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Cell Death in Denervated Skeletal Muscle Is Distinct From Classical Apoptosis

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ABSTRACT

Denervation of skeletal muscle is followed by the progressive loss of tissue mass and impairment of its functional properties. The purpose of the present study was to investigate the occurrence of cell death and its mechanism in rat skeletal muscle undergoing post-denervation atrophy. We studied the expression of specific markers of apoptosis and necrosis in experimentally denervated tibialis anterior, extensor digitorum longus and soleus muscles of adult rats. Fluorescent staining of nuclear DNA with propidium iodide revealed the presence of nuclei with hypercondensed chromatin and fragmented nuclei typical of apoptotic cells in the muscle tissue 2, 4 and to a lesser extent 7 months after denervation. This finding was supported by electron microscopy of the denervated muscle. We found clear morphological manifestations of muscle cell death, with ultrastructural characteristics very similar if not identical to those considered as nuclear and cytoplasmic markers of apoptosis. With increasing time of denervation, progressive destabilization of the differentiated phenotype of muscle cells was observed. It included disalignment and spatial disorganization of myofibrils as well as their resorption and formation of myofibril-free zones. These changes initially appeared in subsarcolemmal areas around myonuclei, and by 4 months following nerve transection they were spread throughout the sarcoplasm. Despite an increased number of residual bodies and secondary lysosomes in denervated muscle, we did not find any evidence of involvement of autophagocytosis in the resorption of the contractile system. Dead muscle fibers were usually surrounded by a folded intact basal lamina; they had an intact sarcolemma and highly condensed chromatin and sarcoplasm. Folds of the basal lamina around the dead cells resulted from significant shrinkage of cell volume. Macrophages were occasionally found in close proximity to dead myocytes. We detected no manifestations of inflammation in the denervated tissue. Single myocytes expressing traits of the necrotic phenotype were very rare. A search for another marker of apoptosis, nuclear DNA fragmentation, using terminal deoxyribonucleotidyl transferase mediated dUTP nick end labeling (the TUNEL method) in situ, revealed the presence of multiple DNA fragments in cell nuclei in only a very small number of cell nuclei in 2 and 4 month denervated muscle and to less extent in 7 month denervated muscle. Virtually no TUNEL reactivity was found in normal muscle. Double labeling of tissue denervated for 2 and 4 months for genome fragmentation with the TUNEL method and for total nuclear DNA with propidium iodide demonstrated co-localization of the TUNEL-positive fragmented DNA in some of the nuclei containing condensed chromatin and in fragmented nuclei. However, the numbers of nuclei of abnormal morphology containing condensed and/or irregular patterns of chromatin distribution, as revealed by DNA staining and electron microscopy, exceeded by 33–38 times the numbers of nuclei positive for the TUNEL reaction. Thus, we found a discrepancy between the frequences of expression of morphological markers of apoptosis and DNA fragmentation in denervated muscle. This provides evidence that fragmentation of the genomic DNA is not an obligatory event during atrophy and death of muscle cells, or, alternatively, it may occur only for a short period of time during this process. Unlike classical apoptosis described in mammalian thymocytes and lymphoid cells, non-inflammatory death of muscle fibers in denervated muscle occurs a long time after the removal of myotrophic influence of the nerve and is preceded by the progressive imbalance of the state of terminal differentiation. Our results indicate that apoptosis appears to be represented by a number of distinct isotypes in animals belonging to different taxonomic groups and in different cell lineages of the same organism. Anat Rec 258:305–318, 2000.

Key words: skeletal muscle; denervation; cell death; apoptosis; muscle fibers

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Innervation is one of the important factors controlling the process of terminal differentiation of skeletal muscle cells (for reviews see Pette and Vrbova, 1985; Grinnell, 1994; Vrbova et al., 1995). Denervation of adult muscle is followed by impairment of its functional capacity. For several months after denervation, muscle tissue undergoes atrophy, losing up to 80% of its mass (Gutmann and Zelena, 1962; Hnik, 1962). Early and recent morphological studies have shown that the structure of denervated muscle undergoes significant changes, the most dramatic of which is a progressive atrophy of muscle fibers (Tower, 1935; Sunderland and Ray, 1950; Gutmann and Zelena, 1962; Pellegrino and Franzini, 1963; Miledi and Slater, 1969; Gauthier and Dunn, 1973; Stonnington and Engel, 1973; Mussini et al., 1987; Anzil and Wernig, 1989; Schmalbruch et al., 1991; Schmalbruch and Lewis, 1994; de Castro Rodrigues and Schmalbruch, 1995). However, despite a large number of descriptive publications concerning the gross and microscopical morphology of denervated muscle, the cellular mechanisms and molecular basis of post-denervation muscle atrophy still remain unknown.

For this reason it is important to study the involvement of cell death in the pathogenesis of post-denervation muscle atrophy. Understanding the mechanism of cell loss in denervated muscle requires differential recognition of apoptotic and necrotic types of cell death. Apoptosis is a fundamental component of normal histogenesis and cell turnover in developing and terminally differentiated tissues and organs (review Steller, 1995). This process has been described in different cell types of a number of invertebrate and vertebrate species, which indicates its early appearance during the course of evolution (Vaux et al., 1994). Necrosis is widely observed in different kinds of pathology, but, unlike apoptosis, it is underlined by irreversible cell injury, rather than by a specific pattern of differential gene expression (for comprehensive bibliography see reviews Wyllie, 1987; Walker et al., 1988). Apoptosis can be activated under a number of experimental and pathological conditions such as changes of hormonal background or deprivation of growth and trophic factors (review Williams and Smith, 1993; Thompson, 1995). The major criteria for identification of apoptotic cells are the following: 1) aggregation and condensation of chromatin with subsequent lobulation and fragmentation of nuclei; 2) increase in density and shrinkage of cytoplasm and frequently observed blebbing of the cell surface; 3) internucleosomal cleavage of DNA by specific endonuclease into multiple fragments with free 3′-OH ends. To this end, the combined use of several independent markers of this process is especially important for its reliable identification.

Condensation of chromatin and nuclear fragmentation can be detected cytochemically after staining of cell nuclei with fluorescent DNA-binding dyes (for literature see Lazebnik et al., 1993; Arends and Harrison, 1994). A significant reduction in cell volume and a dramatic increase of cell density was already observed in early ultrastructural studies of apoptosis; that is why this type of cell death was initially referred to as "shrinkage necrosis" (Kerr, 1971; Kerr et al., 1972; Arends and Wyllie, 1991). Electron microscopy still remains the most reliable method for identification of the structural hallmarks of apoptotic cell death and is instrumental in the differential diagnosis of apoptotic vs. necrotic types of cell death. This is especially important when different types of cell death occur in tissue simultaneously. Ultrastructural study also provides information concerning the differentiated characteristics of cells and fine details of cell structure, thus allowing one to identify the histotype of apoptotic cells in heterogeneous cell populations. In muscle, it ensures reliable identification of muscle fibers, perimysial and endomysial connective tissue and cells comprising the blood vessels.

Another distinctive feature of apoptosis is fragmentation of the genomic DNA into short sequences consisting of 180–200 base pairs with free 3′-OH ends. These fragments accumulate in the nuclei of apoptotic cells, and their presence can be revealed by DNA electrophoresis in agarose gels as a characteristic "ladder" pattern (reviewed by Arends and Harrison, 1994) or by the TUNEL (or *T*erminal deoxynucleotydil transferase-mediated d*U*TP *N*ick *E*nd *L*abeling) reaction in situ (Gavrieli et al., 1992; Wijsman et al., 1993; Gold et al., 1994).

The booming interest in apoptotic cell death that developed in recent years resulted in quick and wide application of these techniques into research practice and a mass outburst of publications claiming the presence of apoptosis in very different tissues under different experimental conditions based only on the use of DNA electrophoresis or free-end DNA labeling in situ. However, a number of recent reports raise doubts about the validity of using DNA fragmentation as the sole criterion to detect apoptosis and, therefore, reliably classify the type of cell death. From one hand, even in well-characterized models of apoptosis, including primary culture of lymphocytes, DNA fragmentation can occur non-specifically during necrosis and without the expression of any structural markers of the apoptotic phenotype (Collins et al., 1992; Ansari et al., 1993; Garcia-Martinez et al., 1993) or even as an artifact (Enright et al., 1994). Internucleosomal DNA cleavage and typical ladder pattern were also reported to occur during necrotic death of several cell types that included hepatocytes, thymocytes and kidney cells (Dong et al., 1997). It was suggested that endonuclease activity itself is not required for activation of the cell death program and that it functions only to degrade DNA in dead cells during cell resorption (Ellis et al., 1991). This finding was further supported by the data from studies of cell death in interdigital tissue of developing rodent limb and insect metamorphosis. In these cases of programmed cell death a ladder of fragmented DNA was generated only after much of cytoplasm had already been destroyed (Zakeri et al., 1993; Woo et al., 1994). On the other hand, there are observations indicating that DNA fragmentation may occur as an early event during apoptosis (for discussion see Arends and Harrison, 1994; Sanders and Wride, 1995). The failure to detect genome fragmentation in cases of cell death whose circumstances and morphological characteristics clearly indicate its apoptotic nature, shows that DNA cleavage appears to be a dispensable feature in at least some cases of programmed cell death (Cohen et al., 1992; Ucker et al., 1992, Falcieri et al., 1993; Oberhammer et al., 1993a,b; Tomei et al., 1993; Zakeri et al., 1993; Schulze-Osthoff et al., 1994). Thus, the role of DNA fragmentation in cell death and its causal and temporal interrelations with the expression of morphological markers of apoptosis are currently unclear.

The purpose of the present study was to investigate the presence of specific markers of cell death in experimen-

tally denervated skeletal muscle tissue. For this purpose, we used the combination of the criteria discussed above to search for the expression of major markers of apoptotic and necrotic types of cell death in rat fast and slow muscles during the course of muscle atrophy induced by denervation.

MATERIALS AND METHODS

Muscle Denervation

The experiments were conducted on adult (4-month-old) male WI/Hicks Car 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 of 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 at 0, 2, 4 and 7 months after denervation.

Processing of the Tissue and Nuclear DNA Staining

The tibialis anterior, the extensor digitorum longus (EDL) and the soleus muscles were excised under anesthesia and immediately fixed in 4% paraformaldehyde. Non-denervated normal muscles served as controls. Tissue samples from 3 normal and 3 denervated muscles at each time point were processed for histology using conventional methods and sectioned at a thickness of 8 μm. To visualize cell nuclei the samples were stained with a 3 μM solution of a DNA-binding dye, propidium iodide, for 5 min. Stained samples were examined in epifluorescent optics with a Nikon Optiphot fluorescence microscope equipped with excitation filter at 510–560 nm. Emission was observed using a barrier filter at 590–615 nm.

Electron Microscopy

The samples of 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 removing 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) 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.). 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.

Detection of Internucleosomal DNA Fragmentation in Nuclei

To detect endonuclease-mediated internucleosomal cleavage of genomic DNA typical of apoptotic nuclei, we employed the TUNEL method (Gavrieli et al., 1992). The labeling target of this technique was the multitude of free 3′-OH DNA ends generated by DNA fragmentation in nuclei of cells undergoing apoptosis. Free 3′-OH ends of DNA fragments were extended with digoxigenin-labeled nucleotides using an enzyme-directed reaction. In apoptotic nuclei, residues of digoxigenin-labeled nucleotides, deoxyuridinetriphosphate (dUTP) and deoxyadeninetriphosphate (dATP), used in this reaction were catalytically added to free 3′-OH ends of genomic DNA by terminal deoxynucleotidyl transferase (TdT). This enzyme catalyzed a template independent addition of deoxyribonucleotide triphosphates to the 3′-OH ends of double or single-stranded DNA. During this reaction, the incorporated nucleotides form a random heteropolymer at the free 3′-OH ends of DNA. These incorporated nucleotides labeled with digoxigenin were visualized using anti-digoxigenin antibody conjugated with fluorescein. Non-apoptotic nuclei did not incorporate the label because of the absence of free 3′-OH DNA ends. We used the reagents and the technique for non-isotopic DNA 3′-OH end-extension in situ and for immunocytochemical staining of the extended DNA sequences from the Apoptag kit (Oncor, Gaithersburg, MD). The fluorescent reaction was observed with Nikon Optiphot fluorescence microscope using an excitation filter at 470–490 nm and an emission barrier filter at 520–560 nm wavelengths. For double labeling experiments we used nuclear DNA counterstaining with propidium iodide. In double stained samples, emission of DNA-binding dye in the same field of view was excited by light at 510–560 nm wavelength and observed with the barrier filter at 590–615 nm.

RESULTS

Identification of Apoptotic Nuclei Using Nuclear DNA Staining

The use of fluorescent cytochemical staining of nuclear DNA with propidium iodide permitted us to visualize cell nuclei and analyze their morphology. This technique was especially effective for the purpose of identification of chromatin hypercondensation and nuclear breakdown. Fluorescent staining of nuclear DNA with propidium iodide in sections of normal and denervated muscle revealed the presence of nuclei containing hypercondensed chromatin in the tissue denervated for 2, 4 and to a lesser extent for 7 months (Fig. 1). As shown in Figure 1, unlike normal myonuclei that had an elongated form, the nuclei containing hypercondensed chromatin were usually rounded and brightly fluorescent. Another morphological manifestation of abnormal nuclear morphology in the denervated muscle is the breakdown of nuclei (Fig. 2). As a result of this process that progresses with the increasing time of denervation, nuclei underwent fragmentation into clusters of several DNA-positiverounded bodies exhibiting bright fluorescence after staining with propidium iodide. Thus, DNA staining allowed us to detect small nuclear fragments with high resolution and clearly differentiate them from the background, which is difficult to do using the technique of non-fluorescent light microscopy without specific DNA labeling.

Expression of Ultrastructural Markers of Apoptosis

Electron microscopic study has shown significant ultrastructural differences between normal and denervated

muscle. Already in 2-month denervated tissue degenerative changes in muscle cells were clearly visible. Besides muscle fiber atrophy, they included the loss of significant amounts of myofibrils and formation of myofibril-free zones which were located mostly at the peripheral areas of sarcoplasm around myonuclei (Fig. 3a). As can be seen from Figure 3a, degenerating organelles, their remnants, secondary lysosomes, residual bodies and lipofuscin granules were more frequently observed in these areas. An important morphological feature of 2-month denervated muscle is the beginning of destabilization of the differentiated phenotype of muscle cells. Disalignment of myofibrils and the loss of their longitudinal orientation were seen only occasionally. Nuclear changes included an increase in the number of nuclei containing higher proportions of heterochromatin over euchromatin (Fig. 5a).

The degenerative changes described above further progressed and were more pronounced by 4 months after denervation. At this stage, the areas of local loss of myofibrils were expanded in some cells (Fig. 3b) and were observed predominantly in subsarcolemmal areas near myonuclei (Fig. 3b). Their contractile system exhibited further disalignment and partial resorption. Many myofibril bundles lost their orientation parallel to the long axis of the muscle cell and were located at different angles to one another. This resulted in the appearance of zones where individual myofibrils and their bundles ran in different directions (Fig. 3c). At all stages after denervation, cell population was heterogeneous in terms of instability of the differentiated phenotype and the levels of atrophy of individual cells. Although single cells with varying levels of degenerative changes were observed in 2-month denervated muscle, they were much more frequent 4 months after denervation. By this time myocytes with different degree of tortuosity and blebbing of the sarcolemma could be found in the denervated tissue (Fig. 4). The presence of myonuclei with increased levels of chromatin condensation that were very rare in normal muscle is a characteristic feature of 4-month denervated muscle (Figs. 4, 5c,d). These nuclei frequently exhibited unusual morphology which included a variety of convoluted, crescent-like and lobulated nuclear patterns (Fig. 5c,d). As can be seen from the presented illustrations, the nuclear material is homogeneously condensed, and only round electron-lucent areas of small diameter intermingled with the areas filled with dense chromatin masses. Deep elongated and rounded protrusions budding from the nuclei (Fig. 5d) and clusters of spatially isolated rounded bodies consisting of condensed nuclear material (Figs. 3a, 6) were observed. After 4 months of denervation, crystal-like laminated structures apparently of a lipoprotein nature were observed in myocytes with well-developed manifestations of involution (Fig. 5d). Dead cells with highly condensed nuclei and cytoplasm were present in 4-month denervated muscle (Fig. 7a) and rarely in 2-month denervated muscle (Fig. 7b). We observed the evidence of muscle cell fragmentation into several electron dense bodies (Fig. 7b). Both at initial and advanced stages of this process the basal lamina, sarcolemma and intracellular organelles did not demonstrate any structural manifestations typical of necrotic injury. The basal laminae of dead muscle fibers frequently showed signs of totuosity and folding (Fig. 7a), which apparently results from shrinkage of the volume of the dying cells. Myocytes with condensed nuclei and cytoplasm were frequently located at short distances from myocytes with normal nuclear and cytoplasmic density (Fig. 7b). This indicates that individual cells rather than cell groups were susceptible to cell death. Macrophages or their cytoplasmic processes can be occasionally seen near degenerating cells at the advanced and final stages of this process (Fig. 7a). After 7 months of denervation, the intensity of degenerative processes was less pronounced than in 4-month denervated muscle. The muscle cells further decreased in cross-sectional diameters, and nuclei with unusual ultrastructure were still present in the tissue (Fig. 5b). As can be seen from Figure 5, at the onset of nuclear changes the cytoplasm of muscle cells still remains intact before beginning to condense. The heterogeneity of the levels of atrophy and degenerative changes in individual muscle cells was apparent at all stages after denervation.

Detection of Apoptosis-Associated Genome Fragmentation Using the TUNEL Method

Detection of another marker of apoptosis, 3′-OH site-specific DNA fragmentation, using the TUNEL method revealed strong immunopositivity in a very small fraction of nuclei in 2 and 4 month denervated muscle and rarely in 7 month denervated muscle. The reactivity for DNA breaks in denervated muscle was found both in morphologically intact and rounded nuclei (Fig. 8A,B) and in fragmented nuclei (Fig. 8C,D). Structural patterns of the TUNEL immunopositivity were similar at all stages after denervation. The number of the TUNEL positive morphologically intact and fragmented nuclei after 2 months of denervation did not exceed 7–12 per longitudinal section in the tibialis anterior, 5–9 in extensor digitorum longus muscles and 5–11 in the soleus muscle and remained at the same level in 4-month denervated muscle. No positive reaction was found in normal control muscle. To investigate to what degree localization of the TUNEL reaction coincided with patterns of nuclear DNA staining with propidium iodide, we performed double staining for DNA fragmentation and for nuclear DNA. We found the TUNEL reactivity in only a fraction of cells expressing the abnormal morphology frequently associated with different levels of hypercondensation of chromatin and nuclear fragmentation (Fig. 9). To find an explanation for this discrepancy, we assessed the numbers of nuclei exhibiting abnormal structure and different degrees of chromatin aggregation after DNA staining and by using the electron microscopy. The numbers of nuclei with abnormal morphology and/or condensed chromatin exceeded the numbers of the TUNEL-positive nuclei by 33–38 times. Thus, only a small fraction of nuclei with different degrees of chromatin condensation expressed the TUNEL reactivity.

DISCUSSION

Our results clearly show that cell death is an important component contributing to the pathogenesis of post-denervation muscle atrophy. Structural peculiarities of this process have much in common with morphological characteristics of apoptosis. A classical and the most profoundly studied experimental model of apoptosis in mammalian cells is death of thymocytes induced

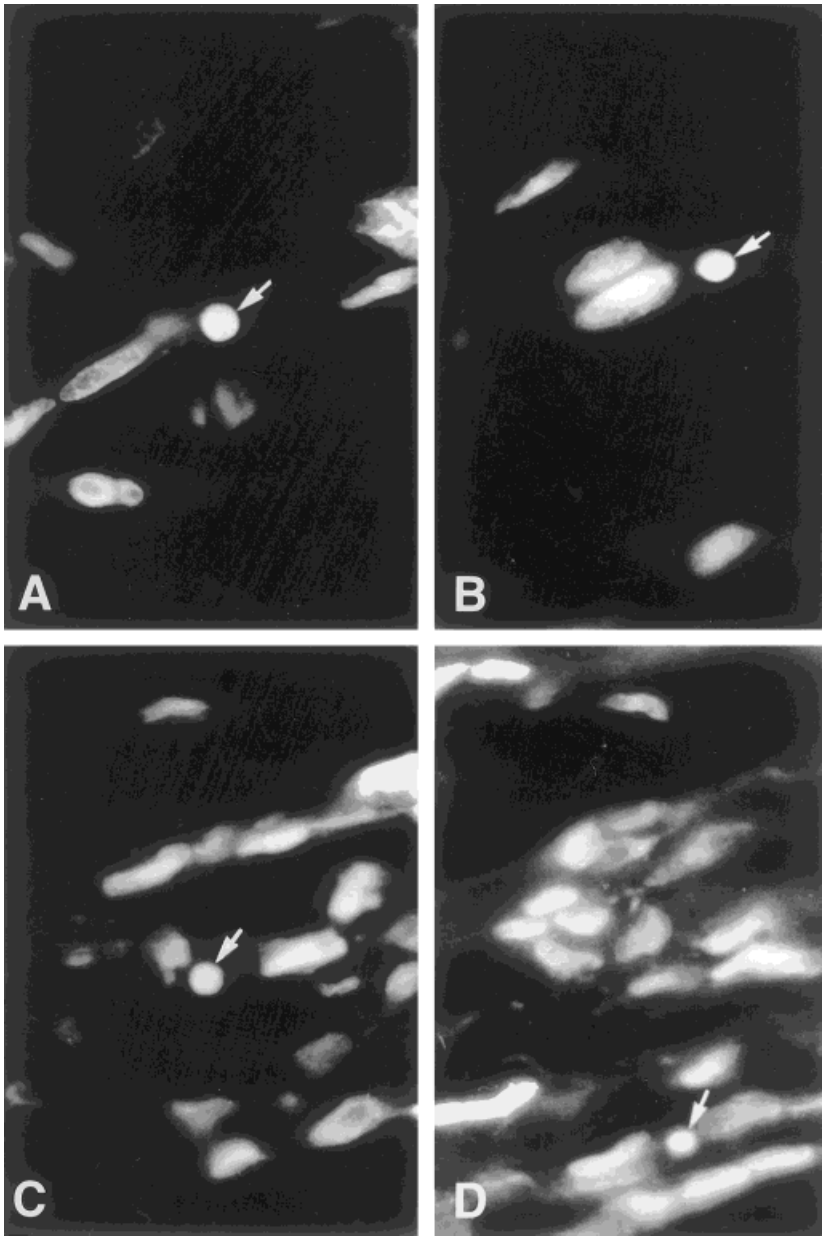


Fig. 1. Early stage apoptotic nuclei showing bright fluorescence of highly condensed chromatin (arrows). Normal myonuclei stained with propidium iodide exhibit a significantly less intense and more heterogeneous DNA staining pattern associated with the presence of euchromatin and heterochromatin. Note the difference between the elongated form of normal nuclei and the rounded apoptotic nuclei. **A,B:** Two-month denervated tibialis anterior and soleus respectively. **C:** Four-month denervated EDL muscle. **D:** Seven-month denervated tibialis anterior. Magnification ×570.

by glucocorticoids. Detailed description of structural aspects of this process was presented in the pioneering works of Kerr, Wyllie and co-workers (Kerr, 1971; Kerr et al., 1972; Wyllie, 1980). Data obtained in studies of hormone-induced thymocyte death led to formulation of criteria for classification of cell death as the apoptotic one. Non-uniform condensation of chromatin, its margination near the nuclear membrane, formation of electron-dense toroid intranuclear structures, deep invaginations of nuclear membraneand formation of crescent-like nuclei were described in several mammalian cell types during apoptosis (for literature see Arends and Wyllie, 1991; Tomei et al., 1993; Arends and Harrison, 1994). Comparison of our illustrations to those considered as classical examples of changes of nuclear structure during apoptosis, as revealed both by fluorescent staining of nuclear DNA and by electron microscopy, shows their very close resemblance. Condensation of

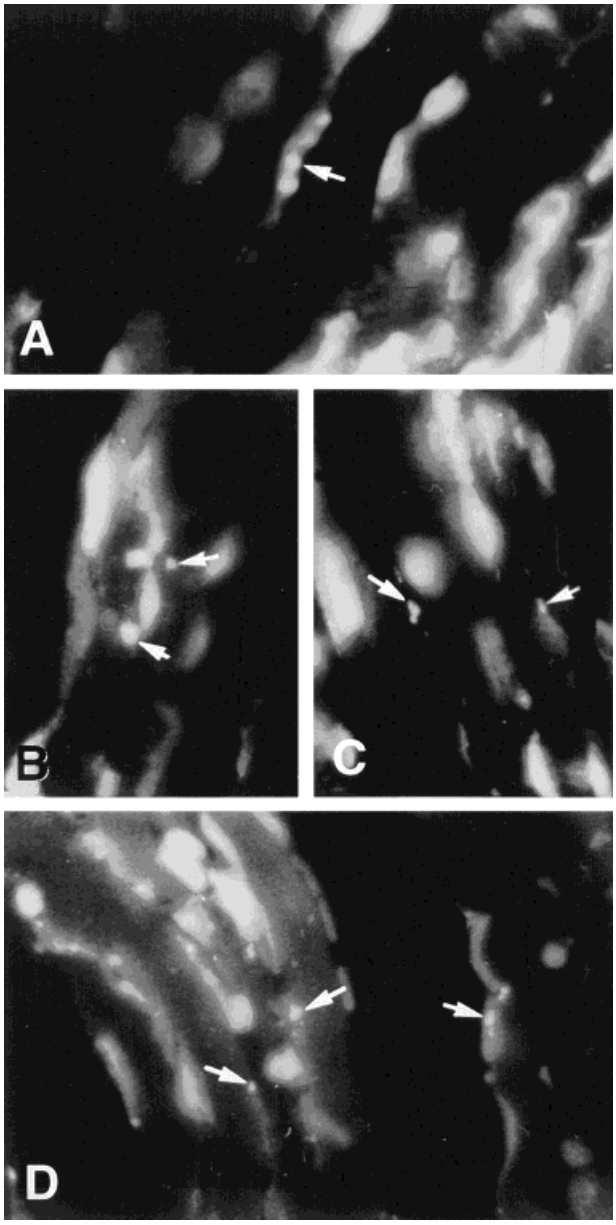


Fig. 2. Advanced stages of nuclear fragmentation in denervated muscle. **A:** Two-month denervated tibialis anterior. **B:** Four-month denervated EDL. **C:** Four-month denervated soleus. **D:** Seven-month denervated EDL muscle. Arrows indicate fragmented nuclei visible as clusters of DNA-positive small bodies with highly condensed chromatin. Nuclear DNA staining with propidium iodide. Magnification ×570.

the cytoplasm and fragmentation of the cell body is typical of advanced and late stages of apoptosis, and we observed similar events in denervated muscle. Thus, the processes of nuclear and cytoplasmic condensation and fragmentation that we found in denervated muscle cells are very similar to classical manifestations of apoptosis.

It is interesting to compare our results to the phenomena of programmed cell death in muscle tissue during metamorphosis in lower vertebrates and insects and in developing muscle of birds and mammals. The purpose of this comparison is to find the answers to two important questions: 1) How conservative phylogenetically are the pathways of muscle cell death in terms of their similarity and differences in animals belonging to different taxonomic groups? and 2) Does the same (or a very similar) mechanism of cell death operate during developmental elimination of some muscle cells in normal histogenesis and in adult muscle after depletion of the trophic support of the nerve? Detailed analysis of degeneration and death of muscle cells during metamorphosis in Lepidoptera has been performed in the laboratories of Lockshin and Schwartz (for comprehensive reviews, see Beaulaton and Lockshin, 1982; Zakeri et al., 1993, 1995; Schwartz, 1992; Lockshin and Zakeri, 1996; Milligan and Schwartz, 1996). It was found that the volume of dying muscle fibers was significantly reduced, their nuclei and cytoplasm were condensed and rounded bodies were occasionally budded at the periphery of the cells. The final stages of muscle cell degradation in insects, as illustrated by Beaulaton and Lockshin (1982), are structurally similar to those observed by us in mammalian muscle undergoing post-denervation atrophy. An apoptosis-like process in skeletal muscle fibers in some aspects similar to our observations (structural disorganization and cell condensation) was also described in the tail muscle of tadpoles during their metamorphosis (Kerr et al., 1974). Dead muscle cells with highly condensed shrunken cytoplasm were found in adult frog muscle after long-term denervation by Anzil and Wernig (1989). Unlike our observations, de Castro Rodrigues and Schmalbruch (1995), in their ultrastructural study of long-term denervated skeletal muscle of neonatal and 5-week-old rats, did not find cells at advanced stages of an apoptosis-like process. However, we also found an increased polymorphism of myonuclear structure in denervated muscles. Ultrastructural evidence of cell death that included cytoplasmic and nuclear condensation was described in developing chicken cardiac and skeletal muscle (Manasek, 1969; McClearn et al., 1995), as well as in skeletal muscle tissue of rat and human embryos (Grim, 1977; Webb, 1977; Abood and Jones, 1991; Fidzianska and Goebel, 1991). Illustrations of developmental cell death in embryonic mammalian muscle presented by Fidzianska and Goebel (1991) and Abood and Jones (1991) strikingly resemble our illustrations of dying cells in denervated muscle. Involvement of macrophages in degradation of dead muscle fibers observed in our experiments has been described during developmental cell death in several types of tissues (Beaulaton and Lockshin, 1982; Abood and Jones, 1991; Lang and Bishop,

Fig. 3. Destabilization of the differentiated characteristics of muscle cells in the denervated tissue. **a:** A myofibril-free zone in the subsarcolemmal area of a muscle cell; arrow indicates a fragment of a degenerating nucleus, small arrowheads show residual bodies. **b:** Large area free of myofibrils near myonucleus. **c:** Disalignment of myofibrils oriented in different directions in 4-month denervated muscle. a–c: EDL muscle 2 months (a) and 4 months (b,c) after denervation. Magnification ×17,500 (a), 5,200 (b), 15,500 (c).

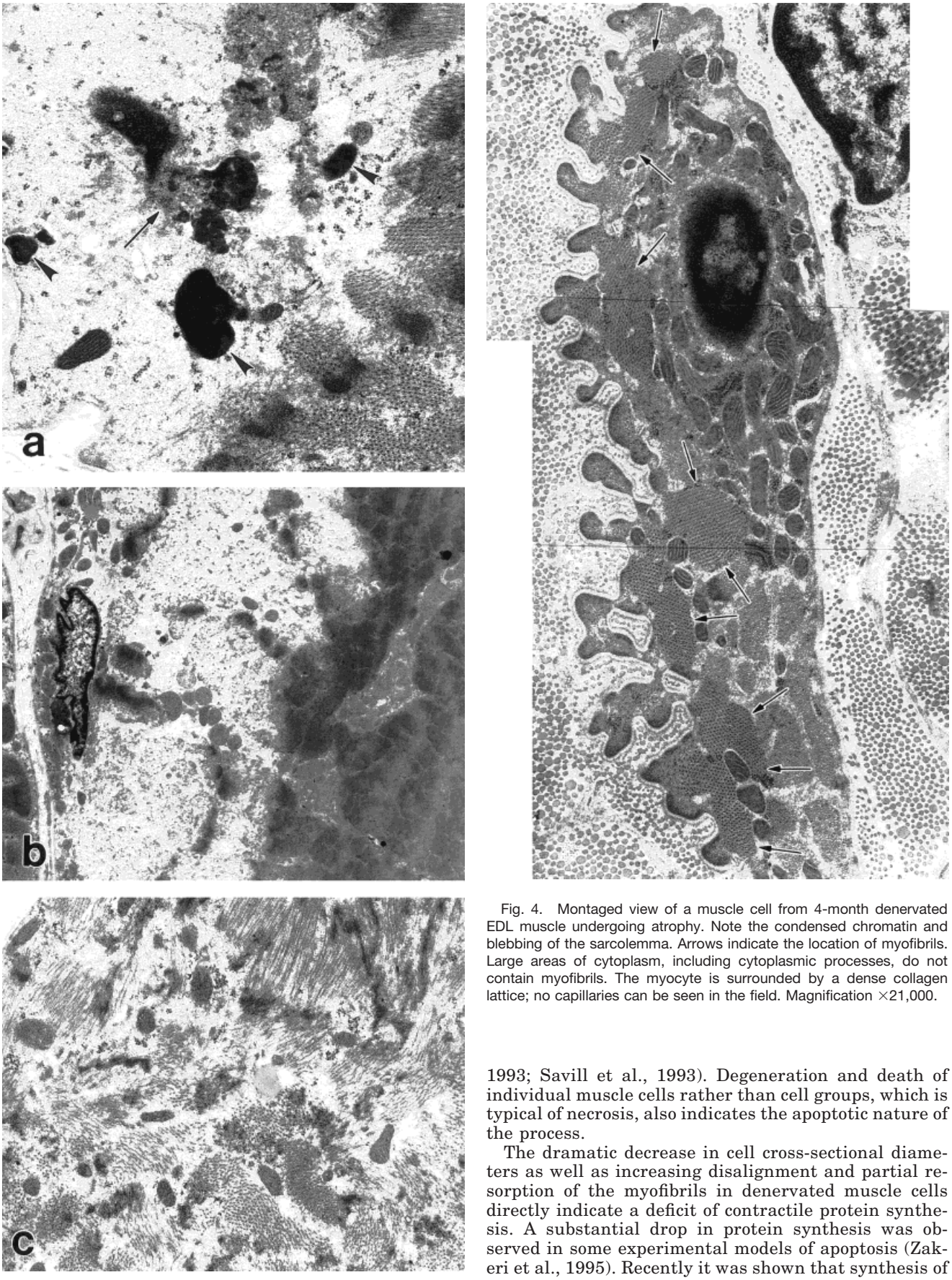


Fig. 4. Montaged view of a muscle cell from 4-month denervated EDL muscle undergoing atrophy. Note the condensed chromatin and blebbing of the sarcolemma. Arrows indicate the location of myofibrils. Large areas of cytoplasm, including cytoplasmic processes, do not contain myofibrils. The myocyte is surrounded by a dense collagen lattice; no capillaries can be seen in the field. Magnification ×21,000.

1993; Savill et al., 1993). Degeneration and death of individual muscle cells rather than cell groups, which is typical of necrosis, also indicates the apoptotic nature of the process.

The dramatic decrease in cell cross-sectional diameters as well as increasing disalignment and partial resorption of the myofibrils in denervated muscle cells directly indicate a deficit of contractile protein synthesis. A substantial drop in protein synthesis was observed in some experimental models of apoptosis (Zakeri et al., 1995). Recently it was shown that synthesis of

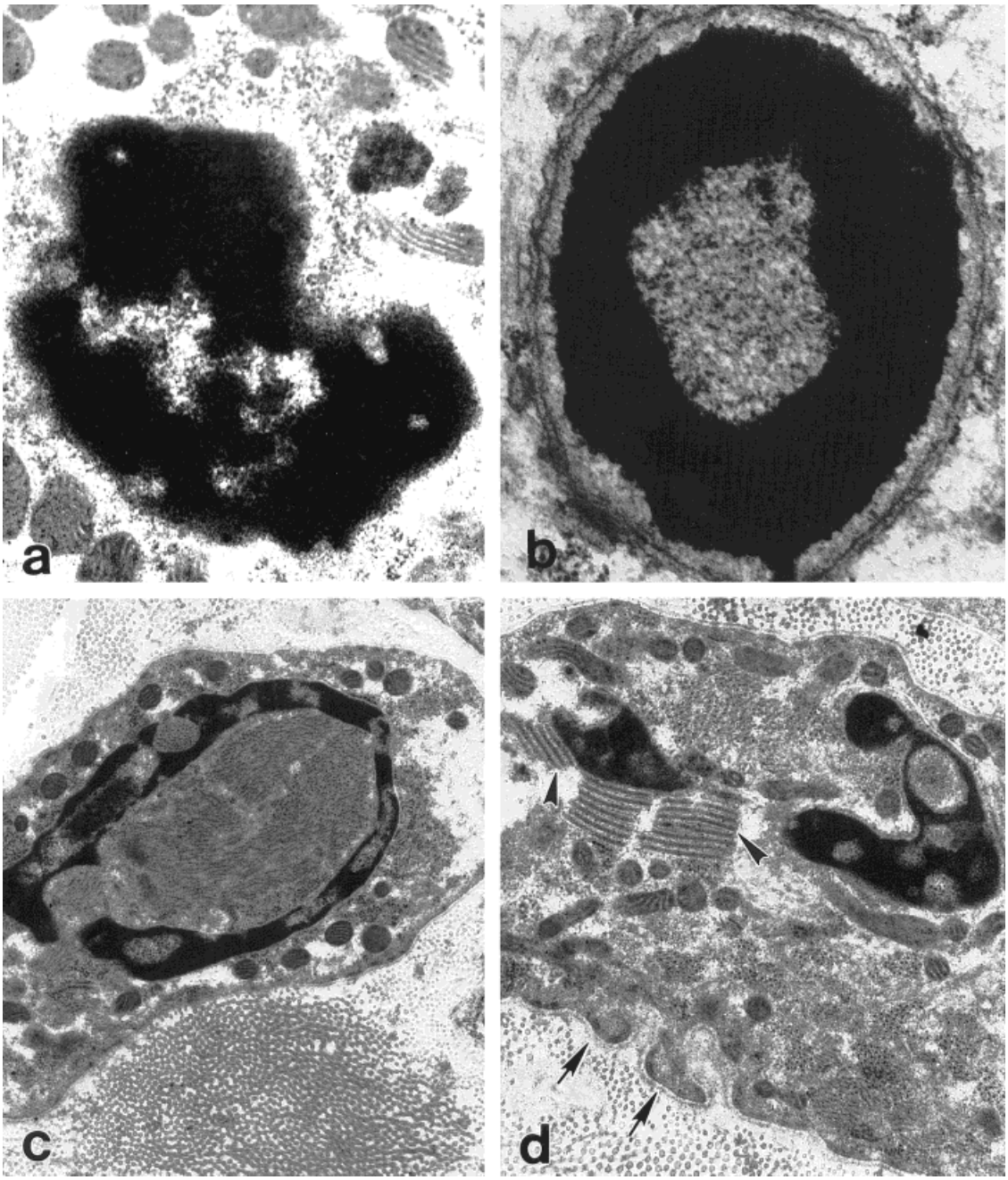


Fig. 5. Nuclear changes in denervated EDL muscle. **a:** Myonucleus with increased level of chromatin condensation in 2-month denervated muscle. **b:** Toroid structure formed by chromatin condensed around the periphery of the nucleus; note the intact nuclear envelope. **c,d:** Crescent-shaped myonuclei with condensed chromatin 4 months after denervation; in (c), nuclear membrane invaginates around crescents of condensed chromatin, but the nucleus is still intact; in (d), an advanced stage of nuclear fragmentation is evident and resorption of a greater part of myofibrils is clearly seen; small arrows indicate blebbing of the sarcolemma, large arrow shows the laminated crystal-like structures located near the nucleus. Magnification ×28,750 (a), 34,200 (b), 15,500 (c), 21,000 (d).

actin and myosin heavy chains is selectively repressed during programmed cell death in insect skeletal muscle (Schwartz et al., 1993b). These findings support the data of ultrastructural studies considering the condensation of chromatin and the nucleoli as morphological evidence of inactivation of the transcription mechanism (for discussion see Beaulaton and Lockshin, 1982; Schwartz, 1993b). Activation of lysosomes and stimulation of autophagocytosis was described in several nonmammalian types of programmed cell death (reviewed

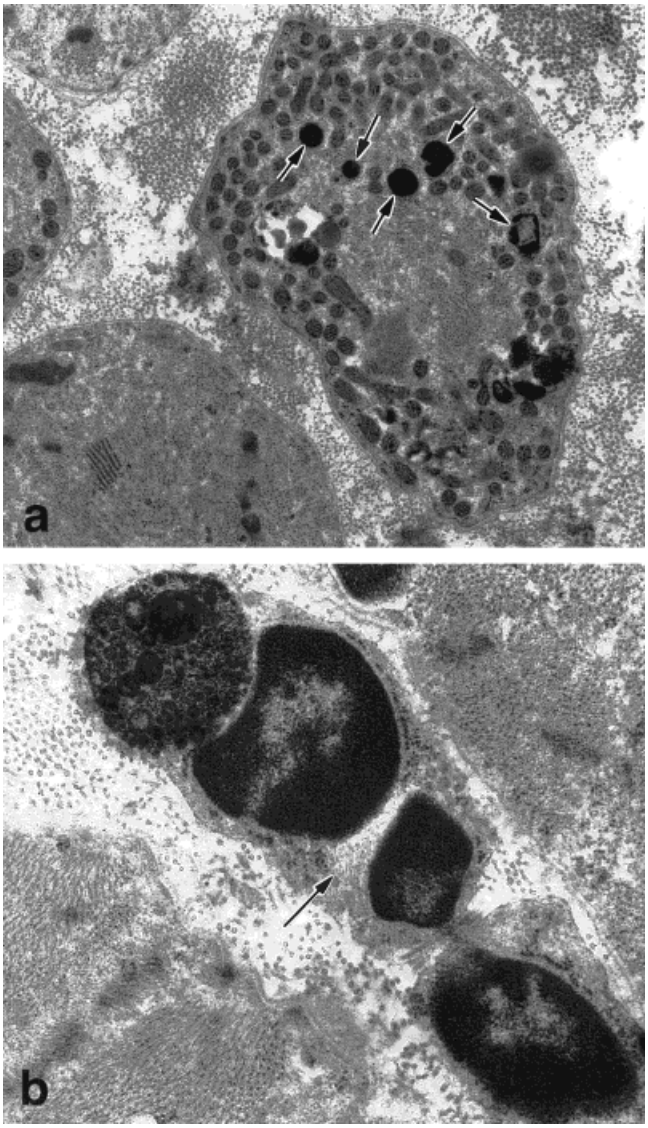


Fig. 6. Fragmentation of nucleus into several small chromatin spheres (arrows) in a muscle cell 4 months after denervation. Magnification ×11,500 (a), 21,000 (b).

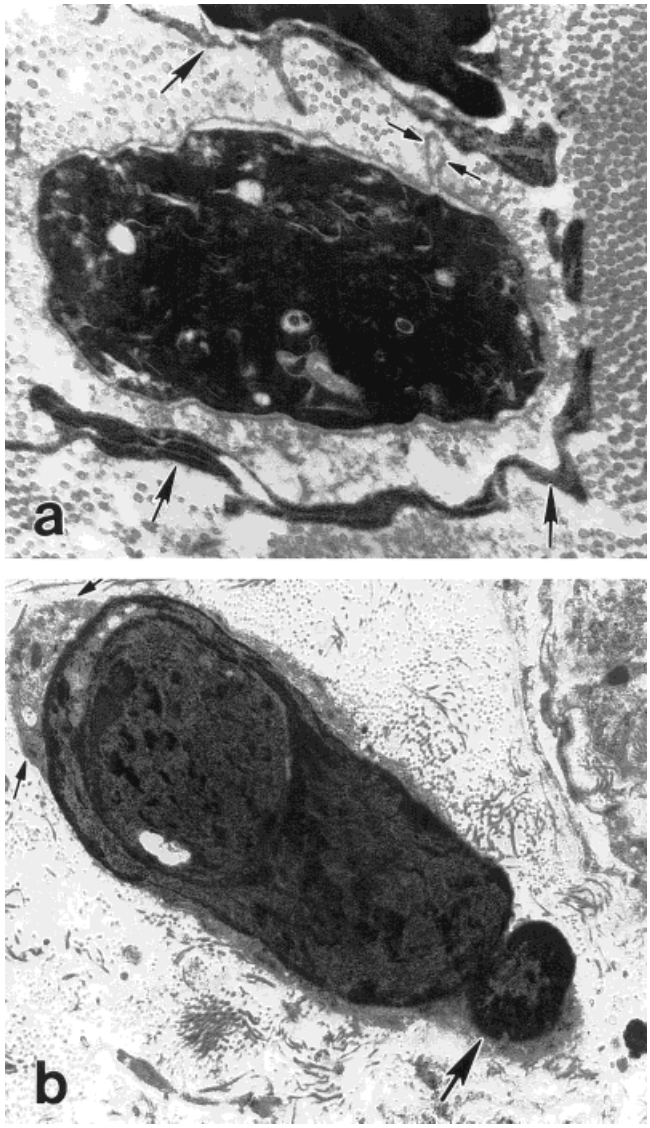
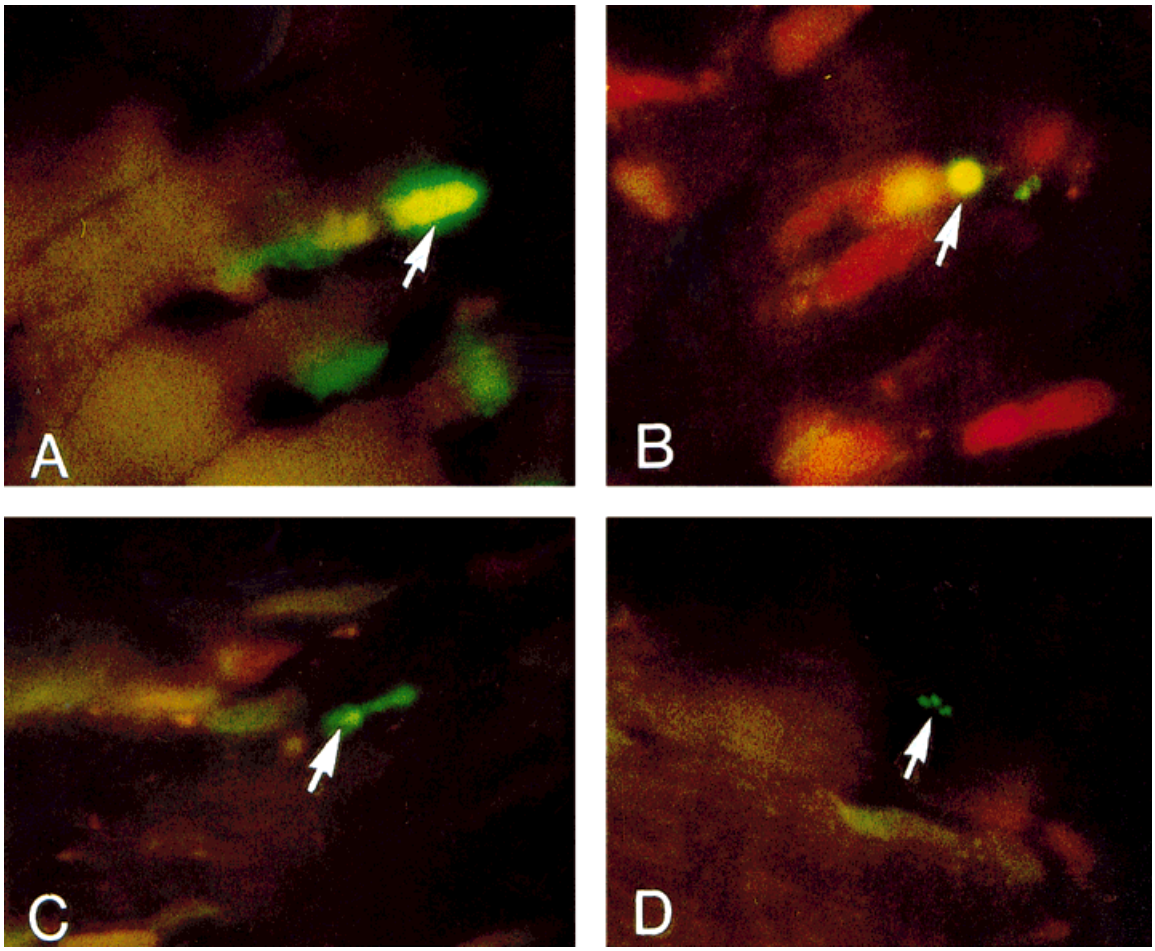
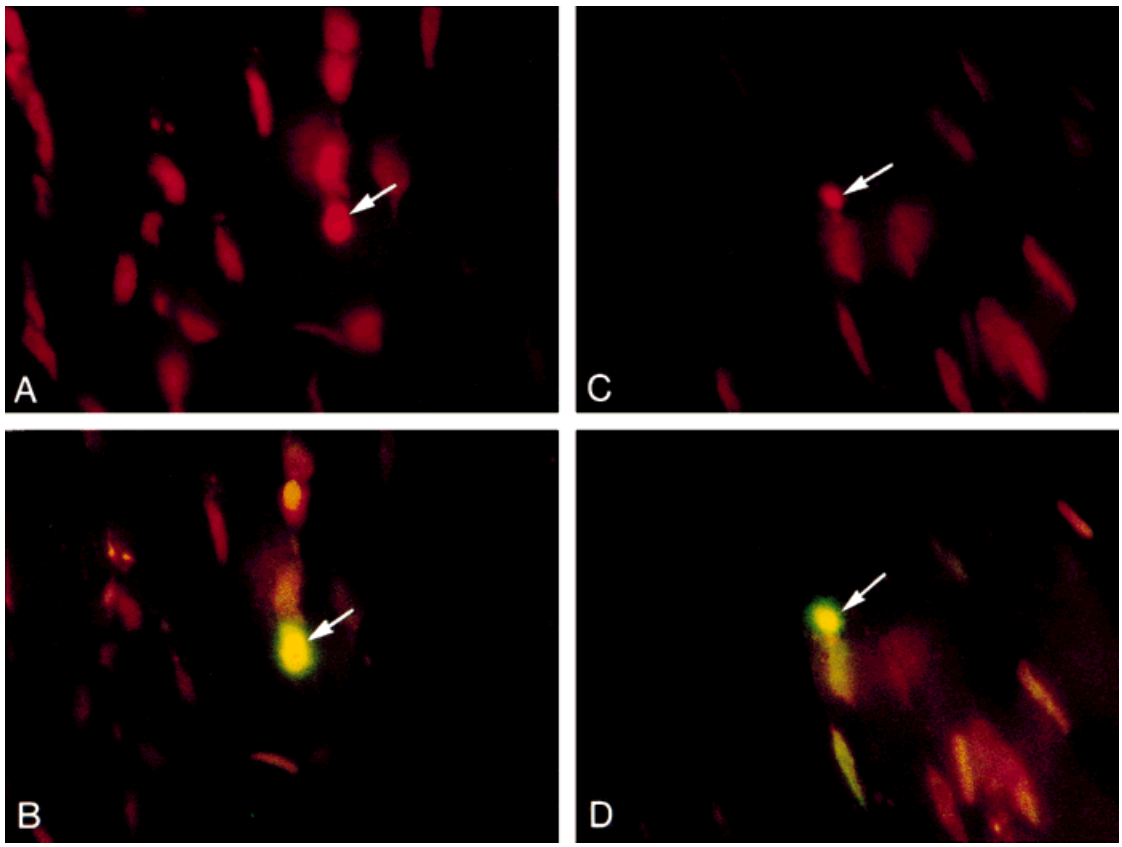


Fig. 7. Dead muscle cells exhibiting highly condensed nucleus and cytoplasm. **a:** The cell is still intact and dilated cisterns of endoplasmic reticulum and vacuoles are visible; large arrows indicate cytoplasmic processes of a macrophage that surround the muscle cell; small arrows indicate a fold of the basal lamina. **b:** Early stage of cell fragmentation into apoptotic bodies; large arrow indicates a budding round structure at the periphery of condensed muscle cell; small arrows indicate cross section of the cytoplasm of a viable cell located beneath the basal lamina of a dying muscle fiber. A viable muscle cell with normal non-condensed cytoplasm is seen in the upper right corner in (b).EDL muscle 2 months (b) and 4 months (a) after denervation.

by Beaulaton and Lockshin, 1982; Lockshin and Zakeri, 1996). A moderate increase in intensity of the histochemical reaction for acid phosphatase, an enzymatic marker of lysosomes, was reported in degenerating muscle cells in the human embryo (Grim, 1978). Some activation of the lysosomal system and autophagocytosis with a moderate increase in histochemical reactivity for acid phosphatase was reported in response to denervation in fetal and neonatal rat muscle (Schiaffino and Hanzlikova, 1972). In our experiments, the number of secondary lysosomes and residual bodies in denervated muscle cells was not unusually high, although they were more frequently observed in samples of denervated muscle than in normal control. We did not find any evidence of participation of lysosomes in the resorption of the contractile system. This indicates that autophagy does not play a major role in the degenerative events in denervated muscle. There is an indication that nuclear changes during apoptosis involve the proteolytic separation of DNA and chromatin from their attachment sites to the nuclear membrane (Weaver et al., 1993). This may explain why the toroids of condensed chromatin are sometimes located at close distance from the





Figures 8 and 9.

nuclear envelope without direct contact to it (Fig. 5b). In normal differentiated muscle fibers, the contractile apparatus of muscle cells is organized in register, so that individual myofibril bundles are well-aligned at the level of Z-bands. A number of structural non-contractile proteins perform the function of cement connecting and binding individual bundles of myofibrils to one another in a well-aligned register (for a review see Franzini-Armstrong and Fischman, 1994). It is apparent that disalignment of the contractile system and loss of myofilaments observed in denervated muscle require the removal of proteins that bind the bundles of myofibrils together. To this end, it is tempting to explain degenerative events in the contractile system of denervated muscle by the involvement of calpain-like proteases. There is more direct evidence indicating activation of calpains during this process. Baker and Margolis (1987) reported activation of calpain-like proteinases 1 week after tenotomy of the soleus muscle in rats, when, according to the authors, myofibril organization is completely disrupted. Kumamoto et al. (1992) observed a progressive increase in concentration of calpains revealed as changes in densities of collodial gold immunolabeling in rat skeletal muscle after denervation during the entire period of their 2 week observations. It is interesting that disalignment of myofibril bundles, resorption of Z-bands and partial dissolution of sarcomeres preceded more dramatic changes in amphibian and insect muscle tissue (Lockshin and Beaulaton, 1979; Beaulaton and Lockshin, 1982).

Of special interest is the small number cells positive for DNA fragmentation that we observed in denervated muscle tissue. The technique of visualization of DNA breaks in situ was introduced into research practice by Modak and Bollum (1972), and during the last several years different modifications of this technique have been extensively used for identification of apoptotic cells. Now it is evident that neither the presence nor the absence of nuclear DNA fragmentation can be considered as a universally reliable indicator of the presence or intensity of apoptotic cell death. Even in a well-studied model of apoptosis, such as cultured rat thymocytes induced to undergo apoptosis by methylprednisolone, many cells that could be identified morphologically as apoptotic did not show a positive reaction for DNA fragmentation after end labeling of nuclear DNA fragments (Ansari et al., 1993). Wood and co-workers (1993) found no correlation between the localization of DNA free end labeling in situ and apoptotic cell morphology in the developing mouse cerebellum. Some degree of cross-reactivity of labeling between apoptotic and necrotic cell nuclei has been described with the use of these methods and different modifications of the technique show different levels of this cross-reactivity (Gold et al., 1994). Using a combination of fluorescent DNA staining and DNA free end labeling, Oberhammer et al. (1993b) reported that cultured hepatocytes induced to undergo apoptosis by TGF-β1 were almost completely negative for DNA fragmentation. However, a closer view revealed that about 5% of the nuclear fragments contained DNA strand breaks. The difficulty of interpretation of these results, according to the authors includes the impossibility of differentiating between apoptosis and secondary necrosis and possible fragmentation of DNA by the lytic enzymes released by dead cells. Gavrieli et al. (1992) failed to detect DNA fragmentation in situ in the involuting mouse mammary gland. Using the same experimental model and essentially the same technique, Strange et al. (1995) found that 30–40% of the cells in post-lactation breast tissue were TUNEL-positive. Earlier studies have shown the presence of light microscopic, ultrastructural and biochemical (DNA ladder) markers of apoptosis (Walker et al., 1989; Strange et al., 1992) in mammary epithelial cells. A similar difference in data occurs in studies of DNA fragmentation during apoptotic cell death in interdigital tissue of chicken and rodent limb buds (Zakeri et al., 1993; Tone et al., 1994; Sanders and Wride, 1995). In another well-documented model of apoptosis, Ansari et al. (1993) observed that, besides labeling of definitely apoptotic cells at the tips of intestinal villi, positive reactivity for DNA fragmentation was present in a number of morphologically normal cells. It was recently reported that TUNEL-positive myonuclei were present in neonatal denervated skeletal muscle (Trachtenberg, 1998). However an earlier report claimed that rat soleus muscle did not show DNA strand breaks 30 weeks after denervation performed during neonatal period as demonstrated by in situ nick translation (de Castro Rodrigues and Schmalbruch, 1995). This agrees with our results indicating that the vast majority of cells undergoing degeneration according to morphologic criteria were immunonegative for DNA fragmentation. Nevertheless, the absence of clear criteria of specificity, sensitivity and universal technical standards used in different studies makes meaningful comparisons among the various methods available to detect DNA strand breaks in situ difficult.

Another important question concerns the nature of the signals triggering apoptosis in denervated muscle and its connection to trophic factors produced by the nerve. The first indications of interdependence of cell death in neurons and their peripheral target organs came from studies of developmental cell death (for literature see Pilar and Landmesser, 1976; Oppenheim, 1991; Raff et al., 1993). Apoptotic cell death can be induced by optic nerve lesion in the eye of the neonatal rat (Rabacchi et al., 1994). Pilar and Landmesser (1976) have shown that neurons in chick ciliary ganglion did not complete differentiation and died

Fig. 8. (At left.) Detection of DNA fragmentation in intact nuclei in denervated muscle using the TUNEL technique. **A:** A TUNEL positive intact nucleus in 2-month denervated tibialis anterior. **B:** A TUNEL-positive condensed rounded nucleus in the soleus muscle 4 months after denervation; **C,D:** Clusters of small nuclear fragments positive for the TUNEL reaction in 4-month denervated EDL and tibialis anterior muscles. Note the morphological similarity of nuclei positive for the TUNEL reaction to those identified as apoptotic after DNA staining with propidium iodide (Figs. 1 and 2). Magnification ×830.

Fig. 9. (At left.) Localization of TUNEL reactivity in nuclei with a high level of chromatin condensation. **A,C:** Fluorescent DNA staining with propidium iodide. **B,D:** DNA fragmentation as revealed by the TUNEL technique. A,B: Tibialis anterior 2 months after denervation. C,D: Soleus 2 months after denervation. Magnification ×830.

if they had been prevented from forming contacts to the target tissue. Of special interest for our discussion is the recent report that axotomy results in delayed cell death and apoptosis of retinal ganglion cells in adult rats (Berkelaar et al., 1994). We have shown that in denervated muscle, cell death also occurs as a delayed rather than immediate consequence after the depletion of the trophic influence of the nerve. Very little is known as yet concerning the trophic factors involved in nerve-muscle interactions, but it is apparent that both cell types are closely interrelated functionally and trophically. For example, the success and completeness of muscle regeneration strongly depend on intact innervation (for review see Carlson, 1991).

Of special interest for our discussion is the recent report that axotomy results in delayed cell death and apoptosis of retinal ganglion cells in adult rats (Berkelaar et al., 1994). We have shown that in denervated muscle, cell death also occurs as a delayed rather than immediate consequence after the depletion of the trophic influence of the nerve. The results of our study demonstrate that cell death in denervated muscle is distinct from classical apoptosis in the form it is described in mammalian lymphoid cells. Three major differences can be observed: 1) myocytes in denervated muscle lose viability long time after the depletion of the support of the nerve; 2) cell death is preceded by a progressive decrease of cell diameter (cytoplasmic atrophy) of muscle fibers; 3) no large-scale nuclear DNA fragmentation is observed. Taking into consideration that the rate of cellular destruction during apoptosis is very rapid and comprises for some cell types 2–3 hours (Bursch et al., 1990a, 1990b), even a small number of apoptotic cells revealed in the tissue can reflect significant regression of tissue mass during long period of time. It is possible that more than one mechanism of cell death is present in denervated skeletal muscle and that individual contributions of these mechanisms to muscle atrophy change during the course of long-term denervation. A similar conclusion concerning the presence of several mechanisms of cell death operating during development was made by Schwartz et al. (1993b).

Morphological peculiarities of cell death in denervated muscle are very similar to those observed during programmed cell death in embryonic and neonatal mammalian muscle. This indicates that a very similar pathway of cell death may be activated to eliminate cells in developing muscle and in adult muscle after its denervation. Thus, taking into account the similarity of basic cellular mechanisms of histogenesis, regeneration and compensatory remodeling of muscle, we conclude that the mechanism of cell death occurring in denervated muscle appears to be very similar to that observed during normal muscle development.

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