Changes in the Basement Membrane Zone Components during Skeletal Muscle Fiber Degeneration and Regeneration

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ABSTRACT The basement membrane of skeletal muscle fibers is believed to persist unchanged during myofiber degeneration and act as a tubular structure within which the regeneration of new myofibers occurs. In the present study we describe macromolecular changes in the basement membrane zone during muscle degeneration and regeneration, as monitored by immunofluorescence using specific antibodies against types IV and V collagen, laminin, and heparan sulfate proteoglycan and by the binding of concanavalin A (Con A). Skeletal muscle regeneration was induced by autotransplantation of the extensor digitorum longus muscle in rats. After this procedure, the myofibers degenerate; this is followed by myosatellite cell activation, proliferation, and fusion, resulting in the formation of new myotubes that mature into myofibers. In normal muscle, the distribution of types IV and V collagen, laminin, heparan sulfate proteoglycan, and Con A binding was seen in the pericellular basement membrane region. In autotransplanted muscle, the various components of the basement membrane zone disappeared, leaving behind some unidentifiable component that still bound Con A. Around the regenerated myotubes a new basement membrane (zone) reappeared, which persisted during maturation of the regenerating muscle. The distribution of various basement membrane components in the regenerated myofibers was similar to that seen in the normal muscle. Based on our present and previous study (Gulati, A. K., A. H. Reddi, and A. A. Zalewski, 1982, Anat. Rec. 204:175–183), it appears that some of the original basement membrane zone components disappear during myofiber degeneration and initial regeneration. As a new basement membrane develops, its components reappear and persist in the mature myofibers. We conclude that skeletal muscle fiber basement membrane (zone) is not a static structure as previously thought, but rather that its components change quite rapidly during myofiber degeneration and regeneration.

The importance of basement membrane (basal lamina) during the orderly regeneration of damaged skeletal muscle has been emphasized by several authors (2, 5, 7, 21, 40, 47, 50). In general, on the basis of the studies just listed, it is believed that the basement membrane of each myofiber persists as an unchanged tube during muscle fiber degeneration and initial regeneration and forms the structural framework within which the new myotubes and myofibers develop. The evidence supporting this structural role of basement membrane is based on electron microscope studies, which demonstrate its presence around the degenerating myofiber (1, 21, 47).

Although the complete macromolecular composition of basement membrane is still not known, its complex biochemical nature has been recently studied extensively (16, 24, 37, 46). Basement membranes are known to consist of several collagenous and noncollagenous components. The most prominent collagenous component of basement membrane is type IV collagen, which has been localized by immunoelectron microscopy in the lamina densa layer (12, 36, 51). Type V collagen, although associated with a few basement membranes, is not an integral component (29) but is localized in the pericellular environment (15, 29).

Several noncollagenous components have recently been characterized and localized within the basement membrane. Laminin, a high molecular weight glycoprotein, is a ubiquitous component of the basement membrane (46). Laminin
has been localized in the lamina lucida (lamina rara) layer of basement membrane (13). Recently, laminin has also been detected in the lamina densa (26, 38). Fibronectin is another glycoprotein which has been associated with the lamina lucida of the basement membrane; however, it is not a universal component and is also present in other regions of the extracellular matrix (8, 30, 34, 44). Heparan sulfate proteoglycan has also been shown to be an important component of basement membranes and has also been localized in the lamina lucida layer (22, 23). Since there is some overlap and co-distribution of various components in different layers, it may be that all components jointly form the basement membrane. Other components that have been associated with different basement membranes include entactin, bullous pemphigoid antigen, chondroitin sulfate, and certain lipids. Additional details regarding the components of basement membrane can be obtained from several review articles (24, 34, 37, 42, 43, 45).

Since it is now known that the basement membrane consists of several unique macromolecules, we investigated possible changes in basement membrane components and related these to the processes of skeletal muscle degeneration and regeneration. Skeletal muscle degeneration and regeneration was induced by autotransplantation of extensor digitorum longus (EDL) muscle in rats. This model has been widely used to study muscle regeneration in vivo (6, 7, 17, 18, 19).

### MATERIALS AND METHODS

**Surgery and Tissue Preparation:** We used male Fischer rats (175-200 g body weight) obtained from the National Institutes of Health (Bethesda, MD) breeding colony. Each animal was anesthetized with chloral hydrate (400 mg/100 g body weight i.p.) and the EDL muscle in each leg autotransplanted according to the procedure described earlier (19). In brief, the proximal tendon of the EDL muscle was cut close to the knee; the muscle was removed from its bed and transplanted back in the same site. No attempt was made to join any blood vessels or nerves to the muscle since it was expected that revascularization and reinnervation would occur from the blood vessels and nerves in the graft site (7). Finally, the overlying muscle and skin were separately sutured. Autografted muscles were removed at 2, 4, 7, 14, 28, and 56 d after surgery and frozen in liquid nitrogen. Six to seven muscles were analyzed at each time interval; in addition, several normal muscles were analyzed. Frozen cross-sections of 6 µm thickness, from different regions of the muscle, were cut serially in a cryostat set at -20°C and mounted on multiple glass slides. Some of these slides were stained with periodic acid-Schiff (PAS)-hematoxylin for histological analysis, whereas the remaining ones were used for immunofluorescent staining.

**Preparation of Antibodies and Immunofluorescent Staining:** Type IV collagen and laminin were purified from Engelbreth-Holm-Swarm murine sarcoma, which is known to produce large amounts of basement membrane matrix (46). Both type IV collagen and laminin were dissolved separately in PBS and emulsified with an equal volume of Freund's complete adjuvant and injected into different rabbits. Antibodies against type IV collagen and laminin were isolated by cross-immunoadsorption. The specificity of these antibodies has been reported earlier (11). Another batch of antibodies against type IV collagen as well as the antibodies against heparan sulfate proteoglycan and type V collagen were provided by Dr. George Martin and colleagues (National Institute of Dental Research, Bethesda, MD). In addition, fluorescein-conjugated concanavalin A (Con A) (Vector Laboratories, Burlingame, CA) was used to demonstrate the muscle fiber cell surface and basement membrane zone during skeletal muscle degeneration and regeneration. Con A and other lectins have been used to demonstrate the pericellular basement membrane zone of myofibers in recent studies (32, 39). The purified antibodies to types IV and V collagen, laminin, and heparan sulfate proteoglycan were applied to adjacent tissue sections (20 µg/ml) and incubated at room temperature (22°C-25°C) for 30 min. The slides were then washed with PBS and incubated with fluorescein-conjugated goat antibody to rabbit IgG diluted 1:20 with PBS (Cappel Laboratories, West Chester, PA). The slides were washed again and mounted in a medium consisting of 90% glycerol and 10% PBS. Some slides at each time interval were incubated directly with fluorescein-conjugated Con A (diluted 1:40), washed with PBS, and mounted as before. All slides were viewed with a Zeiss Ortholux II, epilluminated fluorescent microscope. Controls consisted of sections incubated in preimmune serum only, preabsorbed antibodies with specific antigen, or Con A with mannose, and PBS as the first incubating solution.

### RESULTS

#### Normal Muscle

The normal EDL muscle of the rat consisted of muscle fibers which varied in diameter and staining intensity, and each possessed a peripheral nuclei (Fig. 1). The immunofluorescent localization of types IV and V collagen, laminin, and heparan sulfate proteoglycan was seen as a fine line in the pericellular region of each myofiber corresponding to the basement membrane (Fig. 2). Type V collagen stained more diffusely in the pericellular region of myofibers, and its distribution was similar to that of fibronectin as seen in our previous study (19). The cytoplasm of the myofiber was devoid of any staining for basement membrane components. A similar pericellular binding was also noted in the muscle when stained with fluorescein-conjugated Con A (not shown). Wheat germ agglutinin and Ricinus communis agglutinin 120 also bound in the pericellular region (nonsynaptic), whereas other lectins (soybean agglutinin, Dolichos biflorus agglutinin, Ulex europaeus agglutinin I, peanut agglutinin) either bound very poorly or not at all (A. K. Gulati, unpublished observation). The cell-surface distribution of the various basement membrane zone components and the binding of lectins in normal muscle agreed with the findings of previous reports (9, 31, 32, 38, 39, 44). All control sections lacked fluorescence; a representative control section of normal muscle in which the antibodies were preabsorbed with purified antigen (laminin) lacking fluorescence is shown in Fig. 3.

#### Autotransplanted Muscle

The histological progression of muscle fiber degeneration and regeneration observed after autotransplantation was similar to that described by Carlson and colleagues (6, 7). After 2 d, two distinct zones were visible in the muscle graft: a thin peripheral zone of surviving myofibers (which apparently could receive nutrients from the surrounding tissues) and a remaining zone of ischemic myofibers (6, 7). As the vascularization of muscle grafts was restored, the ischemic myofibers nearest to the surviving myofibers underwent degeneration and were phagocytosed by infiltrating macrophages. At the same time, the myosatellite cells present within these degenerating myofibers differentiated to form myoblasts, which proliferated and fused to form myotubes (41). With the activation of myoblasts and the formation of regenerated myotubes, a new myogenic zone appeared in 4-d autotransplants (Fig. 4). The myogenic zone extended centrally as further revascularization was restored at 7 d and the regenerated myotubes grew in size. By 14 d most of the muscle graft was filled with peripheral original surviving myofibers and the regenerated myofibers and myotubes. The regenerated myofibers had grown in size at 28 and 56 d and become similar to the original surviving myofibers; however, the regenerated myofibers possessed centrally located nuclei, a typical feature.
of regenerated muscle (7, 17).

In a 2-d muscle autotransplant, the distribution of types IV and V collagen, laminin, and heparan sulfate proteoglycan did not change in the basement membrane region of the myofibers. In contrast to this observation, we have shown previously that fibronectin, another basement membrane zone glycoprotein disappears from the pericellular region of the degenerating myofibers within 2 d (19). In 4-d muscle grafts, three distinct zones were visible when the sections were stained for various basement membrane region components (Figs. 5 and 9). A zone of peripheral surviving original myofibers maintained all of the original basement membrane zone components (including fibronectin). The inner zone of ischemic and degenerating myofibers also continued to express the presence of various basement membrane components. The degenerating and ischemic myofibers appeared similar when stained for types IV and V collagen, laminin, and heparan sulfate proteoglycan. In contrast, staining for fibronectin showed differential staining of degenerating and ischemic myofibers, thus resulting in four zones as described earlier (19). In the myogenic zone, the activated myoblasts did not show any staining for basement membrane components; however, after their fusion to form myotubes, staining for basement membrane was seen as small rings around them (Figs. 5 and 9). The small newly regenerated myotubes were initially seen in close approximation to the larger myotubes (Fig. 6) or in some cases close to the outer surface of the basement membrane of degenerating myotubes (Fig. 9). Since regenerated myotubes appear as clusters in the myogenic zone (Figs. 5 and 6), this may mean that the larger myotubes provide a favorable environment for the formation of new myotubes. That these clusters were actually regenerated myotubes and not basement membrane profiles of the degenerated myofibers that they resemble (38) was confirmed by comparing adjacent sections stained with PAS-hematoxylin, which showed centrally located nuclei in the cytoplasm. The new basement membrane acquired by regenerated myotubes persisted throughout maturation, and the entire muscle was filled with peripherally located original myofibers and centrally located regenerated myofibers (Fig. 8). In a few muscle grafts, a central region of necrotic myofibers was seen because of incomplete revascularization after 21 d. The distribution of various basement membrane components was irregular and patchy around these necrotic myofibers (Fig. 7).

The binding of fluorescein-conjugated Con A revealed the remains of the original basement membrane zone of the myofibers undergoing degeneration (Figs. 10 and 11), which was not seen with antibodies against specific basement membrane components (Figs. 5 and 9; and reference 19). In addition to binding to the original basement membrane, Con A also bound to the surface of myotubes. Weak binding was also seen on certain unidentified cells (possibly myoblasts and macrophages) present in the ischemic and degenerating myofibers (Fig. 10).

In a fully regenerated muscle the distribution of all basement membrane zone components was similar to that seen in the normal muscle. In summary, during the process of myofiber degeneration fibronectin disappeared from the pericellular region (19). This was followed by the disappearance of laminin and type IV collagen. Heparan sulfate proteoglycan and type V collagen also disappeared about the same time, leaving behind certain unidentifiable component that had affinity for Con A binding. The regenerated myotubes produced a new basement membrane, the components of which reappeared with fibronectin appearing last.

DISCUSSION

The disappearance and reappearance of types IV and V collagen, laminin, and heparan sulfate proteoglycan, four known components of the basement membrane matrix (16, 24) were used to study the changes in the skeletal muscle basement membrane region during degeneration and regeneration. In normal muscle, these macromolecules and fibronectin (19) were localized in the pericellular region of myofiber corresponding to the basement membrane zone.

The various components of the basement membrane zone did not change initially; however, as the revascularization of the muscle occurred changes in the expression of various basement membrane macromolecules were seen. Fibronectin first disappeared from the basement membrane region of the degenerating myofibers (19); this was followed by the disappearance of other major components of basement membrane. The disappearance of various basement membrane macromolecules may be due to their enzymatic degradation. Enzymes involved in the degradation of basement membrane components may be released by infiltrating macrophages which are known to possess such enzymes (27). Also enzymes present in the activated myoblasts or accumulating extracellular fluid could be responsible for the degradation of basement membrane components. In fact, in several in vivo and in vitro models, alterations and degradation of basement membranes and their components have been shown to result from the action of specific enzymes (3, 27, 33, 52). Surviving cells (like myoblasts) within the muscle graft seem to be essential for the degradation of basement membrane because, in muscle allografts undergoing immune rejection, the basement membrane components persist in spite of the fact that myofiber cytoplasm and myoblast disappear (A. K. Gulati, unpublished observation).

As the activated myoblasts that lack basement membrane fuse to form myotubes, a new basement membrane appears around them. The formation of new basement membrane (zone) involves the sequential appearance of various components, which are probably synthesized by the fusing myoblasts. It has been demonstrated that myoblasts can synthesize various basement membrane matrix components in vitro and can assemble them on the myotube cell surface (3, 14, 25). A similar synthesis of various components may occur with in vivo regenerating myotubes. The new basement membrane enlarges along with growing myofibers and eventually becomes similar to the original basement membrane. The sequential appearance of various basement membrane components, as seen in the present study, has also been reported during embryonic kidney development (10) and in regenerating muscle of the amputated newt limb (20). The disappearance of the original basement membrane zone of the degenerating myofibers and the formation of a new basement membrane zone around the regenerated myofibers seems to be well-regulated phenomenon, since no elaboration or thickening of it was observed in a regenerated muscle. Abnormalities in the basement membrane, as indicated by abnormal formation and thickening, have been seen in various pathological states (31, 34, 35, 47, 48, 49).

Although the major basement membrane zone macromolecules disappeared quite rapidly from the pericellular region of the degenerating myofiber, some unidentifiable component

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FIGURES 9–11  Fig. 9: Cross-section of a 4-d autotransplanted muscle stained with antibodies against heparan sulfate proteoglycan (adjacent to Fig. 5). Three distinct regions are again visible. The long arrows point to the remains of the original basement membrane. The newly regenerated myotubes (short arrow) mostly are seen in close approximation to the original basement membrane but are located on the outside. A similar distribution is also seen for type V collagen. × 220. Fig. 10: Cross-section of a 4-d autotransplanted muscle showing binding of fluorescein-conjugated Con A. Again three distinct zones are visible. The binding pattern of Con A reveals what appears to be the remains of the original basement membrane zone (long arrows) that was not seen in earlier figures (Figs. 5 and 9). Also compare to PAS-hematoxylin stained adjacent section (Fig. 4). Within the remains of the original basement membrane of the ischemic myofibers, a few weakly stained unidentifiable cells are seen (short arrows). × 220. Fig. 11: Higher magnification of Fig. 10 showing the remains of the original basement membrane (short arrow) as revealed by Con A 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 in the muscle. × 480.

did remain as visualized by the binding of Con A. By the use of Con A, a lectin which specifically binds to the mannose or glucose subunit of glycoproteins or glycolipids (32) the irregular and broken nature of what appeared to be the original basement membrane zone (39) could be visualized. In fact, in some locations the remains of original basement membrane zone appeared as a complete ring with myoblasts and myotubes maturing within them. It may be that the persistence of this Con A–binding component is revealed as a dark staining line with an electron microscope and could lead to the conclusion that the original basement membrane remains intact, allowing proper regeneration (1, 27, 47, 50). It is, however, possible that the Con A–binding remnants of the original basement membrane possess enough information for ade-
quatereinnervation and regeneration. It is known that in frog muscle the basement membrane of the degenerating myofibers continues to maintain original synaptic sites and cholinesterase activity, thus allowing proper reinnervation (4, 28, 39). These results show that the major components of basement membrane disappear; it remains to be determined what happens to these components in electron immunohistochemical study. It also remains to be determined whether the breakdown of basement membrane components is essential for regeneration to occur and, if so, what might happen if the process was inhibited.

The results of the present and our previous study (19) indicate that various macromolecular components of basement membrane zone disappear during early phases of myofiber degeneration. A new basement membrane reappears around the regenerated myotubes, with its various components appearing in a sequence. In the regenerated muscle, the various components of the new basement membrane appear to be similar to the original basement membrane. We conclude that the myofiber basement membrane zone is not static but that its components change rapidly during degeneration and regeneration.

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