Distinct Patterns of Dystrophin Organization in Myocyte Sarcolemma and Transverse Tubules of Normal and Diseased Human Myocardium

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Background—Genetic mutations of dystrophin and associated glycoproteins underlie cell degeneration in several inherited cardiomyopathies, although the precise physiological role of these proteins remains under discussion. We studied the distribution of dystrophin in relation to the force-transducing vinculin-rich costameres in left ventricular cardiomyocytes from normal and failing human hearts to further elucidate the function of this protein complex.

Methods and Results—Single- and double-label immunoconfocal microscopy and parallel high-resolution immunogold fracture-label electron microscopy were used to localize dystrophin and vinculin in human left ventricular myocardies from normal (n=6) and failing hearts (idiopathic dilated cardiomyopathy, n=7, or ischemic heart disease, n=5). In control cardiomyocytes, dystrophin had a continuous distribution at the peripheral sarcolemma, with concentrated bands corresponding to the vinculin-rich costameres. Intracellular labeling extended along transverse (T) tubule membranes. Fracture-label confirmed this distribution, showing significantly greater label on plasma membrane fractures overlying I-bands (I-band 4.1±0.3 gold particles/μm; A-band 3.3±0.2 gold particles/μm; mean±SE, P=0.02). Hypertrophied myocytes from failing hearts showed maintained of this surface distribution except in degenerating cells; there was a clear increase in intracellular dystrophin label reflecting T-tubule hypertrophy.

Conclusions—Dystrophin partially colocalizes with costameric vinculin in normal and hypertrophied myocytes, a distribution lost in degenerating cells. This suggests a primarily mechanical role for dystrophin in maintenance of cell membrane integrity in normal and hypertrophied myocytes. The presence of dystrophin in the cardiac T-tubule membrane, in contrast to its known absence in skeletal muscle T-tubules, implies additional roles for dystrophin in membrane domain organization. (Circulation. 2000;101:2586-2594.)

Key Words: proteins □ myocardium □ myocyte □ heart failure

Genetic mutations of dystrophin and dystrophin-associated glycoproteins (DAG), components of the myocyte membrane cytoskeleton, underlie the degeneration of cardiac and skeletal muscle in several inherited myopathies.1 Dystrophin is a 427-kDa protein with binding affinity to cytoskeletal actin; DAG in turn comprises a transmembrane complex linked laterally by β-dystroglycan (formerly known as DAG-43) to α-dystroglycan, a surface membrane receptor for the extracellular matrix component laminin,2 suggesting that dystrophin-DAG provides a structural link between the myocyte cytoskeleton and extracellular matrix. Study of such protein-binding affinities and patterns of muscle degeneration in patients with dystrophin/DAG mutations have opened new perspectives on the pathophysiology of the muscle degeneration in these diseases and in turn permitted progress toward the identification of the subcellular functions of the individual affected proteins.

Investigation into the cellular localization of dystrophin may provide further evidence for the precise functional role of this molecule. Dystrophin distribution in skeletal myocytes has been extensively studied with the use of immunolabeling techniques with light and transmission electron microscopy (EM). Early results suggested a continuous subsarcolemmal layer of dystrophin, with absence in the transverse (T) tubules3–6; subsequent studies, however, report dystrophin in a discontinuous cell surface network involving colocalization with the cytoskeletal-membrane–linking protein vinculin at costameres.7–10 Costameres, which are found in both cardiac and skeletal myocytes, were first defined by the concentration of vinculin in a series of sarcolemmal riblike bands overlying the Z-bands11 and are now known to contain multiple proteins.12 Acting as anchor points between the myofibrils and the plasma membrane, they are implicated in the lateral transduction of contractile force from the myocyte to the extracellular matrix.13 Degeneration of skeletal muscle of patients with dystrophin mutations may occur because disruption of dystrophin at these points of mechanical stress14.
results in mechanical fragility of myocyte membranes. In comparison to skeletal muscle, relatively few studies have focused on cardiac muscle, and little information is available on dystrophin distribution in human cardiomyocytes. In rat heart, dystrophin is localized as a continuous sheet at the sarcolemma and, unlike skeletal myocytes, is also found along T-tubules. The association of dystrophin with cardiomyocyte T-tubules, which are important in excitation-contraction coupling and do not serve in the transmission of contractile force, suggests that dystrophin may serve diverse roles in myocyte membranes. Indeed, mechanisms other than membrane fragility, such as defects in calcium handling, have also been proposed as a primary cause for myocyte degeneration in inherited muscular dystrophy.

In the present study, we aimed to further elucidate the function of this protein complex by application of the complementary techniques of immunoconfocal microscopy and immunogold fracture-label EM to establish the cellular distribution of dystrophin in relation to force-transducing vinculin in normal and remodeled human cardiac myocytes.

Methods

Tissue Acquisition

Normal human left ventricular tissue (n=6) was obtained from 2 patients with cystic fibrosis undergoing heart-lung transplantation, whose hearts were not subsequently used for domino procedures. Such hearts typically demonstrate isolated right ventricular hypertrophy secondary to pulmonary hypertension. Four further specimens were acquired from clinically normal hearts initially harvested for use as transplantation donor organs but rejected for technical reasons. The cause of death was massive subarachnoid hemorrhage (n=3) or head trauma (n=1).

Human left ventricular samples were also collected from the explanted hearts of patients undergoing orthotopic heart transplantation at The Royal Brompton and Harefield NHS Trust Hospitals. All patients were severely symptomatic (New York Heart Association grade III/IV), with poor left ventricular systolic function recorded on echocardiography, multiple gated acquisition scanning, and ventriculography. Patients either had normal coronary arteries and were free of inherited myopathies (idiopathic dilated cardiomyopathy, DCM (n=7), or had severe coronary artery disease (ICM, n=5), with a history of previous myocardial infarction (5 of 5) and/or coronary artery bypass surgery (3 of 5). Patient demographic and clinical details are shown in the Table.

The pathological tissue was dissected in the operating theater immediately after explantation and was processed for immunoconfocal microscopy and for thin-section and fracture-label EM. The normal hearts had been in cardioplegic solution on ice for up to 3 hours before tissue was available. The left ventricular free wall was sampled from the normal hearts and from patients with DCM. The macroscopically homogenous nature of the left ventricles of these specimens made directed sampling less important. In the ICM hearts, large areas of full and partial thickness scarring were easily discernible. Tissue was taken, as far as possible, from the ventricular free wall distant from macroscopic scarring. If such tissue was not available, then specimens were taken from alternative ventricular segments away from infarcted territories. The study was approved by the local ethics committee; individual patient consent was obtained.

Antibodies

The following antibodies were used in the study. Dystrophin: (1) mouse monoclonal antibody (Dr Louise Anderson, University of Newcastle on Tyne) to the last 17 amino acids of the COOH
terminal domain of the dystrophin molecule (Dy8/6C5) was diluted 1:1000 for immunofluorescence and used undiluted for fracture-label; (2) rabbit polyclonal antibody (Dr Henry Klamut, Ontario Cancer Institute, Toronto, Canada) to the same sequence (P1583) was diluted 1:100 for double immunolabeling. Vinculin antibody was obtained from Sigma Immunochemicals (clone No. VIN-11 to 5) and diluted 1:50. β-Dystroglycan: mouse monoclonal antibody (43 Dag1/8D5) produced against the last 16 amino acids of the C-terminal domain (1:50 dilution). The secondary antibodies (immunofluorescence) were (1) goat anti-mouse Cy3 at 1:500 dilution, (2) goat anti-rabbit Cy3 at 1:500 dilution, and (3) goat anti-mouse FITC at 1:25 dilution (all Chemicon International). For fracture-label immunogold labeling, biotinylated goat anti-mouse immunoglobulin 1:50) with 10-nm gold-streptavidin complexes (Amersham Life Sciences) was used to label dystrophin, and goat anti-mouse 15-nm gold complexes (British BioCell International) at 1:50 dilution were used to label vinculin. All antibody dilutions and washes were performed with 0.5% BSA in PBS containing 0.05% sodium azide. All incubations were performed at room temperature.

**Immunofluorescence Microscopy**

Small blocks of tissue were rapidly frozen in liquid nitrogen–cooled isopentane. Ten-micrometer-thick cryosections were either initially fixed in freshly prepared 2% paraformaldehyde for 5 minutes and subsequently blocked in 0.5% BSA/PBS for 45 minutes or thawed directly in the blocking solution. Primary antibody was applied (dystrophin 180 minutes; vinculin and β-dystroglycan overnight), followed by the appropriate secondary antibody for 1 hour. Negative controls were performed by omission of the primary antibody. Dystrophin/vinculin double labeling was performed on unfixed cryopreserved tissue with a procedure modified from that above. Sections were incubated with rabbit polyclonal anti-dystrophin antibody (P1583) overnight, washed, and treated with mouse monoclonal anti-vinculin antibody for 5 hours. A mixed solution of the secondary antibodies was applied for 60 minutes before mounting as described above. Specificity of the labeling was confirmed with appropriate controls. Sections were examined by confocal microscopy with a Leica TCS 4D equipped with an argon/krypton laser and appropriate filter blocks for either single- or dual-channel scanning. All images were recorded within 24 hours.

**Standard Thin-Section EM**

Tissue fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.3, postfixed for 2 hours in cacodylate-buffered 2% osmium tetroxide, was processed for standard thin-section EM. Ultrathin sections were stained with uranyl acetate and lead citrate before examination.

**Fracture-Label EM**

A modification of the fracture-label technique, which facilitates plasma membrane fracturing in cardiac muscle, was used. Tissue was divided into 2-mm blocks, fixed for 5 or 15 minutes in freshly made 2% paraformaldehyde in PBS, incubated overnight in 30% glycerol/PBS for cryoprotection, and processed as described by Stevenson et al.

**Quantitative Analysis of Dystrophin Immunogold Label**

The distribution of dystrophin gold label per unit length of plasma membrane was analyzed in relation to the underlying sarcomeres. Thin-section images of clearly identified fractures of the surface membrane (P-half) showing well-preserved underlying sarcomeres in longitudinal section were recorded at a final magnification of ×35 880. Because of the variable contractile state of the muscle, individual sarcomere lengths were measured and each sarcomere was then divided into a central region half of the length of the full sarcomere (A-band) flanked by 2 equal lengths of membrane (I-band). The number of gold particles per unit length for each defined A-band and I-band was determined with the use of VID3 III image analysis software (Analytical Measuring Systems). Data were expressed as mean±SD for both groups and compared with the use of the Student’s t test. Analysis was performed along the whole length of cleanly fractured membrane to include lengths of high and low labeling.

![Image of dystrophin and β-dystroglycan labeling](http://circ.ahajournals.org/)

**Figure 1.** Immunofluorescent images of normal human ventricular tissue labeled with monoclonal anti-dystrophin (A through C) and β-dystroglycan (D) antibodies. A and B, Transverse and longitudinal sections of myocytes showing dystrophin at peripheral sarcolemma (S) and T-tubules (T); C, tangential membrane view showing dystrophin in dense cross-striations, with finer interconnecting longitudinal strands (L); D, Longitudinal section showing β-dystroglycan distribution mirroring that of dystrophin. Bright spots represent highly autofluorescent lipofuscin.
Results

Dystrophin in Normal Human Myocytes

Immunofluorescent microscopy of normal ventricular tissue revealed consistent patterns of dystrophin distribution. In transverse sections, dystrophin was seen as a thin layer of immunofluorescence at the myocyte sarcolemma, with irregular spokelike extensions penetrating toward the center of the cell (Figure 1A). In longitudinal sections, and at higher resolution with the ×63 lens, the cell surface labeling was visualized as bright, regularly spaced foci of dystrophin label superimposed on a less intense continuous background layer of label (Figure 1B). The intercalated disks were unlabeled. In optical sections including a tangential surface view (en face) of the sarcolemma, periodic broad bands of dystrophin were seen in a direction perpendicular to the long axis of the cell with finer interconnecting longitudinal strands (Figure 1C). Intracellular dystrophin was observed as regular cross-striations corresponding to the spokelike extensions described in transverse sections. This labeling was identified as T-tubular because of its occurrence at the Z-line level penetrating into the cell. A similar pattern of T-tubular labeling was obtained with antibodies against fibronectin, a recognized component of the basal lamina within cardiac T-tubules (data not shown). Dystrophin labeling patterns were unaffected by tissue fixation or with the use of either the monoclonal and polyclonal anti-dystrophin antibodies. Tissue distribution of β-dystroglycan mirrored that of dystrophin (Figure 1D). Continuous labeling with a regular punctate pattern of denser label could be discerned at the myocyte surface membrane; T-tubules were again clearly labeled with no labeling of the intercalated disk.

Figure 2. Longitudinal section of normal human ventricular myocardium showing vinculin labeling at intercalated disks (I), surface membrane in a punctate pattern (S), and T-tubules. En face views of membrane clearly demonstrate regular costameric riblike bands (EF).

Figure 3. Normal ventricular myocardium double-labeled for vinculin (green fluorescence) and dystrophin (red fluorescence). Areas of colocalization are seen as yellow. Clear foci of vinculin label at sarcolemmal surface (S) (costameres) precisely matched punctate pattern observed for dystrophin; intervening vinculin-negative membrane revealed lower although detectable levels of dystrophin (see inset). T-tubules (T) are labeled for both proteins; intercalated disks (I) are labeled strongly for vinculin alone. Highly autofluorescent lipofuscin is easily distinguished from specific immunolabeling (L).
Vinculin immunolabeling revealed sarcolemmal distribution in the characteristic costameric pattern of regularly repeating riblike structures perpendicular to the long axis of the cell, clear T-tubule labeling, and strong labeling of the intercalated disks (Figure 2). Double-labeled cells viewed longitudinally at high resolution showed that the clear foci of vinculin label at the sarcolemmal surface precisely matched the punctate pattern observed for dystrophin, with the intervening vinculin-negative membrane revealing lower though clearly detectable levels of dystrophin (Figure 3). In en face membrane views, we observed colocalization of vinculin and dystrophin over the transverse bands encircling the myocyte, again with fainter dystrophin label occurring as fine longitudinal strands between the dense bands. The T-tubular label for dystrophin coincided with that of vinculin.

**Immunogold Fracture-Label EM**

Thin-section examination of fracture-labeled tissue was used to clarify the distribution of both vinculin (Figure 4) and dystrophin (Figure 5) at the ultrastructural level. In plasma membrane fractures, vinculin labeling was sparse and closely associated with the Z-band/sarcolemmal junction (Figure 4); in contrast, dystrophin showed a wider distribution with more frequent label (Figure 5). There was a visual impression of a higher concentration of dystrophin label over the I/Z-bands, with gold particles occurring in clusters in these regions, compared with single particles in membrane overlying A-bands. There was a much lower level of label along the surfaces of fractures that entered the cytoplasm (cross-fractures); in these cases, the gold particles were observed in association with fractures exposing the T-tubules. Intercalated disks lacked dystrophin but had prominent vinculin labeling.

Fracture-label replicas for dystrophin-labeled tissues, which allow inspection of large expanses of membrane in plan view, showed prominent label of surface membrane fractures with minimal label of cross-fractures (Figure 6). Extracellular (connective tissue) fractures showed negligible label. The positions of Z-bands of myofibrils subjacent to the surface membrane could be identified as ridges; dystrophin labeling patterns confirmed the findings of the thin-section
examination, with gold particles found at higher density in the regions close to Z-bands. Fracture-label controls with omission of the primary antibody showed very low levels of background labeling. Quantitative analysis of dystrophin immunogold distribution (eg, Figure 5) confirmed a statistically significant higher density of gold label over the I/Z-band \( (4.1 \pm 3.4 \text{ gold particles/\( \mu \text{m} \)); standard error = 0.26; \( n = 172 \) ) than the A-band \( (3.3 \pm 2.9 \text{ gold particles/\( \mu \text{m} \)); standard error = 0.23; \( n = 154 \) ).

**Dystrophin in Human Heart Failure**

Histological examination of tissue from human end-stage failing hearts revealed myocyte hypertrophy with varying degrees of interstitial fibrosis irrespective of underlying cause. Occasional islands of fibrosis suggesting previous subsegmental infarction were seen in ICM specimens. Myocyte hypertrophy has been formally measured in an overlapping group of patients in a separate study; we showed a predominant increase in cell length in DCM and predominant increase in cell cross-sectional area in ICM.23

In longitudinally sectioned tissue from both DCM (Figure 7A) and ICM (Figure 7B), dystrophin was seen in a continuous surface membrane distribution with a superimposed regular punctate pattern as described in control tissues. Intracellular dystrophin distribution seen as linear structures showed increased tortuosity and size (Figures 7, C and D) and unlike controls were frequently observed to run in a longitudinal orientation (Figures 7A and 8A). Thin-section EM confirmed that these structures represented T-tubules (Figure 8). There were clear increases in size and number of the T-tubules at EM examination in both DCM and ICM hearts as compared with normal controls, with prominence of longitudinally orientated elements (Figure 8, B through D).

Cardiomyocytes adjacent to larger zones of scarring suggesting previous localized infarction were studied separately from the noninfarcted tissue. In contrast to the noninfarcted tissue, certain cells demonstrated an abnormal distribution of sarcolemmal dystrophin, with loss of the normal punctate pattern seen in longitudinal section (Figure 9).

**Discussion**

Genetic mutations of dystrophin and its associated membrane proteins underlie progressive myocyte degeneration in several inherited cardiomyopathies,1 although the precise role of these proteins in muscle physiology remains under discussion. The novel results of the present study, which help to further our understanding of the function of this protein complex, are that (1) dystrophin is partially colocalyzed with vinculin in the human cardiac myocyte sarcolemma in a costameric distribution but is also present, though at lower density, in the intervening noncostameric surface membrane; (2) dystrophin and vinculin coexist throughout the T-tubular system in normal cardiac myocytes; (3) the distribution of \( \beta \)-dystroglycan, a marker for the DAG complex, mirrors that of dystrophin; (4) cardiac cell hypertrophy is associated with maintenance of sarcolemmal dystrophin labeling and increased intracellular dystrophin in parallel with more extensive T-tubular development; and (5) the normal sarcolemmal distribution is lost in degenerating cells in the failing left ventricle. These features, established by single- and double-label immunofluorescence microscopy, were extended and confirmed at high resolution by fracture-label immunogold EM.

The costameric pattern of surface membrane dystrophin label observed here in human cardiac muscle resembles that reported in skeletal muscle by confocal microscopy.6–9 However, not all studies have found this pattern either in skeletal or cardiac muscle.3–5 This distribution was difficult to discern on initial examination of the 10-\( \mu \text{m} \) sections with conventional epifluorescence microscopy but was clearly seen with the greater resolution of immunofluorescent microscopy. Furthermore, we found that adjustment of the microscope to provide the thinnest optical sections of \( \approx \)0.5-\( \mu \text{m} \) thickness...
optimized the images obtained. These findings may partly explain the earlier reports of human cardiomyocytes showing a continuous pattern of sarcolemmal label. The distribution of dystrophin immunolabeling in the present study is supported by the consistency of results achieved with the use of 2 separate antibodies and varying tissue fixation and extended by the findings of qualitative and quantitative fracture-label EM. Tissue distribution of β-dystroglycan as a marker of the DAG complex was found to be identical to that of dystrophin, again supporting specificity of labeling in the study.

Fracture label is particularly useful for the study of sarcolemma-associated proteins such as dystrophin because the fracturing process exposes hydrophobic internal membrane faces (membrane fractures) and crosses through cells, exposing the underlying cytoplasm (cross-fractures).21 The small absolute differences and high standard deviation in dystrophin label density observed in this study reflect the tendency for fractured hydrophobic surfaces to randomly reassociate into bilayer segments after exposure to aqueous media at the thawing stage.21 Where such bilayer segments occur, access of antibodies to any underlying epitopes are blocked, leaving the areas devoid of label.

Stevenson et al19 recently showed that dystrophin is not confined to costameres in rat cardiac muscle but rather has a continuous and uniform distribution at the cell surface. Our present finding of an increased concentration of dystrophin and β-dystroglycan at costameres with a lower density over the A-band in human cardiac muscle suggests the existence of species differences in cardiac dystrophin distribution between large and small animals. Previous work has shown that skeletal muscle from the mdx mouse model20—which, like the muscle of patients with Duchenne muscular dystrophy, lacks dystrophin—sustains less severe degenerative changes.25,26 The higher stress to which human muscle is exposed because of the larger body mass may therefore be important for myocyte damage in Duchenne muscular dystrophy.8 In a similar way the human heart, being larger and generating a higher wall shear stress according to Laplace’s law, may display clustering of dystrophin over the costameric regions of the membrane (the points of highest shear force) as an adaptation to provide the necessary increased mechanical strength. The functional importance of such membrane localization to cell survival is underlined by preservation of the sarcolemmal pattern in hypertrophied myocytes subjected to higher mechanical stress in human heart failure, whereas other cytoskeletal proteins are known to show disrupted patterns.27

The differences in sarcolemmal colocalization of dystrophin with vinculin in our study and in previous reports in both striated19 and smooth muscle cells28 suggest that the function of dystrophin is not entirely associated with the transmission of force, as has been proposed for costameric proteins.13 Furthermore, a nonmechanical function of dystrophin is suggested by its association with the cardiac T-tubule, a structure not directly affected by membrane distortion during contraction.29 The importance of this association is underlined in the current study by preservation of submembrane dystrophin in the extended T-tubules of hypertrophied cells from failing hearts. T-tubular dystrophin previously has been postulated to be important in maintenance of tubular patency during myocyte contraction.12 However, the absence of dys-

Figure 7. Immunoconfocal single optical sections of human left ventricular tissue from end-stage failing hearts secondary to either DCM or ICM labeled for dystrophin. A and B, Longitudinal sections showing maintenance of punctate sarcolemmal labeling (S); prominent dystrophin-labeled T-tubules are seen orientated both parallel to and transverse to long axis of cells (T). C and D, Transverse sections showing hypertrophied T-tubules penetrating into myocytes, sometimes with clearly defined lumina (T). Longitudinally oriented tubules seen in A can be seen as circular structures.
trophin in the skeletal muscle counterpart (distinguished morphologically by a lack of a basement membrane and the presence of triadic junctional complexes) and the consequent differing mechanisms triggering contraction in these muscle cell types suggest an alternative hypothesis of selective association of dystrophin with distinct functional membrane domains. A role for dystrophin in membrane domain organization is also supported by the existence in skeletal muscle of sarcolemmal and T-tubular membrane subcompartments separable by density-gradient centrifugation with variable contents of dystrophin and the clustering of dystrophin in the troughs of the muscle end-plate at the neuromuscular junction. Accumulating evidence also implicates dystrophin in regulatory interactions with other membrane proteins.

Our results suggest that distinct muscle type and species differences exist for the distribution of the dystrophin membrane cytoskeleton. In human myocardium, dystrophin is distributed at the sarcolemma, with concentration at the lateral costameres suggesting a mechanical role in surface membrane support. The localization of dystrophin in the cardiac T-tubule suggests a cellular function besides that of mechanical support and transmission of force. Further study of dystrophin distribution and molecular interactions is necessary to precisely define the function of this system in human cardiomyocytes in health and disease.

Acknowledgments

We thank the British Heart Foundation for support of this work (grant FS/94073). Dr Cullen is supported by The Wellcome Trust. We thank Professor Sir Magdi Yacoub and Mr Asghar Khaghani at Harefield Hospital for the generous provision of human specimens.

References

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Circulation. 2000;101:2586-2594
doi: 10.1161/01.CIR.101.22.2586

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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