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Interrelations of Myogenic Response, Progressive Atrophy of Muscle Fibers, and Cell Death in Denervated Skeletal Muscle

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ABSTRACT

Little is known concerning the time-course and structural dynamics of reactivation of compensatory myogenesis in denervated muscle, its initiating cellular mechanisms, and the relationship between this process and the progression of postdenervation atrophy. The purpose of this study was to investigate the interrelations between temporal and spatial patterns of the myogenic response in denervated muscle and progressive atrophy of muscle fibers. Another objective was to study whether reactivation of myogenesis correlates with destabilization of the differentiated state and death of denervated muscle cells. It has remained unclear whether muscle fiber atrophy was the primary factor activating the myogenic response, what levels of cellular atrophy were associated with its activation, and whether the initiation and intensity of myogenesis depended on the local and individual heterogeneity of atrophic changes among fibers. For this reason, our objective was also to identify the levels of atrophic and degenerative changes in denervated muscle fibers that are correlated with activation of the myogenic response. We found that the reactivation of myogenesis in the tibialis anterior and extensor digitorum longus muscles of the rat starts between days 10–21 following nerve transection, before atrophy has attained advanced level, long before dead cells are found in the tissue. Formation of new muscle fibers reaches its maximum between 2 and 4 months following denervation and gradually decreases with progressive postdenervation atrophy. The myogenic response is biphasic and includes two distinct processes. The first process resembles the formation of secondary and tertiary generations of myotubes during normal muscle development and dominates during the first 2 months of denervation. During this period, activated satellite cells form new myotubes on live differentiated muscle fibers. Most of the daughter myotubes in 1- and 2-month denervated muscle develop on the surface of fast type parent muscle fibers, and some of the newly formed muscle fibers express slow myosin. Some fast type parent fibers are weakly or, more rarely, moderately immunopositive for embryonic isomyosin. This indicates that reactivation of myogenesis may also depend on the fiber type. The level of atrophy, destabilization of the differentiated myofiber phenotype, and degenerative changes of individual fibers in denervated muscle are very heterogeneous. The myogenic response of the first type is associated predominantly with fibers of average and higher than average levels of atrophy. Muscle cells that undergo a lesser degree of atrophy also form daughter fibers, although with a lower incidence. We did not find any correlation between the size of newly formed fibers and the level of atrophy of parent fibers. The topographical distribution of new myotubes both in the peripheral and central areas of the mid-belly equatorial sections at the early stages following nerve transection indicates that myogenesis of the first type represents a systemic reaction of muscle to the loss of neural control. These data indicate that activation of the myogenic response does not depend on cell death and degenerative processes per se. The second type of myogenesis is a typical regenerative reaction that occurs mainly within the spaces surrounded by the basal laminae of dead muscle fibers. Myocytes of different sizes are susceptible to degeneration and death, which indicates that cell death in denervated muscle does not correlate with levels of muscle cell atrophy. The regenerative process frequently results in development of abnormal muscle cells that branch or form small clusters. Replacement of lost fibers becomes activated between 2 and 4 months following nerve transection, i.e., mainly at advanced stages of postdenervation atrophy, when cell death becomes a contributing factor of the atrophic process. In long-term denervated muscle, the first and second types of myogenesisoccur concurrently, and the topographical distribution of the myogenic response becomes more heterogeneous than during the first weeks following denervation. Thus, our data demonstrate differential temporal and spatial expression of two patterns of myogenesis in denervated muscle that appear to be controlled by different regulatory mechanisms during the postdenervation period. Anat Rec 264:203–218, 2001.

Key words: myogenesis; denervated muscle; myosin isoforms; fiber types; denervation atrophy

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Denervation of skeletal muscle results in progressive impairment of its functional properties, atrophy of muscle fibers, and a rapid loss of up to 70–85% of its tissue mass during the first several months following denervation (Gutmann and Zelena, 1962; Hnik, 1962). Biological and clinical studies of denervated muscle have long focused on the kinetics and structural aspects of overall atrophy as a major visible manifestation of the pathologic process (for literature see Schmalbruch and Lewis, 1994; Lu et al., 1997; Viguie et al., 1997). Meanwhile, it remains unclear whether and to what degree progressive atrophy and functional destabilization of muscle fibers are correlated with initiation of the myogenic response in the denervated tissue. Little information is also available on the time-course and structural patterns of formation of new muscle fibers at different stages following denervation.

Despite a number of descriptive publications concerning the gross, microscopic, and ultrastructural morphology of denervated muscle (for reviews see Vrbova et al., 1995; McComas, 1996), the cellular mechanisms and the molecular basis of postdenervation muscle atrophy are still poorly understood. It is becoming increasingly apparent that the response of muscle to the loss of innervation is pleiotypic and includes many concurrent processes. Up to the present time, the analysis of the dynamic changes in denervated skeletal muscle has been limited to the characterization of single phenomena, and the causal interrelations of different events involved in the response of muscle to the loss of nerve control are still poorly understood. For example, despite the general belief that the majority of denervated muscle fibers survive after extended denervation (for an historic retrospect, see Sunderland and Ray, 1950; Schmalbruch et al., 1991), studies of postdenervation muscle atrophy in amphibia and mammals have shown that cell death is a significant component in the pathogenesis of postdenervation muscle atrophy (Anzil and Wernig, 1989; Schmalbruch and Lewis, 1994; Borisov and Carlson, 2000). In the present study, we examined the issue of whether myogenesis in denervated muscle is activated in response to the loss of muscle fibers or in response to the process of their progressive atrophy. We also investigated the myogenic response with special reference to both of these processes. The interrelations of muscle fiber atrophy, cell death, and reactivation of myogenesis still remain poorly understood. The structural dynamics of the myogenic response also have not been defined as yet in long-term denervated skeletal muscle.

For this reason, it is of interest to test the hypothesis that the myogenesis in denervated muscle is activated when muscle fibers attain a certain critical levels of atrophy and/or destabilization of the terminally differentiated state. Taking into account that regeneration is usually activated in response to the loss of muscle tissue, we investigated the temporal and spatial patterns of myogenic activity in denervated muscle with special reference to the occurrence of cell death. Earlier we have shown that progressive atrophy of muscle fibers is accompanied by extensive remodeling of the vascular bed in denervated muscle that included rapid progressive degeneration of capillaries (Borisov et al., 2000). To this end, it was also interesting to investigate whether the loss of muscle fibers and the myogenic response correlated with the dynamics of the impairment of vascular supply and devascularization of the tissue. Studies of the interrelations of the regenerative and degenerative processes in denervated muscle would provide a clearer understanding of the control mechanisms underlying the pathogenesis of postdenervation muscle atrophy at the cellular level. Thus, the purpose of the present study was to investigate the interrelations of reactivated myogenesis, progressive atrophy, destabilization of the differentiated phenotype, and death of muscle fibers in long-term denervated tibialis anterior and extensor digitorum longus muscles of the rat.

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 denervated by sectioning the sciatic nerve high in the thigh, suturing proximal and distal stumps, and implanting the proximal stump into muscles of the hip 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 euthanized under ether anesthesia 10 days, 1, 2, 4, 7, 12, 14, and 18 months after denervation.

Experimental Design

Tissue samples of the extensor digitorum longus and tibialis anterior muscles were taken from 4 control animals and from 3 experimental animals for each time point. Each muscle was divided into two parts, cryoprotected in sucrose solution, and embedded for transverse and longitudinal sectioning in cryoprotective freezing medium TBS (Triangle Biomedical Sciences, Durham, NC) for immunohistochemical and histological studies. Cross-sections were prepared through the equatorial area of each muscle and mounted in groups of four on histological slides. Three to five slides per each time point were studied from each animal.

Immunohistochemistry and Light Microscopy

After the fixative was washed out, the samples were sectioned into 8-μm longitudinal and cross-sections and immunostained using the technique of indirect immunofluorescence. Primary antibodies to isomyosins and laminin were obtained from the Developmental Studies Hybrydoma Bank (University of Iowa, Iowa City, IA), Chemicon International Inc. (Temecula, CA), American Type Culture Collection (Rockville, MD), and Novocastra Laboratories Ltd. (Newcastle upon Tyne, UK). Fluorescein-conjugated rabbit anti-mouse IgG (Sigma, St. Louis, MO) was used as a secondary antibody for the detection of binding of monoclonal antibodies. TRITC-conjugated goat anti-rabbit IgG (Sigma) was used as a secondary antibody for visualization of binding of polyclonal primary rabbit antibodies. The immunofluorescent reaction was examined with a Nikon Optiphot fluorescence microscope equipped with an excitation filter at 470–490 nm and emission barrier filter at 510–560-nm wavelengths for fluorescein and an excitation filter at 510–560 nm and emission barrier filter at 590–615 nm for rhodamine.

Morphometry

Quantitative morphometric measurements of muscle fiber cross-sectional areas were performed from digital im-

ages of tissue sections immunostained for laminin and slow and embryonic isoforms of myosin. Images were collected, stored. and processed using Adobe Photoshop, version 5.5 software, and measurements were performed using the NIH Image, version 1.62 software program.

Electron Microscopy

Samples of muscle tissue were prefixed immediately after excision from the limb in an ice-cold solution of 2.5% glutaraldehyde and 4% formaldehyde in 0.1 M isoosmotic phosphate buffer, pH 7.4 for 1 hr. The tissue was then 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 postfixed with 1% OsO_4 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., Redding, CA). Ultrathin sections were prepared using a Reichert-Young Ultracut E ultramicrotome and mounted on copper/rhodium mesh grids M150-CR (Electron Microscopy Sciences, Fort Washington, PA). After staining with uranium acetate and lead citrate (Reynolds, 1963), the samples were examined with a Philips CM 100 electron microscope at an accelerating voltage of 60 kV.

RESULTS AND DISCUSSION

Heterogeneous Cellular Response to Denervation: Variability of Rates of Atrophy and Destabilization of the Differentiated State of Individual Muscle Fibers

An important characteristic of the response of skeletal muscle to denervation is considerable heterogeneity of the rates of atrophy of individual muscle fibers. This difference in rates of atrophy caused a polymorphism of sizes of muscle fibers that was already evident 4–5 weeks following denervation (Fig. 1a–d). Further progressive atrophy resulted in a dramatic increase in the range of variation of cross-sectional areas of muscle fibers in 2-month denervated (Fig. 1e,f) and 4-month denervated muscle (Fig. 1g,h). Both light microscopy (Fig. 1) and electron microscopic study (Fig. 2) clearly demonstrated a manifold difference in the sizes of individual fibers within the samples of denervated muscle tissue. As shown in Figure 2, in 4-month denervated muscle the differences of cross-sectional areas between large and small muscle fibers vary greatly, in the range of 4–17 times. These illustrations clearly demonstrate a different sensitivity of muscle fibers to postdenervation atrophy. Unlike denervated muscle, muscle fibers in control normal muscle are a considerably more homogeneous cell population as we observed both at light microscopic (Fig. 1a,b) and electron microscopic levels (see fig. 1a in Lu et al., 1997 and fig. 2a in Borisov et al., 2000).

One of the determinants underlying the rate and the magnitude of the atrophic process in denervated muscle is the type of muscle fiber. Fast and slow types of muscle fibers in the extensor digitorum longus and tibilalis anterior muscles have significantly different rates of atrophy. Slow fibers, as shown by double immunofluorescent staining for slow isoform of myosin and laminin, undergo significantly slower atrophy than do the fast fibers during the first 2 months following denervation (Fig. 1a–f). The levels of atrophy of individual muscle cells vary greatly within the population of fast fibers and are more homogeneous in the population of slow fibers. We found that 2 months following denervation, the levels of atrophy of fast muscle fibers on average are 3–5 times greater than the levels of atrophy of slow muscle fibers (Fig. 1e,f).

The low resistance of fast muscle fibers, in contrast to slow fibers, to denervation atrophy during the first 2 months may be explained by profound differences in their cell biology and a higher dependence of terminal differentiation of fast fibers on neural control. For example, slow and fast muscle fibers differ in the enzymatic activities of intracellular signaling pathways (Hoover et al., 2001). Interestingly, the differentiation of slow fibers in developing muscle, in contrast to fast fibers, can progress in the absence of innervation (Engel and Karpati, 1968). By 4 months, this difference is less dramatically expressed (Fig. 1g,h). Thus, the rate of atrophy of slow muscle fibers accelerates between months 2 and 4 following denervation. The factors controlling this acceleration of the atrophic process of slow fibers remain unclear. Computer-assisted morphometry has shown that cross-sectional areas of fast fibers in the tibialis anterior muscle decreased on average by 45, 69, and 88% from control levels by 1, 2, and 4 months after denervation, respectively (Dedkov et al., in press).

Another type of heterogeneity observed in all studied samples of denervated muscle is considerable variation in susceptibility of individual muscle fibers to destabilization of the differentiated phenotype. The process of postdenervation atrophy is accompanied by significant structural remodeling of muscle cells and a progressive loss of tissue-specific structures. Both immunohistochemical staining of longitudinal sections for sarcomeric proteins (Fig. 3a,b) and electron microscopy (Fig. 4a,b) have shown that the progressive loss of alignment of myofibrils, normally in register at the level of Z-bands typical of mature muscle, is one of the early structural changes in denervated muscle. This process was observed in both fast and slow muscle fibers and was more intense and rapid in fast muscle fibers than in slow fibers. Both fast and slow populations are heterogeneous in terms of the rate of disalignment of the striated pattern of myofibrils in individual fibers at early stages following denervation. The misalignment of the contactile system is followed by the loss of actin and myosin filaments, resorption of sarcomeres, formation of expanding myofibril-free zones, and the progressive loss of mitochondria. The progression and intensity of these processes also variy greatly in individual muscle fibers. We discussed earlier the ultrastructural characteristics and dynamics of atrophic and degenerative changes at different stages following denervation (Lu et al., 1997; Borisov et al., 2000), and for this reason will not illustrate them here. Interestingly enough, the loss of the tissue-specific structures is not necessarily associated with advanced levels of atrophy of individual fibers. We observed muscle cells with a large cross-sectional area that contained significantly disorganized contractile apparatus and relatively small fibers that retain a high stability of the differentiated phenotype. We also found that muscle fibers at very different levels of the atrophic process are susceptible to degeneration and death. The dead cells are sometimes located among well-differentiated non-atrophic or very slightly atrophic muscle fibers that express a high level of

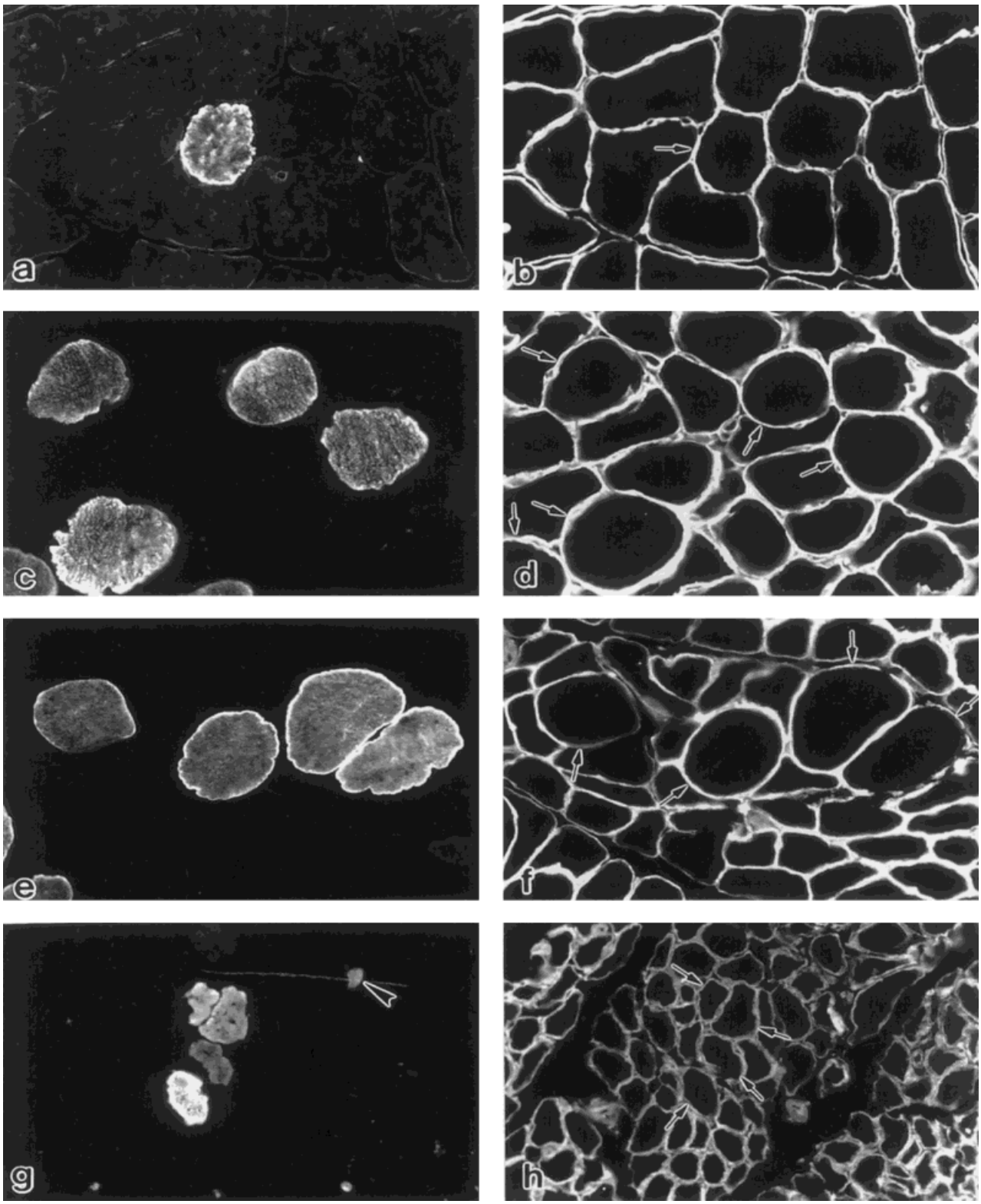

Fig. 1. Differential atrophy of slow and fast muscle fibers as seen in transversely sectioned tibialis anterior muscle. **a,b:** Normal innervated muscle (control); **c–e** and **f–h**: muscle denervated for 1, 2, and 4 months, respectively. Immunostaining for slow isomyosin shows the localization of type I fibers (a,c,e,g), and immunostaining for laminin in the same fields (b,d,f,h) shows the contours of both type I and type II muscle fibers. Arrows in b,d,f, and h indicate the localization of type I fibers shown in a,c,e, and g. The arrowhead in g shows the location of muscle fibers of a very small diameter. 220×.

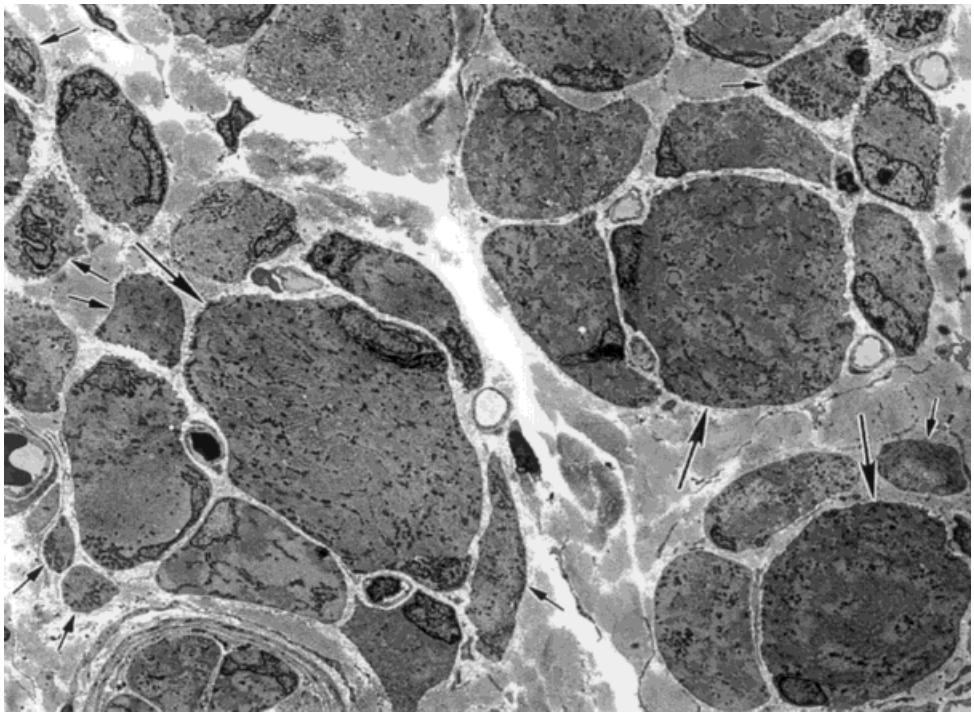

Fig. 2. Heterogeneity of atrophy of muscle cells in the extensor digitorum longus muscle 4 months following denervation. Note the multifold difference in the size of transversely sectioned individual fibers: large arrows indicate large fibers, and small arrows indicate small fibers. 1,650×.

stability of the differentiated state (Fig. 4b). This indicates that degeneration and death of many denervated muscle cells does not require high levels of atrophy or a significant loss of the differentiative markers.

A subclass of slow fibers of small diameter that comprised in some samples up to 10–14% of their population appears in the tissue 2 months after denervation (Fig.1g; see also Fig. 9e,f). These small fibers are intermingled among fibers of medium and large diameters and first appear with a rare incidence as early as 4–5 weeks following nerve transection. Both light and electron microscopic studies have shown that small muscle fibers are abundant at all stages of the atrophic process (Fig. 4c; see also Figs. 6a–e, 8a,b).

Taking into account that we first observed these fibers at stages when the atrophic process had not reached an advanced stage, we suggest that their presence may be explained by the reactivation of myogenesis following denervation. This suggestion is supported by the fact that a transient coexpression of the slow isoform of myosin and embryonic myosin has been reported during normal embryonic development in predominantly fast muscles (Narusawa et al., 1987). An alternative explanation is that small fibers represent a subpopulation of muscle cells easily susceptible to activation of a rapid atrophic process. To elucidate the origin of the small fibers and understand better their structural interrelations with surrounding large fibers, we used immunostaining for embryonic myosin and electron microscopy to study the time-course of the myogenic response and the nature of the stimuli triggering the myogenesis in denervated muscle.

Two Types of Myogenic Response in Denervated Muscle

We observed two distinct structural types of myogenic response that exhibit a different temporal presence during the postdenervation period. The first type of myogenesis resembles the formation of secondary and tertiary generations of myotubes in developing muscle. During this process, formation of new muscle fibers occurs on the surface of live muscle fibers (Fig. 4a,c; see also Figs. 6a–e, 8a). Double immunostaining of transverse and longitudinal sections of denervated muscle for embryonic isomyosin and laminin (Figs. 5a, 6a,b), as well as electron microscopy (Fig. 6c–e) clearly demonstrated that activation of myogenesis of the first type correlates with activation of satellite cells beneath the basal laminae. Interestingly, several daughter myotubes can develop at the same time on the surface of parent muscle fibers during this type of the myogenic response (Figs. 4c; 6c,d). Newly developed nascent myotubes located beneath the basal lamina of the parent muscle fibers usually form new basal laminae that progressively separate themselves from the parent muscle fibers (Fig. 6a,b). Single small myotubes can occasionally be seen as early as 10 days following denervation. This type of myogenesis is the most active between 2 and 4 months following nerve transection and then gradually decreases, possibly as a result of progressive exhaustion of the pool of satellite cells. By 2–4 months following nerve transection, the number of muscle fibers immunopositive for the embryonic isoform of myosin never exceeds 2–3% of the total number of fibers. After 7 months of denervation,

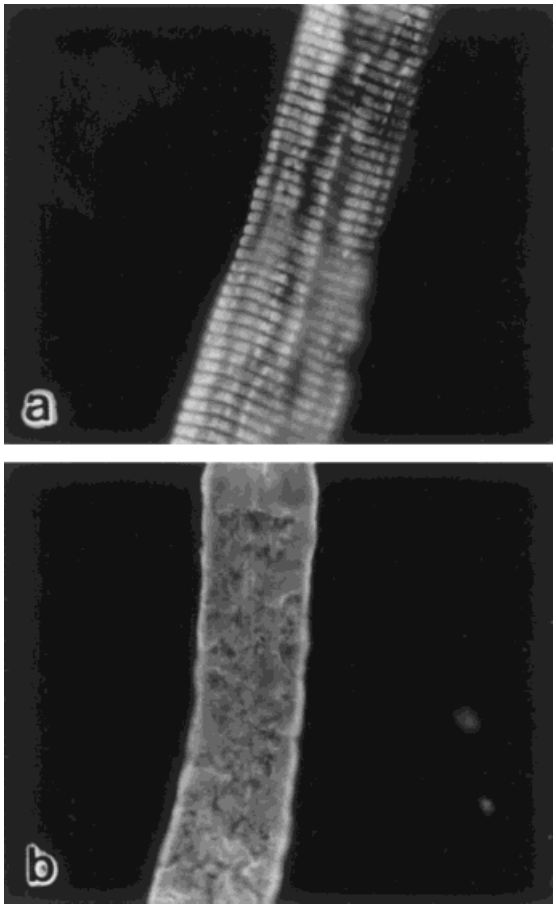

Fig. 3. Transition from a cross-striated well-registered pattern of myofibril alignment in innervated muscle (**a**) to diffuse misalignment of the contractile system 2 months following denervation (**b**).

the population of new myotubes did not exceed 1–1.5%, although it should be mentioned that we observed wide variation of myogenic response in different samples of long-term denervated muscle. At advanced stages of postdenervation atrophy, it was difficult to identify newly formed muscle fibers due to profound atrophy and dedifferentiation of the whole muscle cell population. We should note that in some cases it was difficult to determine precisely the origin of daughter muscle fibers at the light microscopic level. This difficulty occurred in the relatively rare cases when the newly formed myotubes are found between two or more fibers that are located at a very close distance to one another, and the basal lamina separating the parent and daughter fibers are still not formed. However, electron microscopic study allowed us to perform a reliable identification of parent and daughter muscle cells: nascent newly formed muscle fibers were located under the basal lamina of the parent fiber that was clearly visible at the ultrastructural level. To identify the pairs of parent and daughter muscle cells, we used the following morphological criteria: (1) close topographical association of two muscle cells of different size and immunopositivity of one of these cells for the embryonic isotype of myosin, (2) the presence of the common single basal lamina covering both cells that can be detected by immunostaining for laminin and by electron microscopy, (3) a low electron density of the cytoplasm, poor organization of the contractile system, and the presence of nascent myofibrils in daughter cells. Daughter muscle fibers sometimes develop the basal lamina that separates them from the parent fibers (Fig. 6a–c).

The second type of myogenic response is a typical reparative regenerative reaction that consists of the replacement of dead muscle fibers by newly formed muscle cells (Figs. 4b,d, 7a–c). As one can see from Figure 5b, the regenerative process typically occurs within the basal lamina of the degenerated muscle fibers. Activation of the second type of myogenesis occurs between months 2 and 4 of the postdenervation period when degeneration and death of muscle fibers become a contributing factor to the process of postdenervation atrophy. Single nascent muscle fibers (Fig. 5b)or their clusters (Figs. 4d, 7a–c) usually occupy empty spaces between muscle cells in areas where degenerated muscle fibers are located (Fig. 4b). An important structural characteristic of the regenerative process in denervated muscle is the formation of groups of muscle fibers or branching fibers that replace degenerated muscle fibers of a large size (Figs. 4d and 7a). In such cases, each of the new muscle fibers is frequently surrounded by a basal lamina that separates it from its siblings (Fig. 7b,c). The basal lamina of the degenerated muscle fiber does not undergo resorption for some period of time, and such newly regenerated fibers are easily identified by the presence of the double basal lamina (Fig. 7a–c).

The next question that we tried to answer was whether muscle fibers that developed as a result of the second type of myogenesis are able to activate the first type of myogenesis. It appears that regenerated muscle fibers can resume the first type of myogenesis. Some muscle fibers surrounded by a double basal lamina are topographically associated with daughter myotubes (Fig. 6c). We found instances of parent muscle fibers at advanced levels of differentiation that were located close to very small daughter fibers and were surrounded by the double basal lamina (Fig. 6c). Centrally located nuclei (Fig. 6c–e) typical of regenerating fibers show an incomplete terminal differentiation of these cells, whereas the absence of the basal laminae between the parent and daughter muscle fibers may indicate the presence of the first type of myogenesis. At the same time, such structural interrelations between big and small muscle fibers within the double basal lamina may reflect different differentiative properties of myotubes that form during the regenerative process rather than represent fibers that belong to different generations. It was also of interest to investigate the occurrence of intermediate or combined types of myogenesis in denervated muscle in addition to the types described above. Here we should note that formation of several muscle fibers surrounded by a common basal lamina results, at least in some cases, from the first type of myogenic response, rather than the second type. For example, if new myotubes located at a close distance to each other on the parent muscle fiber (e.g., Figs. 4c, 6c,d) continue to grow and differentiate and the parent muscle fiber subsequently dies, the daughter fibers can occupy the empty basal lamina. This process may occur without activation of the regenerative process and formation of new muscle fibers following cell death. On the other hand, it may

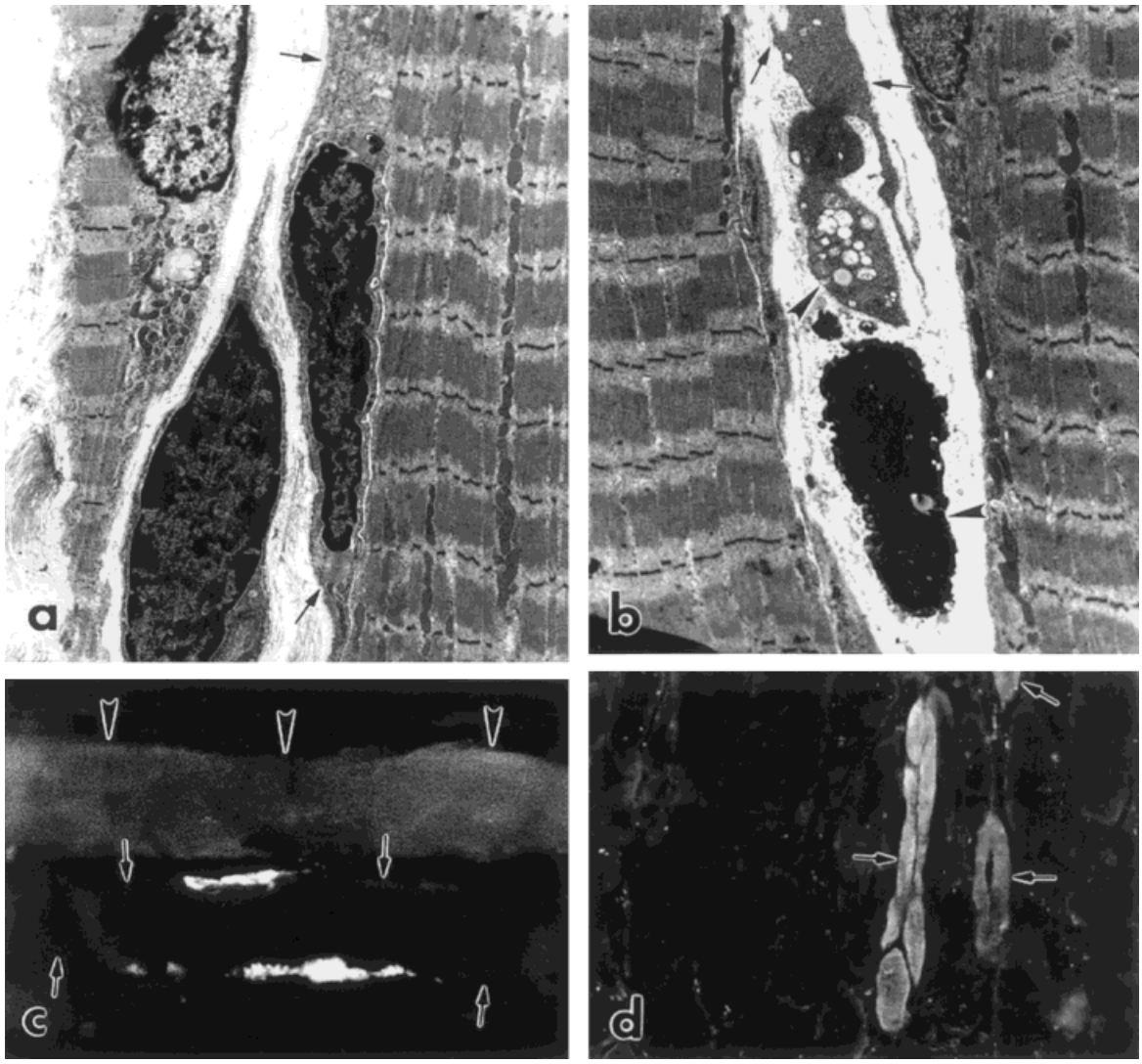

Fig. 4. Activation of myogenesis and cell death in denervated muscle. **a:** Formation of cytoplasmic processes and cytoplasmic growth in an activated satellite cell (arrows) in 2-month denervated EDL muscle. Note that the satellite cell is associated with a muscle fiber that retains a high level of differentiation. **b:** Degenerated muscle fiber localized between two muscle fibers retaining a high level of differentiation in 2-month denervated EDL muscle; arrows show the localization of fragments of the basal lamina; arrowheads show fragmented remnants of a dead muscle cell. **c:** Immunofluorescent staining for embryonic isomyosin demonstrates activation of myogenesis on live muscle fibers in longitudinally sectioned tibialis anterior muscle denervated for 2 months; arrows indicate the boundaries of the parent fiber that contacts two nascent myotubes formed by its satellite cells; arrowheads show the muscle fiber that is weakly immunopositive for the embryonic protein but does not reactivate myogenesis. **d:** Area of longitudinal section that shows the regeneration of individual muscle fibers: arrows indicate compact clusters of small muscle fibers expressing the embryonic isomyosin. A transverse section of such a cluster is presented in Figure 7. Such groups usually form at the sites of localization of dead parent muscle fibers. Note the absence of the myogenic response in the areas surrounding the focus of regeneration shown in d. a,b: 2,600×; **c,d:** 208×.

include additional rounds of myogenesis if additional amounts of activated satellite cells are available. The temporal superimposition of the myogenic response of the first type and progressive reactivation of myogenesis of the second type contributed to the peak of myotube formation observed in muscle tissue between 2 and 4 months following denervation. Thus, our data show that myogenesis in denervated muscle is biphasic and includes two different structural types of myogenic response that have different temporal patterns of expression during the postdenervation period.

Interrelations of the Myogenic Response, Instability of the Differentiated State, Progressive Atrophy and Death of Muscle Fibers

To understand better the regulatory cellular mechanisms that control the interrelations of degenerative and regenerative processes during postdenervation atrophy, we addressed the following four questions: (1) whether the reactivation of myogenesis in denervated muscle was triggered by progressive atrophy of muscle fibers; (2) whether

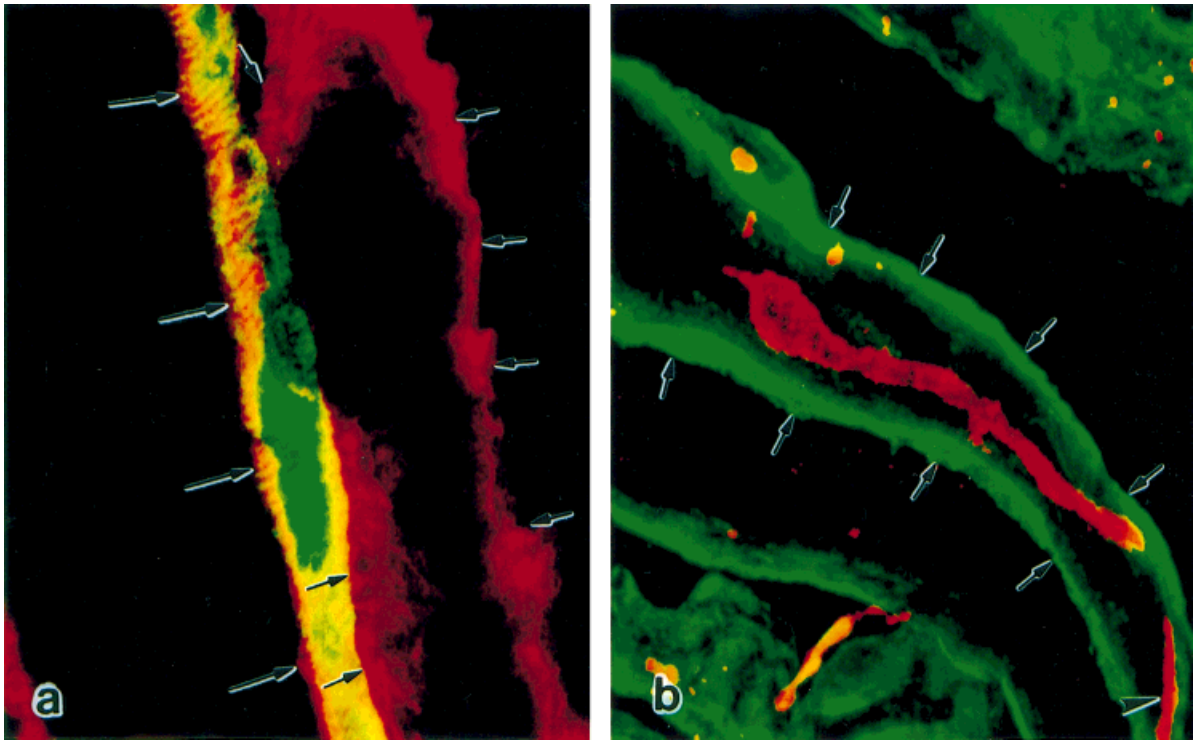

Fig. 5. Confocal images of longitudinal sections of 2-month denervated tibialis anterior muscle illustrating two structural types of myogenesis typical of denervated skeletal muscle. **a:** Formation of muscle fibers on the surface of parent muscle fibers; double indirect immunostaining for embryonic isomyosin (green fluorescence) and laminin (red fluorescence). Note the presence of two basal laminae, one of which covers the surface of the daughter fiber (four long arrows) and the other separates the parent and daughter muscle fibers (three small arrows); four short arrows indicate the basal lamina on the other boundary of the parent muscle fiber. **b:** Formation of nascent muscle fibers within the basal lamina; double indirect immunostaining for NCAM (red fluorescence) and laminin (green fluorescence). Arrowhead in the lower right corner indicates one more cell immunopositive for NCAM (red fluorescence). Small arrows in b indicate the basal lamina of a muscle fiber.

the myogenic response is a "rescue" mechanism activated when muscle fibers attain certain critical levels of atrophy, such as the loss of one fifth, one third, one half, or more of their normal volume, (3) whether a more or less significant destabilization of the differentiated state of muscle fibers is correlated with the reactivation of myogenesis, and (4) whether the myogenic response depends on the functional type of muscle fibers.

We found that reactivation of myogenesis during the first weeks following denervation occurred earlier than any visible structural manifestations of cell atrophy and cell death in denervated muscle. At this stage, the response is represented by the first type of myogenesis described above. This process does not require any significant destabilization of the differentiated phenotype of muscle fibers. As shown in Figures 4a,c and 6a–c, activation of myoblasts and formation of new myotubes usually takes place on the surface of well-differentiated muscle cells that retain a high level of structural organization of the contractile apparatus. Unlike inactive satellite cells in control innervated tissue, activated myoblasts in denervated muscle have a significantly increased volume of cytoplasm and form elongated cytoplasmic processes (Fig. 4a). At advanced stages of the atrophic process, in 2- and 4-month denervated muscle, we observed the development of new myotubes on the surface of fibers that express very different levels of atrophy and destabilization of differentiated characteristics (Fig. 6a–e). Studies of longitudinal sections have shown that fibers carrying myotubes on their surface can be located very close to nonresponding fibers that possess slightly lower or comparable levels of atrophy (Figs. 4c, 6a,b, 8a,b, 9a,b). Measurements of levels of atrophy of parent muscle fibers topographically associated with newly formed daughter muscle fibers show that the reactivation of myogenesis occurs predominantly on the cells representing the average level of atrophy in the denervated population (Figs. 10, 11). Fibers with higher than the average levels of atrophy are also involved in the myogenic response, whereas non-atrophic or slightly atrophic fibers activate myogenesis with a lower incidence. Of particular interest is the fact that fibers at very different levels of atrophy, having lost from 10 to 80% of the cross-sectional area of typical control non-atrophic fibers, are involved in reactivation of the myogenic response on their surface. Thus, we observed no strict correlation among the degree of fiber atrophy, the level of destabilization of the differentiated phenotype, the capacity of satellite cells to activate myogenesis, and the intensity of the first type of myogenic response. Due to severe atrophy, destabilization of the differentiated phenotype and progressive degenerative changes in muscle fibers in 4-month denervated muscle (compare Figs. 1a–f and g,h) it was more difficult to establish the parent-daughter relationship between muscle fibers at later stages following denervation. For

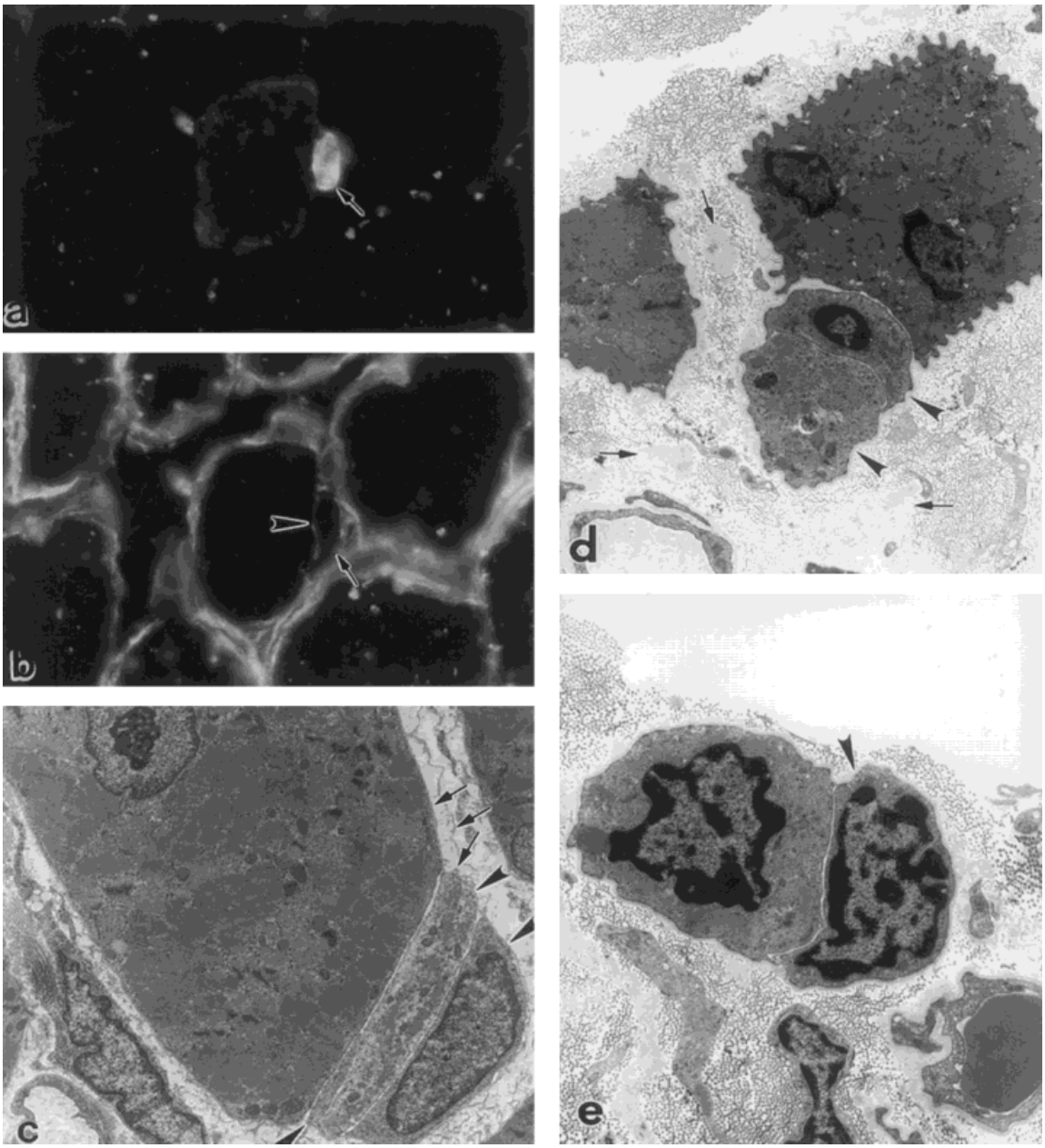

Fig. 6. Formation of new muscle fibers on live parent fibers in denervated muscle. **a:** Cross-section of the tibialis anterior muscle immunostained for the embryonic isomyosin 2 months following nerve transection; arrow indicates the daughter muscle fiber that formed on a parent muscle fiber; **b:** immunofluorescent staining for laminin in the same field shows that the nascent daughter fiber is located beneath the basal lamina of the parent fiber (arrow) and develops the basal lamina (arrowhead) that separates it from the parent fiber. **c–e:** Electron microphotographs illustrating ultrastructure of nascent muscle fibers that appear to correspond to the immunofluorescent images shown in Figures 4c,d, 6a,b, and 7a–c; daughter myotube-like cells are located under the basal laminae (arrowheads) of parent muscle fibers in transversely sectioned extensor digitorum longus muscle 2 and 12 months following denervation, respectively. Note three daughter myotubes on the parent fiber shown in c and two fibers on the parent fiber shown in d; three arrows in d indicate three empty basal laminae that apparently belonged to degenerated muscle cells. Small arrows in c indicate the presence of the double basal lamina. Electron microscopy (c,d) clearly demonstrates that parent fibers do not express any structural manifestations of degeneration or severe atrophy; arrows show three small muscle fibers located under the basal lamina of the parent muscle fiber. e: Two small low-differentiated muscle fibers surrounded by the basal lamina.

this reason, we focused our analysis on 2-month denervated muscle.

Interestingly enough, muscle fibers at the same levels of atrophy frequently form daughter fibers of very different size (Fig. 10a–c). To this end, we investigated the capacity of satellite cells associated with muscle fibers at different levels of atrophy for formation of large and small daughter muscle fibers. A computer-assisted quantitative morpho-

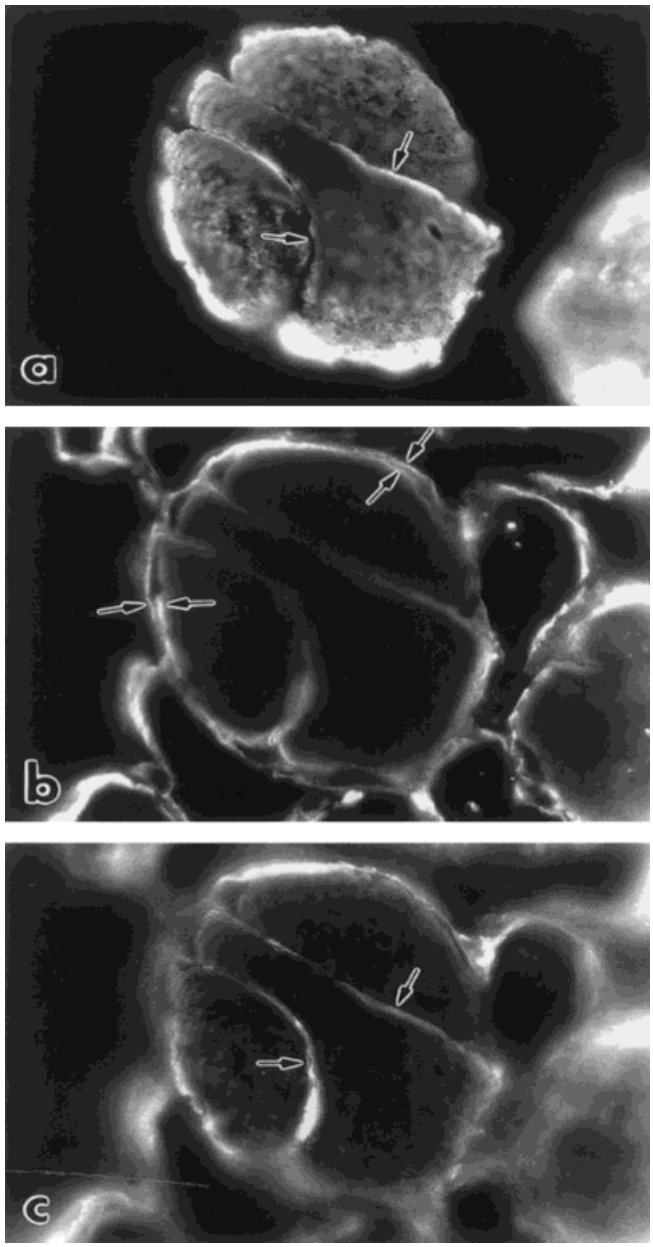

Fig. 7. Formation of new muscle fibers within the basal lamina of a degenerated muscle fiber. **a:** Immunofluorescent staining for slow isomyosin shows three muscle fibers or three branches of the same fiber; arrows indicate boundaries of the fibers in the transverse section. **b,c:** Immunofluorescent staining for laminin in the same field taken at two different focal planes; duplicated basal lamina (arrows) is clearly seen in b, and c shows the basal laminae surrounding each of three muscle fibers. 575×.

metric analysis of sizes of the parent and daughter muscle fibers has shown that there is no direct correlation between the sizes of the parental and daughter muscle fibers (see Fig. 12). The differences in sizes of daughter muscle fibers may reflect either the differences in their level of differentiation or the production in denervated muscle of muscle fibers of distinct sizes. Our data show that, if the size of the daughter fibers reflects the level of their maturity, the capacity of daughter fibers for advanced differentiation does not depend on the level of atrophy of the parent fibers. An important methodological question in this part of our study was to investigate whether the plane of section affects the measurements of fiber sizes. Ultrastructural and immunohistochemical study of longitudinal and transverse sections of our samples for tapering of fibers towards their ends did not reveal any myotendinous and myomyous junctions in our material taken from the midbelly of the muscles. The analysis of serial sections of daughter and parent muscle fibers has shown that their dimensions were not affected by the plane of sectioning. Due to the restricted volume of this paper, we present only two pairs of neighboring serial sections (Figs. 8a–d and 9a–d) rather than the whole series. Our earlier studies of longitudinal sections and individual fibers isolated from denervated muscle also show that the diameters of the parent fibers do not change significantly (see figures in Viguie et al., 1997).

We found that the distribution of newly formed myotubes immunopositive for embryonic isomyosin occurred both in the peripheral and central regions of equatorial sections made through the mid-belly area of muscles at early stages following denervation. Electron microscopic study has shown that myogenesis is activated on muscle fibers that represent a wide spectrum of modulations of the differentiated phenotype and different degrees of destabilization of the differentiative characteristics (Fig. 6c–e). The population of responding cells includes well-differentiated muscle fibers that retain a high level of structural stability (Fig. 4a). Thus, activation of the first type of myogenesis at the early stages following nerve transection occurs before the onset of atrophy and before muscle cells start to degenerate and die.

The next question that we tried to answer was whether muscle cell death and activation of the second type of myogenic response (i.e., regenerative replacement of the lost fibers) correlated with a profound level of postdenervation atrophy. We found that muscle cell death was not limited to the subpopulation of severely atrophic fibers and that, in many instances, the remnants of dead cells were surrounded by muscle fibers that retained very high levels of stability of the differentiated state (Fig. 4b). More illustrations of moribund and dead muscle fibers of different size found in denervated rat skeletal muscle are presented in our earlier publications (Borisov et al., 2000; Borisov and Carlson, 2000). An interesting characteristic of the second type of myogenic response is the trend toward a less uniform distribution of regenerating muscle fibers at advanced stages of postdenervation atrophy. Examples of a heterogeneous topographical distribution of the regenerative response can already be found in 2-month denervated muscle. For example, in Figure 4d, one can see three regenerating fibers (arrows) located at a short distance, whereas the surrounding area does not show a regenerative response of either the first or the second type. Two fibers shown in Figure 4 d represent branching fibers or consist of fibers of smaller size. Interestingly, in 4-month denervated muscle and to a greater extent at later stages, foci of muscle fiber degeneration and regenerative myogenesis within a muscle are also intermingled with nonresponding zones. Since at these stages the topographical distribution of degenerative changes is also not uniform, we can suggest that such

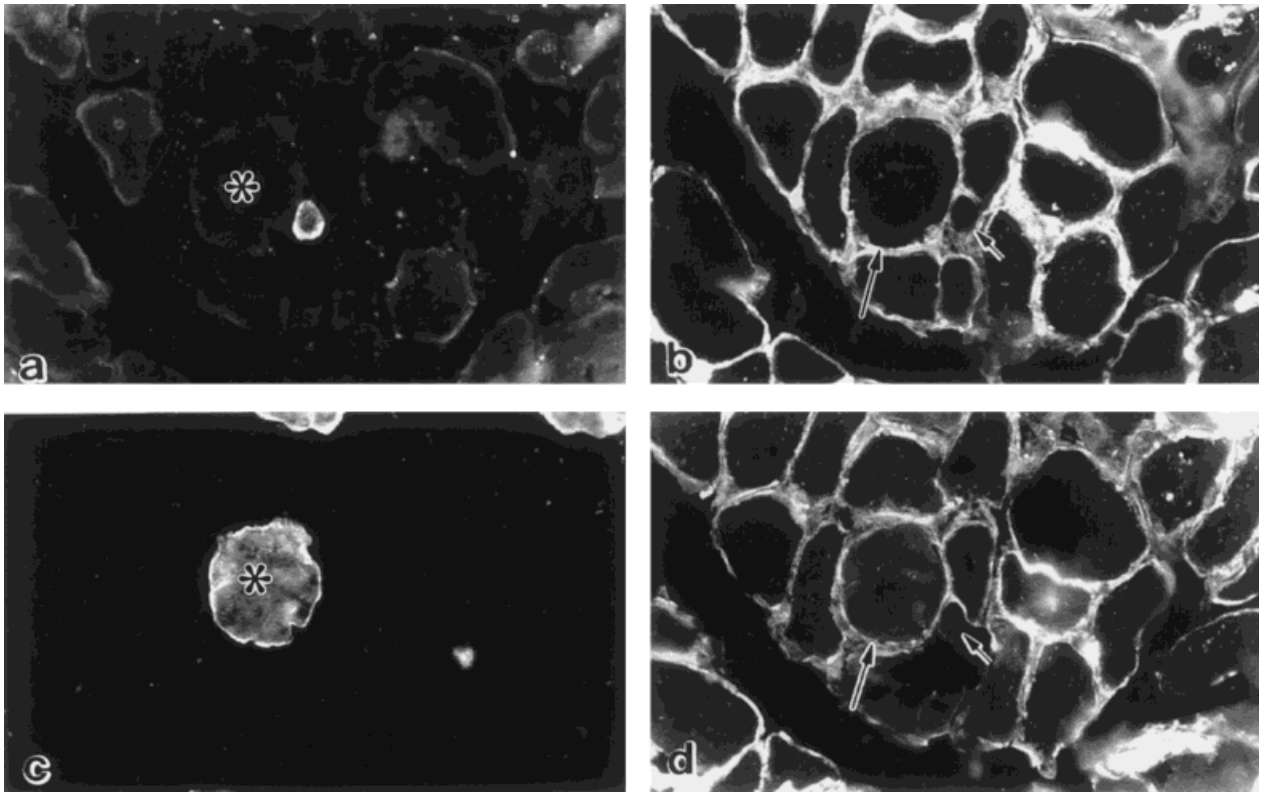

Fig. 8. Formation of daughter muscle fibers in close proximity to parent slow muscle fibers. **a:** Immunofluorescent staining for the embryonic isotype of myosin; **b:** immunostaining for laminin in the same field; **c:** immunostaining for slow isomyosin in the next serial section, **d:** the contours of muscle fibers visualized by immunostaining for laminin in the field shown in c. Arrow in d indicates the location of the slow fiber shown in c; small arrows in b and d indicate the location of the fiber immunopositive for embryonic isomyosin shown in a. a–d: 208×.

factors as different local trophic conditions and different levels of impairment of the vascular bed might explain this difference in the regenerative capacity. Earlier we have found that the vascular bed in denervated muscle underwent a dramatic remodeling during postdenervation period that included rapid progressive loss of capillaries (Borisov et al., 2000). Nonuniform pattern of degeneration of capillaries resulted in formation of avascular zones in the denervated tissue, and such foci of regional hypoxia may lead to death of muscle fibers and induce reactivation of the regenerative response.

Taking into account the different responses of slow and fast muscle fibers to denervation, it was interesting to compare their capacity for the myogenic response. For this purpose, we investigated the colocalization of expression of slow and fast muscle fibers and newly formed muscle fibers on adjacent serial sections of denervated muscle, using double indirect immunofluorescent staining for laminin and slow and embryonic isoforms of skeletal muscle myosin (Figs. 8a–d, 9a–d) We found that during the first weeks following denervation, newly formed myotubes immunopositive for embryonic isomyosin were associated predominantly with the fast type of muscle fibers (Fig. 9). Some slow fibers also formed daughter myotubes (Fig. 8), which indicates that they are also able to reactivate myogenic response following denervation. Fast muscle fibers in our samples of the tibialis anterior and extensor digitorum longus muscles comprised the majority (93–98%) of the muscle fiber population. This correlates well with the data available in the literature that reports the presence of 2.6–5% of slow fibers in the same rat muscles (Edström and Kugelberg, 1968; Eddinger et al., 1985; Staron et al., 1999). A precise quantitative analysis of the myogenic response of fast and slow muscle fibers proved difficult due to the small number of slow fibers in the tissue samples and a variability of the reaction from animal to animal.

Interestingly, a small subpopulation of fast muscle fibers was weakly or moderately immunopositive for embryonic myosin (Figs. 4c, 10 a), and this positive reaction was not correlated with the presence or absence of daughter myotubes on their surface. This phenomenon may be explained by a higher susceptibility of fast muscle fibers to destabilization of the differentiated state and partial dedifferentiation. A multifold range of variations of sizes of fast muscle fibers (Figs. 1c–h, 8c,d, 9c–f) also indicates that they possess a higher phenotypic plasticity and sensitivity of their differentiative properties in the absence of innervation than do slow fibers. Thus, taken together, these results indicate that the loss of neural control alone results in activation of satellite cells and their involvement in additional rounds of myogenesis.

The results of the present study indicate that the myogenic response in denervated muscle precedes the onset of any visible structural manifestations of advanced atrophy

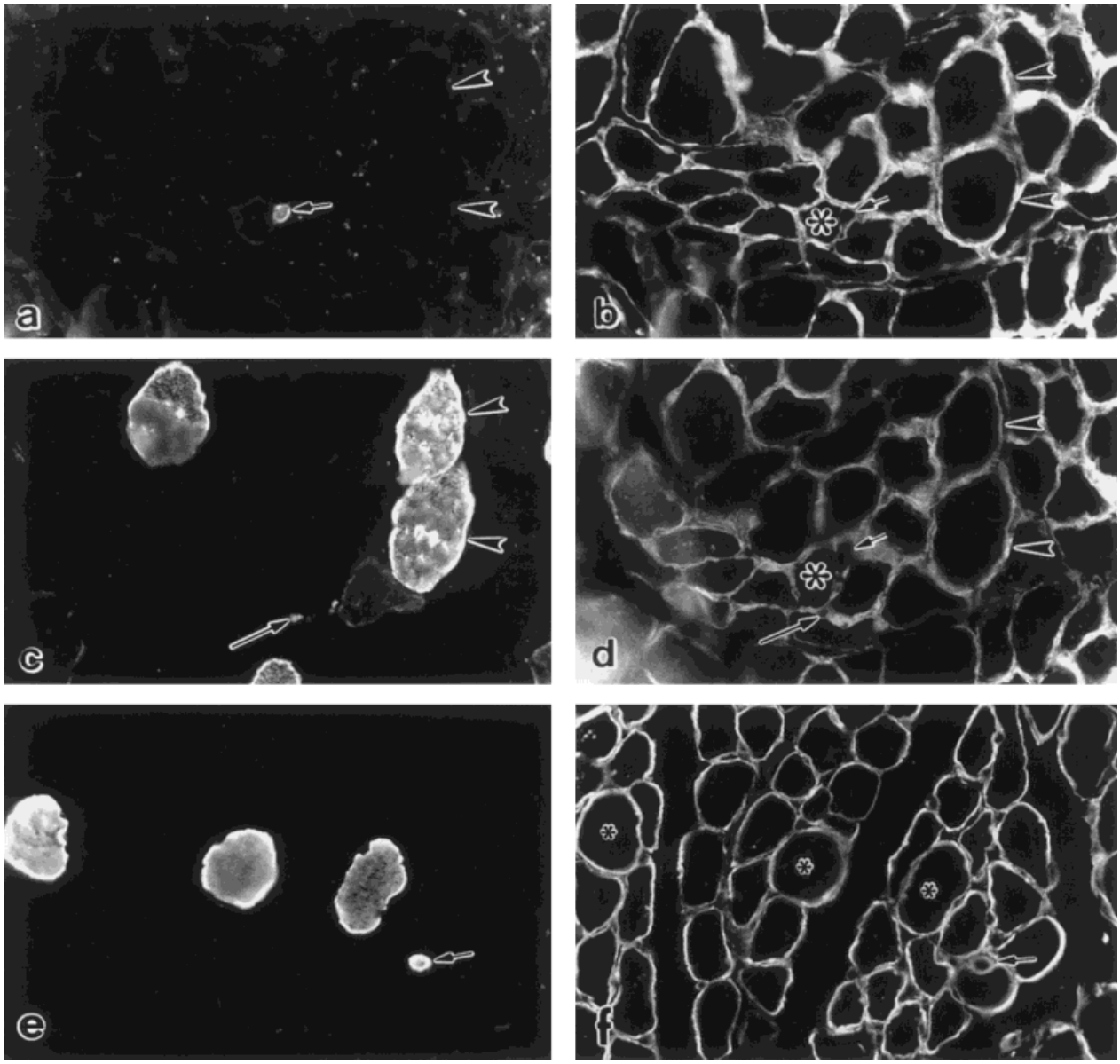

Fig. 9. Formation of daughter muscle fibers expressing the embryonic and slow isoforms of myosin on fast parent muscle fibers. **a:** Expression of embryonic isomyosin in a nascent muscle fiber. **b:** Localization of laminin in the same field shows the contours of muscle fibers. **c:** Same group of muscle fibers in the next serial section immunostained for slow isomyosin. **d:** Basal laminae of muscle fibers shown in c immunostained for laminin. Short arrows in a and b indicate the location of a muscle fiber expressing the embryonic isoform of myosin; arrowheads in a–d show the localization of slow muscle fibers; long arrows in c and d indicate the localization of a small muscle fiber expressing slow isomyosin; asterisks in b and d show the same fiber in two serial sections, which appears to be a parent fiber to the myotube expressing the embryonic isoform of myosin. This fiber is morphologically similar to the fiber shown in Figure 6a,b and, as seen at a higher magnification, develops the basal lamina separating it from the daughter fiber (not shown). **e,f:** Formation of slow fibers on parent fast fibers; arrow in e indicates a slow fiber of a very small diameter, and immunostaining for laminin demonstrates topographical association of this fiber with the parent fast fiber (arrow in f). 208×.

or destabilization of the differentiative characteristics of muscle fibers. Our data also show that, unlike the classical reparative regeneration, reactivation of myogenesis in denervated muscle does not require the death of muscle fibers. This indicates that the satellite cells appear to respond by initiation of myogenesis to the loss of the electric or trophic regulation of the motor nerve rather than to the loss of the mass of muscle tissue during the atrophic process of muscle fibers. Reactivation of myogenesis at early stages following denervation appears to be a systemic reaction of whole skeletal muscle to the loss of motor innervation rather than the response to local changes in trophic environment or changes in intercellular interactions in its different topographical regions. This conclusion is also supported by the central and peripheral distribution of newly formed myotubes within the trans-

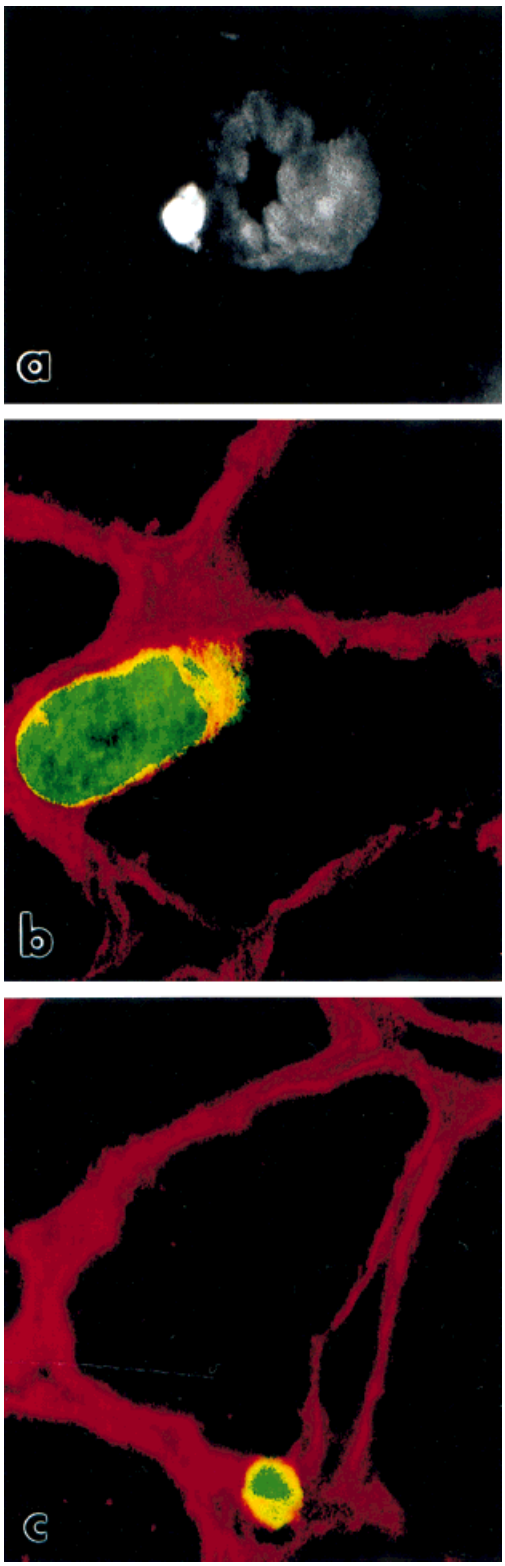

Fig. 10. Formation of daughter muscle fibers of different size on parent muscle fibers. **a:** Moderate level of expression of embryonic myosin in a parent muscle fiber. **b,c:** Parent fibers at the similar levels of atrophy form daughter fibers of different size.

verse mid-belly sections of denervated muscle and by the complete absence of foci of cell death and severe atrophy during this period.

Of special interest for our discussion is the nature of the signals that activate the myogenic response. It is important to mention that significant physiological changes in denervated muscle occur very rapidly, within the first few hours and days after cutting the motor nerve (for review see Vrbova et al., 1995). Among these changes are an immediate fall in resting membrane potential, changes in conductance of ions and ion channel characteristics, the induction of insulin resistance, changes in acetylcholine receptors, and the development of hypersensitivity to acetylcholine and insulin resistance. These changes result in profound physiological changes in the properties of denervated muscle. For this reason, one can suggest that satellite cells react to early changes in muscle fibers by activation of the myogenic response. It remains unclear why only some satellite cells are involved in this response on fast and slow muscle fibers, and what factors block the compensatory regenerative process in progressively atrophying denervated muscle. Among possible factors affecting asynchronous activation of satellite cells in denervated muscle are local heterogeneity of humoral and vascular supply or intrinsic heterogeneity of satellite cell population. Such an explanation is supported by recent observations concerning the heterogeneity of local capillary densities in denervated skeletal muscle (Borisov et al., 2000) and the different capacity of satellite cells of normal adult muscle to reactivate DNA synthesis in vitro (Schultz, 1996). The presence of nonresponding zones may also be explained by nonuniform distribution of growth-promoting molecules within the tissue.

In this study, we found that myogenesis in denervated muscle is presented by two structural types: (1) myogenesis on the surface of live intact muscle fibers that retain the structural characteristics of the differentiated phenotype and (2) regenerative reaction to replace dead muscle fibers. Of particular interest is that the structural characteristics of the first type of myogenic response in denervated muscle recapitulate the formation of new generations of secondary and tertiary muscle fibers during normal muscle development. The comparison of photographs illustrating the formation of new generations of secondary and tertiary myotubes in developing muscle presented by Duxson et al. (1989), Harris et al. (1989), Kelly and Rubinstein (1994), and McComas (1996) to our illustrations presented in this paper clearly shows a close similarity of these processes.

Regeneration following injury and formation of new muscle fibers during hypertrophy induced by functional overloading represent two classical examples of reactivation of myogenesis in adult skeletal muscle. During regeneration, activated myogenic satellite cells proliferate and differentiate into new muscle fibers replacing the lost necrotic tissue at the site of injury. The structural characteristics of myogenesis in overloaded muscle are different from the regenerative reaction. Unlike the regeneration, activation of this process does not appear to depend on or require cell death. At the same time, overloaded muscle activates cytoplasmic cell growth, and both preexisting and newly formed fibers increase their size to the levels that exceed the sizes of myocytes in the normal adult tissue. It is important to note that the first type of myogenesis that we found in denervated muscle is very

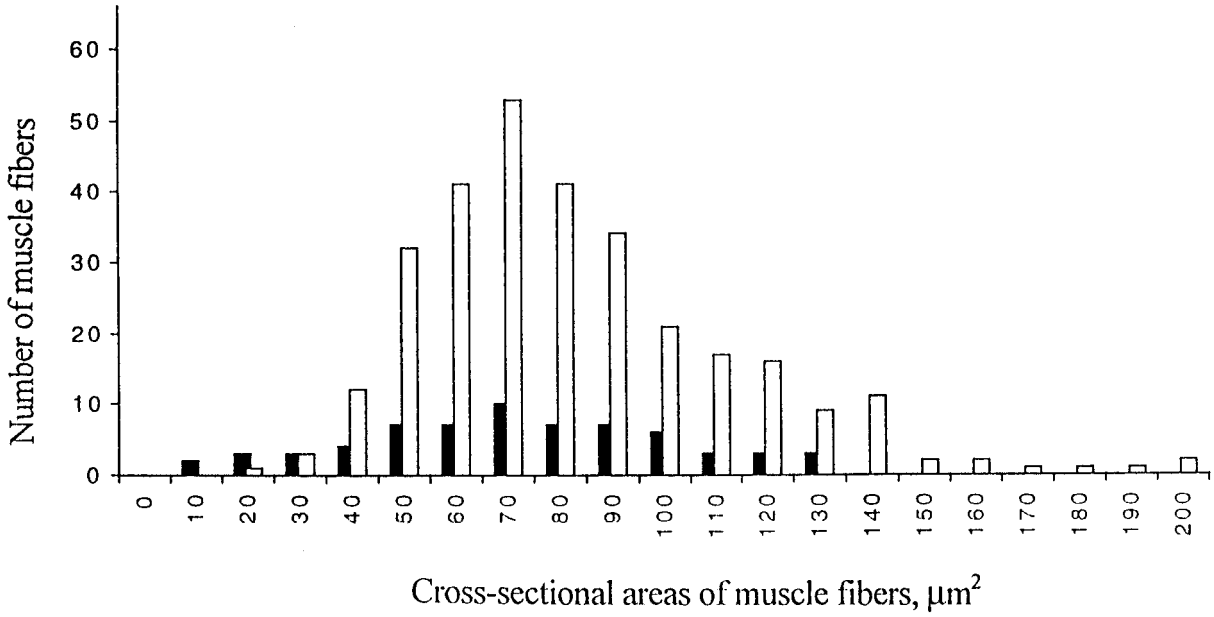

Fig. 11. Levels of atrophy of muscle fibers carrying daughter nascent fibers on their surface (black columns) and fibers that do not form daughter fibers (white columns) in 2-month denervated tibialis anterior muscle. Abscissa: Cross-sectional areas (CSA) of muscle fibers measured in μm^2.

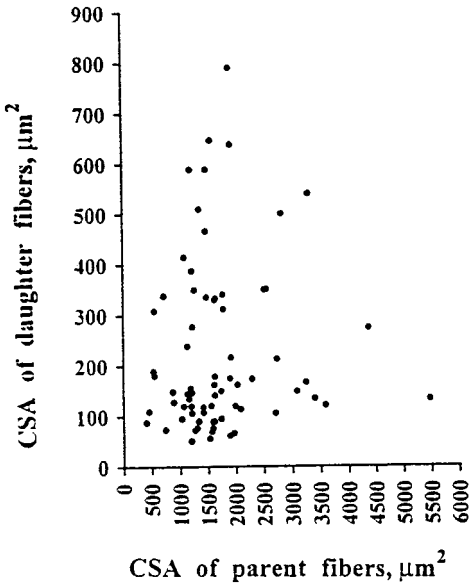

Fig. 12. Dependence of the sizes of daughter muscle fibers on the levels of atrophy of parental muscle fibers in 2-month denervated tibialis anterior muscle. Abscissa: Cross-sectional areas of the parent muscle fibers. Ordinate: Cross sectional areas of daughter muscle fibers associated with parent muscle fibers. Cross sectional areas (CSA) have been measured in μm^2.

similar to the mechanism of formation of new muscle fibers described during hypertrophy of overloaded muscle. Interestingly enough, denervated muscle activates both of these mechanisms: the mechanism typical of overloaded muscle at early stages and the typical regenerative reaction at later stages following denervation. Thus, under different functional conditions skeletal muscle can activate different types of compensatory response.

Of special interest is also the similarity of the first type of new fiber formation to secondary myogenesis during development. On the one hand, there are indications that in embryonic muscle secondary myotubes develop in association with neuromuscular junctions (Duxson et al., 1989). On the other hand, there is evidence that secondary myotubes are distributed all along the lengths of primary myotubes without any visible topographical association with the synapses (Ontell et al., 1988; Hughes et al., 1992). In our material, we found that formation of new myotubes frequently occurs outside the areas of the neuromuscular junctions. This may indicate that the cellular mechanisms of formation of new generations of muscle fibers are not identical in the embryonic muscle and adult denervated muscle.

The data concerning the regenerative process in denervated muscle are fragmentary and contradictory. Several studies have been focused on the changes of the number of myogenic satellite cells in denervated muscle. Satellite cells are the reserve of myoblasts for muscle regeneration, and for this reason their number reflects the regenerative potential of the tissue. On the other hand, a numeric increase of the satellite cell pool may be considered as an indirect characteristic of activated regenerative status of the tissue. The common consensus is that there is an increase in the number of satellite cells following denervation. Ontell (1974, 1975) reported a 5–6-fold increase in number of satellite cells following denervation, and Murray and Robbins (1982) reported reactivation of DNA synthesis in satellite cells in denervated muscle. Snow (1983) found that in mouse muscle the number of satellite cells increased 4 weeks following denervation and then re-

turned to the control level by 9–10 weeks following denervation. This author also described the presence of regenerating fibers 4 weeks following nerve transection. Schmalbruch and Lewis (1994) reported that denervation of the rat soleus muscle for 70 days increased the percentage of satellite cells in some muscles and in others caused a decrease to half of normal. They also observed ultrastructural evidence of a regenerative process in muscles denervated for 6–10 months. Viguie et al. (1997) and Lu et al. (1997) found an activation and progressive 4-fold increase in the number of satellite cells in the rat extensor digitorum longus muscle by 2 months following denervation and then described a progressive decrease of the satellite cell number. At the present time, the mechanisms that activate the myogenic response, the time course of involvement of satellite cells in myogenesis, and the relation of the regenerative process to cell atrophy in long-term denervated muscle remains unclear.

The results of biochemical and immunocytochemical studies of the expression of the embryonic isoforms of contractile proteins are controversial. Different researchers used anatomically different muscles of taxonomically different species taken at various stages after denervation. Some authors found that denervated skeletal muscles of adult chicken and rats expressed only adult isotypes of myosin heavy chains, and no re-expression of embryonic isoforms was found (Matsuda et al., 1984; Carraro et al., 1985). At the same time, Matsuda and coworkers (1984) observed that the expression of tropomyosin, troponin, and myosin light chain in chicken pectoral muscle reverted to embryonic isotypes. Despite the absence of any biochemically detectable expression of embryonic myosin, Carraro et al. (1985) found the morphological evidence of muscle regeneration in chronically denervated rat hemidiaphragm. Others reported the presence of neonatal and embryonic isoforms of myosin following denervation (Schiaffino et al., 1988; Jakubiec-Puka, 1992), and our observations support these data. However, none of these studies concentrated on the structural aspects of myogenesis during long-term denervation and on the interrelations of this process with the dynamics and topographic characteristics of the atrophic process in denervated muscle fibers. An interesting characteristic of the regenerative process in denervated muscle is the formation of branching fibers or clusters of small fibers covered by the same basal lamina (Figs. 4d and 7a–c). This phenomenon can be explained by the presence of mechanical obstacles for normal myogenesis within the space under the basal lamina such as collagen fibers or incompletely resorbed fragments of degenerated muscle fibers.

The current understanding of the cellular kinetics and myogenesis in denervated muscle is that this process appears to represent repeated cycles of degeneration and regeneration (Schmalbruch et al., 1991; Schmalbruch and Lewis, 1994). Ultrastructural studies of the rat denervated soleus muscle led Schmalbruch and Lewis (1994) to the conclusion that the slow and non-synchronized breakdown of denervated soleus muscle fibers induces proliferation of satellite cells as in necrotized muscles. In the present study, we have shown that the loss of the ability to control the initiation of additional rounds of myogenesis on the surface of parent muscle fibers is an important characteristic of the myogenic events in long-term denervated extensor digitorum longus and tibialis anterior muscles. Thus, the response of muscle to the loss of regulatory support of the nerve results in the loss of control over activation of satellite cells and recapitulation of the type of myogenesis typically observed in the embryonic muscle.

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