Journal of Cellular Biochemistry 27:337–346 (1985)
Extracellular Matrix: Structure and Function 137–146

Basement Membrane Component Changes in Skeletal Muscle Transplants Undergoing Regeneration or Rejection

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The basement membrane of myofibers plays an important role during orderly regeneration of skeletal muscle after injury. In this report, changes in various basement membrane components were analyzed in skeletal muscle grafts undergoing regeneration (autografts) or immune rejection (allografts). The immunofluorescence technique using specific antibodies against laminin, types IV and V collagen, heparan sulfate proteoglycan, fibronectin, in combination with binding of concanavalin A (ConA) was used to monitor basement membranes. In normal muscle, these components were localized in the pericellular region of myofiber corresponding to its basement membrane. After transplantation, the majority of myofibers underwent degeneration as a result of ischemic injury, followed by regeneration from precursor myosatellite cells. Various components of basement membrane zone disappeared from the degenerating myofibers, leaving behind some unidentifiable component that still bound ConA. A new basement membrane appeared around the regenerated myotubes which persisted during maturation of the regenerating muscle. In rejected skeletal muscles, the immunoreactivity of various components persisted even after the disappearance of myotubes and myofiber cytoplasm. In addition, an accumulation of fibronectin was seen throughout the rejected muscle with the onset of immune rejection. These results demonstrate that the major basement membrane components disappear and reappear sequentially during myofiber degeneration and regeneration. Such a turnover is not seen in rejected skeletal muscles. Thus, the myofiber basement membrane is not a static structure as previously thought but one which changes chemically during degeneration and regeneration. This feature of basement membrane may be important in the orderly regeneration of skeletal muscle after injury.

Key words: laminin, fibronectin, basement membrane, regeneration, immune rejection, skeletal muscle

The basement membrane of myofibers, which constitutes an important component of extracellular matrix, is believed to persist after injury to skeletal muscle and acts as a scaffold for subsequent regeneration [1–6]. This role of basement membrane

Received June 13, 1984; revised and accepted November 1, 1984.

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in orderly regeneration is based on extensive electron microscopic observations where its presence is seen around the degenerating myofibers. In the past few years, the complex biochemical nature of basement membranes has become evident and several unique macromolecules have been isolated and localized in this structure [7,8,9]. The major identifiable components of the basement membrane are laminin, type IV collagen, and heparan sulfate proteoglycan. In addition, type V collagen and fibronectin are associated with some basement membranes but are also present in the extracellular matrix. Other components of basement membranes include entactin, bullous pemphigoid antigen, and certain lipids [8,10].

The present report describes basement membrane component changes in two types of skeletal muscle transplants: (1) Autotransplants: skeletal muscle transplants in the same animal; such transplants invariably undergo degeneration and, upon revascularization, subsequent regeneration from precursor myosatellite cells [4,5,11]. (2) Allotransplants: skeletal muscle transplants between genetically different animals of the same species; such transplants are immunologically rejected. The experimental model of transplanting the entire extensor digitorum longus (EDL) muscle in the rat was used to induce regeneration or rejection [4,5,12–14]. Basement membrane component changes were monitored by immunofluoresence with the aid of monospecific antibodies and ConA binding.

METHODS

Surgery for Muscle Autotransplants

Male Fischer (FR) rats weighing abount 200 gm each were obtained from the NIH breeding colony. After chloral hydrate anesthesia (40 mg/100 mg body weight, i.p.), the EDL muscle was exposed and its proximal tendon cut. The muscle was then removed from its bed and transplanted back in the same site and the cut tendons sutured back together. The surgical procedure was similar to that used earlier and has been detailed elsewhere [12,13].

Surgery for Muscle Allotransplants

For this part of the study, isogeneic FR and Buffalo (BF) rats obtained from NIH breeding colony, weighing about 200 gm each were used. These two rat strains differ in major and multiple minor loci derived transplantation antigens and elicit a rapid and destructive immune response from the host after transplantation [14]. The FR rats served as hosts and received bilateral EDL muscles taken from BF rats. The surgical procedure was similar to that for autotransplants, but in this case the host EDL muscle was removed and discarded. A donor EDL muscle was then obtained from a BF rat and placed into the graft site. The donor EDL tendons were sutured together to the cut remnants of the host tendons.

Muscle Transplant(s) Analysis and Tissue Preparation

Both auto- and allotransplanted muscles were removed at 2, 4, 7, 14, 28, and 56 days after surgery and immediately frozen in liquid nitrogen. Frozen sections of 6 μm thickness were prepared from different regions of each muscle and mounted on multiple glass microscopic slides. Some of these slides were stained with periodic acid-Schiff (PAS-hematoxylin) for histological analysis, whereas the remaining slides were used for immunofluorescence staining.

Preparation of Antibodies and Immunofluorescence Staining

Fibronectin was isolated from freshly obtained rat plasma and antibodies were prepared in rabbits as described in detail elsewhere [12,15].

Type IV collagen and laminin were purified from EHS murine sarcoma and again antibodies prepared in rabbits [16].

Antibodies against type V collagen and heparan sulfate proteoglycan were kindly provided by Dr. George Martin and colleagues (National Institute of Dental Research).

In addition, fluorescein-conjugated concanavalin A (ConA), obtained from Vector Laboratories (Burlingame, CA) was used to further study the myofiber cell surface and basement membrane zone during myofiber degeneration and regeneration [17].

The indirect immunofluorescence staining procedure was similar to that used earlier [12,13]. In brief, purified antibodies (20 μg/ml) against various basement membrane components were applied to adjacent tissue sections, rinsed in phosphate buffered saline (PBS), and incubated with fluorescein-conjugated goat antibody to rabbit IgG (Cappel Laboratories, West Chester, PA). Some slides were directly incubated in fluorescein-conjugated ConA. Controls consisted of sections incubated in preimmune serum only, antibodies preabsorbed with specific antigen, ConA inhibited by mannose, or PBS as the first incubating solution. As shown previously, none of these controls exhibit any nonspecific fluorescence [12,13].

RESULTS

Normal Muscle

The normal rat EDL muscle in cross-section consisted of myofibers of varying diameter and staining intensity (Fig. 1). The immunofluorescence localization of laminin, types IV and V collagen, heparan sulfate proteoglycan, fibronectin, and binding of ConA were seen in the pericellular region of each myofiber corresponding to the basement membrane zone (Fig. 2). The cytoplasm of the myofibers was devoid of these components. Similar distribution of various basement membrane components and collagens has been reported earlier [12,13,18].

Autotransplanted Muscle

After muscle transplantation, the majority of myofibers, except a thin rim of peripheral myofibers, undergo degeneration as a result of devascularization injury. As these grafts become revascularized, the myosatellite cells become activated, undergo proliferation and fusion to form new myotubes, and mature into myofibers. Thus, in early grafts (4 days), three distinct regions were visible: a thin rim of peripheral surviving myofibers; just interior to this area, a small myogenic zone consisting of activated myoblasts and small myotubes; and the remaining central region of ischemic myofibers yet to undergo degeneration (Fig. 3). The myogenic zone extended centrally at 7 days, and the regenerated myotubes grew in size; by 14 days the regeneration process was complete. The regenerated myotubes and myofibers matured in long-term grafts.

Changes in the basement membrane components during the initial myofiber degeneration were quite striking. The expression of fibronectin became fragmented and slowly disappeared from the pericellular region of the degenerating myofibers (Fig. 4). Laminin, types IV and V collagen, and heparan sulfate proteoglycan

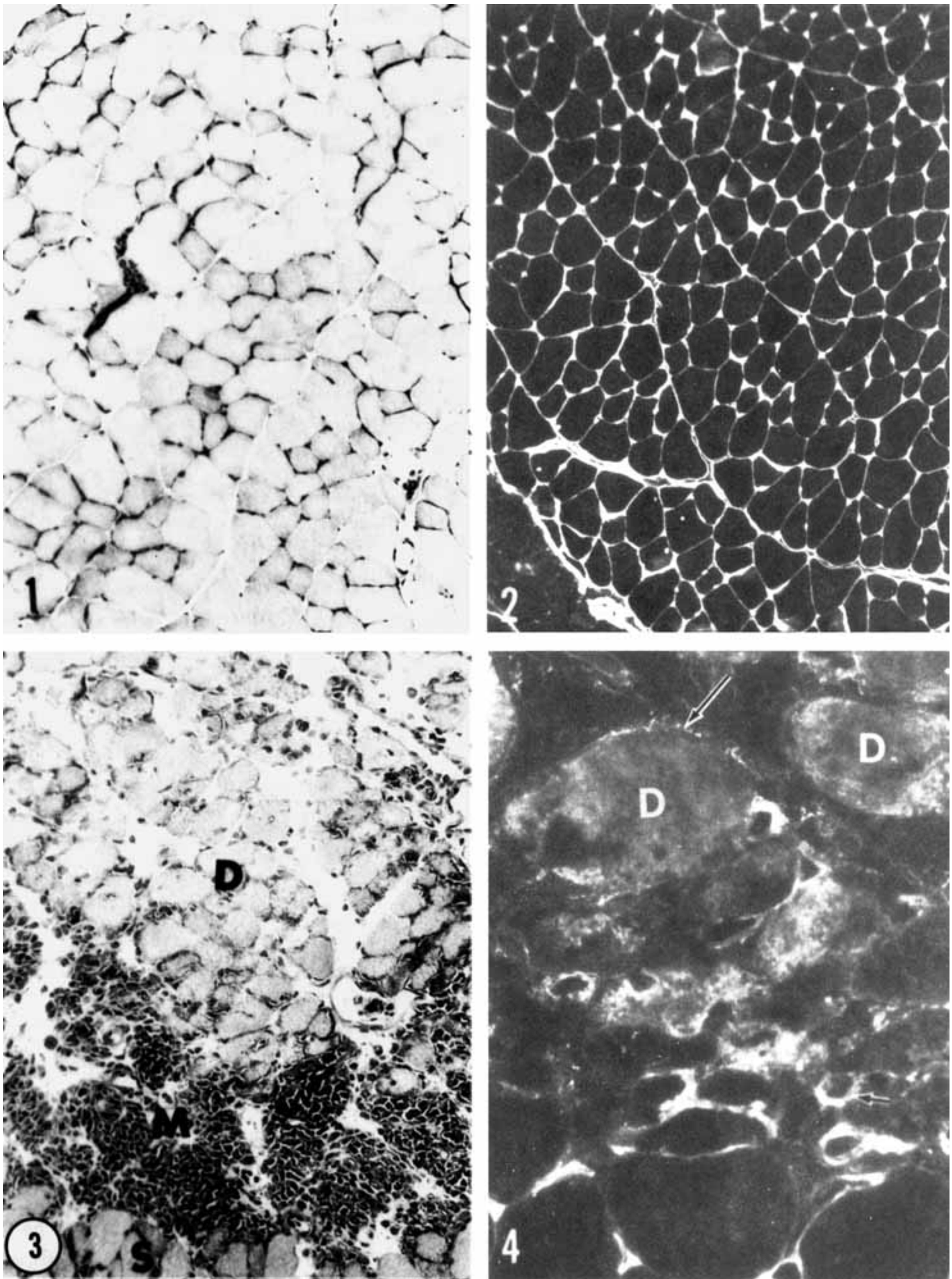

Fig. 1. Cross-section of a normal rat muscle. The muscle consists of myofibers of different sizes and staining intensity. PAS-hematoxylin. ×130.

Fig. 2. Cross-section of a normal EDL muscle stained with antibodies against laminin. Laminin is located in the pericellular region of myofibers corresponding to the region of basement membrane. A similar localization is also seen for other basement membrane components analyzed. ×130.

persisted longer than fibronectin but also eventually disappeared (Fig. 5). Although the major components of basement membrane disappeared, certain unknown component(s) did persist which bound to ConA (Figs. 8, 9). This binding of ConA may be due to persistence of other minor basement membrane components such as entactin, nidogen, or other molecules.

A new basement membrane first appeared around the clusters of regenerated myotubes as a small, ringlike structure (Fig. 6) and was not seen on the cell surface of activated and proliferating myoblast cells. Various components of basement membrane reappeared around the regenerated myotubes, with fibronectin appearing last of all. This new basement membrane enlarged with the maturing myotubes and myofibers and, by 56 days, the expression of all the basement membrane components and ConA binding pattern was similar to that seen in normal muscle (Fig. 7).

Allotransplanted Muscle

Early muscle allotransplants underwent changes similar to those seen in autotransplanted muscles, and an initial attempt at regeneration was evident at 4 and 7 days after transplantation. In later grafts, the regeneration process ceased because of the onset of immune rejection and all the original myofibers and the regenerated myotubes were slowly rejected and disappeared. Thus, by 28 days, a thin muscle was visible consisting of connective tissue matrix, with unidentifiable cells that were probably host macrophages and fibroblasts (Fig. 10). In spite of the disappearance of the muscle cells, their basement membranes remained and continued to express immunoreactivity for basement membrane components (Fig. 11). In 56-day transplants, the immunoreactive staining was seen again in the basement membranes, but they appeared further distorted and collapsed.

The expression of fibronectin in the rejected allotransplants was quite interesting and unexpected. With the onset of immune rejection, fibronectin was seen throughout the muscle, and its staining intensity increased in long-term rejected muscles (Fig. 12).

DISCUSSION

These results describe immunocytochemical changes in the basement membrane components in skeletal muscle transplants undergoing regeneration or rejection. Previously, the fate of the basement membrane has been monitored with the use of the electron microscope. In those studies the basement membrane is described as persisting unchanged during myofiber degeneration and initial regeneration and acting

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Fig. 3. Cross-section of a 4-day autotransplanted EDL muscle. Three zones are visible: a peripheral zone of original surviving myofibers (S), a myogenic zone (M), consisting of newly regenerated myotubes and proliferating myoblasts, and a central zone of ischemic myofibers yet to undergo degeneration (D). PAS-hematoxylin. ×130.

Fig. 4. Cross-section of a 4-day autotransplanted EDL muscle stained with antibodies against fibronectin. There is a loss of pericellular localization of fibronectin in the degenerating myofibers (D). A few degenerating myofibers present a patchy pericellular distribution (long arrow). Also note that the sarcoplasm of the degenerating myofibers shows increased staining for fibronectin, the reason for which is unknown at present. The short arrow points to a small, ringlike expression of fibronectin around the regenerated myotube. ×480.

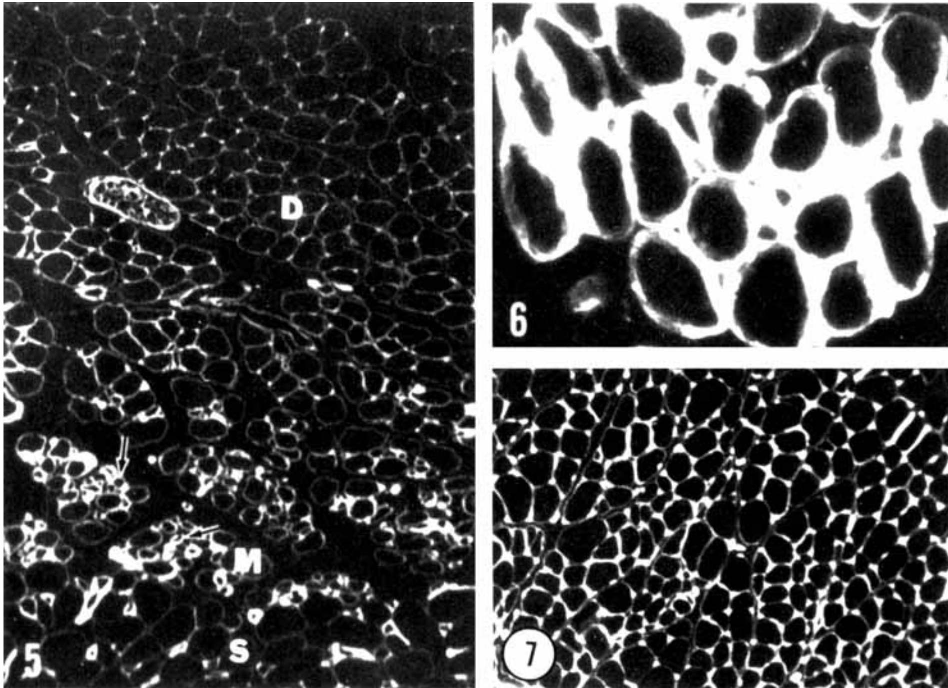


Fig. 5. Cross-section of a 4-day autotransplanted muscle stained with antibodies against laminin (adjacent section to Fig. 3). Three distinct regions are visible: a peripheral zone of original surviving myofibers (S), a myogenic zone (M) consisting of regenerated myotubes, and a central zone of degenerating myofibers (D). The small regenerated myotubes (arrows) present in the myogenic zone appear in clusters and possess a continuous ring of laminin. ×130.

Fig. 6. A cluster of the regenerated myotubes from Figure 5 at higher magnification. Laminin staining of new basement membrane is first seen as small rings on the surface of regenerated myotubes after myoblast fusion. ×480.

Fig. 7. Cross-section of a 28-day autotransplanted muscle stained with antibodies against laminin. The entire muscle is filled with regenerated myofiber(s), and the localization of various basement membrane components is similar to that seen in normal muscle (compare to Fig. 2). ×130.

as a scaffold for orderly regeneration [2–5]. The present results show that although a certain component(s) of this basement membrane persist for some time, other major components disappear quite rapidly from the basement membrane zone of the degenerating myofibers. The disappearance of major components may be due to enzymatic degradation. Such enzymes may be released from the infiltrating macrophages [19] or activated myoblasts. The myoblast could be the source of such enzymes because in rejected muscle allografts, which lack myoblasts (immunologically rejected), the basement membranes continue to express immunoreactivity for various components. It is also noteworthy that in regenerating skeletal muscle, the basement membrane does not disappear completely. A certain component persists, and it may be that the remaining component(s) act as a scaffold for orderly regeneration [2,3,20].

Activated myoblasts lack basement membranes, and the formation of a new basement membrane occurs only after they fuse to form myotubes. The formation of

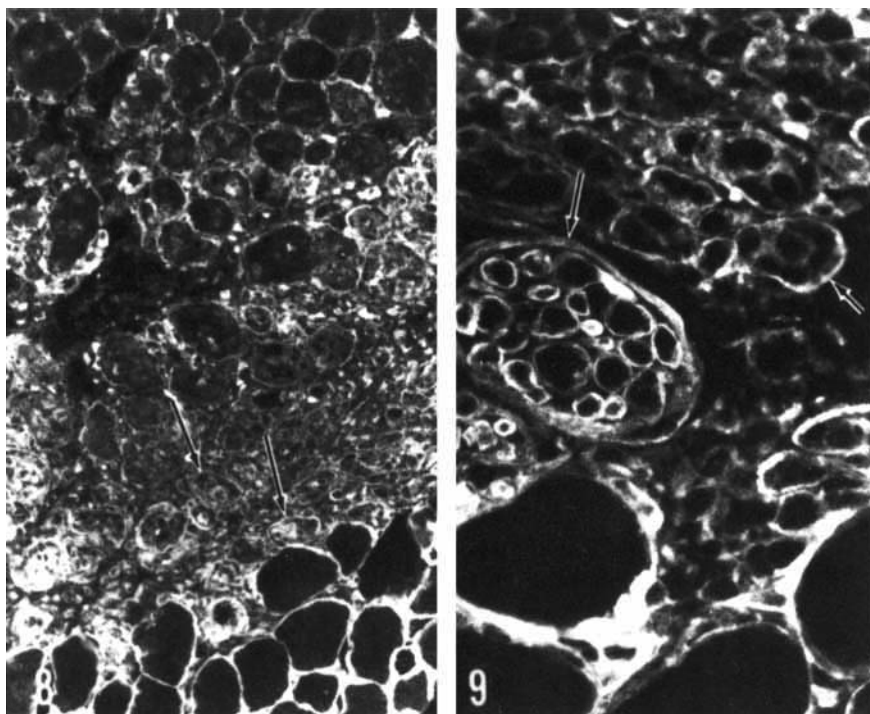
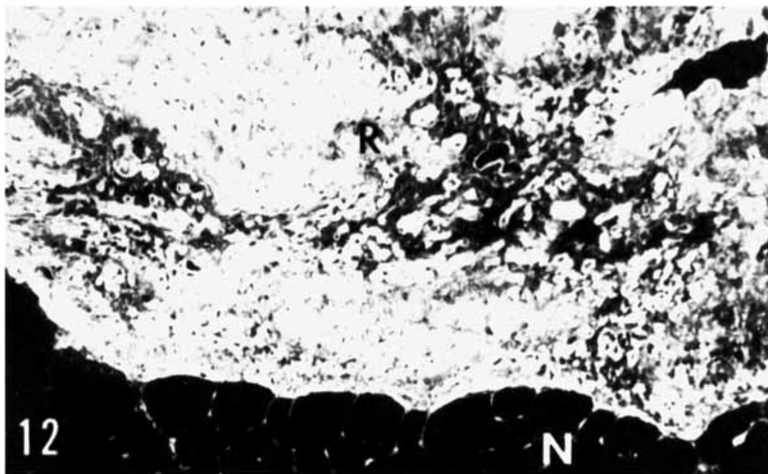


Fig. 8. Cross-section of 4-day autotransplanted muscle stained with fluorescein-conjugated ConA. Again, three distinct zones are visible, as mentioned in Figures 4 and 5. The binding pattern of ConA reveals the remains of the original basement membrane zone (long arrows), that was not seen in sections stained with specific antibodies against major basement membrane components (compare Figs. 5 and 8) [from 13; reproduced through the courtesy of J. Cell Biol. and the Rockefeller Press]. ×220.

Fig. 9. Higher magnification of Figure 8 showing the remains of the original basement membrane (short arrow) as revealed by ConA binding. Small rings of regenerated myotubes are seen within the remains of the old basement membrane (short arrow). The long arrow points to the perineurium of a myelinated nerve present within the muscle [from 13; reproduced through the courtesy of J. Cell Biol. and the Rockefeller Press]. ×480.

the new basement membrane involves the sequential reappearance of its various components. In muscle culture studies, formation of new basement membrane has been described. It has been shown that myoblasts can synthesize basement membrane components but only start assembling them on their surface after their fusion and formation of myotubes [21,22]. The present in vivo results and previous in vitro results [21,22] are in agreement and demonstrate that the formation of a new basement membrane is a terminal step during muscle regeneration.

In contrast to autotransplants, where there is a rapid disappearance and reappearance of basement membrane, in allotransplants no such turnover is seen. Although the basement membranes undergo an extensive change in their shape, the immunoreactivity for various basement membrane components remains unchanged. In addition, the accumulation of fibronectin in allotransplants with the onset of immune rejection is intriguing and is deserving of further studies to determine its exact functional significance. The presence of fibronectin in rejected muscle may



Figs. 10, 11, 12

have a chemotactic effect on fibroblasts and macrophages and as an opsonin which is necessary for the ingestion of degenerating foreign tissue [14,23–25].

In summary, the changes in the basement membrane components undergoing muscle regeneration or rejection, as observed here, suggest a more dynamic role for the basement membrane. The changes in various basement membrane components may be important in initiating and controlling the process of orderly degeneration and regeneration of skeletal muscle after injury.

ACKNOWLEDGMENTS

The author thanks Drs. Andrew A. Zalewski, A.H. Reddi, and Mathew Daniels for helpful discussions and comments, and Connie Benson and Sandra Dunn for the skillful preparation of the manuscript.

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Fig. 10. Cross-section of a 28-day allotransplanted EDL muscle, which has undergone rejection. The rejected muscle (R) consists of connective tissue with an occasional myotube and infiltrated mononuclear cells. Since the overall size of the allotransplanted muscles was reduced, they were encased in the slabs of normal muscle (N) to facilitate their sectioning [from 14; reproduced through the courtesy of Anat. Rec. and Alan R. Liss, Inc.]. PAS-hematoxylin. ×120.

Fig. 11. Cross-section of a 28-day allotransplanted EDL muscle stained with antibodies against laminin, adjacent to Figure 10. The basement membrane region (arrows) of the rejected myotubes are immunoreactive for laminin, although they appear small and distorted. Similar immunoreactivity was also seen for type IV collagen [from 14; reproduced through the courtesy of Anat. Rec. and Alan R. Liss, Inc.]. ×120.

Fig. 12. Cross-section of a 28-day allotransplanted EDL muscle stained with antibodies against fibronectin. An intense fluorescence for fibronectin is seen throughout the rejected muscle (R). Encasing normal muscle (N) shows the distribution of fibronectin in the pericellular region of the normal myofibers [from 14; reproduced through the courtesy of Anat. Rec. and Alan R. Liss, Inc.]. ×120.

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