Muscular dystrophies proteins evaluation by western blott and immunofluorescence

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Abstract

The muscular dystrophies are a diverse group of inherited muscle disorders characterized by progressive muscle weakness and wasting with characteristic histological abnormalities such as degeneration, necrosis, and regeneration of muscle fibers. Many muscle-wasting diseases are caused by defect in genes for muscle proteins. Most of these proteins appear to play a role in supporting the structure of muscle fibers, although some may play a role in the biochemical processes that go on in muscle fibers. DMD and DMB are X-linked allelic myopathies caused by dystrophin deficiency while LGMD 2A is caused by mutations in the calpain 3 gene.

The aim of our studies is to evaluate the expression of dystrophin and calpain by Western blotting. The study of calpain 3 protein in muscle at this time can only be carried out by Western blotting since the antibodies that are available have no immunoreaction on the sections.

Keywords*:* muscular dystrophy, dystrophin, calpain 3, western blot.

Introduction

Muscular dystrophies are a diverse group of inherited muscle disorders characterized by progressive muscle weakness and wasting. They can be subdivided into several groups, including congenital forms, in accordance with the distribution of predominant muscle weakness: Duchenne and Becker; Emery-Dreifuss; distal; facioscapulo-humeral; oculopharyngeal; and limb-girdle which is the most heterogeneous group. The genes and their protein products that cause most of these disorders have now been identified. [12].

The gene defective in DMD/ BMD was isolated in 1986 and was called dystrophin.

Although the disease is caused by mutations in the dystrophin gene, the precise molecular mechanisms leading to muscle pathology are poorly understood. The dystrophin gene is 3,000 kb in size and consists of 79 exons encoding a 14-kb mRNA. The gene encoding dystrophin is the largest identified in humans, occupying approximately 1% of the X chromosome [10]. Dystrophin is thought to play a structural role by providing a link between the intracellular actin cytoskeleton and the extracellular matrix *via* its interaction with a complex of peripheral and integral membrane proteins called “the dystrophin-glycoprotein complex” (DGC). Disruption of this linkage results in membrane instability and renders dystrophic muscle fibers highly susceptible to contraction-induced injury. Several members of the DGC play a role in cell signaling rather than contributing to mechanical stability. [10].

Duchenne muscular dystrophy (DMD) is a common X-linked recessive disorder inherited disease with a worldwide incidence of 1 in 3,500 male births. The affected individuals are wheelchair-bound by the age of 12 and succumb to cardiac or respiratory failure in the mid to late 20s.

Becker muscular dystrophy (BMD) is distinguished from DMD by delayed onset, later dependence on wheelchair support and has a slower rate of progression; affected individuals remain ambulatory beyond the age of 16 y, and a few may lead near-normal lives [7].

Limb-girdle muscular dystrophies are another subgroup of muscular dystrophy in which type 2 A (LGMD2A) is most common and represent a heterogeneous group of genetically determined progressive disorders of skeletal muscle, [6] caused by mutations in the gene for calpain 3 (a muscle-specific protease enzyme). Calpain 3 (p94) is a muscle – specific nonlysosomal cysteine proteinase. Patients show phenotypes similar to other recessive dystrophies. [30]

The aim of our studies is to evaluate the expression of dystrophin and calpain by Western blotting. The study of calpain 3 protein in muscle at this time can only be carried out by Western blotting since the antibodies that are available have no immunoreactions on the sections.

Materials and methods

Human Muscle Samples from Normal and Disease Control Patients

Muscle biopsies (gastrocnemius) were taken from patients as a part of the routine diagnostic procedure. Muscle samples from normal control subjects were also obtained, with consent, from legs amputated at the knee (gastrocnemius). The biopsies samples were frozen in isopentan cooled in liquid nitrogen.

Protein analysis in the diagnosis of muscular dystrophies is based on an understanding of the mutated proteins associated with different forms of muscular dystrophy. The two main methods used in our studies are: immunofluorescence and immunoblotting (western blotting). Both tehniques use labelled antibodies to the specific muscle protein that is abnormally expressed in a particular muscular dystrophy.

Immunofluorescence

Seven micrometers thick tissue sections were incubated with primary antibodies diluted in BSA (bovine serum albumine) in PBS. After repeated washes with phosphate buffered saline (PBS), secondary antibody conjugated to biotin from SANTA CRUZ as applied. Following washes with PBS, the antibodies were visualized with FITC-Streptavidine (Sigma). The samples were evaluated by fluorescent microscopy.

We used three mouse monoclonal antibodies against three domains of N-terminal-, rod-domain- and C-terminal- dystrophin (NCL-DYS1, NCL-DYS2, NCL-DYS3) and monoclonal antibody against utrophin (NCL-DRP 2), from Novocastra.

A total of 40 muscle biopsies from patients with clinical diagnosis of LGMD were tested for dystrophin, utrophin, sarcoglycans (α, β, γ) and merosin. Of these 40 patients, 8 these patients have a normal expression of above mentioned muscle proteins, and it was necessary to investigate calpain 3.

The study of calpain 3 protein in muscle at this time can only be carried out by Western blotting since the antibodies that are available have no immunoreactions on the sections.

Polyacrilamide gel electrophoresis and Western blotting

The Western blot analyse that we use was modified in order to separate the large proteins, more then 200 kDa (egg. dystrophin) from the others under 150 kDa (egg. calpain 3). In this method the polyacrylamide gel system is a biphasic one.

By this system, the large proteins are separated in the top part of the gel while and smaller proteins, in the bottom. After electrophoresis the gel was blotted with a current of a constant amperage calculated with respect to BioRad instructions (mA=L gel x l gel x 3), for 1 hour. We use a nitrocellulose membrane and Towbin buffer as a transfer buffer [2].

Frozen muscle samples (20 to 50 mg) were weighed and homogenized for 15 minutes in a Potter homogenizer with buffer containing Tris/ HCl buffer, glycerol, SDS, urea, mercaptoethanol. After that, it was centrifuged at 8000 x g for 15 minutes before 30µl aliquots of the supernatant were applied to each lane.

The myosin heavy chain band on post-blotted gel, stained with Coomassie blue was used to indicate how much muscle protein (as opposed to fat and fibrous connective tissue) had been loaded in each sample lane 11.

For Western blotting we used two mouse monoclonal antibodies against three domains of dystrophin: rod-domain- and C-terminal- (DYS1 and DYS2) and monoclonal antibody against calpain-3 (CAPN 2C4), from Novocastra.

The monoclonal primary antibodies cocktail was applied on the same gel and the protein expression was visualized by using a chromogen method with Western Breeze.

With commercially available antibodies, calpain 3 is only detectable on blots and produces a characteristic pattern of bands.

Results and discussion

Immunofluorescence analyses

By immunofluorescence analysis, dystrophin is present at the sarcolemma of skeletal muscle fibers in unaffected muscle biopsies, but is absent or severely reduced in DMD. Utrophin (an autosomal protein that has considerable, sequence homology with dystrophin) is localized in normal skeletal muscle at the membrane of neuromuscular junction. This complete loss of expression is also seen in western blotting analysis.

In some biopsies of DMD, low levels of dystrophin can be detected on several fibers, and a few fibers may have near normal expression. The latter are known as ‘‘revertant’’ fibers. Utrophin is extended from the neuromuscular junction (NMJ) at surface of whole sarcolemma in all the analysed cases.

In contrast, BMD patients often have some residual staining at the sarcolemma of the muscle fibers and a change in the quantity or size of the protein is often seen by Western blot.

At patients diagnostics with LGMD2A by immunohistochemical analysis of the proteins analyzed (dystrophin- for three antibodies: Rod-, N-terminus and C-terminus domains) have a normal expression. Utrophin was localized at the membrane of neuromuscular junction.

Western blot analyses

On normal control subjects, when Dys C-terminal antibody was applied, a single band (427kDa) was detected, while for the rod domain of the protein we obtained a doublet (427 kDa and 400 kDa) plus lower molecular mass metabolites. At normal patients, calpain 3 is detected at 94-kDa and 30 kDa.

Fig 2. shows a blot labeled for the Dys1 and Dys2 and Calpain 3, which was obtained after western blot analyses of five patients

We considered as normal bands whose intensity was the equivalent of control levels and abnormal those with a diminished intensity or bands that were absent.

- On lanes 1A and 1B (Fig 2) we observed a normal expression for dystrophin and loss of expression for calpain. For this reason, the patient was diagnosed with LGMD2A.
- On lanes 2A and 2B – is an other patient with the same features as the one from lines 1A, B which was also diagnosed LGMD2A.

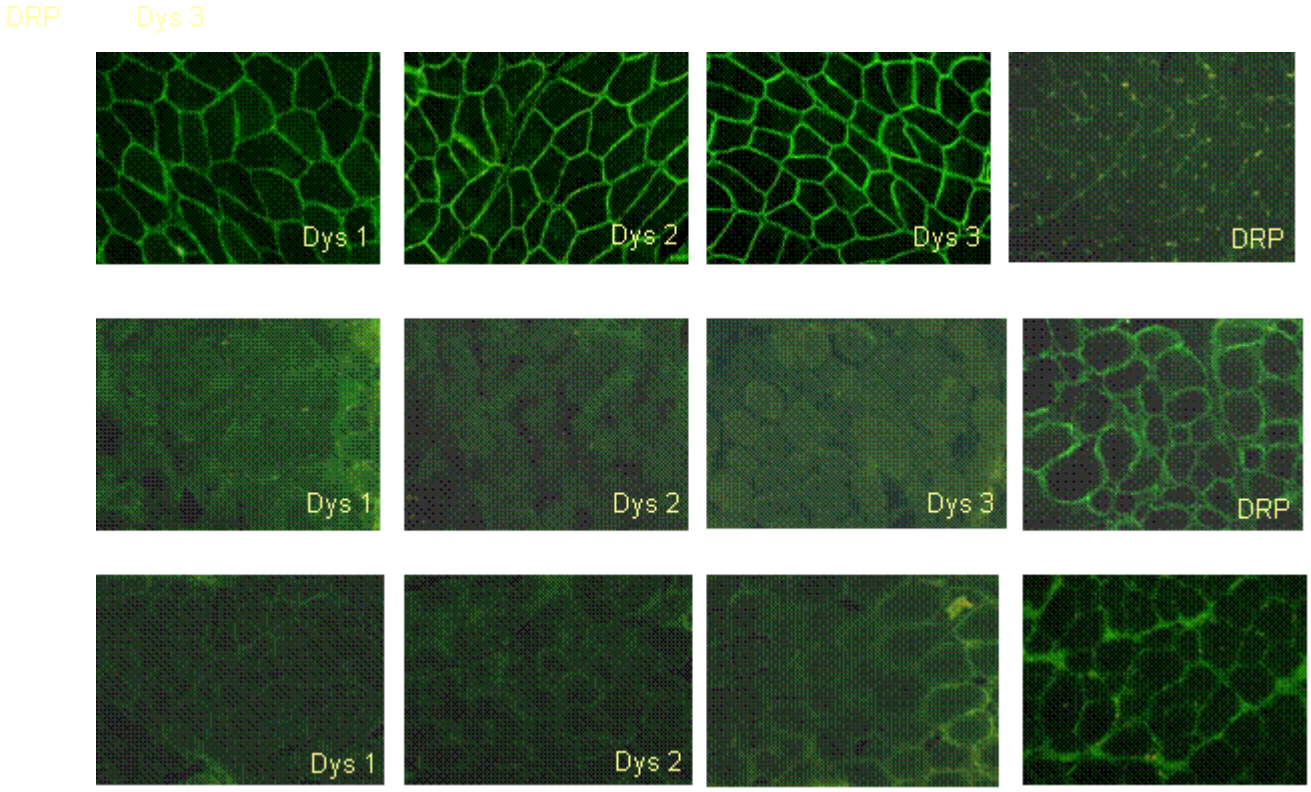

Figure 1. Immunofluorescence analysis

A) **Control-** showing normal sarcolemmal labeling of dystrophin for all antibodies used (Dys 1 –against to the rod-domain, Dys 2- against to the C-terminus, Dys 3- against to the N-terminus and Utrophin) in all fibers.

B) **A case of Duchenne dystrophy** with an absence of dystrophin and extended for Utrophin from the neuromuscular junction (NMJ) at surface of whole sarcolemma

C) **A case of Becker dystrophy** with uneven and reduced labeling of dystrophin and a reduced expression for utrophin.

- On lanes 3A and 3B we noticed a complete absence of dystrophin expression for both antibodies –rod- and C-terminus domains. This patient was diagnosed with DMD.
- On lanes 4 A and 4 B we can notice a complete absence of dystrophin for both rod- and C-terminus domain of DYS and an abnormal expression for calpain 3 (reduced). The patient was diagnosed with DMD with reduced calpain 3.

On our experiments the results obtained on patient from lines 4 indicate a very rare DMD, because comparatively with normal features where Calpain 3 is unmodified, this patient has a massive reduction of Calpain 3 expression. The previous studies suggested that in Limb Girdle Muscular Dystrophy 2A DYS has a normal expression and calpain 3 is absent. At this moment we cannot explain the reduction of Calpain 3 until futures genetics studies of CAPN3 will give us more information about this case. The fact that it is an enzyme and not a structural protein like the dystrophin-associated protein complex means that different factors may affect its analysis. Anderson L.V.B., in his paper published in *American Journal of Pathology* in 2000 [1], concerning the dysferlinopathies, observed by Western blot a reduction of Calpain 3 in 5 patients with DMD out of 200.

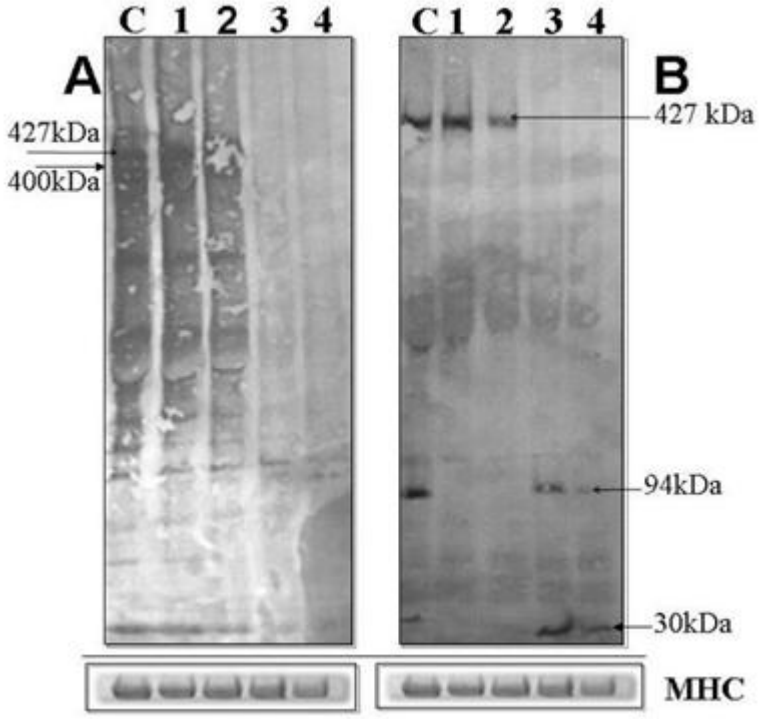

Figure. 2 Western blotting analyses of muscle homogenates from four patients with muscular dystrophy

A. Blot labeled with Dys1: C-normal control; lane 1-LGMD 2A, absence of calpain 3, normal expression of dystrophin labeling; lane 2-LGMD 2A; lane 3-DMD, absence of dystrophin labeling; lane 4-DMD,absence of dystrophin;

B. Blot labeled with Dys2+ calpain 3: C-normal control; lane 1-LGMD 2A, normal expression of dystrophin labeling, absence of calpain 3; lane 2-LGMD 2A; lane 3- DMD, absence of dystrophin labeling; lane 4-DMD, absence of dystrophin, reduced expression of calpain 3;

MHC – corresponding myosin heavy chain bands on the post-blotted gel, stained with Coomassie blue

Conclusions

Protein analysis using Western blotting with specific antibodies is obvious a method for differential diagnosis of dystrophinopathies and for physiopathology elucidation of each genetic disorder involved.

DMD and BMD are associated with secondary reductions in the expression of other proteins from DGC complex. Dystrophin immunolabeling on blots is particularly useful in BMD patients because bands of abnormal size and abundance are easily detected comparatively with immunohistochemistry techniques.

Analysis of muscular proteins on Western blot are necessary as a previous analysis before genetic analysis. Dystrophin immunoreactivity as detected by western blotting and immunofluorescence has also been important in establishing diagnosis for muscular dystrophy and particularly in distinguishing the allelic forms of Becker and Duchenne muscular dystrophy. The combination of these techniques allowed us to demonstrate a variable dystrophin expression and the presence of two different dystrophins, normal- or reduced-sized in muscle fibers of DMB patients.

The study of calpain 3 protein in muscle at this time can only be carried out by Western blotting since the antibodies that are available have no immunoreactions on the sections.

The successful identification of the genes and gene defects in these muscular dystrophies has oriented our research toward two major goals: understanding the structure and the function of normal muscle and the pathogenesis of these diseases.

Definitive diagnosis of patients with inherited muscle disorders is essential for the establishment of an accurate prognostic and genetic counseling.

References

1. ANDERSON LV, DAVISON K, MOSS JA, RICHARD I, FARDEAU M, TOME FM, HUBNER C, LASA A, COLOMER J & BECKMANN JS . Characterization of monoclonal antibodies to calpain 3 and protein expression in muscle from patients with limb-girdle muscular dystrophy type 2A. *American Journal of Pathology*, 153: 1169

2. ANDERSON L.V.B. K. DAVISON, Multiplex Western Blotting System for the Analysis of Muscular Dystrophies Proteins. Am. J. Pathol., 154 (4), 1999, 1017-1022. 1179, (1998)

3. BLAKE D.J. Function and Genetics of Dystrophin and Dystrophin - Related proteins in Muscle. *Physiol Rev*. **82**, 291 – 329 (2002)

4. BUSHBY K.M.D**.**. The limb – girdle muscular dystrophies – multiple genes, multiple mechanisms. *Human Molecular Genetics*, **8** (10): 1875 – 1882.(1999)

5. COHN, R.D. AND CAMPBELL, K.P Molecular basis of muscular dystrophies. Muscle Nerve 23, 1456–1471 (2000)

6. FROSK P, WEILER T, NYLEN E, SUDHA T, GREENBERG CR, MORGAN K, FUJIWARA TM, WROGEMANN K. Limb-girdle muscular dystrophy type 2H associated with mutation in TRIM32, a putative E3 ubiquitin-ligase gene. Am J Hum Genet; 70:663–72, (2002).

7. H. SORIMACHI, N. TOYAMA-SORIMACHI, T.C. SAIDO, H. KAWASAKI, H. SUGITA, M. MIYASAKA, K. ARAHATA, S. ISHIURA, K. SUZUKI, Muscle-specific calpain, p94, is degraded by autolysis immediately after translation, resulting in disappearance from muscle, J. Biol. Chem. 268 10593–10605, (1993)

8. HOFFMAN, E.P. et al. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 51, 919–928, (1987)

9. K. KINBARA, H. SORIMACHI, S. ISHIURA, K. SUZUKI, Skeletal muscle specific calpain, p94, Biochem. Pharmacol. 56 415–420, (1989)

10. KOENIG M, HOFFMAN EP, BERTELSON CJ, MONACO AP, FEENER C, KUNKEL LM Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. *Cell* 50: 509-517, (1987)

11. FARDEAU, B. EYMARD, C. MIGNARD, F. M. S. TOMB, I. RICHARD, J. BECKMANN. Chromosome 15-linked limb girdle muscular dystrophy — clinical phenotype *Neuromuscular Disorders*, *Volume 6, Issue 2*, *March*, *Page S7, (*1996)

12. MOREIRA ES, WILTSHIRE TJ, FAULKNER G, NILFOROUSHAN A, VAINZOF M, SUZUKI OT, VALLE G, REEVES R, ZATZ M, PASSOS-BUENO MR, JENNE DE. Limb-girdle muscular dystrophy type 2G is caused by mutations in the gene encoding the sarcomeric protein telethonin. Nat Genet 24:163–6. (2000).

13. NICHOLSON LVB, JOHNSON MA, GARDNER-MEDWIN D, BHATTACHARYA S, HARRIS JB: Heterogeneity of dystrophin expression in patients with Duchenne and Becker muscular dystrophy. Acta Neuropathol , 80:239–250, (1990).

14. NIGRO V. Molecular bases of autosomal recessive limb-girdle muscular dystrophies. Acta Myol; 22:35–42. (2003).

15. PAULA F, VAINZOF M, PASSOS-BUENO MR, PAVANELLO RCM, MATIOLI SR, ANDERSON LVB, NIGRO V & ZATZ M. Clinical variability in calpainopathy: What makes the difference? *European Journal of Human Genetics*, 10: 825-832. (2002)

16. RICHARD I, BROUX O, ALLAMAND V, FOUGEROUSSE F, CHIANNILKULCHAI N, BOURG N, BRENGUIER L, DEVAUD C, PASTURAUD P, ROUDAUT C, et al. Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. Cell; 81:27–40.(1995)

17. ROBERDS SL, LETURCQ F, ALLAMAND V, PICCOLO F, JEANPIERRE M, ANDERSON RD, LIM LE, LEE JC, TOME FMS, ROMERO NB, et al. Missense mutations in the adhalin gene linked to autosomal recessive muscular dystrophy. Cell; 78:625–33.(1994)

18. STARLING A, KOK F, PASSOS-BUENO MR, VAINZOF M, ZATZ M. A new form of autosomal dominant limb-girdle muscular dystrophy (LGMD1G) with progressive fingers and toes flexion limitation maps to chromosome 4p21. Eur J Hum Genet; 13:264.(2005)

19. SORIMACHI H., ISHIURA S., SUZUKI K. Structure and physiological function of calpains. J. *Biochem.* 328 (Pt 3), 721 – 732.(1997)

20. ZATZ M, DE PAULA F, STARLING A, VAINZOF M. The 10 autosomal recessive limb girdle, Neuromuscular Disord; 13:532–44.(2003)

21. VAINZOF M**.,** DE PAULA F., TSANACLIS A.M., ZATZ M. The effect of calpain 3 deficiency on the pattern of muscle degeneration in the earliest stages of LGMD 2A. *J. Clin. Pathol.* 56, 624 – 626.(2003)