



EXPRESSION OF DYSTROPHIN-ASSOCIATED GLYCOPROTEINS AND UTROPHIN IN CARRIERS OF DUCHENNE MUSCULAR DYSTROPHY

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Abstract—The expression of dystrophin, the dystrophin-associated proteins and utrophin has been studied immunocytochemically in three young, manifesting carriers of Duchenne muscular dystrophy, aged 3, 5 and 12 yrs, one adult manifesting carrier, aged 60 yrs, and one presumptive carrier with a raised serum creatine kinase, aged 24 yrs, the mother of the 5-yr-old manifesting carrier. The manifesting carriers had variable degrees of weakness; the presumptive carrier had no weakness. Morphological abnormalities were also variable and were most marked in the young manifesting carriers. The three young manifesting carriers and the presumptive carrier had a mosaic pattern of dystrophin-positive and dystrophin-negative fibres. All the dystrophin-associated proteins were reduced in the dystrophin-deficient fibres, giving a similar mosaic pattern to dystrophin. Expression of dystrophin and the dystrophin-associated proteins was normal in the adult manifesting carrier. Utrophin was detected on the sarcolemma of fibres both with and without dystrophin and the dystrophin-associated proteins. Thus, dystrophin and utrophin are co-expressed in several fibres in carriers. The results emphasize the close association between dystrophin and the glycoprotein complex and their role in the pathogenesis of muscle damage. In addition, the presence of utrophin in fibres with greatly reduced glycoproteins suggests that very little of the glycoprotein complex may be required to anchor the amount of utrophin expressed at the sarcolemma in these particular cases.

Keywords: Duchenne muscular dystrophy, carriers, dystrophin, dystrophin-associated proteins, utrophin, dystrophin-related protein.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder characterized by progressive muscle wasting and weakness, and a defect in the gene that encodes a 427 kDa protein, dystrophin [1, 2]. Dystrophin shows sequence homology to the cytoskeletal proteins α -actinin and spectrin [3] and in normal muscle it is localized to the cytoplasmic face of the sarcolemma [4-7]. Dystrophin exists in a large oligomeric complex associated with three transmembrane glycoproteins of 35 kDa, 43 kDa and 50 kDa (35DAG, 43DAG, 50DAG), a transmembrane protein of 25 kDa, an extracellular glycoprotein of 156 kDa (156DAG) and a cytoskeletal protein of 59 kDa (59DAP) [8-11]. The 43DAG and 156DAG are encoded by a single gene and the 156 kDa component has been shown to bind to the extracellular matrix

constituent, laminin [11]. It has been proposed that the dystrophin-glycoprotein complex provides a link between the subsarcolemmal cytoskeleton and the extracellular matrix, and that disruptions of the complex lead to muscle fibre necrosis [12-14].

In contrast to its sarcolemmal localization in normal muscle, dystrophin is absent, or greatly reduced, in DMD [4-6]. Similarly, the expression of all the dystrophin-associated proteins is also greatly reduced [13,15]. Dystrophin expression is also abnormal in a proportion of heterozygote carriers of DMD. Some, particularly those that manifest the disease, show a variable number of dystrophin-deficient fibres [16-18], while asymptomatic carriers show only minor disruption with immunolabelling, or a slight reduction in the amount of dystrophin on western blot [19]. Dystrophin shows considerable homology to the autosomal, chromosome 6 encoded protein,

Table 1. Clinical details of the carriers studied and the expression of dystrophin, dystrophin-associated proteins (DAPs) and utrophin in their biopsies.

Case	Age at Biopsy (years)	Affected relative	CK*	Weakness†	Dystrophin	DAPs	Utrophin	
<i>Manifesting carriers</i>								
1	SM	3	Brother	19,465	+	- ve fibres	↓	n/d
2	CW	5	Mother with ↑ CK	6810	++++	- ve fibres	↓	All fibres + ve
3	EB	12	Sister and mother with ↑ CK	5740	+++	- ve fibres	↓	n/d
4	MM	60	Son	700	++	Normal	Normal	1 + ve fibre
<i>Presumptive carrier</i>								
5	JW	24	Daughter (case 2)	1490	none	- ve fibres	↓	Many + ve fibres

*IU l⁻¹ (upper limit of normal = 170 IU l⁻¹)

† + - minimal weakness; ++ = mild weakness; +++ = moderate weakness; ++++ = severe weakness (≡ DMD).

- ve = negative; + ve = positive; ↑ = raised; ↓ = reduced; n/d = not done.

utrophin [20-22]. Utrophin has similar domains to dystrophin but, unlike dystrophin, is expressed in several visceral tissues in addition to muscle and neural tissues [23, 24]. In normal adult muscle utrophin is absent from the extrajunctional sarcolemma and is confined to the neuromuscular junction and vascular components [24-27], and has also been reported at the myotendinous junction [28]. It is however, expressed on the sarcolemma of regenerating fibres in a variety of neuromuscular disorders [26, 27] and on human fetal fibres until 26 weeks gestation [29]. In diseased muscle it is also detected on several mature fibres in Xp21 dystrophies and inflammatory myopathies [27, 28] and in severe childhood autosomal recessive, Duchenne-like, muscular dystrophy (SCARMD), which is characterized by a deficiency of the 50DAG [30]. It has recently been reported that utrophin is associated with a similar glycoprotein complex to that of dystrophin in mouse muscle [31].

Reports on the expression of utrophin and the dystrophin-associated glycoproteins in carriers of DMD are limited. Utrophin expression was found to be normal in one obligate carrier [28], whilst a single case who was suspected to be a symptomatic carrier showed that all the glycoproteins were reduced in the dystrophin-deficient fibres [32]. We report here the expression of the dystrophin-associated proteins and utrophin in four manifesting carriers of DMD and a presumptive carrier with raised serum creatine kinase.

METHODS

Needle biopsies were obtained under local anaesthesia from the quadriceps of four manifesting carriers with muscle weakness, aged 3, 5, 12 and 60 yrs (cases 1-4), and one presumptive asymptomatic carrier aged 24 (case 5) (Table 1). The manifesting carriers had varying degrees of weakness ranging from minimal to very severe and were identified by their family history, clinical presentation, raised CK and the presence of dystrophin-deficient fibres in their muscle biopsies (except case 4) [18]. The presumptive carrier had a raised CK and dystrophin-deficient fibres, but no weakness. She was the mother of the 5-yr-old manifesting carrier (case 2). The expression of dystrophin, the dystrophin-associated proteins and P-spectrin was studied in all cases and utrophin was examined in the 5-yr-old manifesting carrier and her mother, and the adult manifesting carrier.

Antibodies

All antibodies have been characterized previously. Dystrophin was localized using a mouse monoclonal antibody to the C-terminal domain (V1A4:), applied neat [9,12, 13,15], and a rabbit polyclonal antibody (P6) corresponding to the rod domain (amino acids 2814-3028), diluted 1:1000 [33]. Sheep polyclonal antibodies to the 59DAP, 50DAG, 43DAG and 35DAG were used at a dilution of 1:20 and that to the 156DAG used neat. A mouse monoclonal antibody to the 50DAG (IVD3,) was diluted 1:50 [9, 12, 13, 15]. Utrophin was detected with the mouse monoclo-

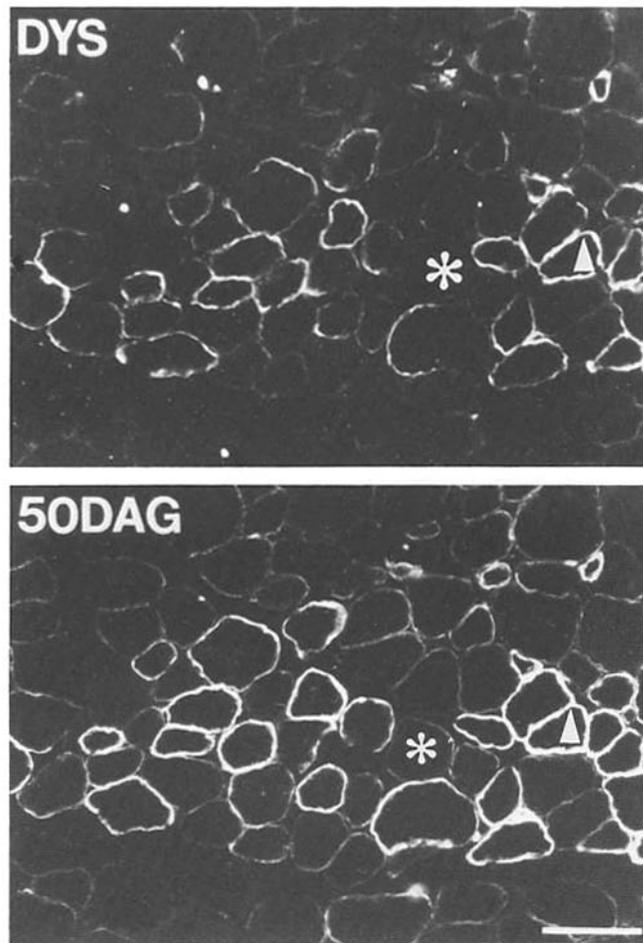


Fig. 1. Serial areas from the biopsy of the 5-yr-old manifesting carrier (case 2) immunolabelled with antibodies to dystrophin and the 50 kDa dystrophin-associated protein showing a similar mosaic pattern with both antibodies, with some fibres deficient in both dystrophin and the dystrophin-associated protein (*) whilst others have normal expression (▲). Bar=50 μ m.

nal antibody MANCHO 7 [34], diluted 1:5, and β -spectrin with a mouse monoclonal antibody raised against human erythrocyte β -spectrin (56A), diluted 1:1 [35].

Immunocytochemistry

Samples were frozen in isopentane cooled in liquid nitrogen and stored at -80°C or in liquid nitrogen. Cryostat sections (6 μ m) were incubated with primary antibodies for 2 h (VIA4₂, DAG and DAP antibodies) or 30 min (P6, utrophin, β -spectrin). For the sheep DAG and DAP antibodies this was followed by a biotinylated anti-sheep secondary antibody (1:500) for 1 h, and fluorescein-conjugated streptavidin (1:1000) for 30 min. The mouse monoclonal antibodies, VIA4₂ and IVD3₁, were visualized with fluorescein-conjugated anti-mouse IgG-

IgM (1:200) for 1 h. The P6 antibody to dystrophin and antibodies to utrophin and p-spectrin were visualized with the appropriate biotinylated secondary antibody (1:200) followed by streptavidin conjugated to Texas-Red (1:200) for 15 min. All washings and dilutions were in phosphate buffered saline. Sections from a control biopsy and a DMD were immunolabelled for dystrophin and the DAPs on the same slide as those from the carriers. Control sections were labelled without primary antibody and were consistently negative, except for autofluorescence.

RESULTS

Dystrophin-associated proteins

Biopsies from the three young manifesting

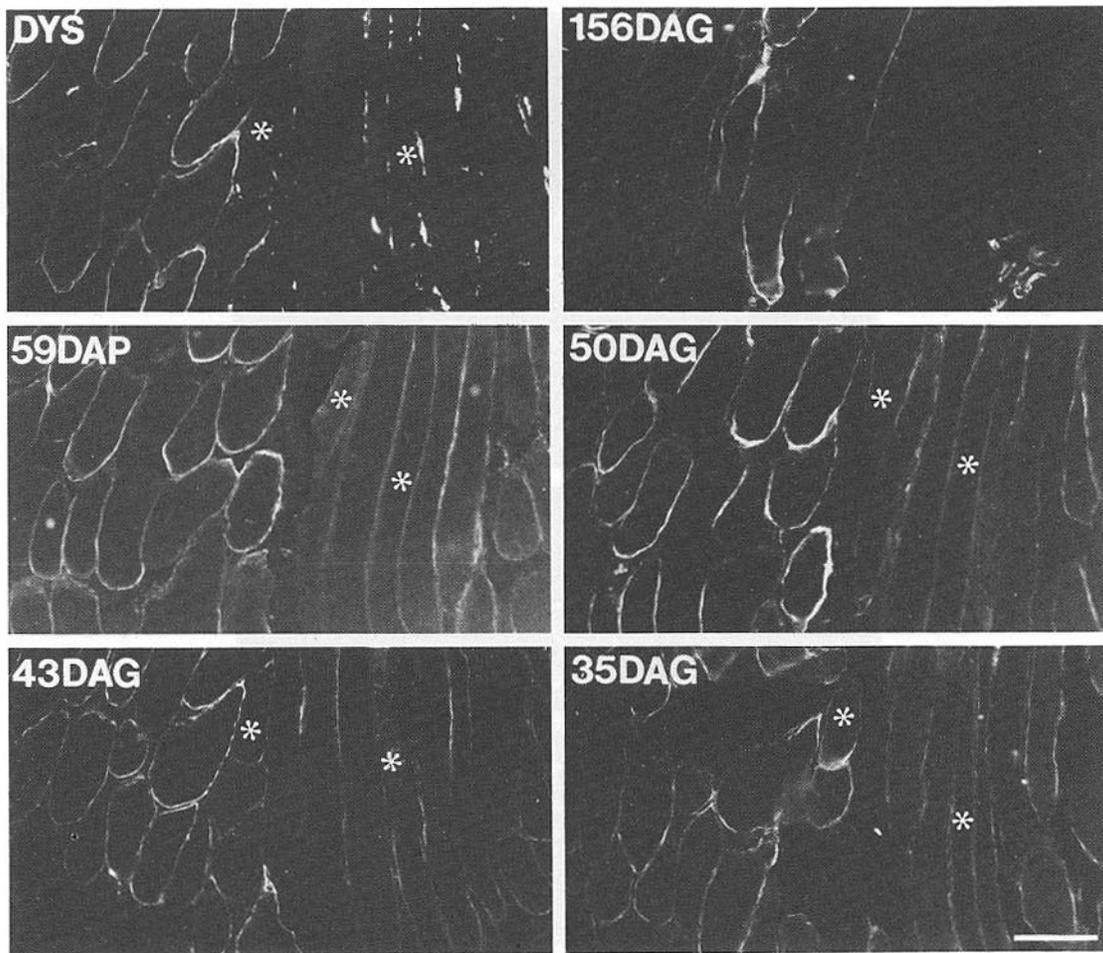


Fig. 2. Sections from the biopsy of case 2 immunolabelled with antibodies to dystrophin and the dystrophin-associated proteins showing a reduction of all the dystrophin-associated proteins in dystrophin-deficient fibres (*). (The section labelled with the antibody to the 156 K component is not serial.) Fibres sectioned longitudinally show a reduction in all components along the length of the fibres. Bar = 50 μ m.

carriers showed marked dystrophic changes with variation in fibre size and an increase in fat and connective tissue. All three had dystrophin-deficient fibres and a deficiency of the dystrophin-associated proteins in the same fibres (Fig. 1), in addition to fibres with normal expression of dystrophin and the dystrophin-associated proteins. All components of the glycoprotein complex were reduced, although the proportion of affected fibres varied (Fig. 2). Some residual immunolabelling of the glycoproteins could be seen in the dystrophin-deficient fibres (Fig. 2). The presumptive carrier (case 5) showed groups of dystrophin-positive and dystrophin-deficient fibres and a marked reduction in the dystrophin-associated glycoproteins in fibres deficient in dystrophin (Fig. 3).

The adult manifesting carrier showed relatively minor pathological changes with only slight variation in fibre size and no significant

increase in fat or connective tissue. Immunolabelling for dystrophin, the dystrophin-associated proteins and spectrin was normal in all fibres.

Utrophin

In control muscle, utrophin expression was confined to the capillaries and blood vessels (Fig. 4a). In the 5-yr-old manifesting carrier (case 2) utrophin and β -spectrin were detected on the sarcolemma of *all* fibres (Fig. 4b, d), in contrast to the mosaic pattern of dystrophin (Fig. 4c) and the proteins of the dystrophin-associated complex. This patient's mother (case 5), a presumptive carrier, also had uniform expression of utrophin on many fibres, both with and without dystrophin and the dystrophin-associated proteins (Figs 3 and 5). Fibres in one area of the biopsy, however, which had normal expression of dystrophin, did not express utrophin.

Although regenerating fibres were present in

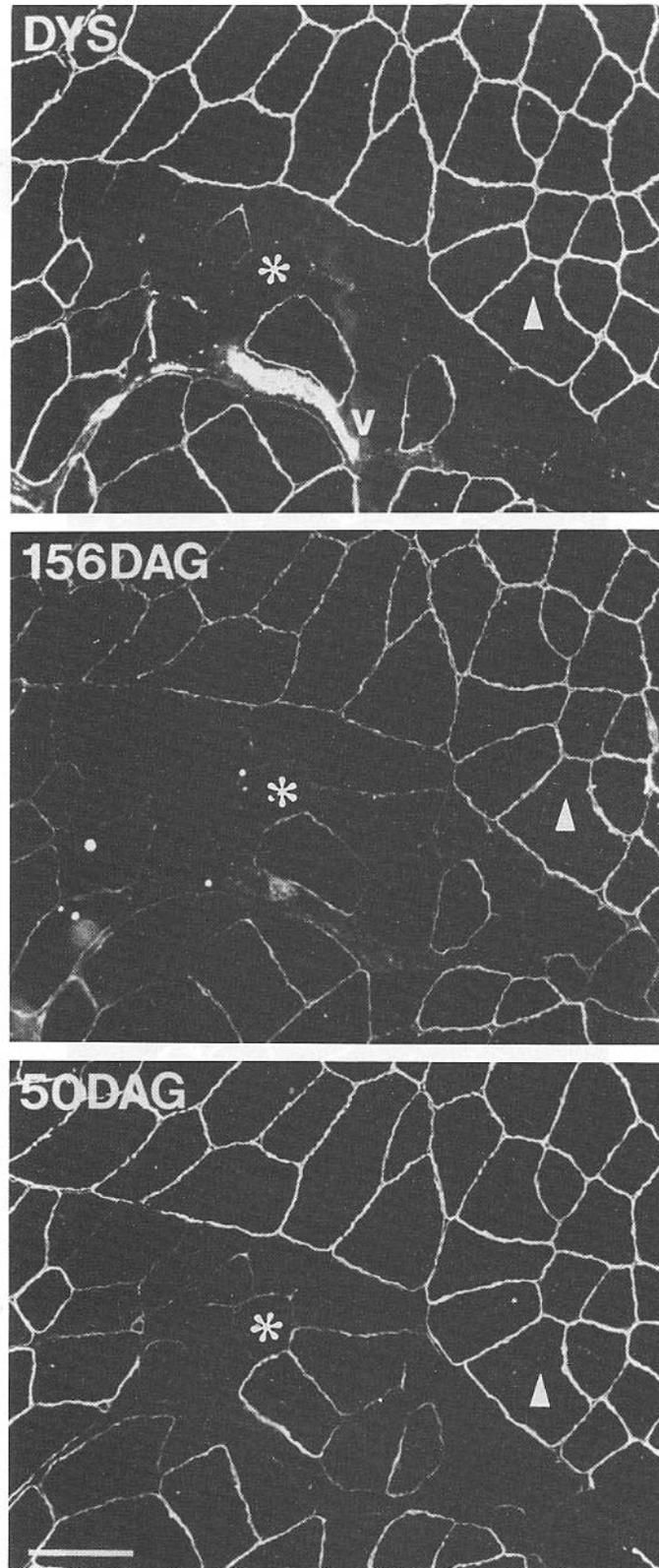
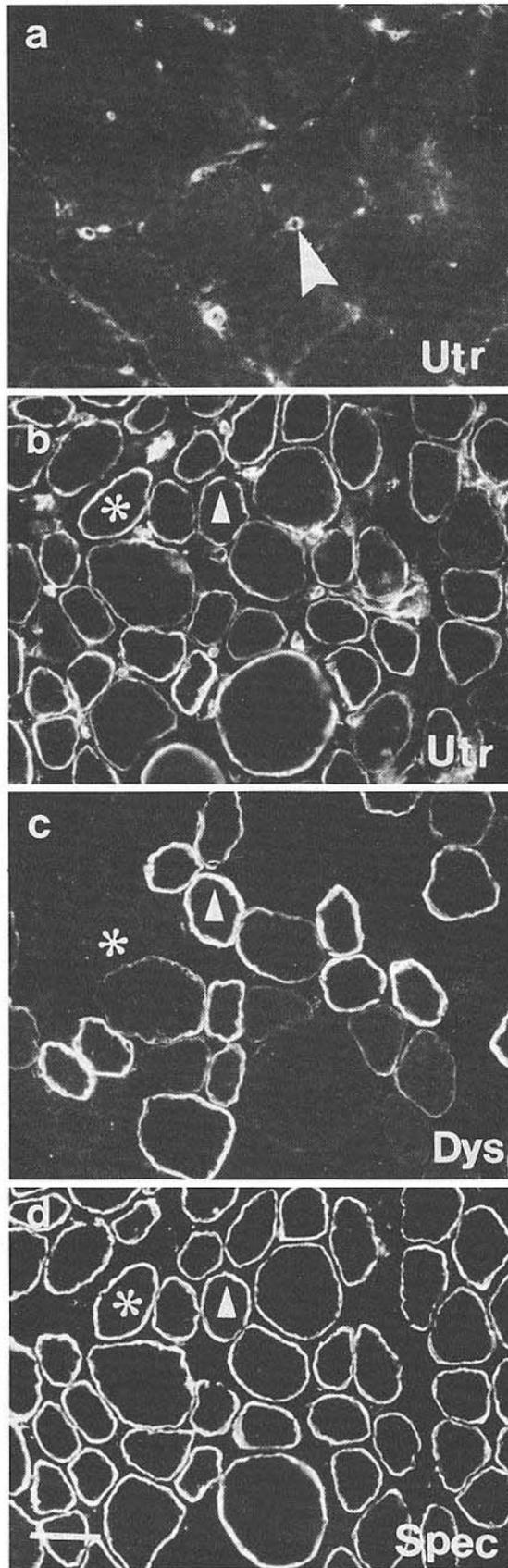


Fig. 3. Serial sections from the biopsy of case 5, the presumptive carrier, immunolabelled with antibodies to dystrophin, the 156 kDa and the 50 kDa dystrophin-associated glycoproteins showing a reduction of all three in the same fibres (*) but normal expression in the remaining fibres (▲). Note also labelling of a blood vessel (v) with the antibody to dystrophin. Bar = 50 μ m.



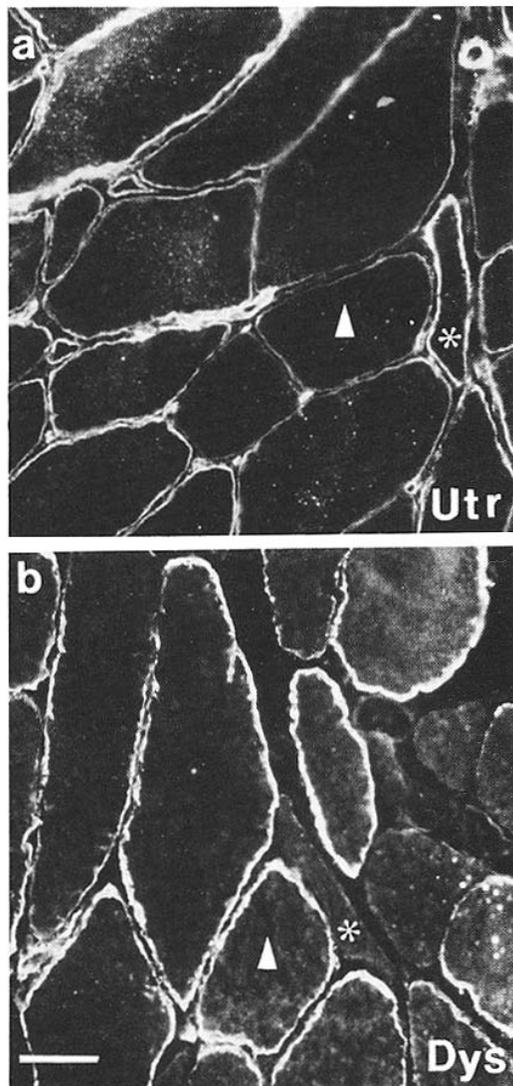


Fig. 5. Expression of (a) utrophin (Utr) and (b) dystrophin (Dys) in the biopsy of the presumptive carrier (case 5) showing expression of utrophin on dystrophin-positive (\blacktriangle) and dystrophin-negative fibres (*). Bar = 50 μ m.

the biopsy from the 5-yr-old manifesting carrier, several fibres that expressed utrophin were large in size and did not express proteins associated with immaturity, such as fetal myosin and N-CAM. Similarly, several of the fibres in the presumptive carrier that expressed utrophin were mature.

In the adult manifesting carrier (case 4), with

no dystrophin-deficient fibres, only one fibre expressing utrophin was observed.

DISCUSSION

The results show that dystrophin and the dystrophin-associated proteins co-localize in the same fibres in carriers of DMD. Thus, in cases with dystrophin-deficient fibres there is a concomitant loss of the dystrophin-associated proteins and a mosaic pattern is observed for both dystrophin and the dystrophin-associated proteins. All components of the dystrophin-glycoprotein complex are affected, including the laminin-binding 156 kDa component. This is similar to findings in DMD patients themselves in which all the glycoproteins are reduced, in parallel with dystrophin [13]. The results highlight the role of dystrophin in anchoring the glycoprotein complex, and emphasize its role in the pathogenesis of muscle damage. The biopsies of the young manifesting carriers showed marked pathological changes, similar to those seen in DMD patients [18], presumably resulting from a similar instability of the membrane involving both dystrophin and the glycoproteins.

In the manifesting carrier with apparently normal expression of dystrophin, the dystrophin-associated proteins were not affected. Manifesting carriers reported previously have shown a variable proportion of dystrophin-deficient fibres [17, 18], in contrast to asymptomatic carriers in which only minor changes are usually found [19]. Our observation in this manifesting carrier, aged 60 yrs, may relate to an age-related decrease in dystrophin-deficient fibres, as discussed previously [18, 19]. Pathological changes such as a wider variation in fibre size, however, were evident in this case. This has also been noted in a series of asymptomatic carriers who showed only minor abnormalities in dystrophin expression immunocytochemically [19]. Thus, morphological abnormalities in some carriers cannot be explained on the basis of any appreciable reduction in either dystrophin or the dystrophin-associated proteins. It is possible, however, that, as many fibres are probably a hybrid of normal and abnormal active nuclei, dystrophin and the dystrophin-associated proteins may diffuse to dystrophin-negative areas

Fig. 4. Immunolabelling of utrophin (Utr) in (a) control muscle and (b) the manifesting carrier, case 2. Serial areas in case 2 immunolabelled with antibodies to (c) dystrophin (Dys) and (d) β -spectrin (Spec). Note the expression of utrophin confined to capillaries in the control (arrow) but expression of utrophin and β -spectrin on all fibres in case 2 and a mosaic pattern of dystrophin-positive (\blacktriangle) and dystrophin-negative fibres (*). Bar = 50 μ m.

and give dystrophin-positive areas. Immunolabelling would then appear normal. Sarcolemmal expression of utrophin was markedly increased in the young manifesting carrier and her mother. It was detected on fibres with and without dystrophin; thus, an absence of dystrophin is not a prerequisite for the expression of utrophin. This is similar to the colocalization of utrophin and dystrophin at the sarcolemma in inflammatory myopathies, in normal fetal fibres, and fibres in severe childhood autosomal recessive muscular dystrophy [26, 27, 29, 30]. Utrophin was most marked in the manifesting carrier with a high proportion of dystrophin-deficient fibres, but in the biopsies from both her and her mother, the presumptive carrier, utrophin was detected on several fibres that expressed dystrophin, and not only those deficient in dystrophin. This concomitant expression of utrophin with dystrophin in carriers suggests that utrophin is not merely expressed as a result of the absence of dystrophin. However, the possibility that utrophin could diffuse from positive to negative areas, in a similar manner to dystrophin, and that utrophin may be overexpressed in dystrophin-negative areas, cannot be excluded.

The functional significance of utrophin in dystrophic muscle, however, remains to be elucidated. It would have been of interest to examine the amount of utrophin in the manifesting carriers in relation to the severity of their weakness, especially in the young carrier with minimal weakness and many dystrophin deficient fibres. Unfortunately, insufficient material remained to study utrophin in all the cases presented. In addition, quantitation of utrophin using immunoblots is difficult because of the contribution of both vascular components and regenerating fibres to total utrophin. Both these vary from sample to sample but must be taken into account in quantitative determinations of utrophin.

The significance of detecting utrophin on a single fibre in the manifesting carrier with normal dystrophin is unclear, but controls consistently show no labelling of mature fibres. Further studies are in progress to determine if fibres expressing utrophin occur in asymptomatic carriers and whether it could be an additional marker of abnormality that may be of diagnostic value in assessing biopsies from potential carriers.

Recent studies have shown that utrophin is associated with the same glycoprotein complex

as dystrophin [31]. Our Duchenne carriers, however, show that utrophin is present even in fibres with a reduced level of glycoproteins. This suggests that very little of the glycoprotein complex may be required to anchor the amount of utrophin expressed in these particular cases. The amount required, however, remains to be determined. It should be noted that immunolabelling with the antibodies to the dystrophin-associated proteins was not completely negative and our studies are qualitative and not quantitative.

In summary, we have shown that the dystrophin-associated proteins co-localize with dystrophin in DMD carriers and that utrophin is expressed on the sarcolemma of mature fibres regardless of the expression of dystrophin and the dystrophin-associated proteins.

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