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Dystrophin Deficiency Is Associated with Myotendinous Junction Defects in Prenecrotic and Fully Regenerated Skeletal Muscle

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The myotendinous junction (MTJ) is the major site of force transmission from myofibrils across the muscle cell membrane to the extracellular matrix. The MTJ is thus an appropriate model system in which to test the hypothesis that dystrophin, the gene product absent in Duchenne muscular dystrophy, functions as a structural link between the muscle cytoskeleton and the cell membrane. We studied changes in MTJ structure in dystrophin-deficient* mdx *mice during periods of growth and aging that spanned prenecrotic, necrotic, and regenerative phases of postnatal muscle development in* mdx *mice. Prenecrotic animals were found to exhibit structural defects at MTJs that were similar to those described previously in animals at the peak of necrosis, including a reduction in lateral associations between thin filaments and the MTJ membrane. These defects therefore occur before necrosis and may be directly related to the absence of dystrophin. Observations of regenerating and fully regenerated MTJs in adult animals show that the defects are still present, indicating that normal thin filament-membrane associations are never formed in dystrophin-deficient muscle. However, in prenecrotic as well as regenerated adult* mdx *muscle, the MTJ membrane is only slightly less folded than in age-matched controls. This indicates that* mdx *muscle possesses some dystrophin-independent mechanism that allows for the initial formation of MTJs, despite the absence of dystrophin. The presence of the defect in normal, lateral, thin filament-membrane associations in* mdx *muscle, regardless of age, supports the hypothesis that dystrophin functions as a structural link between thin filaments and the membrane. (Am J Pathol 1993, 142:1513–1523)

Dystrophin is the gene product that is absent or altered in the Duchenne and Becker forms of muscular dystrophy.[1] A current hypothesis concerning the function of dystrophin in normal skeletal muscle states that the protein acts to support the mechanical integrity of the plasma membrane and/or is involved in cytoskeleton-membrane interactions. This hypothesis is based on a) sequence similarities between domains of dystrophin and those of the cytoskeletal proteins spectrin and α-actinin,[2] b) immunolocalization of dystrophin at the muscle cell membrane,[3–6] and c) the apparent association of dystrophin with a glycoprotein complex that spans the plasma membrane[7,8] and has binding affinity for laminin.[9] If dystrophin functions as an essential mechanical link between cytoskeleton and membrane, then the absence of dystrophin could result in the inability of the muscle cell cytoskeleton to maintain its association with the membrane under physiological mechanical loads. Such a defect would likely be detectable morphologically, especially at sites where cytoskeleton-membrane interactions experience high mechanical stress.

The skeletal muscle myotendinous junction (MTJ) is the primary site at which muscular force is transmitted from myofibrils across the plasma membrane to the collagen fibrils of the tendons. The interface between cell and substratum at the MTJ is highly folded and interdigitated, an adaptation for reduc-

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tion of mechanical stress on the junctional membrane.[10,11] Actin filaments extend into the terminal muscle cell processes at the MTJ and are bundled into a subsarcolemmal dense plaque that is also thought to be a specialization for adhesion to and force transmission across the cell membrane.[12] Talin, vinculin, and paxillin, proteins that function in actin-membrane associations in the focal contacts of cultured fibroblasts,[13–15] are also concentrated at MTJs.[15–17] In addition, several recent studies have demonstrated that dystrophin is present at MTJs, as well as at nonjunctional muscle cell membranes.[5,18,19] Thus, the morphological specializations and molecular composition of the MTJ make it an excellent model system in which to study cytoskeleton-membrane interactions, vis-à-vis the function of dystrophin.

We recently identified a defect in MTJ morphology in hind limb muscles of 4-week-old *mdx* mice, which lack dystrophin,[1] that is not seen in age-matched control mice in which dystrophin is present.[20] Specifically, we noted that 4-week *mdx* MTJs show much less of the membrane-folding characteristic of normal MTJs and lack normal, lateral associations between thin filaments and the junctional membrane. These observations support the hypothesis that dystrophin mediates lateral associations between thin filaments and the membrane and that those associations are necessary for MTJ membrane folding. We further speculated that reduction of membrane folding could result in mechanical damage to these sites, leading to muscle fiber necrosis.

In the present investigation, MTJ structure is compared in *mdx* and control mice at various ages, to enable the following questions to be answered. 1) Is the defect in thin filament-membrane association seen in 4-week-old mice also present in *mdx* muscle that has not yet undergone degeneration and necrosis? If MTJs appear normal in 1-week-old *mdx* muscles that display no signs of necrosis, that will indicate that MTJ defects are secondary to some other pathological change resulting from dystrophin deficiency. If, however, the junctions display defects in lateral associations between thin filaments and the membrane, that will support the hypothesis that dystrophin mediates thin filament-membrane lateral associations. 2) Does MTJ morphology of adult *mdx* mice resemble that of controls? If reduction in MTJ membrane folding results in mechanical failure at the junction followed by fiber necrosis, then the successfully regenerated fibers in adult *mdx* muscle must possess some mechanism for generating and maintaining MTJ membrane folding in the absence of dystrophin.

Materials and Methods

Mdx mice and age-matched C57BL/10SnJ mice were killed by cervical dislocation at 1, 4, 6, and 23 weeks of age. Hind limbs were skinned and muscles were fixed *in situ* for 1 hour by immersion in 1.4% glutaraldehyde in 0.2 mol/L cacodylate buffer, pH 7.2. Soleus and extensor digitorum longus muscles were then dissected free and fixed for an additional hour. Muscles were dissected into blocks, postfixed in 1% OsO_4, then dehydrated, and embedded in epoxy resin. Longitudinal thin sections were stained with uranyl acetate and lead citrate. Sections were viewed and photographed using a Zeiss EM10AG transmission electron microscope (Carl Zeiss, Oberkochen, FRG). Because this study is directed toward identifying structural defects specific to the absence of dystrophin, we primarily compared fibers from all four age groups that showed no signs of general degeneration, such as Z-disc streaming, hypercontraction, or dilation of sarcoplasmic reticulum. Because such apparently normal fibers were atypical in *mdx* muscle at 4 and 6 weeks of age, we have also included a description of MTJ morphology that is more representative of the samples from those age groups.

Results

One Week

Tissue from both *mdx* and control mice at 1 week of age displayed signs that the muscle fibers were immature (Figure 1). Fiber profiles had uniformly small diameters in both groups (10 to 20 μ), and fibers from both had narrow, although well-organized, myofibrils interspersed with a large number of mitochondria, free ribosomes, and myonuclei. Mononucleated cells were clustered at the MTJs of fibers from both groups, in approximately equal numbers. These cells had the extended cytoplasmic processes and abundant rough endoplasmic reticulum characteristic of tendon fibroblasts.[21] No qualitative difference in muscle cell membrane folding at MTJs was seen at 1 week. However, fibers that showed no ultrastructural signs of the onset of degeneration in nonjunctional regions displayed a structural anomaly in some MTJ regions (Figure 1A). The terminal thin filaments were bundled as in control MTJs, but the lateral associations between these filament bundles and the junctional plasma membrane that are present at control MTJs were absent in the 1-week *mdx* tissue. These defects in

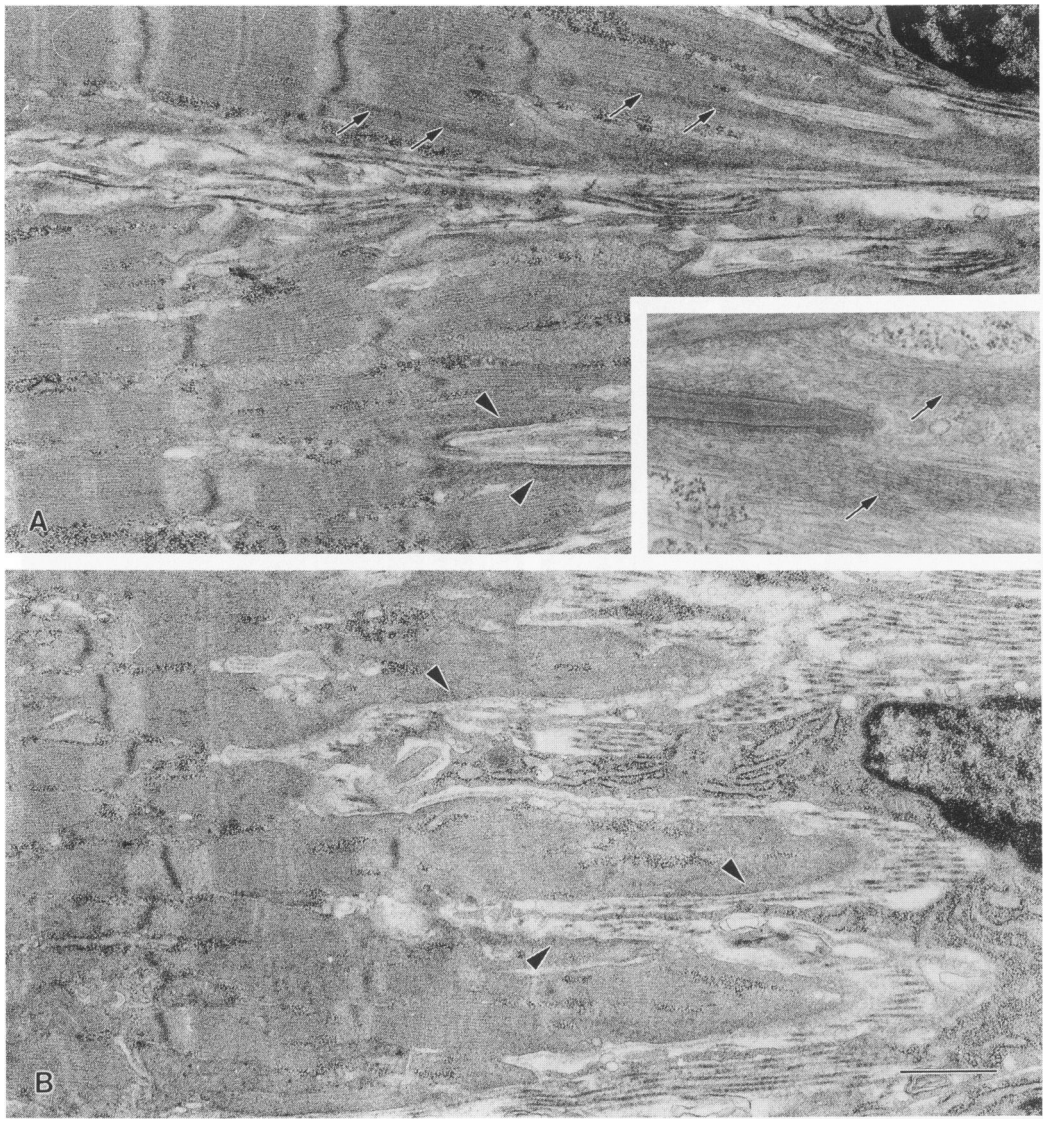

Figure 1. *Electron micrographs of MTJs from hind limb muscle of mice aged 1 week.* **A:** mdx. *The MTJ membrane is highly folded, and terminal cell processes interdigitate with collagen fibrils. Myofibrils extend into terminal muscle cell processes, where most of the terminal thin filaments are bundled and associate laterally with junctional plasma membrane (arrowheads). However, several terminal filament bundles are present without laterally-associated membrane (arrows). These filaments seem to be anchorerd to membrane only at their ends. A fibroblast is seen adjacent to the MTJ. Inset: high magnification of two terminal thin filament bundles lacking lateral association with the cell membrane.* **B:** *control. MTJ membrane is also highly folded in the control fiber, and all terminal thin filament bundles seem to be associated laterally with junctional membrane (arrowheads). Fibroblast processes interdigitate between the muscle cell processes. Scale bar = 1 μ.*

cytoskeleton-membrane association, although definitely present in these prenecrotic samples, were not widespread; terminal thin filament bundles that appeared normally associated with folded plasma membrane were also seen in the 1-week *mdx* fibers. The defect was not seen in any 1-week control samples (Figure 1B). No qualitative differences in MTJ structure were detected between samples from soleus and extensor digitorum longus muscles, in *mdx*, or control mice within each age group studied.

Four Weeks

Muscles from *mdx* and control animals were examined, and the results confirm and extend those of our initial study.[20] Previous studies (e.g., ref. 22) have shown 4 weeks to be the peak of muscle fiber necrosis in *mdx* mice, and our results qualitatively confirm the presence of widespread degeneration and necrosis at this age, absent in control tissue. MTJs of fibers in advanced states of degeneration exhibited Z-disc streaming, dilation of sarcoplasmic

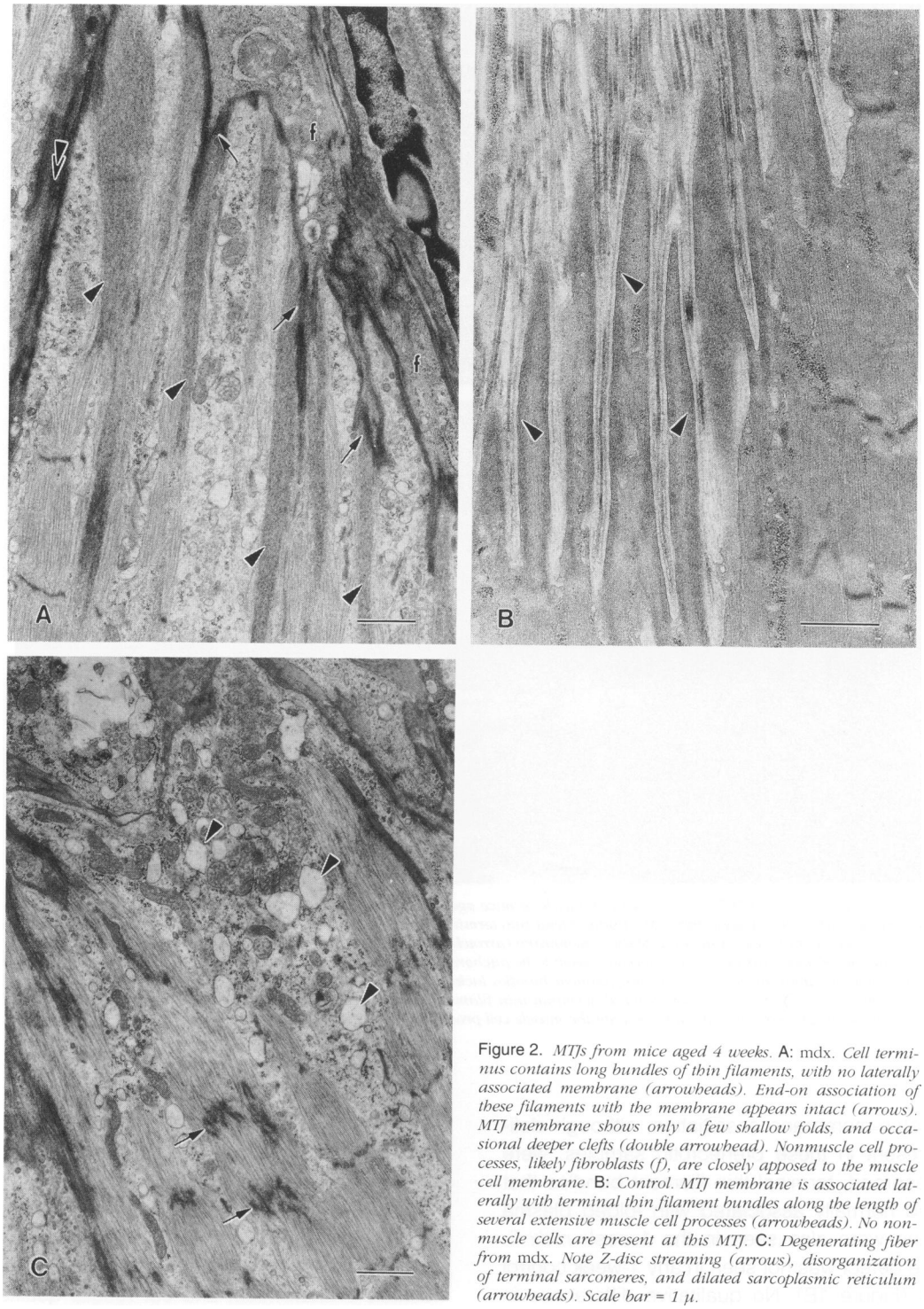

Figure 2. *MTJs from mice aged 4 weeks.* A: mdx. *Cell terminus contains long bundles of thin filaments, with no laterally associated membrane (arrowheads). End-on association of these filaments with the membrane appears intact (arrows). MTJ membrane shows only a few shallow folds, and occasional deeper clefts (double arrowhead). Nonmuscle cell processes, likely fibroblasts (f), are closely apposed to the muscle cell membrane.* B: *Control. MTJ membrane is associated laterally with terminal thin filament bundles along the length of several extensive muscle cell processes (arrowheads). No nonmuscle cells are present at this MTJ.* C: *Degenerating fiber from* mdx. *Note Z-disc streaming (arrows), disorganization of terminal sarcomeres, and dilated sarcoplasmic reticulum (arrowheads). Scale bar = 1 μ.*

reticulum, and disorganization of myofibrils (Figure 2C). However, in cells that appeared otherwise normal in nonjunctional regions, terminal thin filament bundles lacking lateral associations with the plasma membrane were present at MTJs of *mdx* animals (Figure 2A) and were not seen in controls (Figure 2B). These defects were much more prevalent in the 4-week tissue than in the 1-week samples. The MTJ membrane of *mdx* fibers also seemed to be less folded than that of control fibers. Junctional regions in both control and *mdx* tissue were populated by mononucleated cells whose elongated shape, extended cytoplasmic processes, and abundant rough endoplasmic reticulum identified them as fibroblasts. However, these cells were more closely apposed to the muscle cell termini in the *mdx* tissue (Figure 2A), and the dilated state of their rough endoplasmic reticulum along with the presence of prominent nucleoli (not shown) indicated that they were more metabolically active than similar cells at control MTJs.

Six Weeks

Mdx muscles are known to undergo a period of active regeneration that peaks at approximately 6 weeks of age.[22] Examination of *mdx* tissue at 6 weeks confirmed the presence of a large number of fibers that seemed to be actively regenerating. Many fibers displayed chains of nuclei, some at or near the MTJ (Figure 3C), that were located in the middle of the fiber rather than at the periphery. Fibers also frequently contained small-diameter myofibrils (<0.5 μ), with intervening mitochondria and vesiculated cytoplasm (Figure 3, C and D) that were not seen in age-matched control tissue. Myofibrils were often disorganized at MTJs of 6-week *mdx* fibers, exhibiting Z-disc streaming, myofilaments out of lateral register, and terminal sarcomeres without Z-discs (not shown; cf. Figure 2C). Overall, junctional membrane folding in *mdx* fibers appeared much reduced, even more so than at 4 weeks. The few fibers that appeared otherwise normal ultrastructurally displayed the defect in thin filament-membrane association at the MTJ seen in 4-week fibers (Figure 3A). This defect was not seen in the control tissue (Figure 3B). No *mdx* fiber at 6 weeks, regardless of its state of degeneration or regeneration, showed normal thin filament-membrane associations at the MTJ. Also, as in the 4-week tissue, junctional regions were heavily populated by mononucleated cells in both *mdx* and control muscle. However, the mononucleated cells at control MTJs had the same characteristic appearance and abundance as the fibroblasts seen in the 1-week and 4-week controls, whereas the mononucleated cells at *mdx* MTJs were more numerous and more closely apposed to the muscle cell membrane (Figure 3, A, C, and D). Many of these cells also were more rounded, had less rough endoplasmic reticulum and more cytoplasm than fibroblasts, and contained an assortment of lysosomes and small vesicles (Figure 3, C and D). These characteristics indicate that the cells present at 6-week *mdx* MTJs were likely monocytes/macrophages,[23] or possibly neutrophils.[24] Less frequently, cells with the crystalline granules characteristic of eosinophils[25] were also seen near MTJs in the 6-week *mdx* tissue (not shown).

Twenty-Three Weeks (Adult)

Fibers from adult *mdx* tissue were ultrastructurally more like their age-matched controls than were fibers from either the 4- or 6-week muscles. Most adult *mdx* myofibrils had normal diameters and were in lateral register, although Z-disc streaming, sarcoplasmic reticulum dilation, and immature myofibrils were observed in some cells (not shown), as were centrally located chains of nuclei, an indicator of regeneration[26,27] (Figure 4C). The junctional membrane appeared more folded than at 4 or 6 weeks. However, the defect in lateral association between terminal thin filament bundles and the MTJ membrane was still prevalent in adult *mdx* fibers and was not seen in controls (Figure 4, A and B). Fewer mononucleated cells were located near adult control MTJs than at the MTJs of younger controls. Those mononucleated cells present possessed extended cell processes and rough endoplasmic reticulum characteristic of normal tendon fibroblasts (Figure 4B). Mononucleated cells were present in greater numbers at the adult *mdx* MTJs than at the age-matched control MTJs but not as many as were seen in 4- and 6-week *mdx* tissue. Most of these had the characteristic morphology of fibroblasts, although mononucleated cells located at MTJs of regenerating fibers were often macrophages or neutrophils (Figure 4C).

Discussion

Identification of dystrophin as the gene product absent or altered in Duchenne and Becker muscular dystrophy[1] led immediately to speculation regarding the function of the protein in normal skeletal

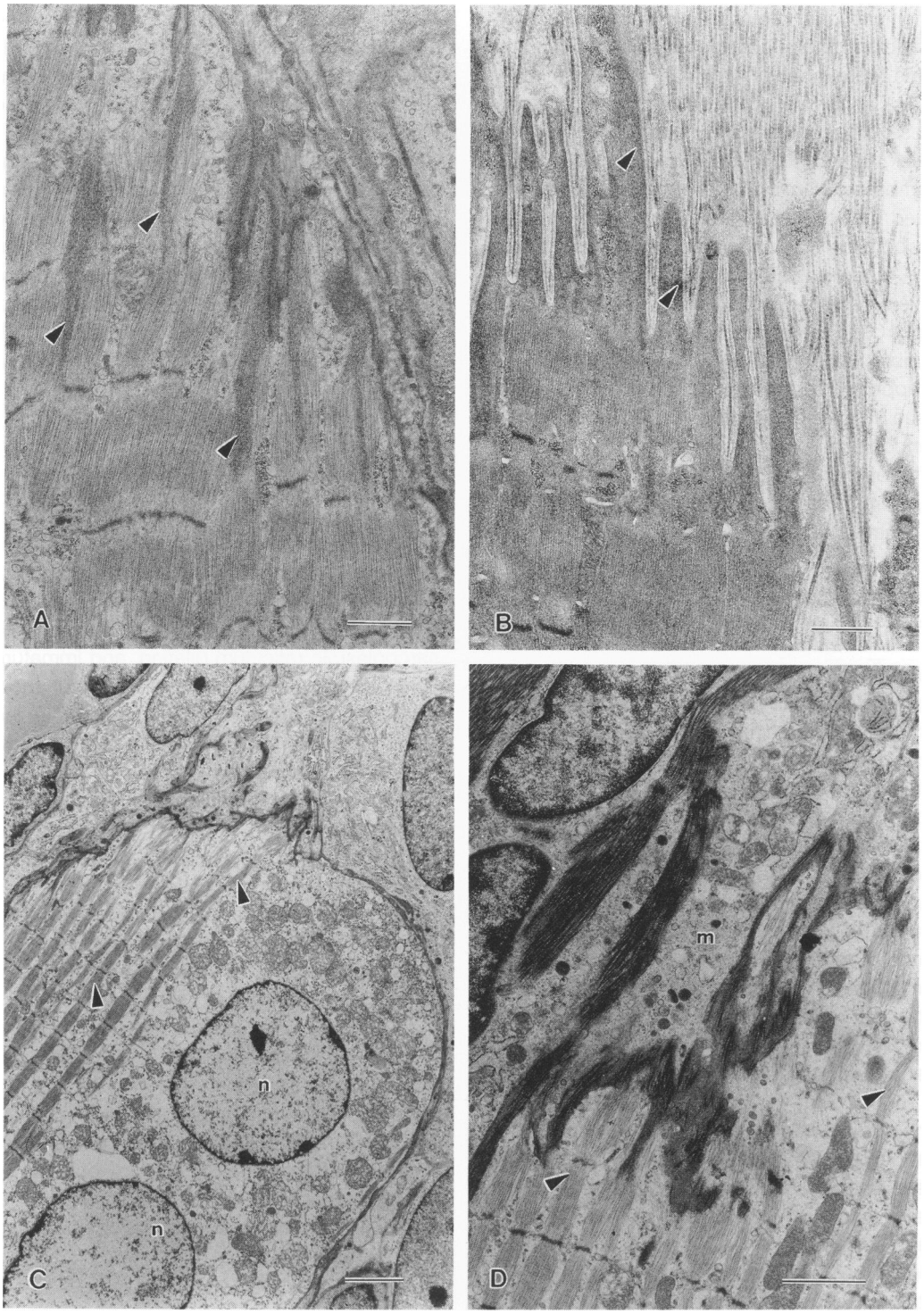

Figure 3. *MTJs from 6-week-old animals.* **A:** mdx. *Several terminal thin filament bundles are shown that lack lateral associations with plasma membrane (arrowheads). Junctional membrane folding appears reduced, similar to that seen in* mdx *fibers from 4 weeks. A nonmuscle cell is closely associated with MTJ. Scale bar = 1 μ.* **B:** *control. Junctional membrane is highly folded and laterally associated with terminal thin filaments, as in previous controls (arrowheads). Scale bar = 1 μ.* **C:** *regenerating* mdx *fiber. Cell terminus includes longitudinally-aligned nuclei with dispersed chromatin (n), and nascent myofibrils (arrowheads) associated with unfolded terminal cell membrane. Scale bar = 3 μ.* **D:** mdx *fiber containing immature myofibrils (arrowheads), closely associated at the MTJ with a macrophage (m). Scale bar = 2 μ.*

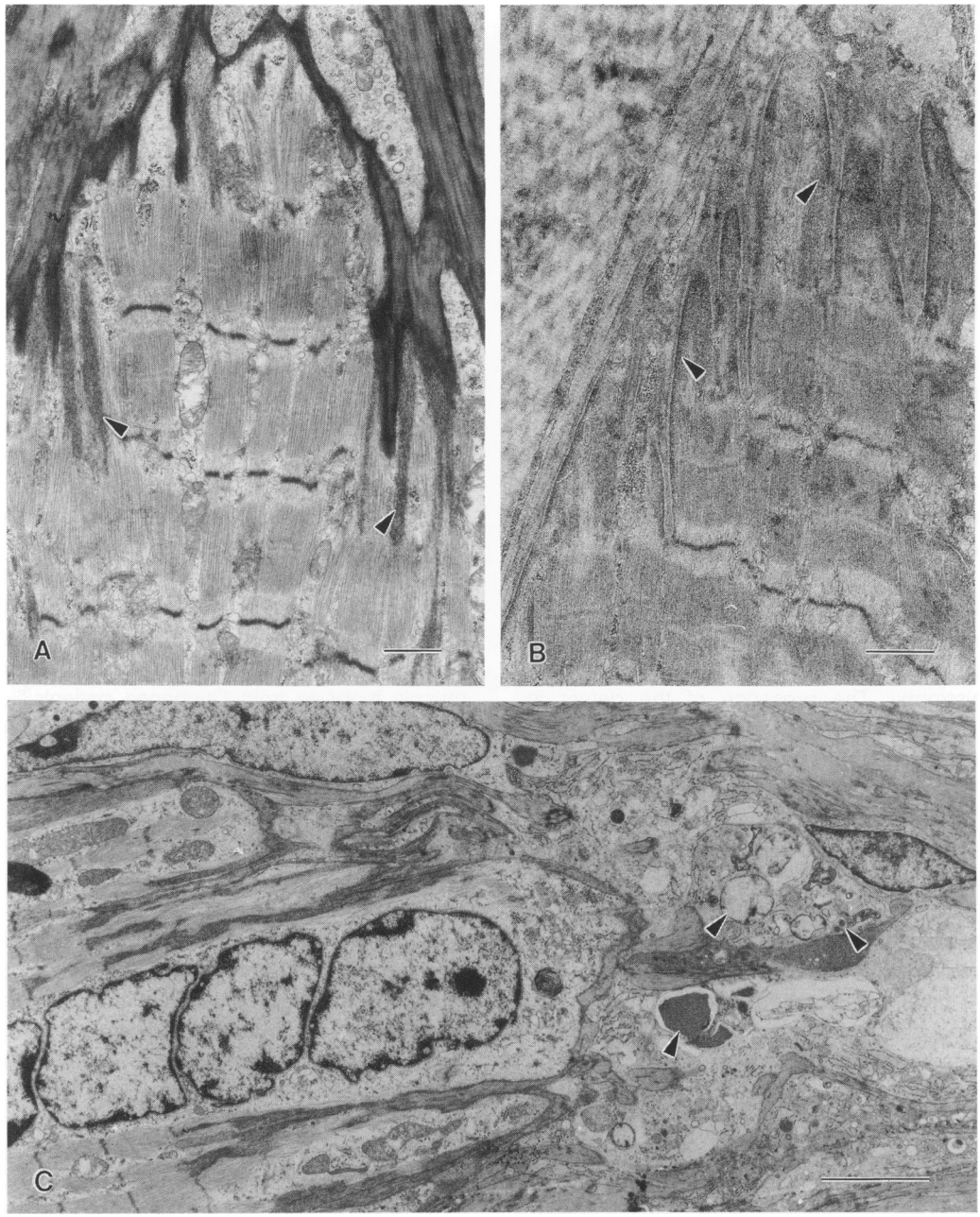

Figure 4. *MTJs from 23-week-old (adult) mice.* **A:** mdx. *Although junctional membrane is folded to some extent, the depth and number of folds appears reduced compared to the control, as in the 4- and 6-week tissue. Again, terminal thin filament bundles lack lateral associations with junctional membrane (arrowheads). Scale bar = 1 μ.* **B:** *control. Membrane folding and thin filament-membrane associations (arrowheads) are qualitatively similar to controls from the three younger age groups. Scale bar = 1 μ.* **C:** *regenerating* mdx *fiber with a chain of myonuclei at the cell terminus. Adjacent macrophages contain a heterogeneous assortment of phagocytic inclusions (arrowheads). Scale bar = 3 μ.*

muscle. Sequence analysis showed a high degree of similarity between the N-terminal domain of dystrophin and the actin-binding domains of α-actinin and spectrin and between the central rod domain of dystrophin and that of spectrin.[2] These comparisons suggest cytoskeletal roles for dystrophin similar to those of α-actinin and spectrin. Dystrophin copurifies with a complex of membrane-associated proteins, some of which span the lipid bilayer.[7,8] One of these dystrophin-associated proteins has binding affinity for the basement membrane protein, laminin.[9] Although these data, together with the subplasmalemmal location of dystrophin,[3,4,6] suggest that dystrophin mediates cytoskeletal-

membrane associations, little experimental evidence exists to establish the function for dystrophin in normal muscle.

Our previous study[20] demonstrated a defect in lateral associations between terminal thin filament bundles and the MTJ plasma membrane in 4-week-old *mdx* mice. Although those results were consistent with a structural role for dystrophin at the MTJ and the defect was observed in fibers that otherwise appeared prenecrotic, it was feasible that the defect is an early sign of necrosis that is not specific to the absence of dystrophin. The present study demonstrates the presence of the defect in *mdx* MTJ structure at 1, 4, 6, and 23 weeks, supporting the hypothesis that dystrophin functions in normal thin filament-membrane associations in skeletal muscle. Critical to this study is the examination of tissue from *mdx* mice that have not yet undergone the initial degeneration seen at 4 weeks to ascertain whether the defect in thin filament-membrane associations is a direct result of the absence of dystrophin, rather than simply an early manifestation of the general pathogenesis of muscular dystrophy. The presence of the defect in 1-week tissue supports our hypothesis that dystrophin mediates lateral associations between thin filaments and the membrane. However, the observation that these lateral associations are present in some prenecrotic *mdx* fibers indicates that dystrophin is not essential for formation of these associations.

In addition to the dystrophin-specific defect in cytoskeleton-membrane interaction at the MTJ, there are other ultrastructural changes in the *mdx* myotendinous region that are consistent with previous descriptions of degeneration and regeneration in nonjunctional regions of *mdx* muscle fibers.[22] Many fibers at both 4 and 6 weeks have obvious signs of pathology at the MTJ, including Z-disc streaming, hypercontraction, and general disorganization of the myofilament lattice. Also, many 6-week fibers have centrally located nuclei near the fiber termini and are often accompanied by the presence of narrow myofibrils resembling those seen in 1-week animals. These signs of degeneration[27] and regeneration[26] are not specific to muscular dystrophy.[26,28,29] Previous investigations have shown increased numbers of mononucleated cells at MTJs following muscle injuries or modified muscle use[30–32] and during embryonic and neonatal muscle growth.[11,21,33,34] Our results show that at 1 week of age, MTJs of *mdx* and control animals are associated with many fibroblasts, presumably involved in the rapid growth experienced at this age. However, mononucleated cells in *mdx* mice 4 weeks and older are more numerous than in controls and in much closer proximity to the MTJ membrane. These cells are a mixture of active fibroblasts, macrophages, neutrophils, and occasional eosinophils. The latter three cell types are phagocytic and probably function in the removal of cellular debris resulting from the muscle cell necrosis that peaks at 4 weeks. This is consistent with previous reports of the presence of phagocytic cells in degenerating muscle from Duchenne muscular dystrophy patients[35] and in muscle beginning to regenerate from thermal injury.[26] The active fibroblasts are likely involved in remodeling the extracellular matrix during regeneration. It is unclear what influence the tendon fibroblasts have on the morphogenesis and maintenance of MTJ structure, although fibroblast cytoplasmic processes are often deeply interdigitated with the junctional muscle cell processes in regenerating fibers, much like the relationship between the two cell types seen in developing muscle[21] and in experimentally overloaded muscle.[32] The separation and duplication of basal lamina reported in muscle biopsies from Duchenne patients[36,37] was not seen in the prenecrotic or regenerated *mdx* muscle. Separation of basal lamina was seen occasionally at 4 and 6 weeks, but only in necrotic cells and cells that seem to be actively regenerating (data not shown). This suggests that defects in cell-substratum attachment may occur only after degeneration has begun and during the period when longitudinal growth of regenerating fibers is accompanied by high turnover of cell-substratum attachments.[38] Such basal lamina separation may be related to the reduction in dystrophin-associated glycoprotein concentration seen in *mdx* muscle,[39] given the affinity of at least one of these glycoproteins for the basal lamina component, laminin.[9]

Although present at all ages studied, the MTJ defect is much rarer in 1-week *mdx* tissue than at 4, 6, or 23 weeks. Also, the reduction in folding of MTJ membrane in *mdx* muscles relative to controls is most severe at 4 and 6 weeks, less so at 23 weeks, and not discernible in 1-week samples. A possible explanation for this is that a second, dystrophin-independent mechanism mediating thin filament-membrane associations is also present in muscle and that this parallel mechanism is transiently disrupted during the stages at which *mdx* fibers experience peaks in degeneration and regeneration. For example, talin, paxillin, and vinculin are known to mediate actin-membrane associations at focal contacts of cultured fibroblasts and are normally present at MTJs.[14–17] Associations mediated by

these proteins may suffice to maintain membrane folding in immature 1-week fibers but may be disrupted in more mature fibers by one of several possible mechanisms. Early studies on Duchenne muscular dystrophy indicated that calcium concentrations are elevated in muscle samples from affected individuals,[40] and more recent studies have demonstrated that muscle cells from *mdx* mice are less able to regulate intracellular calcium concentration than control cells,[41,42] and a resulting increased intracellular calcium level may activate endogenous proteases leading to degradation of contractile proteins.[43] Talin and vinculin are known substrates for one of these calcium-dependent proteases,[44,45] and their degradation may lead to the degeneration seen in *mdx* mice aged 3 to 4 weeks. A second possible mechanism for the age-dependence of the onset of degeneration in *mdx* muscle[20] is that MTJ membrane defects develop as the junction experiences progressively larger mechanical stress as fibers differentiate to express adult isoforms of contractile proteins. The postulated parallel system of cytoskeleton-membrane linkage mediated by talin, paxillin, and vinculin may suffice to maintain normal MTJ morphology under the lower stresses induced by the immature contractile protein isoforms but fail to do so under the higher intrinsic forces produced by adult isoforms.[46] Our hypothesis that dystrophin-deficient MTJs can bear loads generated by contractile protein isoforms characteristic of immature muscle is consistent with the observation that muscle in Duchenne muscular dystrophy patients renews expression of the fetal myosin heavy chain isoform during regeneration[47] and with reports that dystrophin-deficient muscle is more susceptible to injury related to contractile activity than control muscle.[48]

Alternatively, the integrity of MTJ morphology in *mdx* muscle may depend on developmentally regulated expression of cytoskeleton-membrane linkers other than dystrophin. Recent studies have shown that an autosomal gene product related to dystrophin is present at neuromuscular junctions and MTJs in both normal and *mdx* mouse muscle.[49–51] Furthermore, the expression of the dystrophin-related protein is developmentally regulated, peaking perinatally, and thereafter decreasing, reaching the low level present in adult tissue at approximately 3 weeks of age, the stage at which degenerative activity is highest in *mdx* tissue.[51] A recent study[52] showed the concentration of dystrophin-related protein was higher in *mdx* muscle than in controls, and it was distributed generally over dystrophin-deficient muscle cell membranes rather than at MTJs and neuromuscular junctions alone. If this protein is able to substitute in part for dystrophin at the MTJ, its relatively high level of expression in early postnatal animals may explain the rarity of defects in cytoskeleton-membrane association at the MTJs of 1-week-old *mdx* mice noted here.

It is intriguing to note that the severity of the MTJ defects shown here coincides with the reduction in normalized force generation seen in 3- to 4-week-old *mdx* muscle.[53,54] However, further study of the structure and contractile properties of prenecrotic *mdx* muscle is required to determine if the observed physiological abnormalities result directly from the absence of dystrophin or indirectly from the degeneration and necrosis seen at 3 to 4 weeks.

In summary, the documentation in the present study of a defect in cytoskeleton-membrane associations at all of the ages examined, and especially in prenecrotic muscles from 1-week *mdx* mice, supports the hypothesis that dystrophin functions in normal, lateral associations between thin filaments and the membrane at the MTJ. The presence of substantial MTJ membrane folding in prenecrotic and adult *mdx* muscle suggests that, at these stages of development, *mdx* mice may compensate for the absence of dystrophin by using dystrophin-independent mechanisms to link the cytoskeleton to the MTJ membrane.

Acknowledgments

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