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Spatial distribution of β-spectrin in normal and dystrophic human skeletal muscle

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Abstract Spectrin, a major component of the erythrocyte membrane skeleton, has previously been shown to form a two-dimensional lattice in erythrocytes, and in avian or chicken skeletal muscle. Those results were mainly obtained with antibodies against α-spectrin. Using immunofluorescence of semithin cryosections and single muscle fiber preparations, we show here that β-spectrin forms a costameric network which covers the plasma membrane of human skeletal muscle. These spectrin costameres are correlated with the Z-bands. They are longitudinally connected by fine strands and interrupted by myonuclear lacunae. Under mechanical stretching, the costameres retained their correlation to the Z-bands in normal and dystrophin-deficient muscle, up to the point at which the sarcolemma was disrupted. In stretched muscle, in some regions of the stretched fibers in which the costameres seemed to form double strands, the usually 1:1 correlation of spectrin to the Z-bands changed to a 2:1 relation. In dystrophin-deficient muscle, the costameric scaffold of spectrin in the well-preserved fibers appeared normal, indicating that spectrin can be correctly localized in the absence of dystrophin and that the subcellular spectrin organization does not primarily depend on dystrophin expression. The regular organization and the correlation of spectrin costameres to the Z-bands was notable even in stretched Duchenne muscular dystrophy (DMD) muscle. On the other hand, single teased muscle fibers of DMD muscle showed various degrees of morphological alterations of the costameric network, ranging from a focal disarray to complete loss of costameric organization. Because these findings indicate that the costameric spectrin scaffold undergoes secondary changes during the course of the dystrophic process in dystrophin-deficient muscle, spectrin staining of isolated muscle fibers may also serve as a tool to monitor the effect of gene therapy experiments at the single fiber level.

Key words Spectrin · Dystrophin · Ultrastructure · Duchenne muscular dystrophy

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Introduction

Spectrin is a major component of the erythrocyte membrane skeleton [20]. Various spectrin homologues are present in erythrocytes as well as in non-erythrocyte tissues including brain, terminal web of chicken intestinal brush border, and muscle [1, 10–12, 27]. The various erythroid and non-erythroid spectrin isoforms are commonly referred to as members of a group of proteins termed the spectrin superfamily [8]. In addition to the spectrins, other cytoskeletal proteins such as dystrophin, its autosomal homologue 6q dystrophin-related protein or utrophin [18, 19], and α-actinin are considered members of this family due to the shared sequence homologies [7, 13, 17]. Little is known about the function of spectrin in skeletal muscle [31–33]. In erythrocytes, spectrin is considered to maintain the elasticity and stability of the erythrocyte plasma membrane, to modulate membrane protein movement and to organize integral membrane proteins in the plasmalemma [3, 4]. The presence of α- or β-spectrin in the skeletal muscle membrane has been demonstrated using specific antibodies in human and various animal muscle tissues, suggesting a similar role for spectrin in the muscle submembrane cytoskeleton as in the erythrocyte [2, 5, 22, 23]. However, most monoclonal antibodies raised against components of erythrocyte α-spectrin do not react with similar proteins in human skeletal muscle [1], indicating differences in epitopes or organization of muscle and erythrocyte spectrin. In normal chicken and

human skeletal muscle, spectrin is organized in a two-dimensional lattice at the sarcolemma overlying the Z-band [6]. The transverse elements of this lattice, seen in longitudinal sections as a periodic punctate staining pattern, are called costameres [6]. Most of the previous studies on muscle spectrin focused on α-spectrin in avian or hamster skeletal muscle. The purpose of the present study was to define more closely the spatial distribution of β-spectrin in normal and dystrophic human skeletal muscle.

Material and methods

Patients

Human skeletal muscle samples were obtained from diagnostic muscle biopsies usually of the quadriceps femoris muscle. Normal control muscle was obtained by orthopedic surgical procedures undertaken for reasons not related to neuromuscular disease. Patients were classified as suffering from Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD) or limb girdle muscular dystrophy (LGMD) according to dystrophin assessment, and molecular genetic and clinical analysis. Altogether we examined muscle from five control patients without any neuromuscular disease, one patient with BMD and five patients with DMD. Single teased fibers were prepared from four muscle samples from DMD patients, who were shown by immunofluorescence and Western blot to lack dystrophin in their skeletal muscle. The diagnosis for six patients with severe Duchenne-like or milder LGMD was established on clinical grounds and on the basis of histopathological evidence of muscular dystrophy but normal dystrophin expression.

Antibodies

β-spectrin was detected using two different monoclonal antibodies: spec1 and spec2 (Novocastra, Newcastle upon Tyne, UK) directed against the 235-kDa β-spectrin isoform. We used spec1, which also recognizes regenerating fibers (data not shown), for fetal muscle tissue, and spec2 for Western blot analysis and immunofluorescence of muscle tissue from children and adults.

Dystrophin assessment was carried out as described [30] using a panel of monoclonal (dys1, dys2, dys3; Novocastra, Newcastle upon Tyne, UK) or polyclonal (60-kDa anti-dystrophin, a kind gift of Dr. Eric Hoffmann) antibodies. As secondary antibodies for spectrin, monoclonal IgG subclass-specific goat anti-mouse for spec1 (IgG2b) and spec2 (IgG1) antibodies (Amersham, Braunschweig, Germany) were used. For double staining, monoclonal antibodies against spectrin were combined with polyclonal antibodies against α-actinin (Amersham) and anti-goat/sheep polyclonal antibodies against 60-kDa anti-dystrophin. As secondary antibodies for α-actinin, we used biotinylated fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit as well as biotinylated non-conjugated donkey anti-rabbit antibodies. For polyclonal 60-kDa antibodies against dystrophin, we used polyclonal donkey anti-goat/sheep (Amersham) and FITC-conjugated rabbit anti-sheep antibodies (ICN Biomedicals), followed by Texas Red and FITC visualization (Amersham). For nuclear staining, propidium iodide was used.

For double-staining procedures, care was taken to ascertain that no cross-reactions occurred between the various primary and secondary antibodies by omitting single steps. If the two antigens were at the same subcellular location, i.e. dystrophin and spectrin, controls included all double-staining steps performed on dystrophin-negative DMD muscle. Immunofluorescence was viewed and photographed using a photomicroscope equipped for epifluorescence (Zeiss III RS; Zeiss, Oberkochen, Germany).

Western blotting

Proteins from total muscle homogenates were resolved by SDS-PAGE on linear 4–20% gradient gels using a 3.5% stacking gel, and tank blotted onto nitrocellulose sheets as described [30]; 100 µg of protein were loaded per lane. Western blots were probed with the antibodies described above, using peroxidase/diaminobenzidine as the detection system [30].

Semithin cryosections

Muscle specimens were prepared for semithin cryosections as described [29]. Sections, 900 nm thick, were cut on a RMC MT 6000 Ultracut at –60°C, placed on polylysine (Sigma)-coated glass slides and stained as described [29, 30].

Single teased muscle fibers

Single teased muscle fibers were prepared using a modified version [28] of the protocol described by Moisescu and Thieleczek [25]. Teased fibers were placed on gelatine-coated glass slides and immunostained as indicated for the semithin cryosections.

Stretched muscle fibers

To prepare stretched muscle fibers, fresh muscle was dissected under the stereomicroscope into small bundles consisting of about ten muscle fibers. These were put on parafilm-coated cork platelets which had millimeter divisions. The muscle bundle was covered by a drop of Pancuronium to impede air-drying and to relax the muscle fibers. After measurement of resting length during pancuronium-induced relaxation, the fibers were stretched 50–70% over their resting length, and fixed in this position with insect needles 000 (Welabo, Düsseldorf, Germany). Fixation and preparation for immunofluorescence was carried out as described for unstretched muscle.

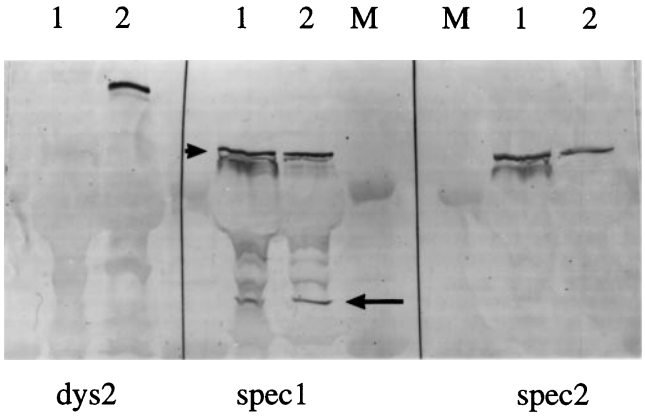

Fig. 1 Western blot. A total muscle homogenate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and tank-blotted. To show the lack of dystrophin in a Duchenne muscular dystrophy (DMD) patient (*dys2, lane 1*), the blot was stained with dys2. The normal muscle (*lane 2*) shows a band at 430 kDa. Antibodies spec1 and spec2 stain a clear band at 230 kDa (*arrowhead*) in both DMD (*lane 1*) and normal (*lane 2*) muscle. Spec1 stains an additional band at 120 kDa in normal muscle as well as in DMD muscle (*arrow*). The molecular mass marker (*lane M*) shows a myosin band at 200 kDa

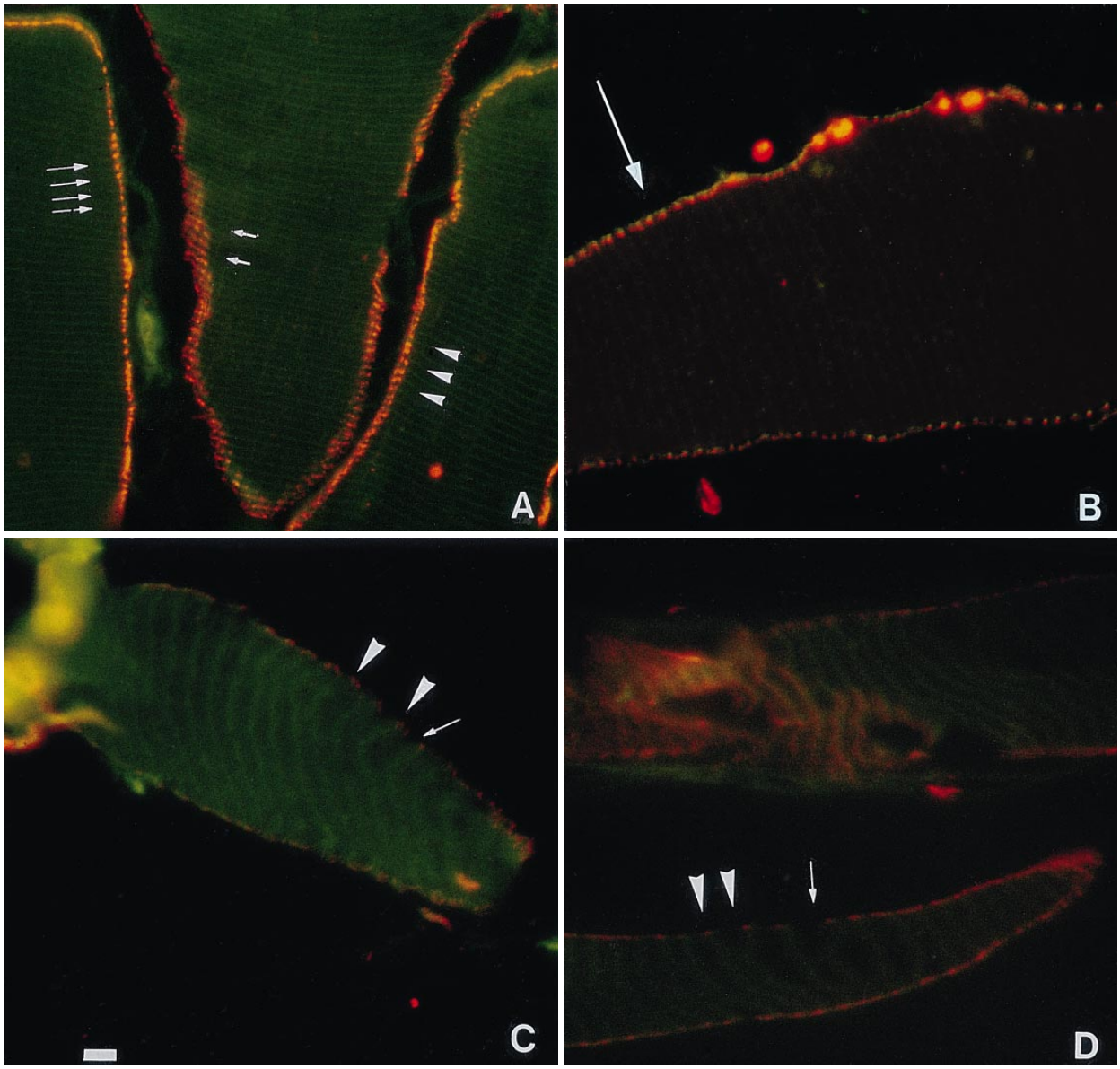

Fig. 2 **A** Spectrin staining of semithin cryosections of normal skeletal muscle. On longitudinal semithin cryosections, spectrin (spec2, Texas Red) reveals a punctate staining pattern with regular intervals (*arrows*). Double staining with (-actinin [fluorscein isothiocyanate (FITC)-conjugated] shows that these spectrin foci are correlated to the Z-bands (*arrowheads*). In tangentially cut areas of the sarcolemma, spectrin overlies the Z-bands as transverse costameres (*short arrows*). **B** Double staining of spectrin and dystrophin on normal human skeletal muscle. Double labeling of spectrin (spec2, Texas Red) and polyclonal anti-dystrophin antibodies, which cross-react with α-actinin (FITC), shows colocalization of spectrin and dystrophin (*arrow*) and demonstrates the focal accumulation of both proteins at the end of the Z-bands. The partial two-color appearance (*arrow*) of single dots is caused by the different filters used for Texas Red and FITC. **C**, **D** Stretched human muscle fibers of normal skeletal muscle (**C**) and DMD skeletal muscle (**D**). Semithin cryosections of stretched muscle fibers were double stained with spectrin (spec2, Texas Red) and α-actinin (FITC). Stretching leads to deviation and sometimes to partial disruption of the sarcolemma (*arrow*). Even after this mechanical stress, the spectrin foci still maintain their relation to the Z-bands (*arrowheads*), though sometimes slightly displaced laterally, giving the aspect of each Z-band being flanked by two spectrin foci. These results are observed in normal muscle (**C**) as well as in DMD muscle (**D**). *Bar* = 5 μm

Results

Western blot

On Western blots of total muscle homogenates, antibodies against both spec1 and spec2 stained a distinct band of 230 kDa for normal and dystrophin-deficient human skeletal muscle (Fig. 1). In addition, spec1 stained a further band, of approximately 120 kDa, in both Duchenne muscle and normal muscle (Fig. 1). The identity of this protein is not known.

Semithin cryosections

Immunofluorescence staining of β-spectrin on longitudinal semithin sections of normal human muscle revealed a punctate staining pattern with regular intervals (Fig. 2A). The periodicity of the punctate staining pattern suggested

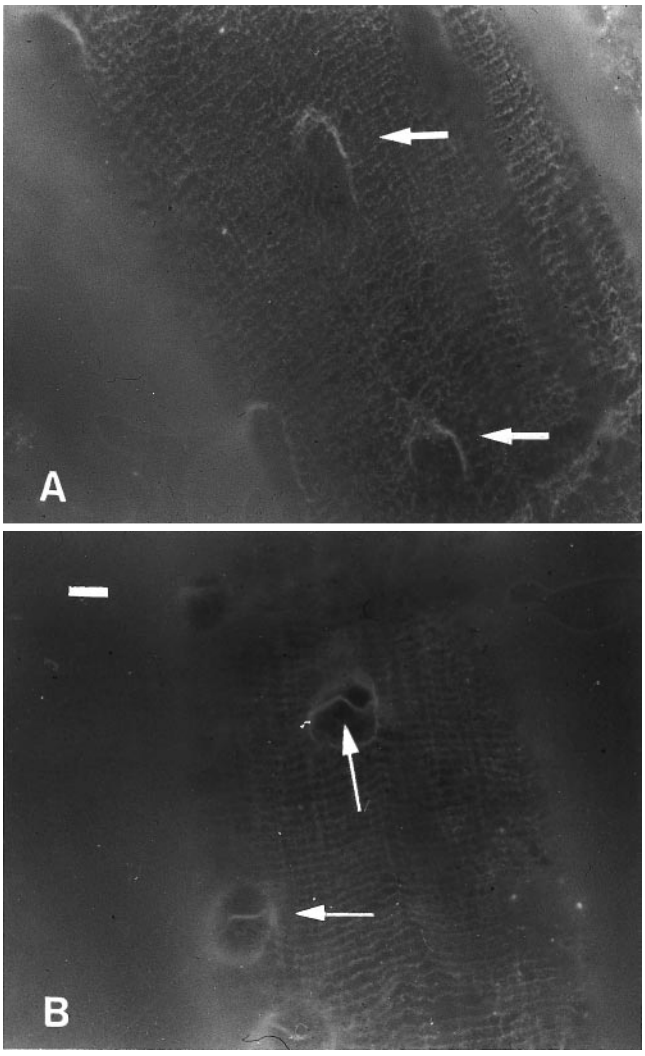

Fig. 3 A, B Myonuclear lacunae on single muscle fibers. **A** A ring of spectrin molecules (spec2, Texas Red) surrounded the myonuclear lacunae (*arrows*) into which transversal elements insert. **B** Sometimes these lacunae are covered by a fine veil of spectrin molecules (*arrows*). *Bar* = 5 μm

that there may be a relation between spectrin and other regular structures in skeletal muscle, i.e., components of the sarcomeres. Double labeling of spectrin and α-actinin indeed revealed a close spatial correlation between focal spectrin accumulations and the Z-bands (Fig. 2A). If the plane of section happened to cut tangentially through the plasma membrane, spectrin appeared to overlie the muscle fibers as transverse costameres (Fig. 2A). In some of these sections, a faint additional immunofluorescence signal was detectable between the costameres. Double staining of spectrin and dystrophin using polyclonal dystrophin antibodies, known to cross-react with a muscle-specific α-actinin isoform, showed colocalization of dystrophin and spectrin to a degree which precluded further distinction at the light microscope level (Fig. 2B).

Using semithin sections of DMD muscle, the staining intensity for spectrin was sometimes reduced but a substantial proportion of the fibers retained a regular periodicity indistinguishable from normal muscle (see below, Fig. 3A). Because of the close spatial relationship of both spectrin and dystrophin with the Z-band, even in DMD muscle, and because of the potential function of mechanical stabilization suggested for dystrophin [15], we were curious to see if and how the subsarcolemmal scaffold would be affected by mechanical stress. Even enormous mechanical stretching of normal muscle fibers, which in some fibers led to mechanical disruption of the sarcolemma, did not alter the regular distribution of spectrin along the plasmalemma and the gross relation of spectrin to the Z-bands (Fig. 2C). However, the spectrin foci were sometimes slightly displaced laterally with respect to the Z-band or appeared as doublets flanking the Z-bands on both sides. Interestingly, mechanically stretched DMD muscle fibers showed the same pattern as normal muscle fibers (Fig. 2D)

Single fibers

Single teased fibers of normal human muscle were covered by a β-spectrin network, similar to the one described for α-spectrin in chicken muscle and for dystrophin in normal human muscle. This network was composed of more predominant transversal elements (costameres), which seemed to encircle the whole fiber perpendicular to its longitudinal axis and at regular intervals (Fig. 3A).

These transverse elements were connected with each other by fine longitudinal strands of variable orientation. In normal muscle, the vast majority of fibers was covered by a regular costameric network, but a few fibers showed some degree of disruption, probably due to the mechanical teasing.

Myonuclear lacunae were apparently randomly scattered throughout the muscle fiber. In double-staining experiments each myonuclear lacuna was shown to harbor a nucleus (not shown). These lacunae were demarcated by a ring of spectrin molecules into which the transversal elements inserted. Sometimes the myonuclear lacunae were covered by a fine veil of ordered spectrin molecules (Fig. 3B).

In contrast, single fibers teased from DMD muscle showed a higher frequency and degree of altered costameric organization. The DMD fibers with the best preservation showed a spectrin network indistinguishable from normal fibers (Fig. 4A). In other fibers the costameric network was less regular, and was marked by loss of scaffold regularity and different longitudinal orientation of various regions of the fiber surface (Fig. 4B). Further muscle fiber damage was characterized by partial loss of the fine interconnections and frequently occurring longitudinal clefts, running along the long axis of the fiber and emanating from the myonuclear lacunae. In fibers with even poorer preservation, the loss of costamere regularity spread over large regions of the fiber, giving the impression of a windblown curtain (Fig. 4C). The most marked alteration was characterized by complete loss of costameric organiza-

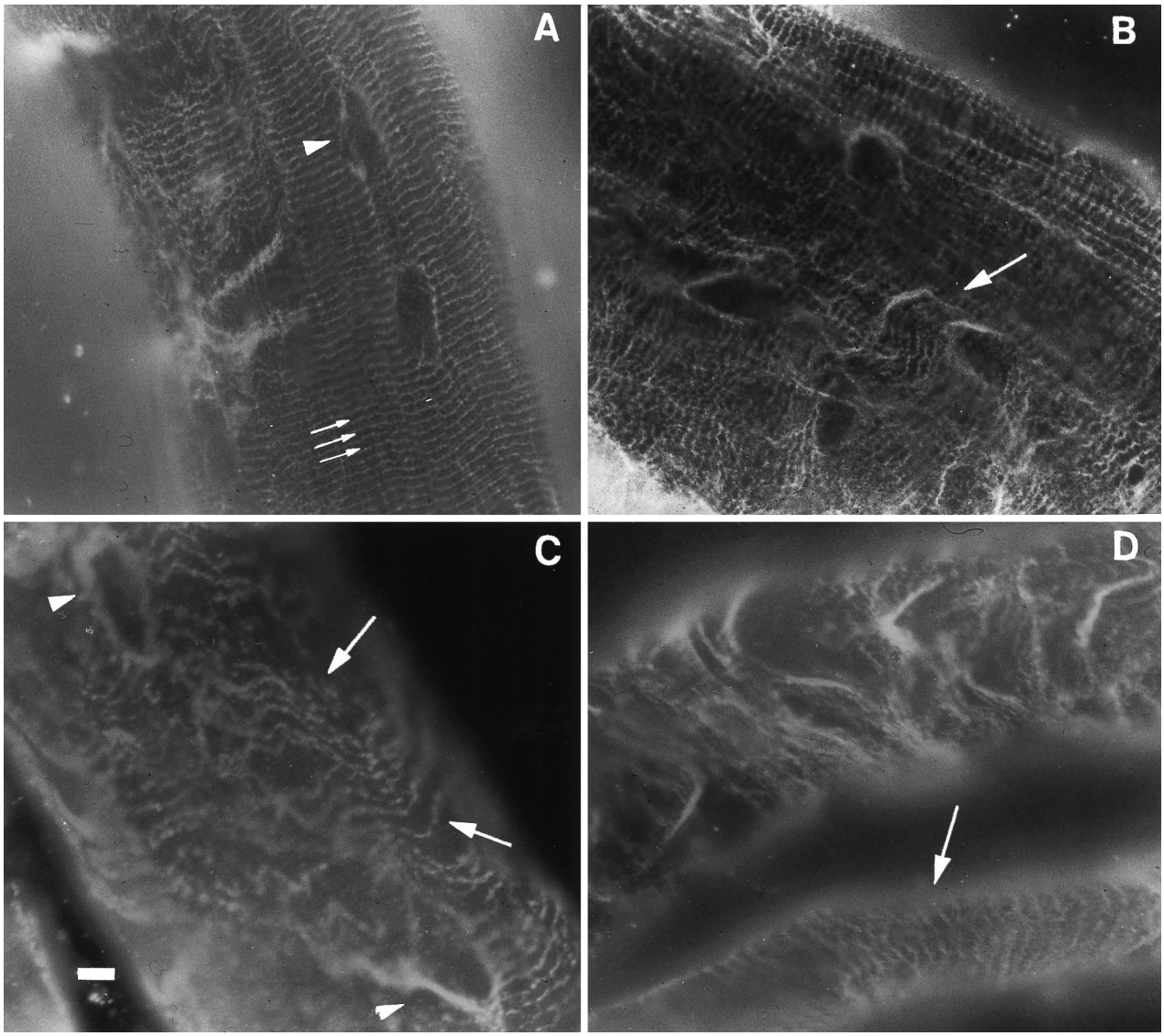

Fig. 4A–D Alterations of the spectrin network on single human DMD muscle fibers. Spectrin (spec2) was visualized on single muscle fibers of DMD patients by Texas Red. **A** A DMD fiber with good preservation of the spectrin network, which is indistinguishable from normal muscle fibers. Predominant transversal elements encircle the fiber perpendicular to its longitudinal axis, and are connected to each other by fine longitudinal strands (*arrows*). The myonuclear lacunae (*arrowhead*) are surrounded by a ring of spectrin molecules. **B** In the next stage of alteration of the spectrin scaffold, the smooth surface of the fiber is lost. This results in a more regional organization of costameres, which makes it impossible to follow one costamere around the surface of one fiber (*arrow*). **C** In the next stage the regular transverse and longitudinal connections (*arrows*) are interrupted, periodicity is lost and the costameres are out of alignment. However, the myonuclear lacunae (*arrowheads*) remain preserved, arguing against a preparation artifact. **D** In the last step of disintegration all regularity of the costameric spacing is lost. Single strands of the former costameric network seem clustered in some areas, and lost in others. Note the preserved costameres on the neighboring fiber (*arrow*), again arguing against a preparation artifact. *Bar* = 5 μm

tion, with loss of contact between transversal rings and interconnections (Fig. 4D). These different degrees of pathology occurred in different fibers of specimens and, importantly, in neighboring fibers so that normal appearing and severely damaged fibers could be found next to each other (Fig. 4D).

Discussion

According to Craig and Pardo [6] costameres represent attachment regions of the myofibrils to the sarcolemma. We have previously described the costameric organization of the dystrophin lattice [28]. This focused our interest on the organization of spectrin in DMD muscle which lacks dystrophin.

The results of our immunocytochemical studies on semithin cryosections of human skeletal muscle showed that β-spectrin is aligned at the plasma membrane of normal human skeletal muscle in a chain-like pattern of punctate foci with regular intervals. In double-labeled

semithin sections, these regularly organized foci of spectrin were spatially related to the end of the Z-bands. The findings in single teased muscle fibers revealed that these spectrin foci are part of a costameric network with predominant transversal rings and fine longitudinal interconnections. This result is in agreement with previous studies on α-spectrin organization [6], but also proves that cross-connections exist for spectrin as they do for dystrophin [28]. In addition, this technique is the first to detected another element of the spectrin network, namely randomly scattered myonuclear lacunae. All these findings show that the β-spectrin organization and subcellular localization in human skeletal muscle is similar to the recently described costameric organization for dystrophin [28].

Little is known about the alterations of the subsarcolemmal scaffold of normal human muscle exposed to mechanical stress. We exposed single muscle fibers to mechanical stress up to the point of rupture, and observed that spectrin foci were maintained in relation to the Z-bands, even if the localization was sometimes displaced laterally with respect to the Z-band, indicating tight mechanical linkage.

Lack of dystrophin did not preclude or alter this tight linkage significantly because similar results were obtained with stretched DMD muscle fibers. It is not precisely understood if and how spectrin interacts with other proteins of the costameric network or with proteins related to the Z-bands. However, the presence of an actin-binding domain at the C terminus of β-spectrin makes it likely that β-spectrin may be linked to the Z-band via F-actin, in a manner similar to that assumed for dystrophin [16, 17]. Furthermore, correct subcellular positioning of spectrin and the formation of a costameric scaffold is possible in the absence of dystrophin.

Nevertheless, structural disintegration of spectrin in dystrophin-deficient muscle does occur. The frequency and degree of such lesions were significantly higher in DMD than in normal muscle. We observed different degrees of alterations of the spectrin scaffold in dystrophin-deficient muscle. This further corroborates previous studies which showed quantitatively decreased and irregular expression of spectrin on cryosections of human skeletal muscle using immunofluorescence and Western blotting [24]. Moderate to gross changes of the spectrin network in DMD muscle suggest that the function of this protein as well as of other proteins which form a part of this network may be severely impaired, even though the expression of the protein remains preserved. This is important in view of the fact that the expression of proteins, such as sarcoglycan, is frequently used to monitor the success of gene therapy experiments [9]. Our studies would indicate that, in addition to protein expression, correct subcellular organization may represent an even more sensitive criterion for successful restoration of a muscle fiber as a result of a gene transfer.

Together with dystrophin [6, 26, 28], vinculin [21] and, as we have recently shown, β-dystroglycan [14], spectrin organization seems to form part of a general scaffolding principle of skeletal muscle. The regular spacing of the costameres in relation to the Z-bands suggests that defined regions of the plasma membrane will correspond to defined sarcomeres during cycles of contraction and relaxation of the muscle fiber. This costameric scaffold extends from the submembrane cytoskeleton (dystrophin, spectrin) through the plasma membrane (β-dystroglycan) into the extracellular matrix (α-dystroglycan). Transmembrane or membrane-associated proteins such as the sarcoglycan complex or the syntrophins are tightly connected to proteins of the costameric scaffold. It seems reasonable to believe that this scaffold serves, among other things, as a network anchoring other proteins with important membrane functions, such as signal transduction at defined regions of the muscle fiber surface. In this case, further knowledge of proteins binding to, or interacting with, β-spectrin would shed more light, if indirectly, on the functional role of this protein in skeletal muscle.

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