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Different utrophin and dystrophin properties related to their vascular smooth muscle distributions

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Abstract Monoclonal antibodies used to distinguish between dystrophin and utrophin were systematically applied to skeletal muscles containing arteries and veins. Small arteries were found to contain long forms of both utrophin and dystrophin, while small veins contained only long forms of utrophin. In addition, all sizes of vascular smooth muscles were demonstrated to contain another related M_r 80 kDa protein (possibly a short utrophin transcript). Regardless of their tissue distributions, we assumed that each of these molecules had distinct properties, i.e. dystrophin with a mechanical function and utrophin with an architectural function. This difference in the roles of dystrophin and utrophin could reduce the efficiency of protection against muscle membrane degeneration when utrophin overexpression is programmed.

1. Introduction

Molecular events leading to Duchenne muscular dystrophy (DMD) result from a deficiency in dystrophin, a protein encoded at the Xp21 locus. The function of this molecule in the cytoskeletal-membrane system is not yet fully characterized, whereas its primary structure has long been established [1]. Dystrophin has extensive homology with other rod-like structures present in alpha-actinin and spectrin [2]. The N-terminal portion of this molecule shares homology sequences with both of the above proteins and an actin binding domain is known to be present [3]. The C-terminal end is specific to dystrophin and is part of a complex with glycoproteins [4–10].

Different proteins show homology with dystrophin, particularly with sequential C-terminal parts [11–16]. The existence of long and short molecules in this dystrophin family, such as utrophin (DRP1) and DRP2 [15,16], suggests that each member of the 'dystrophin group of proteins' may have a specific function in different tissues [17,18]. It would thus be important to determine the specific functional differences between dystrophin and utrophin. This prompted us to investigate vessel tissues which naturally contain dystrophin and utrophin [19]. This study was carefully conducted on the basis of known differences between arteries and veins. Arteries mainly have a mechanical role, i.e. they are structurally able to resist blood flow propulsed by cardiac systole, and this force is propagated as an elastic response. In contrast, veins are more extensive (2-fold more veins than arteries), and their paths generally vary more than those of arteries. Vein diameters can increase 5-fold, while arteries can only dilate 2-fold. However, the major difference is that arteries are responsible for active work, while veins have a passive function with respect to blood transit.

This study focused on possible differences in the presence of dystrophin versus utrophin in both kinds of vessels (arteries and veins). This included: (i) conducting a detailed analysis to compare dystrophin/utrophin distributions in these different smooth muscles relative to those in skeletal muscles, and (ii) characterizing the protein size (long or short forms) in each vessel. We identified the protein using region-specific monoclonal antibodies directed against each member of the dystrophin family. Two complementary approaches were used: immunofluorescence detection on cryostat sections and Western blot analyses of various crude protein extracts from different tissues. Our results provided indirect evidence of functional differences between dystrophin and utrophin.

2. Materials and methods

Rabbit skeletal muscles at the adult development stage (12 months after birth) were examined. Various leg striated muscles were analyzed for their vessel contents (arteries and veins). Arteries and veins were differentiated on the basis of size. Both structures were easily isolated due to the clear shape of large arteries (round) and large veins (more elongated).

2.1. Immunofluorescence detection

Serial 10 μm transverse cryostat sections of rabbit skeletal muscle containing vessels were labelling with different specific antibodies. The following panel of antibodies was used: monoclonal dystrophin-specific antibodies 5G5 and 12G9 (both produced in our laboratory [20–22]), and Dys 2 (Novocastra); monoclonal utrophin-specific antibodies, 5B1 (produced in our laboratory), and 12B6 (NCL-DRP1, Novocastra); a specific monoclonal antibody (5F3) directed against the last 31 amino acids of Dp 71 [23]; a universal antibody (5A3) detecting both dystrophin and utrophin (produced in our laboratory [20–22]). Specific labelling of each antibody on sections was detected with fluoresceine-conjugated goat anti-mouse IgG (1/100, from Sigma Immunochemical).

2.2. Immunoblot analysis

Tissues were homogenized in buffer containing 5% SDS, 5% β-mercaptoethanol, 1 mM PMSF, 0.1 mg/ml soybean trypsin inhibitor, 0.1 mg/ml leupeptine, 1 mM iodoacetamide, 15% glycerol, 0.001% bromophenol and 50 mM Tris-HCl (pH 9.0). Fifty milliliters of each sample was denatured by boiling for 2 min and loaded on 0.75 mm SDS–polyacrylamide 8% gels with stacking gel. After SDS-PAGE migration, the proteins were electrotransferred onto nitrocellulose sheets overnight (30 V, 100 mA) in the presence of 0.1% SDS in the transfer buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS and 20% methanol). Nitrocellulose membranes (0.2 μm) were blocked with 3% BSA dissolved in TBST buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 8) for 30 min at room temperature. Blots were incubated at room temperature for 1 h with specific monoclonal (5B1, 12G9, 5A3) or polyclonal antibodies (CUT specific to the last seven amino acids of utrophin, and previously characterized [24]). The use of secondary antibody coupled to phosphatase alkaline (1:5000

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Abbreviations: DRP1, dystrophin-related protein number 1; DRP2, dystrophin-related protein number 2; Dp 71, dystrophin protein with 71 kDa molecular mass

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dilution, Jackson ImmunoResearch Laboratory) allowed visualization of the protein band with *p*-nitroblue tetrazolium, and 5-bromo-4 chloro-3 indoylphosphate substrate, as previously described [24–27]. The following proteins were used as molecular mass markers in all cases: myosin (199 kDa), β-galactosidase (120 kDa), bovine serum albumin (87 kDa) and ovalbumin (48 kDa) from BIORAD.

3. Results

3.1. Comparative immunofluorescence analyses

On similar rabbit skeletal muscle cross-sections, we compared the expression of vascular smooth muscle dystrophin and utrophin in two ways: (i) large versus small vessels and (ii) arteries versus veins.

In large vessels, labelling was successful with all antibodies in arteries and veins; dystrophin and utrophin were present in both structures (Fig. 1). With no first antibody, only the natural fluorescence of elastine was visible in the large artery present in the cross-section (Fig. 1A). The 5B1 antibody faintly stained artery walls, but more positively revealed the presence of utrophin in the vein walls (Fig. 1B). With a more intense image, 12B6 allowed detection of utrophin in veins and more convincingly in the artery walls (Fig. 1C). The monoclonal antibody 5G5 (as well as 12G9) showed similar positive images concerning the presence of dystrophin in both structures (Fig. 1D). The cumulated presence of both utrophin and dystrophin, in accordance with the specificity of the 5A3 antibody, was clearly revealed in both vascular structures (Fig. 1E).

When small veins and arteries were analysed (Fig. 2), the comparative images differed markedly from those presented in Fig. 1. Utrophin, detected with the specific monoclonal antibodies 5B1 or 12B6, was found in both small vessels (Fig. 2C), while dystrophin detected with 5G5 was present in small arteries, but absent from small veins, as presented in Fig. 2B. The pooled image was clearly shown (Fig. 1E) when using 5A3 antibody which detects dystrophin and utrophin (Fig. 2D).

3.2. Comparative Western blot analyses

For more detailed analysis of these results, crude protein extractions were carried out on isolated large arteries and veins (it was impossible to separate small vessels from one another and from the muscle structure in order to obtain pure small artery and vein extracts). The comparative Western blot analyses are presented in Fig. 3. An M_r 400 kDa protein band was detected with all antibodies in each extract, and only the specific utrophin antibody 5B1 cross-reacted with a new M_r 80 kDa protein band present in artery and vein extracts. This new protein band (Fig. 4, lane 2) was also detected with the CUT utrophin-specific polyclonal antibody, which also identified a 400 kDa protein in vessel-specific crude extracts, corresponding to the full-length DRP1. In rabbit sciatic nerve crude extracts, the CUT polyclonal antibody identified only a 400 kDa protein band corresponding to DRP1, particularly abundant in this tissue (Fig. 4, lane 1). This new 80 kDa protein band was not revealed with 5A3, which detected both utrophin and dystrophin C-terminal parts, or with 12G9 specific to the C-terminal part of dystro-

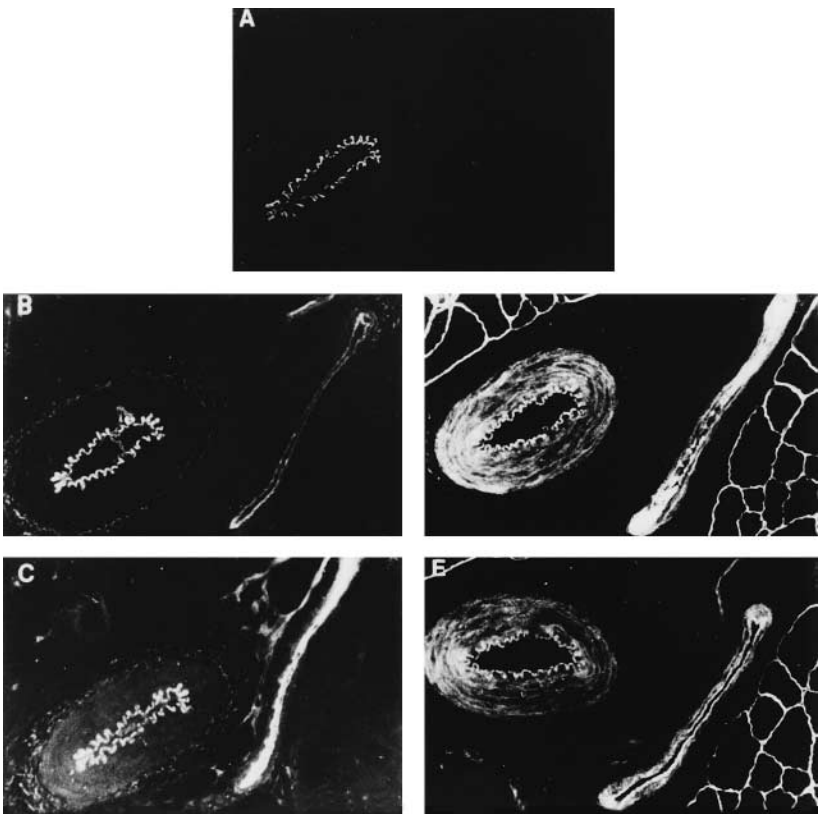

Fig. 1. Comparative detections of dystrophin and utrophin in large artery and vein walls. A: Muscle cross-section showing both artery and vein blood vessels after incubation with only the second fluorescent antibody. Seriated sections were then viewed in (B) and (C) with two different utrophin specific antibodies (5B1 and 12B6), in (D) with a dystrophin antibody (5G5), and in (E) with a universal antibody detecting both dystrophin and utrophin (5A3). Bar = 20 μm.

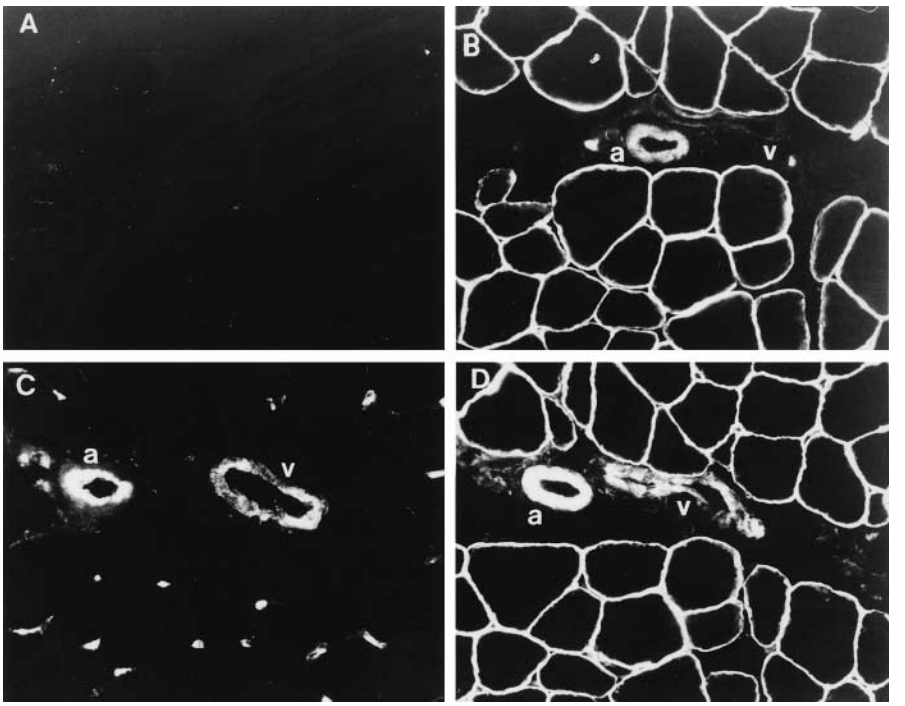

Fig. 2. Comparative detections of dystrophin and utrophin in small artery and vein walls. Panel (A) corresponds to the control, panel (B) to dystrophin monoclonal antibody 12G9, panel (C) to utrophin monoclonal antibody 5B1, and panel (E) to utrophin and dystrophin monoclonal antibody 5A3. Small arteries are indicated as (a) and small veins as (v). Bar = 20 μm.

phin. In addition, neither Dys 2, specific to the dystrophin C-terminal end, nor 5F3, specific to the Dp 71 C-terminal part [23], were able to reveal this new protein band.

4. Discussion

Systematic analysis of dystrophin and utrophin expression in artery and vein walls with a panel of specific antibodies highlighted a clear difference between large and small vessels. Dystrophin and utrophin were both expressed in large arteries and veins. Conversely, dystrophin was absent in small veins, whereas it was always present in small arteries and utrophin appeared in all small vessels.

These results indicate that dystrophin is actively involved in

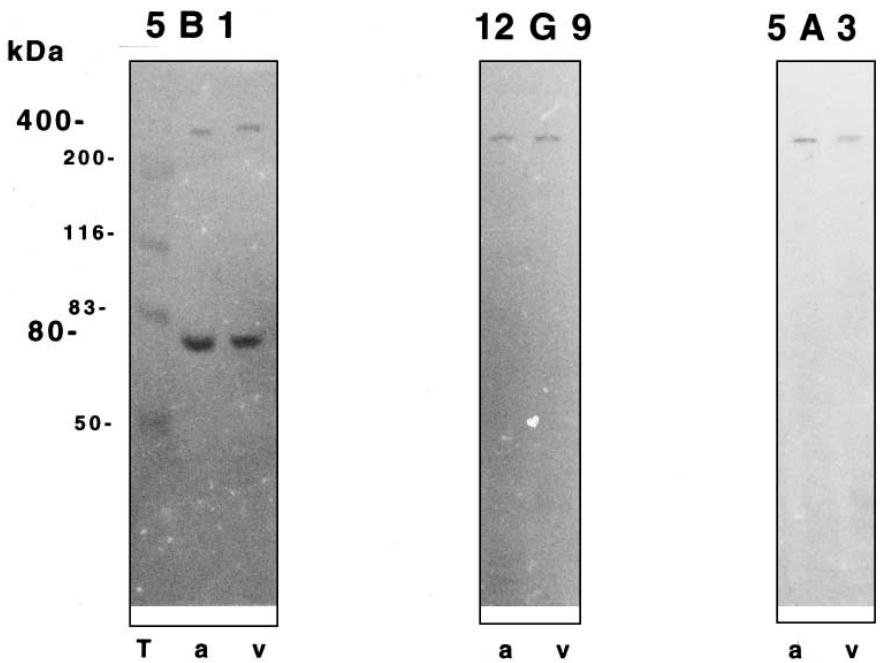

Fig. 3. Western blot analysis of crude protein extracts from isolated large artery and large vein smooth muscles. Immunoblot detections with specific monoclonal antibodies directed against utrophin (5B1), dystrophin (12G9) and dystrophin/utrophin (5A3). Identifications were obtained in all crude extracts from isolated arteries (a) or veins (v) for 400 kDa proteins. With 5B1, another new 80 kDa protein band was detected in these extracts in both tissues. T corresponds to high molecular mass standard proteins obtained from BIORAD.

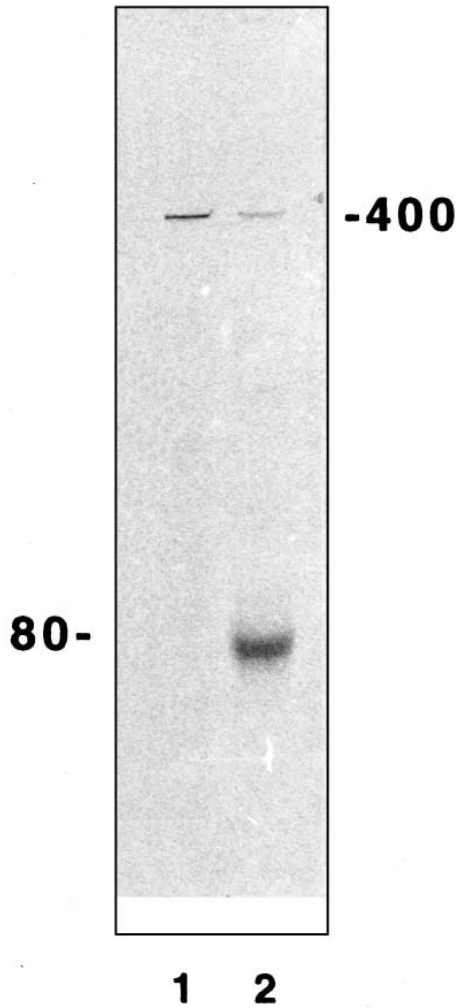

Fig. 4. Comparative Western blot analysis in nerve and vessel crude extracts. Crude extracts from isolated sciatic nerves (1) and vessels (2) were revealed with the CUT utrophin-specific polyclonal antibody directed against the last amino acid of utrophin. Only vessel extracts were found to contain the new 80 kDa protein band.

mechanical resistance of the muscle membrane, which is essential in large arteries, large veins and small arteries. These vessels have an active role in blood circulation and have contractile properties, in contrast to small veins which passively drain blood. Dystrophin's mechanical role could involve its actin binding capacities, which (as recently reported [28]) can be increased by calmodulin–calcium binding, but also modulated by specific phosphorylation of the dystrophin molecule [29]. This dystrophin property clearly differs from those of utrophin, which binds F-actin. However, this latter complex is dissociated by calmodulin–calcium complex formation [30], leading to inefficient reinforcement of the F-actin network during contraction. There is a clear difference in the actin binding properties of dystrophin and utrophin.

In contrast, utrophin may have an important role in the correct assembly of muscle membrane proteins, while also having an architectural function in this structure. Utrophin was detected in all vascular structures, i.e. in the walls of all sizes of arteries and veins, as well as microvessels. The structural role of utrophin presented here is in full agreement with its synthesis as a dystrophin precursor, at least in skeletal muscles, but with progressive replacement by dystrophin during development [31,32]. It is also known that even overexpression of utrophin [33,34] in the muscles of DMD patients cannot provide the membrane with efficient long-term resistance when dystrophin is absent [18,28,29]. This overexpression may delay degeneration of the muscle membrane, but not compensate for an absence of dystrophin, which has also been reported for cardiac muscle [24]. Recently, utrophin was overexpressed in transgenic mdx mice [35], thus bringing serum creatine-kinase levels back to normal. Our results showed that utrophin was less efficient than dystrophin in terms of its participation in the active work required for blood circulation through arteries and large veins. Recorded measurements at muscle contraction will be required to determine whether full recovery of muscle performance in transgenic mdx mice overexpressing utrophin has been obtained, as described in muscles with mini-dystrophin expression [36].

The new 80 kDa protein band, only detected with the 5B1 monoclonal antibody and CUT polyclonal antibody, may be a new short product belonging to the utrophin family according to the specificity of the antibodies used. The CUT polyclonal antibody, which was directed against the last seven amino acids of utrophin, revealed that the 80 kDa protein contained this specific C-terminal sequence and could be considered as a short utrophin product. This is supported by the multitude of previously reported short utrophin products detected using specific utrophin monoclonal antibodies [37]. However, cDNA sequencing will be required before clear membership to this protein family can be established. The roles of such related short proteins should be studied in detail.

In conclusion, the present analysis of dystrophin and utrophin content in small arteries and veins sheds further light on the specific function of these two homologous proteins. The combination of immunofluorescence and Western blot analyses provided complementary information. We assume, from the data summarized in Fig. 5, that dystrophin has a mechanical role in muscle membranes, i.e. participating in the contractile performance of artery and large vein walls, while utrophin is a structural protein essential for correct construction of all vessels. Structure/function relationships in each type of muscle membrane system (skeletal and vascular smooth muscles) should be clarified to determine the molecular pathogenesis of Duchenne muscular dystrophy. The results of this study provide background information that will be critical for successful therapy.

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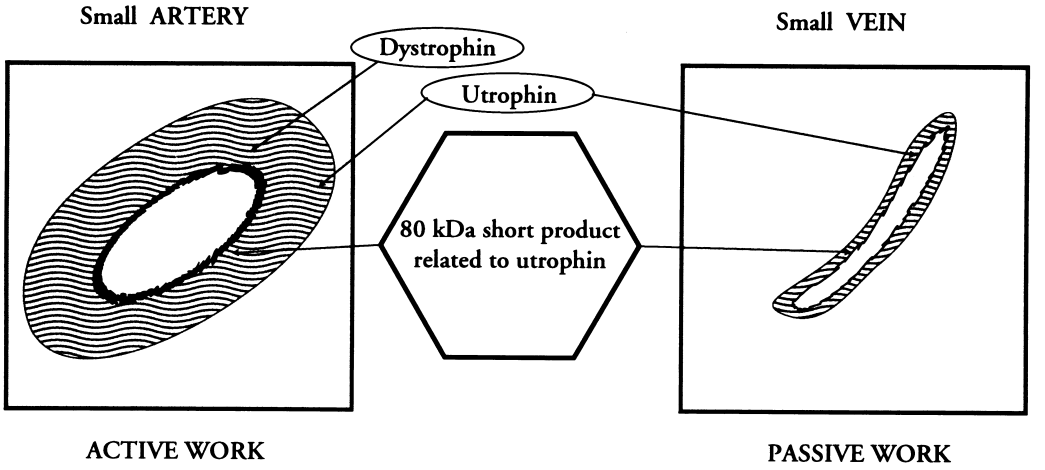

Fig. 5. Schematic representation comparing dystrophin-family proteins present in vessel walls of small arteries and veins. Vessel walls from small arteries (and all other arteries) are schematically represented with a round shape, while similar vein structures are more elongated with no specific geometrical shape. Both structures contained utrophin and a short utrophin related M_r 80 kDa protein. Dystrophin was present in small arteries, but undetectable in small veins. This data was correlated with active (in small arteries) and passive (in small veins) work.

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