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Quantitative analysis of dystrophin in fast- and slow-twitch mammalian skeletal muscle

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The relative tissue content of dystrophin has been evaluated in the slow-twitch soleus (primarily type I fibers) and fast-twitch vastus lateralis (primarily type IIb fibers) muscles of the rat and mouse, as well as in human biopsy samples from the vastus lateralis and gluteus maximus muscles, using a sensitive immunochemical assay. The dystrophin content of the soleus muscle was approximately twofold higher than in the vastus lateralis muscle. This difference is not entirely explained by the higher total sarcolemmal surface of the smaller soleus muscle type I fibers, and is therefore attributed to a higher content of dystrophin in the type I fibers compared to type IIb fibers. PCR analysis of the dystrophin transcript levels in the two muscle types indicated no significant differences. Analysis of human muscle biopsies revealed a twofold higher dystrophin content in the vastus lateralis muscle compared to the gluteus maximum muscle. It is concluded that the tissue content of dystrophin may vary significantly among physiologically different skeletal muscle types.

Dystrophin; Skeletal muscle; Muscle fiber; Muscle biopsy

1. INTRODUCTION

Dystrophin, the protein product of the human Duchenne and Becker muscular dystrophy (DMD/BMD) gene [1], is a large, 427 kDa membrane-associated cytoskeletal protein [2–5]. A significant effort has been devoted to the study of the cellular, molecular and genetic aspects of this inherited human muscular disorder, yet the precise cellular function(s) of this protein, whose absence results in a degenerative response in the muscle fiber, is not clear.

A curious feature of the dystrophic condition in both humans (DMD) and mice (mdx) is the possible early and preferential involvement of specific muscle fiber types in the degenerative process. Using an antibody against an isoform of alpha-actinin as a specific probe for type IIb muscle fibers Minetti et al. [6] have recently provided convincing evidence for the early and preferential degeneration of this particular fiber type in DMD patients. Webster and colleagues [7] have concluded that regeneration of type IIb fibers occurs before slow (i.e. type I) fibers are even affected by the disease. These investigators therefore suggested that dystrophin may have a specific function in type II fibers, which are capable of relatively higher rates of contraction compared to type I fibers. Hoffman and Gorospe [8] have presented an attractive interpretation for the observed differences between the effect of DMD on different fiber types based on the various factors which are involved in cellular calcium homeostasis.

Despite a lack of definitive experimental evidence indicative of a specific and early fiber-type involvement in the degenerative process characteristic of muscular dystrophy it is noteworthy that mdx muscle fibers are more vulnerable to necrosis, induced by lengthening contractions, compared to normal animals [9]. This observation is of particular significance in view of reports indicating that fast-twitch muscle fibers are preferentially damaged by eccentric contractions [10,11]. That a specific skeletel muscle fiber-type is more or less susceptible to the effects of dystrophin deficiency remains an open question which warrants further investigation. The objective of the present series of experiments has been to quantitatively evaluate the relative amount of dystrophin and its corresponding mRNA transcript in the slow-twitch soleus (SOL) and superficial portion of the fast-twitch vastus lateralis (SVL) muscles of the rat and mouse. Relative dystrophin content was also determined in biopsy samples from human vastus lateralis and gluteus maximus (GM) muscles.

2. MATERIALS AND METHODS

2.1. *Tissue homogenates*

The SOL and SVL were dissected from C57BL 105 °/. female mice and Sprague–Dawley rats, snap-frozen in liquid nitrogen and stored at −80°C until use. Tissue homogenization was carried out in 10 vols. of sample buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 3.3% SDS, 10% glycerol and 40 mM dithiothreitol) supplemented with a protease inhibitor combination [12]. The human muscle tissue was obtained by surgical biopsy and immediately prepared for electrophoretic analysis

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[12]. The mass of total protein in the tissue homogenates was determined by the filter paper dye-binding technique [13].

2.2. *Gel electrophoresis and quantification of immunoreaction products*

The tissue samples containing 10 μg of protein were subjected to SDS-PAGE electrophoresis according to the method of Laemmli [14] with the following modifications [12]. A 7–15% linear gradient resolving gel and a 6% stacking gel were used. The ratio of acrylamide to bisacrylamide was 30:0.15 in the stock solution. Electrophoresis was performed in a minigel apparatus (Bio-Rad, Canada) with 1 mm-thick spacers, and fractionated proteins were electroblotted onto polyvinylidene membranes (Millipore Corp., Canada) using a Bio-Rad mini-transblot electrophoretic transfer cell. Two monoclonal antibodies to dystrophin were obtained from Novocastra Laboratories Ltd., (Newcastle upon Tyne, England). These particular antibodies, designated NCL-DYS 1 and NCL-DYS 2, are directed against the mid-rod domain (amino acids 1,181–1,388) and a synthetic peptide corresponding to the last 17 amino acids of the C-terminal region, respectively, of the human dystrophin sequence. The secondary antibody was an affinity-purified goat anti-mouse IgG conjugated to alkaline phosphatase, and was diluted 1:10,000 (BIO/CAN Scientific, Canada). The antigen–antibody complex was revealed by chromogenic development using 5-bromo-4-chloro-3 indolyl phosphate and nitroblue tetrazolium (BCIP/NBT).

The dystrophin immunoreaction product, the myosin heavy chain subunit of stained gels and stained post-transfer gels were digitized using the Loats Associates Research Analysis System (Amersham Corp., Canada). The digitized images were quantitatively analysed using the GL 1000 program. Values are expressed as integrated optical density (IOD) and are corrected for background. The relative levels of dystrophin in samples were standardized according to the mass of protein loaded into each gel lane, the quantity of the myosin heavy chain subunit in the pre- and post-transfer gels. Each method of standardization gave identical results.

2.3. *Isolation of RNA*

Total RNA was isolated from rat skeletal muscle tissue according to Chomczynski and Sacchi [15], and quantified spectrophotometrically by absorbance at 260 nm.

2.4. *Reverse transcriptase (RT) and polymerase chain reaction (PCR)*

The oligonucleotide primers described by Tanaka and Ozawa [16] were synthesized using a Biosearch model 8700 DNA synthesizer (New Brunswick Scientific). The primers, identical (5'TGAAATAATGGAGGAGAGACTCGG3') and complementary (5'GCAGGCCATTCCTCTTTCAGGAAA3') to the mouse cDNA, corresponded to nucleotides 3,124–3,147 and 3,415–3,438, respectively, of human cDNA [17]. Oligonucleotides were purified on a 7 M urea/15% polyacrylamide gels. The predicted length of the amplification product was 314 base pairs [16].

RT and PCR were carried out as previously described [18] in an automatic DNA thermal cycler (Perkin-Elmer, Cetus, Norwalk, CT). Briefly, total RNA was mixed (total volume of 20 μl) with a solution containing 100 ng of complementary primer, PCR buffer 1×, 1 mM of each of dNTPs (Pharmacia-LKB Ltd.), 40 U of RNAsin (Promega Biotech Inc.) and 20 U of RAV2 reverse transcriptase (Amersham Corp.). The resulting cDNA was either used undiluted, or serially diluted in water, and then used as the substrate for the PCR reactions. The cDNA solutions (total volume of 200 μl) were supplemented with 500 ng each of the identical and complementary primers, 0.2 mM of each dNTP, PCR buffer 1× and 5 U of *Taq* polymerase (Perkin-Elmer, Cetus). The amplification was carried out for 15 and 20 cycles as previously described [18].

To determine the size of the PCR products, 20 μl of each reaction sample was run with DNA markers (DNA ladder, Bethesda Research Laboratories) on 1.5% agarose gels, which were subsequently stained with ethidium bromide. The PCR products in the agarose gel were blotted onto Hybond-N membranes (Amersham Corp.) and then hybridized with a specific oligonucleotide probe (5'GCACCTTCAGAAATATGCCAG3'). This probe is located between the two primers and corresponds to nucleotides 3,224–3,255 of the human cDNA sequence. The probe was labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Negative controls consisted of samples in which the reverse transcriptase reaction was omitted. The intensity of the developed signals on the autoradiograms was analysed quantitatively by image analysis as described for the immunoblotting experiments. The bands on the membrane corresponding to the hybridization bands were excised, and radioactivity measured by liquid scintillation spectrometry. Background radioactivity was determined using unexposed areas of the membrane.

3. RESULTS

3.1. *Immunochemical specificity of DYS 1 and DYS 2*

The electrophoretic protein profiles of the muscle homogenates and the immunochemical specificity of the two monoclonal antibodies directed against dystrophin are shown in Fig. 1. This particular SDS-PAGE system provides a significant degree of resolution of the very high molecular mass proteins of skeletal muscle. In all muscle tissue homogenates the DYS 1 antibody reacts with dystrophin, as well as with a protein of slightly higher electrophoretic mobility, whereas the antibody DYS 2 recognizes only the dystrophin band (Figs. 1 and 2).

3.2. *Quantification of dystrophin*

The relative levels of dystrophin were determined in the SOL and SVL muscles of the rat and mouse using

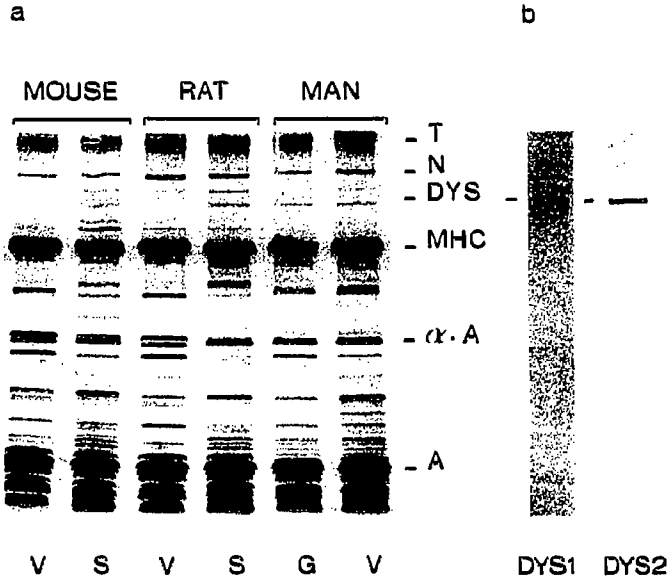

Fig. 1. Immunochemical specificity of dystrophin antibodies. The protein profiles of the human and rodent muscle tissue (V, vastus lateralis; S, soleus; G, gluteus maximus) homogenates are illustrated in panel a. Antibody DYS 1 recognizes dystrophin, as well as a protein of higher electrophoretic mobility, while antibody DYS 2 reacts specifically and only with dystrophin (panel b). T, titin; N, nebulin; DYS, dystrophin; MHC, myosin heavy chain subunit, α-A, alpha-actinin; A, actin.

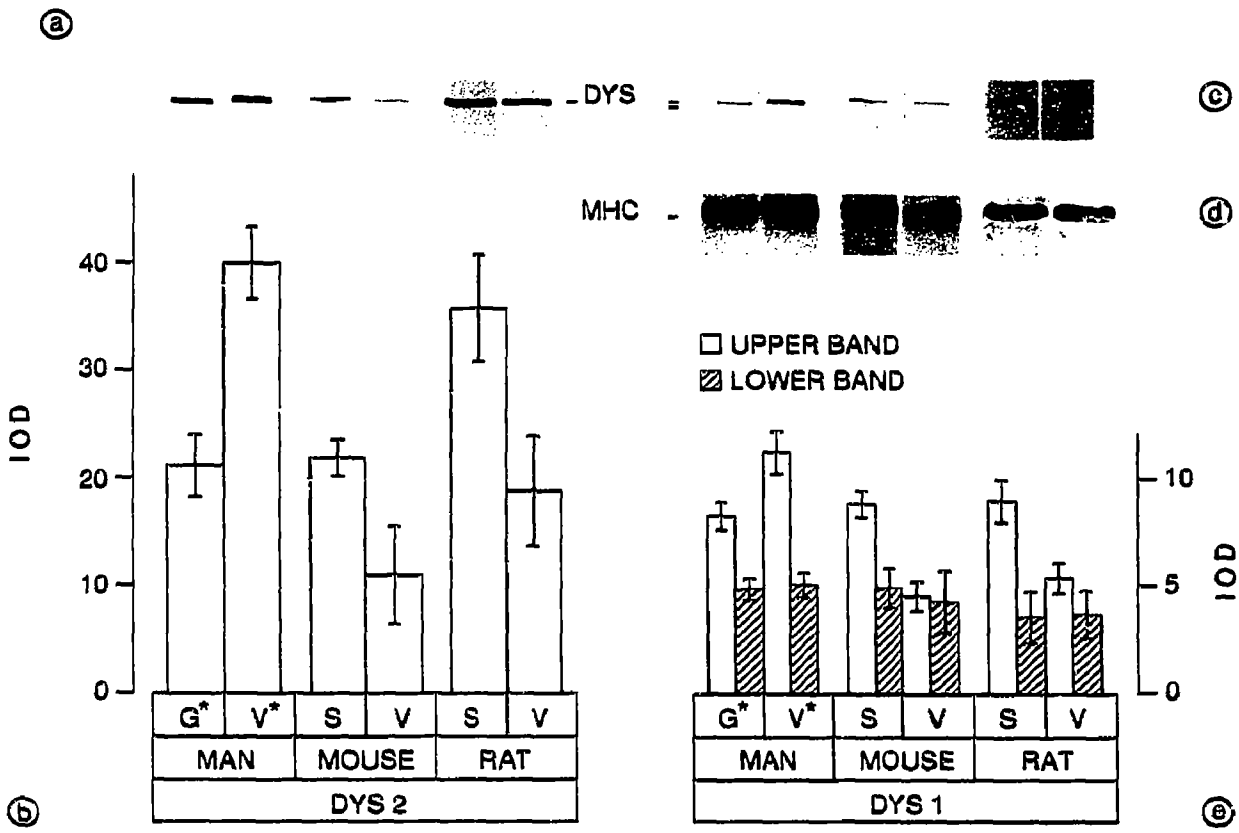

Fig. 2. Immunochemical quantification of dystrophin in rat, mouse and human skeletal muscles. An example of the immunoblots using antibodies DYS 1 and DYS 2 are shown in panels a and c, respectively, with the corresponding relative quantification (IOD, integrated optical density units) provided in panels b and e. Values represent the means ± SD of four separate experiments or *four determinations in the case of the human biopsy samples. The myosin heavy chain subunit (MHC) region of a stained post-transfer gel is shown for reference (d).

a quantitative immunoblotting method described previously [12]. In both rat and mouse the dystrophin content is approximately twofold greater in the SOL muscle compared to the SVL muscle (Fig. 2). This result was obtained using either of the two monoclonal antibodies. However, relative tissue content of the lower molecular weight protein recognized by the antibody DYS 1 does not vary significantly between the two muscles (Fig. 2e).

Dystrophin was also immunoassayed using DYS 1 and DYS 2 in two human muscle biopsy samples. It was found that the SVL muscle has approximately a twofold greater dystrophin level than the GM muscle, while the content of the lower molecular weight protein detected with DYS 1 does not differ significantly between the two muscles (Fig. 2e).

3.3. *Analysis of dystrophin transcript levels*

Having demonstrated that the relative tissue levels of dystrophin varied up to twofold between the slow-twitch SOL and fast-twitch SVL muscles the second objective was to determine if the observed differences in protein content could be accounted for by differences in the cellular levels of the dystrophin transcript. In the first series of experiments total RNA was serially diluted (0.01, 0.1, 1.0, 2.0 and 3.0 μg) prior to RT. The cDNA derived from the RT was used as substrate for PCR, and the amplification reaction was carried out in the same tube. These experiments revealed that there is no significant difference between the two muscles with respect to the relative levels of dystrophin mRNA (Fig. 3a and b).

To further verify the accuracy of the quantification of the PCR products a second and alternative series of experiments were undertaken wherein a fixed amount of total RNA was used in the RT reaction and the resulting cDNA was serially diluted prior to the PCR reaction. The results of these experiments indicate that similar dystrophin transcript levels are found in mouse SOL and SVL muscles (Fig. 3c and d).

4. DISCUSSION

The results of the experiments reported here demonstrate that the cellular content of dystrophin is approximately twofold greater in the SOL muscle of the rat and mouse compared to the SVL muscle. The rat SOL is composed primarily of type I fibers while the SVL is primarily type IIb fibers [19]. Using published data for

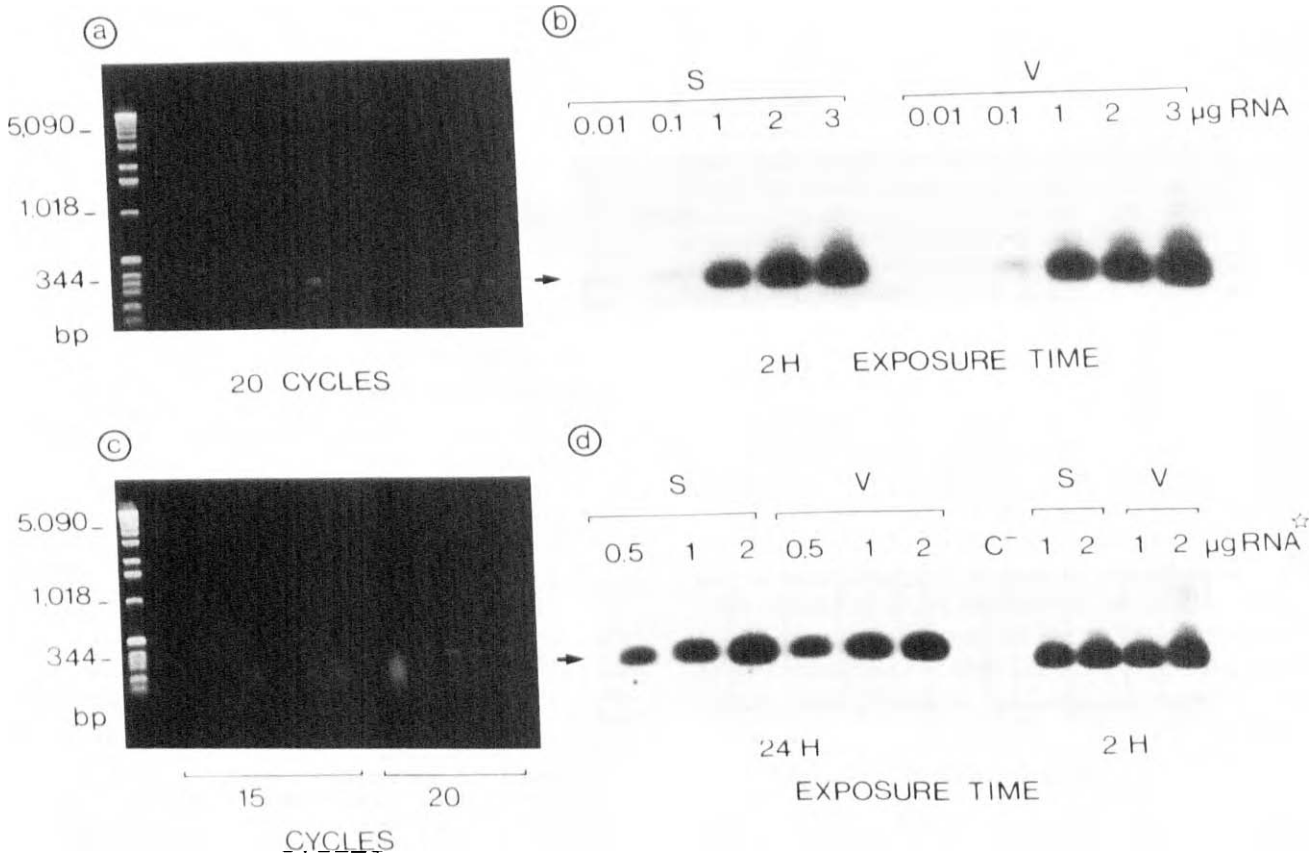

Fig. 3. Analysis of dystrophin mRNA levels in rat soleus (S) and vastus lateralis (V) muscles by PCR. The PCR products obtained following serial dilution of the template RNA are visualized in the ethidium bromide-stained agarose gel following 20 cycles of amplification (panel a), and in the corresponding autoradiogram (panel b) following hybridization with a specific oligonucleotide probe. The PCR products of a second experiment using serially diluted cDNA are shown in panel c, with the corresponding autoradiogram in panel d. The exposure times of 24 h and 2 h correspond to 15 and 20 cycles of amplification, respectively. C^- indicates a control where the RAV2 was omitted. RNA* indicates the corresponding mass of RNA in the PCR reaction following dilution of the cDNA.

percent fiber-type composition and mean cross-sectional areas of the fibers in the two muscle types [20] and our data for the mouse muscles (unpublished results), we predicted that the SOL muscle of both the rat and mouse would contain a 40% greater content of dystrophin than the SVL muscle. The validity of this calculation is based on the assumption that the cellular distribution of dystrophin is uniform in both fiber types and restricted to the cytoplasmic face of the sarcolemma. This leaves approximately 60% of the observed difference in dystrophin content unaccounted for. We therefore concluded that rodent type I fibers contain higher cellular quantities of dystrophin than type IIb fibers. Furthermore, recent results obtained using various other antibodies indicate that fiber-type specific differences in the cellular content of cytoskeletal proteins are not restricted to dystrophin (unpublished observations). Several other investigations have revealed that filamin, spectrin and vinculin are found in greater quantities in avian slow muscle fibers than in fast muscle fibers [21–23]. Based on the data presented here, and on the observations of other studies, it is tempting to speculate that the greater ability of slow-twitch muscle compared to fast-twitch muscle to maintain force over time [24] may be functionally related to differences in cytoskeletal protein content.

The protein of lower molecular weight detected by the DYS 1 antibody probably corresponds to the putative dystrophin isoform identified by Byers et al. [25] which lacks a portion of the C-terminus region of the molecule. Since the DYS 2 antibody employed in the present study was generated using a synthetic peptide corresponding to the last 17 residues of the dystrophin sequence it would not be expected to react with this particular isoform (Fig. 2e). Efforts are currently underway to positively identify this putative dystrophin isoform, since the relative cellular abundance of this polypeptide does not show fiber-type specific differences that would be expected of a protein which, like dystrophin, is restricted to the fiber periphery [26].

The finding that fibers from human SVL muscle contain twice as much dystrophin compared to fibers in the GM muscle was unexpected in view of several previous reports suggesting that the levels of dystrophin are

probably similar in all types of human skeletal muscles [27]. We attribute the detection of this difference to improved quantitative immunoblotting methodology for dystrophin [12]. The biopsy samples used in these experiments were taken from two skeletal muscles which are known to possess a similar range of proportions of type I and II fibers in humans [28]. However, the diameter of human type I and II fibers can vary significantly [28], which may partially account for the observations reported here. It therefore remains to be clearly determined if a significant difference in the cellular content of dystrophin exists between type I and type II fibers in humans. Nevertheless, these results may have important implications for the interpretation of immunoblotting tests in Becker muscular dystrophy (BMD), and for the analysis of female carriers whose muscle fibers may show a mosaic expression of dystrophin. It is generally accepted that the clinical severity of BMD is more closely correlated with the relative quantity of dystrophin than dystrophin quality [27,29]. The data presented here using the human muscle biopsy samples suggest that a comprehensive investigation of this aspect of dystrophin analysis could probe to be important with respect to clinical diagnostic procedures.

The relative levels of the dystrophin transcript in the mouse SOL and SVL evaluated by RT-PCR were not significantly different. In view of the clear difference in the levels of the dystrophin protein in these two muscles, the data concerning dystrophin transcript levels could indicate that other control mechanisms operating, perhaps, on the translational process serve to regulate the intracellular levels of dystrophin. This information should be carefully considered when the relative levels of dystrophin and the corresponding transcripts are to be evaluated for quantitative purposes.

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