism in denervated muscle. In chicken gastrocnemius muscle, denervation results in a delayed stimulation of glycogenolysis and glycolysis, which is accompanied by decreasing activity of oxidative enzymes (Asotra et al., 1986). Sesodia and co-authors (1994) reported that the activity of oxidative enzymes gradually decreased in anaerobically regenerating denervated muscle and the activity of glycolytic enzymes, as shown for lactate dehydrogenase, increased with increasing time of denervation. These authors showed that the activity of oxidative enzymes recovered after re-innervation of the muscle. Thus the studies of energetic metabolism in denervated muscle indicate the activation of an anaerobic glycolytic pathway and a decrease of enzyme activities associated with aerobic oxidative phosphorylation. This also agrees well with our observations concerning the degeneration of mitochondria and the presence of large amounts of glycogen in long-term denervated muscle. For our discussion, of special interest is an understanding of the mechanisms underlying different sensitivity to denervation of oxidative and glycolytic fibers in the same muscle.

Better vascularization of oxidative muscle fibers in normal muscle has been described earlier (for literature and discussion see Hoppeler et al., 1991; Degens et al., 1992, 1994).

Also it was shown that there is a lower heterogeneity in capillary distribution in the soleus, a slow, oxidative muscle than in the extensor digitorum longus, a fast mixed muscle (Egginton et al., 1988). For these reasons, the lower susceptibility of oxidative muscle fibers to atrophy during the first weeks after denervation may be explained, among other factors, by their better vascularization and/or higher capacity to retain capillaries after denervation as compared to fast fibers.

Thus, we found evidence that hypoxia developing in denervated muscle is a contributing factor to the loss of the mass of the tissue occurring from month 1 to month 7 after denervation. Also our data may provide a possible explanation of why initially less vascularized type II muscle fibers are less resistant to denervation at early stages of the atrophic process. Insufficient vasculariza-

tion and accumulation of collagen in denervated muscle may also affect its compensatory regeneration. Using immunocytochemical and ultrastructural markers, we observed clear manifestations of regenerative process at different stages following denervation including the time points discussed in the present paper (Borisov and Carlson, 1997; Borisov et al., 1999). However, terminal differentiation of newly formed muscle fibers and capillaries appears to be blocked in denervated muscle. For this reason, these abortive attempts of regeneration do not provide significant functional compensation for the progressive loss of tissue mass and microvascular degeneration. Of special importance is the question whether the progressive loss of capillaries and atrophy of muscle fibers are controlled by the same factors, or if muscle atrophy and loss of capillaries are relatively independent events. The answer to this question should be found in the future studies.

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