Dystrophin and Dystrophin Homologue in the Uterus: Diversity of Dystrophin Isoforms in Smooth Muscles

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Abstract

Monoclonal antibodies, which recognize 6 different epitopes of the dystrophin molecule expressed in normal human skeletal muscle, were used to characterize dystrophin expression in human and mouse uterus. Two different dystrophins or dystrophin homologues were detected in the uterus: one was expressed at the periphery of the myometrial cells and the other at the periphery of the vessels.The vessel-associated dystrophin homologue was also expressed in the vessels of a DMD patient lacking the first 52 exons of the Xp21 dystrophin gene. Therefore it must have been translated from a different gene transcript. Myometrial-associated and main skeletal muscle dystrophins are structurally different at 2 positions within the dystrophin central spectrin-like domain. These 2 muscle-specific dystrophins must result from 2 different transcripts, presumably generated by different alternative splicing of the Xp21 gene.

Key words : dystrophin, isoform, homologue, uterus, smooth muscle.

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Dystrophin is the protein product of a gene located at the Xp21 locus [8,15]. This protein has a relative molecular mass (Mr) of 400K as determined by Western blotting, and is expressed at the periphery of normal skeletal muscle fibers according to immunocytochemical analysis [2,3,4,21,23,24]. This gene is affected in Duchenne Muscular Dystrophy (DMD) patients and *mdx* mice via mutations which disrupt the reading frame of the transcript, thus preventing dystrophin expression in the corresponding skeletal muscles [1, 8, 20, 21]. Dystrophin or homologues have been detected on the inner face of the plasma membrane of all normal striated, visceral and vascular smooth muscles [9, 14, 16]. The existence of smooth muscle-specific dystrophin(s), that differ from skeletal muscle-specific form(s), was initially suspected based on minor differences in electrophoretic migration [9,16]. This muscle specificity of dystrophin was further supported by the detection of differences at the 3' ends of dystrophin transcripts prepared from smooth and skeletal muscles. Two different transcripts would thus result from smooth-muscle-specific alternative slicing of the Xp21 gene [7]. Meanwhile, Love et al. [13] identified a 13 kb long chromosome 6-encoded transcript in which one 1.5 kb long cDNA (B3 cDNA) sequence exhibited nucleic acid identity of approximately 65 % with the terminal 3' end of the Xp21 encoded dystrophin transcript. Using polyclonal antibodies against a recombinant fragment issued from this chromosome 6-encoded cDNA, Khurana et al. [11] detected a 400K-sized protein in striated and smooth muscles and in kidney, gut and stomach tissues, etc... in both normal and *mdx* mice. This dystrophin-related protein (DRP) was also detected in the skeletal muscles of DMD patients. Using polyclonal [6] and monoclonal [19] antibodies which recognize different regions of the dystrophin molecule, a dystrophin homologue (Mr 400K) was recently observed at the neuromuscular junction (NMJ) of a DMD patient lacking the first 52 exons of Xp21 dystrophin gene and in *mdx* mice. This dystrophin homologue could be related to the DRP [19]. In this study we investigated the possible expression of different forms of dystrophins or dystrophin homologues or DRP within the uterus. Like most other organs containing smooth muscle, the uterus is composed of both visceral smooth muscle (myometrial fibers) and vascular smooth muscle (arteries and veins). Using a set of recently produced monoclonal antibodies (Mabs) raised against 2 parts of the dystrophin central spectrin-like rod-shaped domain and against the carboxy terminus, we addressed the following questions : Is the dystrophin expressed in visceral smooth muscles (myometrial fibers) different from that expressed in vascular smooth muscles? Do dystrophin forms of skeletal and smooth muscles differ immunologically?

Materials and Methods

Tissue Samples.

Small pieces of human gravid myometrium were obtained by caesarean section, immediately stored at 4° C and then

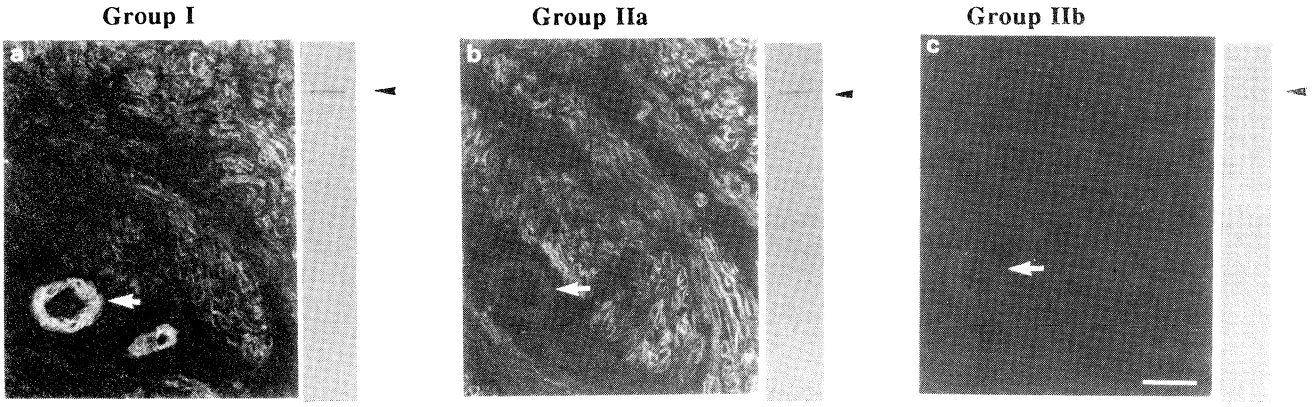


Figure 1. Comparative immunoanalysis of a normal human uterus from a 26 year old female. Serial sections were incubated with 6 Mabs raised against different domains of human and chicken skeletal muscle dystrophin. Corresponding extracts were processed by Western blot analysis using the same set of Mabs. Each panel shows 2 vessels (see white arrows) surrounded by myometrial fibers and the corresponding Western blot on the right (an arrow indicates the 400K level). The 6 antibodies reacted in 3 different ways (a, b, c respectively) and were classified into 3 categories : group I, IIa, IIb. The scale marker (panel c) corresponds to 20 μm.

frozen in liquid nitrogen-cooled isopentane at -150° C as soon as possible.

Two *mdx* mice and 2 BL10 control mice were killed by cervical dislocation. The uteri were immediately removed and then frozen as described above.

DMD skeletal muscle samples were taken from a 9 year old male who had severe DMD and was also mentally retarded. According to the genetic diagnosis, this DMD patient had a deletion spanning at least the first 52 exons of the 75+ which form the Xp21 transcript. The control muscle biopsy was obtained from a patient with no apparent dystrophin deficiency.

Monoclonal antibodies

Five monoclonal antibodies (Mabs) were recently obtained by lymphocyte hybridizations from 3 different mice immunized with recombinant dystrophin fragments spanning 3 different regions of human and chicken skeletal muscle dystrophin [10, 18].

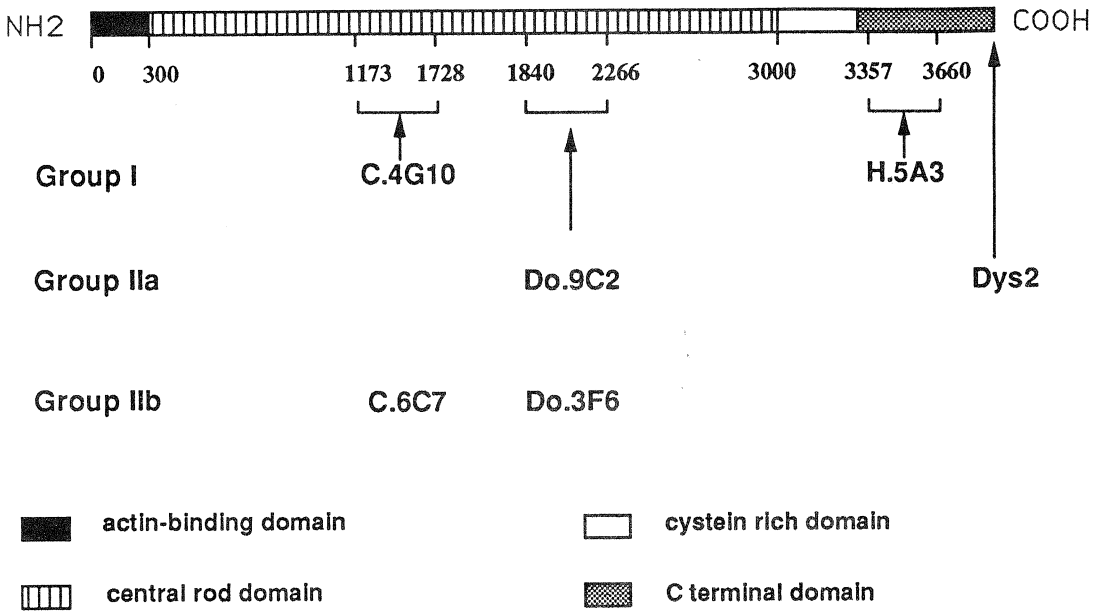
- Mabs C.6C7 and C.4G10 were obtained using a fragment of the central spectrin-like rod-shaped domain of chicken skeletal muscle dystrophin (residues 1173 to 1728) as immunogen.
- Mabs Do.9C2 and Do.3F6 were obtained using a fragment from the central spectrin-like rod-shaped domain of human skeletal muscle dystrophin (residues 1840 to 2266) as immunogen.
- Mab H.5A3 was obtained using the entire carboxy-terminal domain of chicken skeletal muscle dystrophin (residues 3357 to 3660) as immunogen.

Mab Dys 2 (Dy8/6C5) was a generous gift from L. Nicholson. It was obtained by lymphocyte hybridization from a mouse immunized with a synthetic peptide consisting of the last 17 amino acids at the C-terminus of human skeletal muscle dystrophin.

All of these Mabs detect their own antigens in ELISA. They all stained the fiber periphery of normal human skeletal muscle in immunofluorescence experiments. They also detected a Mr 400K protein which was present in extracts of control human skeletal muscle, according to Western blot analyses.

Immunofluorescence and Western blotting.

Indirect immunofluorescence was performed on 10 μm thick cryostat sections of mouse and human muscles frozen in isopentane as described in Dechesne [5]. For Western blotting, 20 serial 10 μm thick cryostat sections, adjacent to the sections sampled for the immunofluorescence studies, were homogenized in 40 μl of gel loading SDS-containing buffer according to the Western blotting microprocedure described in [5]. 10μl samples were applied on 0.75 mm SDS-polyacrylamide gels, using a 2.5% to 7.5% gradient gel containing 25% glycerol but no stacking gel [12]. The amount of muscle extracts applied were *a posteriori* controlled by measuring the myosin content detected by Coomassie staining. Transfer and immunostaining procedures were performed as described in [22]. Alkaline phosphatase labelled anti-mouse antibodies (Miles) were used for labelling.



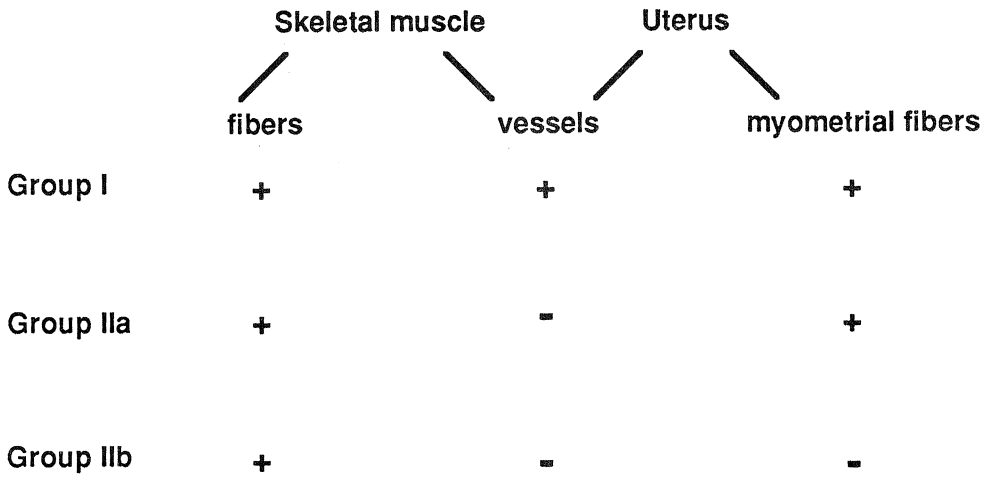


Figure 2 Classification and epitope localization of the 6 anti-dystrophin monoclonal antibodies. On the upper part of the figure, Mabs are represented along the schema of the dystrophin molecule according to the location of the epitopes they recognize. The scale was not proportional for different parts of the molecule. In the lower part of the figure, Mabs are classified into 3 groups (I, IIa, IIb) according to their reactivity in uterus sections (myometrial fibers and vessels, see Fig.1), all of them stained the periphery of normal skeletal muscle fibers.

Results

Six monoclonal antibodies, prepared from recombinant proteins or peptide spanning 3 different regions of human and chicken skeletal muscle dystrophin, were used in immunofluorescence and Western blotting experiments to characterize dystrophin expression in human and mouse uterus. These monoclonal antibodies recognized the 400K dystrophin expressed in normal human skeletal muscle.

The antibodies were applied to serial cryostat sections of normal adult human uterus and analysed by indirect immunofluorescence. The corresponding muscle extracts were simultaneously analysed by Western blotting. The 6 antibodies reacted in 3 different ways:

- Two Mabs (H.5A3 and C.4G10) stained the periphery of myometrial fibers and vessels. Both detected a 400K band in muscle extracts (Fig.1a).
- Two Mabs (Do.9C2 and Dys 2) stained only the periphery of myometrial fibers. The vessels were not stained. They also detected a 400K band in muscle extracts (Fig.1b).
- Two Mabs (C.6C7 and Do.3F6) unexpectedly stained neither myometrial fibers nor vessels.They did not detect any immunoreactive band in muscle extracts (Fig.1c).

Thus the antibodies were classified into 3 different categories according to their reactivity differences in human uterus: group I, IIa and IIb respectively. This Mab classification is summarized in Fig.2 with respect to location of the corresponding antigens along the dystrophin molecule.

To further determine specificities of the antibodies, we analyzed Xp21 dystrophin-deficient muscles from *mdx* mice or human DMD patients (Fig.3). According to Western blotting (Fig.3A), group I antibodies detected a Mr 400K band in extracts from the uterus of normal and *mdx* mice. In contrast, group IIa antibodies detected a 400K band only in uterus extracts from normal mice, but not in those from *mdx* mice. The immunoanalysis was confirmed by comparing reactivity of the antibody set on cryostat sections of human skeletal muscle from a non-dystrophin-deficient control and from a DMD patient with a deletion spanning at least the first 52 exons of the 75+ which form the Xp21 transcript (Fig.3B). Positive immunoreactivity was observed at the periphery of vessels from the DMD patient and at the periphery of control vessels and skeletal muscle fibers using group I antibodies. In contrast, both group IIa and IIb antibodies stained the periphery of control muscle fibers but not the vessels of the control or of the DMD patient.

To closely compare the immunological differences of dystrophin forms expressed in myometrial and skeletal muscle fibers, group IIa and IIb antibodies were applied on cryostat sections and on the corresponding extracts from uterus and normal skeletal muscles (Fig. 4). As expected from Figs. 1 and 3, group IIa antibodies reacted at the periphery of myometrial and skeletal muscle fibers and with a 400K band in both muscle extracts. In contrast, group IIb antibodies did not react in the uterus. They only reacted at the periphery of normal skeletal muscle fibers and with a 400K band in the corresponding muscle extract.

Discussion

These results demonstrate that human and murine uterus contain at least 2 different dystrophins or dystrophin homologues: one is expressed at the periphery of myometrial cells while the other is expressed at the periphery of vessels. We came to this conclusion by observing reactivity differences using several new anti-dystrophin antibodies. The 6 antibodies, which reacted only with a 400K band and at the fiber periphery in normal human skeletal muscles, were directed against 6 different epitopes along the dystrophin isoform encoded by the Xp21 transcript and mainly expressed in normal skeletal muscles. The immunoanalyses of DMD and *mdx* mouse muscles, that have deletions within the gene located at the Xp21 locus, revealed that 2 out of 6 antibodies (group I) still reacted with a 400K band located at the periphery of vessels in skeletal muscles and uterus. This vessel-associated protein was not an isoform of an Xp21-encoded dystrophin resulting from alternative splicing because the vessel protein was detected in muscle from a DMD patient with a substantially deleted Xp21 locus. Such a mechanism could have been hypothesized for the vessel protein detected in *mdx* mouse uterus, since the gene defect is caused by a simple point mutation in this strain [20]. This vessel-associated dystrophin homologue was detected by 2 Mabs (group I) which were generated by immunisation with fusion proteins obtained with cDNAs from 2 distinct regions of chicken skeletal muscle dystrophin. Both of the corresponding epitopes, one in the dystrophin spectrin-like domain and the other in the C-terminal domain, are consequently shared by the Xp21 dystrophin isoforms encoded in the human skeletal and smooth muscles and by the vessel-associated dystrophin homologue. The 4 other epitopes recognized by group IIa and IIb antibodies, 3 in the spectrin-like domain and 1 in the C-terminal domain of Xp21-encoded dystrophin, thus correspond to structural differences between the vessel-associated dystrophin and the 2 Xp21 dystrophin isoforms. Structural homologies and differences are therefore spread over a wide region of the molecule, suggesting that the vessel-associated dystrophin homologue appears as a kind of dystrophin isoform encoded by a gene not located at the Xp21 locus.

The vessel-associated dystrophin homologue has many structural similarities with the dystrophin homologue associated with the neuromuscular junctions (NMJ), which is also expressed at the NMJ of Xp21-encoded dystrophin-deficient muscles. Mab H.5A3 like Mab Dy18 [19], which are both generated by immunisation with the same fusion protein corresponding to the C-terminal region of chicken skeletal muscle dystrophin, reacts with the recombinant bacterial protein issued from the chromosome 6 encoded cDNA constructed by Khurana et al. [11] (not shown). The two dystrophin homologues thus seemed to share at least two common epitopes with the DRP and with the Xp21-encoded dystrophin. Therefore, the vessel-associated dystrophin could also be derived from the chromosome 6-encoded 13 kb long transcript [13]. However it might be a member of the family of dystrophin homologues which could be encoded by a gene that has not yet been identified. Monoclonal antibodies specific for the chromosome 6 protein or the NMJ or vessel homologue, or isolation and sequencing of the different dystrophin homologues are essential to distinguish between these different homologous proteins and to define their original genes.

Another conclusion of the present study is that two different Xp21-encoded dystrophins are expressed at the periphery of myometrial cells and skeletal muscle fibers. Their common genetic origin (Xp21 gene) is shown by their lack of express-

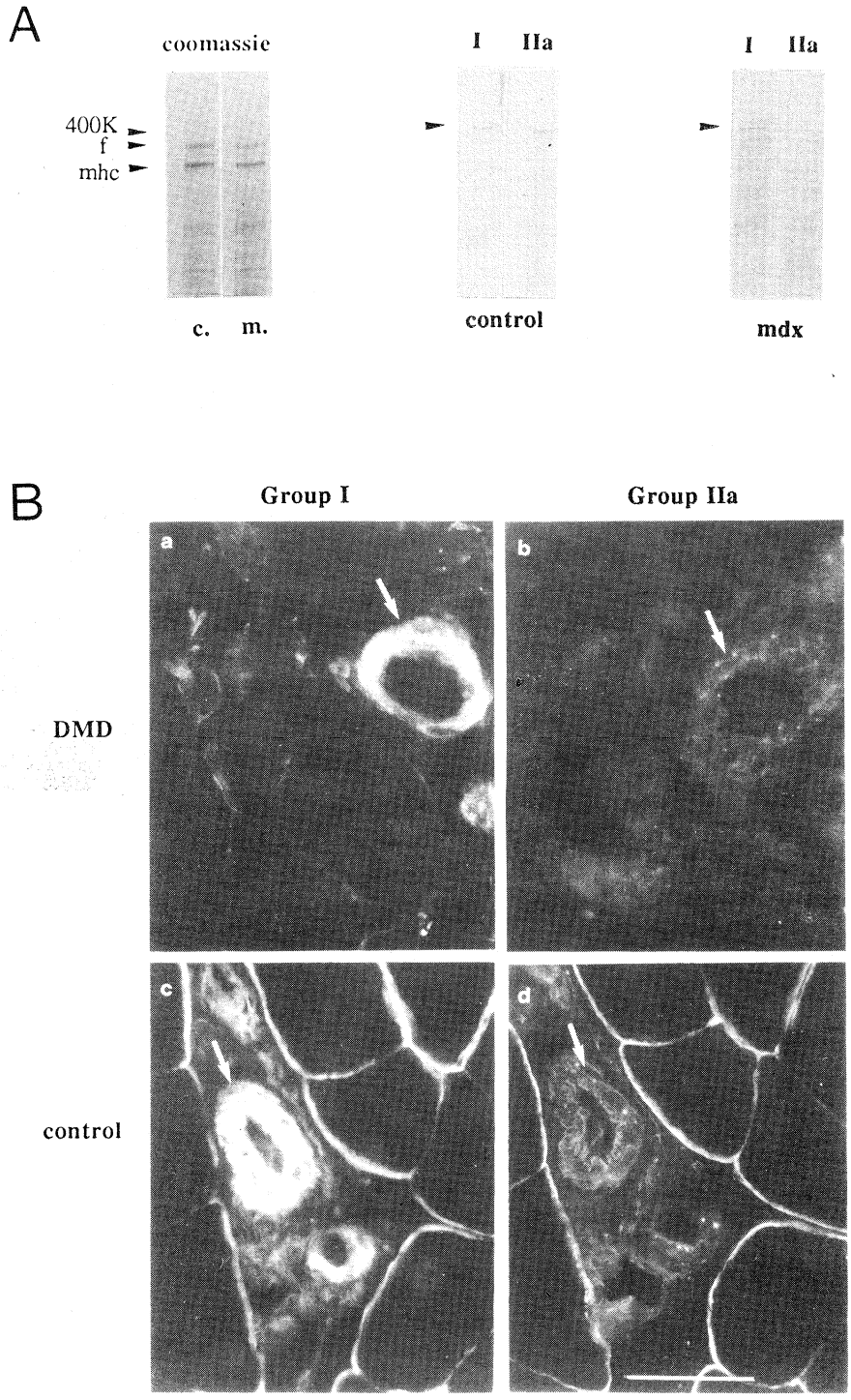


Figure 3.Immunoanalysis of control and dystrophin-deficient muscles.

A.Western blot analyses of control and mdx mice uterus extracts, using Group I and IIa antibodies. Coomassie blue staining of myosin heavy chains shows that the muscle protein content of both extracts is equivalent and the same amounts of extracts were applied in all panels. A black arrow indicates the 400K level, corresponding to the dystrophin immunodetection.

B.Comparative immunofluorescence analysis of human skeletal muscle from a DMD patient lacking the first 52 exons of the Xp21 locus and from a non-dystrophin-deficient control. Cryostat sections from the DMD patient (panel a and b) were incubated with antibodies from groups I and IIa respectively. Panels c and d show control muscle sections that were processed like panels a and b. On each panel blood vessels are indicated by a white arrow and the scale marker (panel d) corresponds to 20 μm. Groups IIa and IIb had the same reactivity on skeletal muscle sections, data with group IIb are not shown.

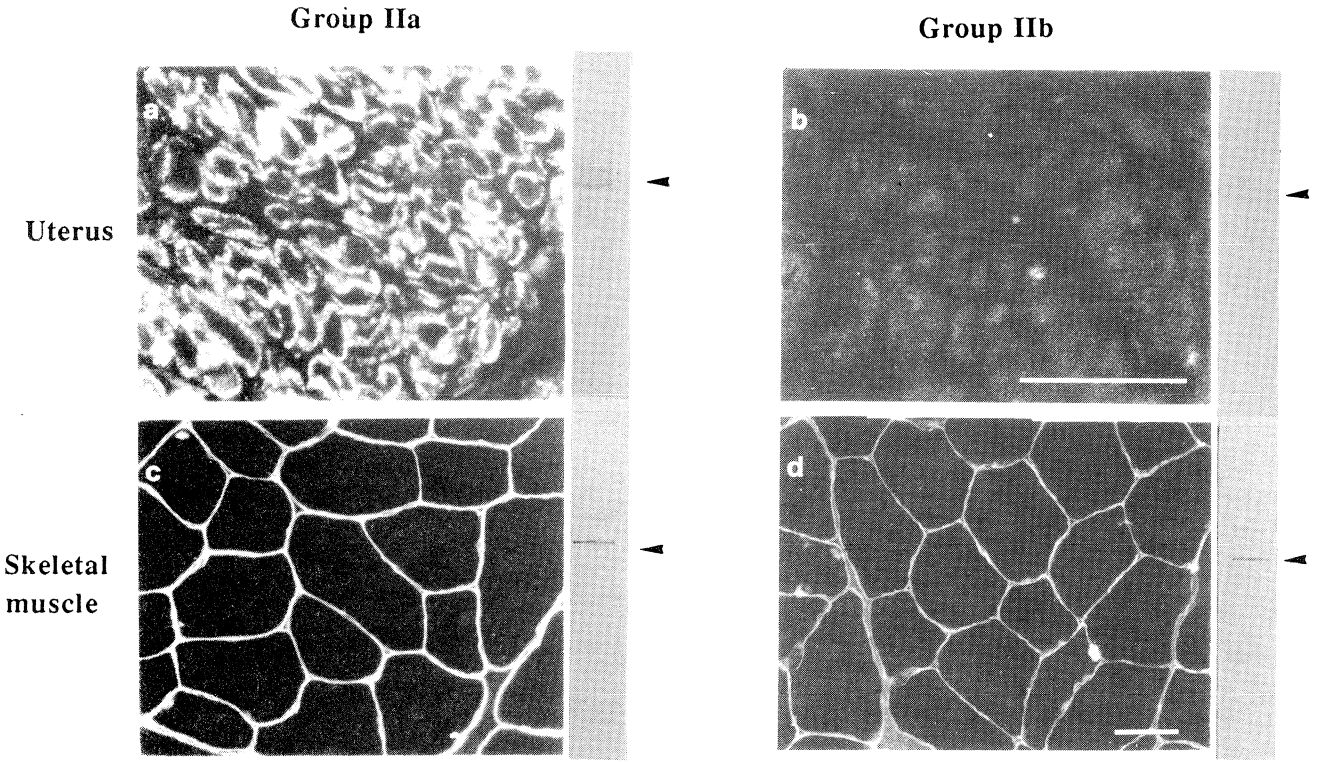


Figure 4. Comparative immunoanalysis of human myometrial and skeletal muscle fibers using antibodies from groups IIa and IIb. Each panel represents a cryostat section and an extract which are both processed with the same antibodies. Panels a and b represent myometrium processed with group IIa and group IIb antibodies respectively. Panels c and d show skeletal muscle sections that were processed like panels a and b. Each scale marker (panel b and d) corresponds to 20 μm.

ion in human and murine dystrophin deficient tissues. Both muscle-specific Xp21 dystrophins differ structurally in at least 2 positions with the central spectrin-like rod-shaped dystrophin domain (group IIb antibodies). They also share two similar epitopes, one in the central domain and one in the C-terminal domain (group IIa antibodies). This is direct evidence, at the protein level, of the existence of muscle-specific Xp21 dystrophin isoforms. Both proteins must be encoded by distinct dystrophin transcripts presumably generated by alternative splicing of the Xp21 gene. The structural differences we observed in the central domain of Xp21 dystrophins (group IIb antibodies) were unexpectedly not detected at the mRNA level in smooth and striated muscles, according to amplification experiments of transcripts from both muscles using primers obtained from the same coding regions of Xp21 dystrophin (see Fig 1b and c in Feener [7]). Besides, although smooth muscle-specific dystrophin appeared to be reduced by more than 250 amino-acids at the C-terminal end from the results of amplification experiments, all dystrophin isoforms detected here by Western blotting were roughly the same size (400K). Furthermore Mab Dys2, which recognizes an epitope within the last 17 carboxy-terminal dystrophin residues, reacted with the Xp21 smooth muscle-specific dystrophin isoform. However under slightly different experimental conditions, dystrophin has often been seen as doublets which could correspond to isoforms of different sizes or to some proteolysis [9,16].

The present results that indicate the existence of two different dystrophin isoforms or homologues in the uterus extend previous studies that have demonstrate the presence of dystrophin in the tunica media of blood vessels [14, 9]. The polyclonal serum used unexpectedly failed to detect any dystrophin in the blood vessels of *mdx* mice. Using a Mab directed against the central domain of mouse skeletal muscle dystrophin, Nicholson et al. [16] showed the presence of an immunoreactive 400K band in the vas deferens and in blood vessels of the rat. This suggests that other dystrophin isoforms or homologues could also be co-expressed in both vascular and visceral smooth muscles in addition to the isoforms detected here using the available Mabs set. In any case, Western blot analyses of smooth muscle extracts and of extracts from any other tissue that were previously performed with polyclonal antibodies which inevitably recognize different Xp21 and not Xp21-encoded dystrophins, should now be re-evaluated. Vascular tissues are present in any organ and the contribution of the vascular dystrophin isoform to other dystrophins or homologues should be redetermined. The use of immunocytochemistry and Western blotting with specific Mabs will be crucial.

The genetic origin (Xp21 gene) of myometrial dystrophin, if identical in the other visceral smooth muscles, could explain some of the stomach and gut troubles often observed in Xp21 dystrophin-deficient Duchenne and Becker patients [17]. It would be very informative to know whether any deletion in the Xp21 gene simultaneously alters the expression of smooth muscle-specific dystrophin and skeletal muscle-specific dystrophin. The alternative splicing mechanism could be responsible for differential expression of the different muscle-specific dystrophins, which dramatically modulate the final effects of gene deletion.

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