The Concomitant Use of Dystrophin and Utrophin/ Dystrophin Related Protein Antibodies to Reduce Misdiagnosis of Duchenne/Becker Muscular Dystrophy

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Antibodies to dystrophin have increased accuracy in the diagnosis of Duchenne/Becker muscular dystrophy (D/BMD). Both typical and 'atypical' presentations of this disease can be confirmed by demonstrating qualitative and quantitative defects in the expression of dystrophin protein. However, owing to the propensity for dystrophin degradation *in vitro,* caution needs to be applied while performing and interpreting antibody-based dystrophin analysis. Here we identify two cases where *in vitro* protein degradation caused diagnostic confusion. We demonstrate the use of utrophin/ dystrophin related protein (DRP) as sensitive control for sample degradation, since it is more labile than dystrophin. We suggest that the concomitant or sequential usage of antibodies specific for dystrophin along with utrophin/DRP can help reduce the misdiagnosis of D/BMD. © 1997 Academic Press

Most patients with Duchenne/Becker muscular Dystrophy (D/BMD) present in a clinically well defined manner. However, careful clinical observation, coupled with widespread use of genetic and antibody based testing has led to the recognition that the phenotypic range of this disease is considerably wider than previously believed[1–7]. Current DNA-based tests can detect genetic mutations in only ~65% of D/BMD cases while the sensitivity of antibody-based tests that analyzes the protein, approaches 100%. Thus the laboratory verification of D/BMD in ~35% of cases, relies solely upon the demonstration of a dystrophinopathy: a quantitative and/or qualitative abnormality in the expression of dystrophin at the protein level. While dystrophin antibody-based immunoblot analysis is thought to be extremely specific, unrecognized technical artifacts may lower the specificity. Indeed, it is possible that in some cases abnormal protein levels may simply reflect *in vitro* degradation of dystrophin rather than a disease process *per se.* Dystrophin having been previously demonstrated to be extremely labile in muscle biopsies after solubilization[8,9]. In this report we identify two cases where in vitro degradation of dystrophin and DRP was recognized [10–12]. Further, we experimentally determined the time course of dystrophin and DRP degradation *in vitro.* We demonstrate that DRP represents a sensitive marker for protein degradation and show how the use of DRP antibodies resolved the diagnostic dilemma presented by these cases. We propose that DRP may be used as an additional protein standard, while analyzing dystropin, in order to reduce misdiagnosis of D/BMD.

MATERIALS AND METHODS

Case A. An eight year old boy presented with an awkward gait, poor running ability and easy fatigability. His symptoms had progressed slowly over several years. Family history indicated that for three maternal generations there had been various undiagnosed complaints involving the neuromuscular system. Physical examination revealed normal muscle bulk and strength, however, deep tendon reflexes were hypoactive. Laboratory evaluation included electromyography which was normal and histopathological studies on quadriceps muscle biopsy which revealed mild type 1 fiber grouping on light microscopy. No significant ultrastructural abnormalities were noted on electron microscopy. Serum chemistry revealed a mild elevation of Creatine kinase 58 IU (normal <30 IU; EC 2.7.3.2). No deletions of the D/BMD gene were detected by PCR-based genetic deletion analysis[13,14].

Case B. A thirty week old female spontaneous abortus. There was no family history of neuromuscular disorders. The fetus had a cytogenetically visible chromosomal abnormality of chromosome 4, however, the chromosomes encoding both dystrophin (chromosome

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X) and DRP (chromosome 6) were normal. A sample of quadriceps muscle was found to have normal appearance on histochemistry and muscle fiber differentiation was appropriate for gestational age. Over 10 hours passed between the abortion and processing of tissue.

Control muscle. Previously characterized muscle biopsies from patients with unrelated neuromuscular disorders. Controls had normal dystrophin and DRP levels. *mdx* B10 mice were sacrificed, muscle rapidly removed and flash-frozen, prior to analysis.

In vitro degradation. Flash-frozen muscle was rapidly solubilized in 20 volumes sample buffer (10% SDS, 0.1 M Tris [pH 8.0], 10 mM EDTA, bromophenol blue and 50 mM DTT) using a teflon coated pestle. Muscle was incubated at 37°C for varying periods of time after which samples were rapidly frozen on solid carbon dioxide.

RESULTS

Case A. To exclude the possibility of sub-clinical or an unusual presentation of D/BMD, dystrophin testing was performed on a portion of a preexisting diagnostic muscle biopsy from this case. No dystrophin was detected on immunoblots using the 60 kDa dystrophin antibody[15]. Since the laboratory finding that suggested DMD was discordant with the patient's clinical presentation, we repeated the immunoblot analysis with the originally tested sample and a fresh sample from the same biopsy. The repeat test revealed the absence of a full size dystrophin band and the presence of multiple degradation fragment bands of lower molecular mass on using d6-10, a different dystrophin antibody (Figure 1A; Lane 2)[16]. On testing a fresh aliquot of the biopsy, a faint band of appropriate size for dystrophin ($\sim$400 kDa) was noted (Figure 1A; Lane 3). A parallel blot was probed with DRP antibodies revealing a lack of the co-migratory DRP band in the original sample and an extremely faint band in the fresh aliquot (Figure 1B; lanes 2–3).

Case B. Muscle from the abortus was tested by immunohistochemistry and immunoblotting with 60 kDa and DRP antibodies. Upon histochemistry, normal dystrophin labeling was detected, however, no DRP labeling was seen, suggesting that DRP degradation occurred before dystrophin degradation in this biopsy[16,17]. Immunoblotting revealed subtle degradative changes in both proteins as well (Fig. 2).

Control muscle. Since DRP appeared to be a sensitive indicator of muscle sample integrity, we analyzed the time course of DRP and dystrophin degradation in a previously characterized muscle biopsy. The sample was solubilized and analyzed at various time points after thawing. Full length dystrophin was undetectable by approximately 45 minutes at 37°, while DRP degradation occurred with a faster time course such that DRP was undetectable by 30 minutes (Figure 3). Similar studies on muscle from dystrophin deficient *mdx* mice, were used to exclude dystrophin cross reactivity and a similar time course of degradation was observed (data not shown).
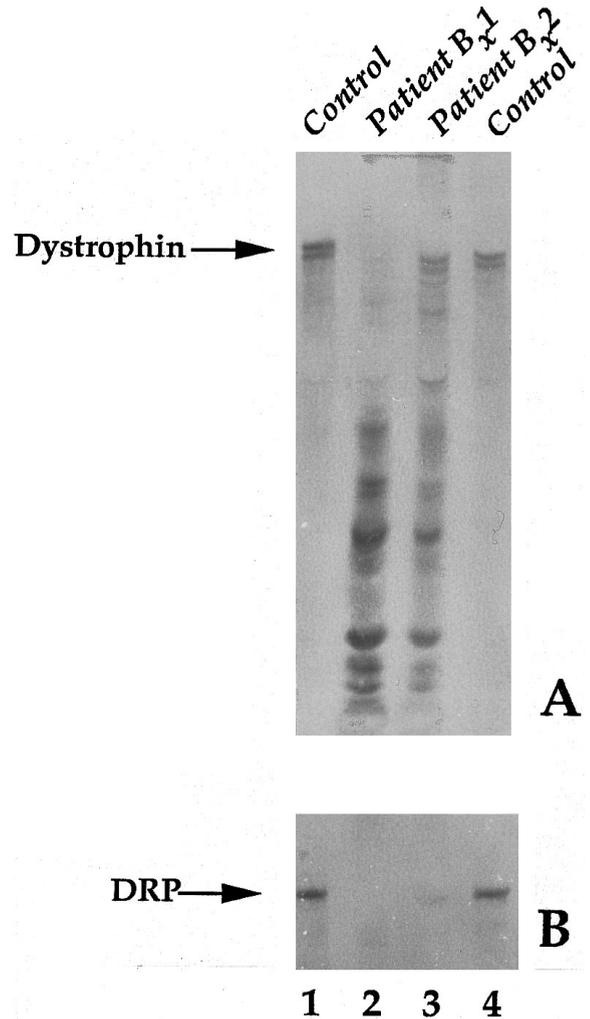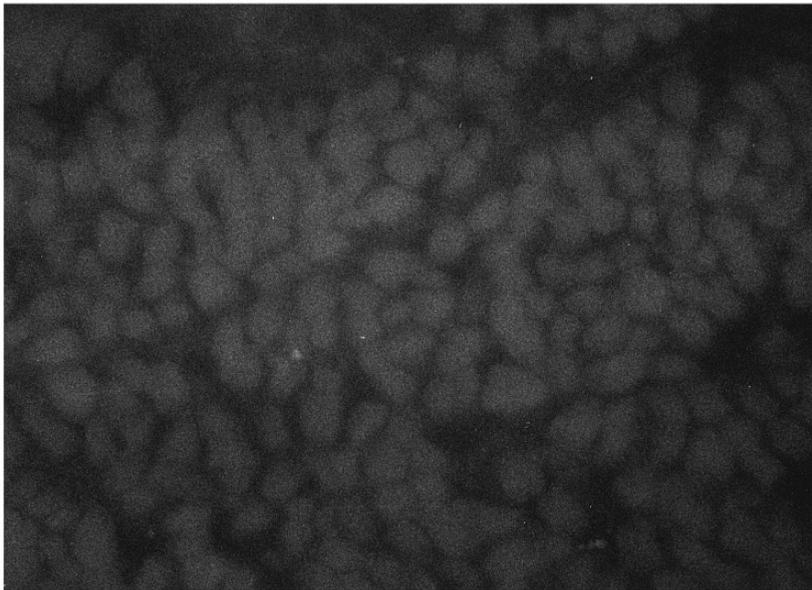

FIG. 1. Immunoblot analysis of dystrophin and DRP in patient and control muscle specimens. Muscle was weighed and solubilized in sample buffer. Tubes containing solubilized muscle were boiled, proteins quantified, electrophoresed and immunoblotted in parallel with either anti-dystrophin antibodies (Panel A) or anti-DRP antibodies (Panel B) as previously described[11,15]. Lanes 1 and 4 contain two different control samples, lanes 2 and 3 contain two pieces of the same biopsy from case A, described in materials and methods. Lane 2 contains the originally tested portion and demonstrates presence of multiple degradation bands and absence of DRP and dystrophin. Lane 3 demonstrates less severe protein degradation as evidenced by discernible, though fainter, dystrophin and DRP staining and fewer degradation bands.

DISCUSSION

Dystrophin testing has become an integral part of the diagnostic workup of a patient with possible D/BMD. The demonstration of a dystrophinopathy along with clinical and histopathological findings, are considered indicative of X-linked D/BMD[1]. In unusual, albeit rarer presentations of D/BMD such as congestive cardiac failure, or limb weakness in females, dystrophin abnormalities may in fact be the only objective evidence of the disease[3–7]. Al-
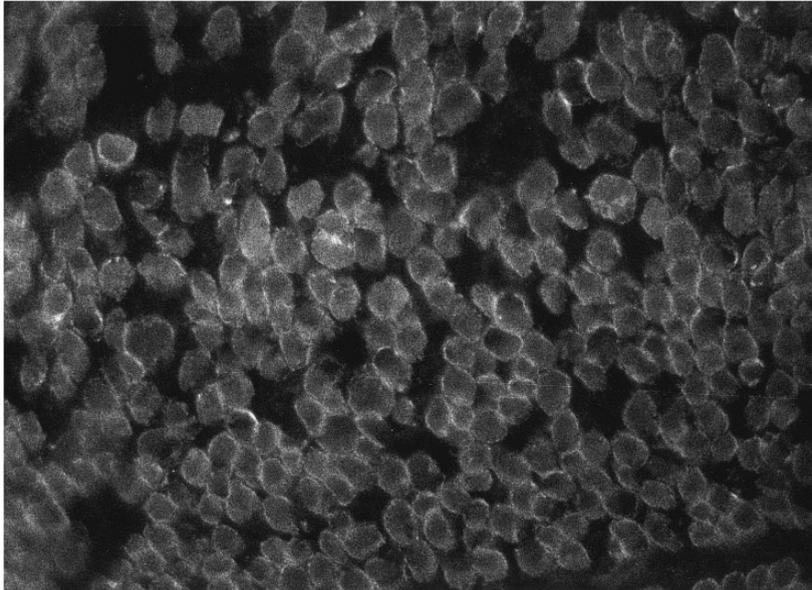
DYS

DRP

FIG. 2. Immunohistochemical analysis of dystrophin and DRP in a 30-wk-old abortus. Cryostat sections were cut from fetal quadriceps muscle and labeled with dystrophin and DRP antibodies and visualized by indirect immunofluorescent microscopy as described for adult tissue. Top panel demonstrates the sarcolemmal labelling obtained using dystrophin (DYS) antibodies. Absence of DRP labeling is evident in the lower panel. Total magnification is 440×.

though, the dystrophin immunoblot is an objective test, unrecognized *in vitro* degradation of dystrophin may result in a false positive diagnosis of D/BMD. Thus, to determine that the absence of dystrophin is indeed due to

a primary genetic cause, there must be some assurance about the integrity of the biopsy being tested.

For purposes of dystrophin analysis adequate integrity can be assumed provided that care has been taken to
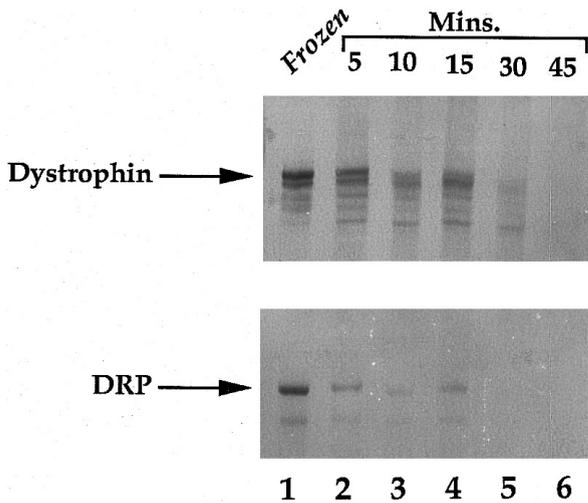
FIG. 3. Time course of *in vitro* degradation of dystrophin and DRP in control muscle. Control flash-frozen muscle was rapidly solubilized in sample buffer. Muscle was incubated at 37°C for varying periods of time after which samples were rapidly frozen on solid carbon dioxide. After collecting samples representing all time points, the tubes were boiled, proteins quantified, electrophoresed and immunoblotted in parallel with dystrophin and DRP antibodies. Top panel shows the time course of dystrophin degradation. Full length dystrophin is undetectable by 45 minutes. The lower panel demonstrates that DRP degrades slightly faster: Full length DRP is undetectable at 30 minutes.

rapidly freeze the specimen and avoid freeze-thaw cycles during storage of the muscle biopsy or processing the sample for immunoblotting. Some degree of reassurance about protein integrity is provided by the nature of the test: Dystrophin degradation itself serving as an internal control since immunoblots often reveal the sequelae of *in vitro* degradation in the form of degradation bands (e.g. Fig. 1A). Unfortunately since these bands have variable expression, are prone to misinterpretation, and may not be apparent during early stages of degradation, they are neither sensitive nor consistent enough to qualify as ideal markers of dystrophin degradation. In selecting the ideal marker for dystrophin degradation it is important that the marker should be of a similar size and continue to be expressed in D/BMD. Moreover the marker should obviously degrade as fast or faster than dystrophin itself. In the past connectin, nebulin and spectrin have been used. However, while useful, these proteins are of a different size, degrade slowly and altered expression of some of these proteins have been noted in patients of D/BMD[5,9,18]. In this study we evaluated the use of DRP as a marker for protein degradation in order to avoid a false positive diagnosis based on dystrophin testing. The absence of DRP signal in some poorly preserved biopsies, and our demonstration that DRP loss appears to occur faster than dystrophin itself, suggest that DRP is a sensi-

tive marker for protein degradation. In addition DRP is known to be of a similar size and expressed in a majority of patients of D/BMD, hence fulfilling the criteria for a degradation marker[11,12,17,19].

In this study we used DRP as a marker to determine that the probable cause of absence of dystrophin in one patient was *in vitro* degradation rather than a genetic mutation, hence avoiding a potential misdiagnosis. Since DRP evaluation can even be performed on old, previously processed dystrophin immunoblots, it should be possible to correct previous false positive D/BMD diagnosis without either a repeat biopsy or long term follow up. We therefore suggest that the concomitant or sequential usage of DRP antibodies along with dystrophin antibodies while analyzing unusual/atypical cases of D/BMD can reduce misdiagnoisis of this disease.

ACKNOWLEDGMENTS

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