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ORIGINAL PAPER

Maziar Assadi · Markus Müntener

Utrophin is lacking at the neuromuscular junctions in the extraocular muscles of normal cat: artefact or true?

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Abstract Extraocular muscles (EOM) are typically spared in Duchenne muscular dystrophy. We hypothesized that this might be due to different patterns of utrophin expression. The expression of utrophin was examined in EOM of normal cats using immunohistochemical methods and Western blot. For detecting acetylcholine receptors (AChR), we used α-bungarotoxin. Surprisingly, α-bungarotoxin failed to stain the AChR and no expression of utrophin could be detected at the neuromuscular junctions. Our study could indicate that the expression of utrophin is dependent on the structure of the AChR.

Keywords Extraocular muscle · Utrophin · Acetylcholine receptor · Immunohistochemistry · Duchenne muscular dystrophy

Abbreviations AChE: Acetylcholinesterase · AChR: Acetylcholine receptors · DMD: Duchenne muscular dystrophy · EOM: Extraocular muscles · NMJ: Neuromuscular junctions

Introduction

Duchenne muscular dystrophy (DMD) is one of the most common inherited neuromuscular diseases and occurs in about 1:3,500 live male births (Worton 1995). DMD is characterized by the almost complete absence of dystrophin either due to promoter defects or nonsense mutations and deletions leading to an unstable transcript or protein. In DMD, extraocular muscles (EOM) remain unaffected during the course of the disease (Kaminski et al. 1992; Karpati et al. 1988; Khurana et al. 1995; Ragusa et al. 1996). The compensatory factors or mechanisms that allow EOM to escape the consequence of dystrophin deficiency are unknown. One hypothesis is that an alternative expression of a protein that is structurally and functionally homologous to the defective dystrophin could save EOM by assuming the role of dystrophin. This would prevent the loss of other vital components of the transmembrane protein complex, thereby stabilizing the sarcolemma and providing myofiber survival. A possible candidate for such a function is utrophin, which has a ubiquitous expression pattern in the brain (Khurana et al. 1990), muscle (Nguyen et al. 1991) and other tissues (Love et al. 1991). In normal mature striated muscle, utrophin expression is detected at the neuromuscular (Nguyen et al. 1991; Takemitsu et al. 1993) and myotendinous junctions (Khurana et al. 1992), in the wall of endomysial capillaries and other blood vessels and in the perineurium and Schwann cells of the intramuscular nerves (Karpati et al. 1993). During ontogeny (Lin and Burgunder 2000) and regeneration (Lin et al. 1998), it is found at the whole sarcolemma. The utrophin and dystrophin genes exhibit a marked degree of homology although the utrophin gene is smaller (Brown 1997). Both dystrophin and utrophin proteins have the same four domains: the amino terminus, the rod domain, the cysteine-rich domain and the carboxy terminus. Both proteins have almost the same molecular weight (dystrophin 427 kDa, utrophin 420 kDa) and the same associated proteins (dystroglycans, sarcoglycans, and syntrophins).

There are three animal models of DMD: mdx mouse (Bulfield et al. 1984; Cooper 1989; Hoffmann 2001), dog (Cooper et al.1988a, b; Kornegay et al. 1988) and cat (Carpenter et al. 1989; Gaschen et al. 1992; Winand et al. 1994). They all lack dystrophin in skeletal muscle tissue because of loss-of-function mutations in the highly

M. Assadi (✉) · M. Müntener
Institute of Anatomy, University of Zürich-Irchel, Zürich, Switzerland

M. Müntener
Department of Chemistry and applied Biosciences, ETH, Zürich, Switzerland

M. Assadi
F. Hoffmann-La Roche, 93/8.08, 4070 Basel, Switzerland
E-mail: maziar.assadi@roche.com

conserved dystrophin gene. The clinical phenotype of each animal model differs from the human disease and from one another. The fact that they show differences in phenotype is valuable in that they provide insight into the secondary consequences of primary dystrophinopathy.

Our hypothesis for the sparing of EOM in DMD was that in normal EOM, utrophin is naturally overexpressed at the extrajunctional sites and replaces dystrophin. Because cat is one of the animal models of DMD, we studied the expression of utrophin in EOM and leg muscles of normal cats using immunohistochemical methods and Western blot.

Materials and methods

Tissue

Three domestic cats and one Wistar rat without apparent myopathy were obtained from the department for veterinary medicine of the University of Zürich. The cats and the rat were killed with a high dose of anesthetics in accordance with the guidelines of the local animal care committee. Samples of EOM and leg muscles (quadriceps muscle) were taken immediately after death and were snap-frozen in isopentane cooled in liquid nitrogen. Until the analysis, the biopsies were stored at −70°C.

Histology

Detection of utrophin and acetylcholine receptors (AChR)

Peroxidase immunohistochemistry For the histological procedures, the muscle specimens were sectioned in a cryostat at 7 μm and dried overnight at room temperature. Processing of sections for detecting utrophin with peroxidase immunohistochemistry was performed as described by Lin et al. (1998). A monoclonal antibody against the amino terminus of utrophin was used (NCL-DRP2, Novocastra Laboratories Ltd.). The second antibody was a biotinylated sheep antimouse antibody (RPN1001, Amersham Biosciences) followed by peroxidase-conjugated streptavidin (016-030-084, Jackson ImmunoResearch Laboratories Inc.). Peroxidase activity was visualized using DAB tablets (DO426, Sigma).

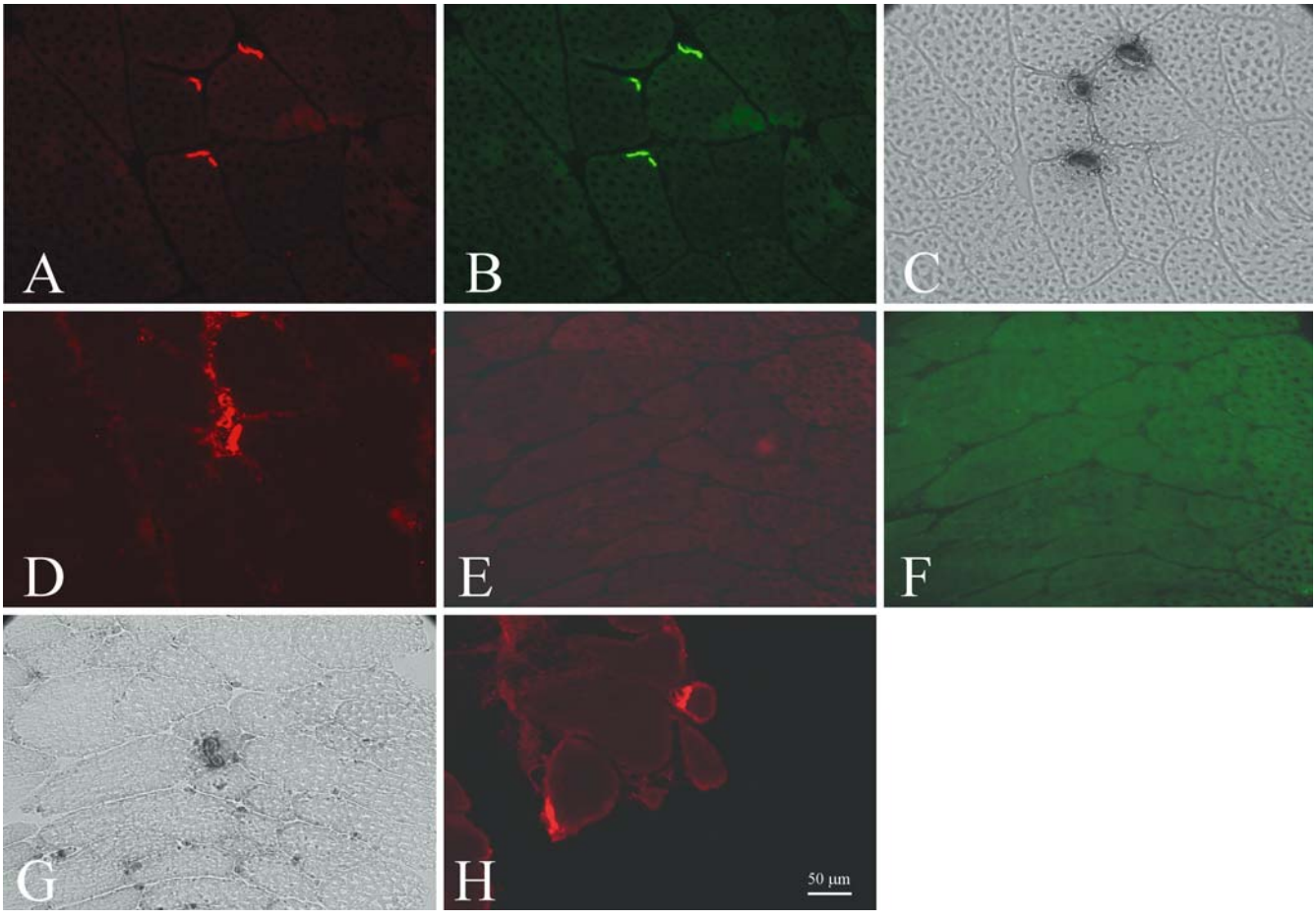

Fig. 1 Neuromuscular junctions (NMJ) were stained in leg muscles (**a**–**d**) and extraocular muscles (EOM) (**e**–**h**) of cat. Double staining with α-bungarotoxin/utrophin showed a positive staining for both utrophin (**a**) and α-bungarotoxin in leg muscles (**b**). In EOM, neither utrophin (**e**) nor α-bungarotoxin (**f**) could be detected. By using the method of Karnovsky on consecutive sections, NMJ could be shown in leg muscles (**c**) and EOM (**g**). NMJ could also be detected with rapsyn in leg muscles (**d**) and EOM (**h**)

Fluorescence immunohistochemistry The same protocol as for peroxidase immunohistochemistry was adapted for fluorescence immunohistochemistry. Sections were double-stained either with utrophin/α-bungarotoxin or with rapsyn/α-bungarotoxin. α-bungarotoxin binds to the extracellular part of the AChR whereas rapsyn interacts with the cytoplasmatic part of the AChR. α-bungarotoxin was conjugated with Alexa Fluor 488 (B-13422, Molecular Probes). Primary antibodies were: (1) monoclonal antibody against the amino terminus of utrophin (NCL-DRP2, Novocastra Laboratories) and (2) rabbit polyclonal antibody against rapsyn (see Acknowledgments). Utrophin was detected with donkey antimouse antibody conjugated with Texas Red (715-075-150, Jackson ImmunoResearch Laboratories). For detection of rapsyn, sections were incubated with a biotinylated goat antirabbit antibody (B-2770, Molecular Probes) followed by Texas Red conjugated streptavidin (RPN1233V, Amersham Biosciences).

Staining of acetylcholinesterase (AChE)

Neuromuscular junctions (NMJ) were demonstrated by staining for AChE activity using the method of Karnovsky (Karnovsky and Roots 1964). All the preparations were examined with a Polyvar 2 microscope (Reichert-Jung) equipped with a CCD camera (Visitron Systems). The digital pictures were processed with Adobe Photoshop 7.

Western blot

For the Western blot, we used a protocol established by Anderson and Davison (1999). As primary antibody, a monoclonal antibody against the amino terminus of utrophin (NCL-DRP2, Novocastra Laboratories) was used. Goat antimouse IgG conjugated with peroxidase (A-2554, Sigma) was detected with ECL Western blotting detection reagents (RPN2109, Amersham Biosciences).

Results

Muscle tissue

In leg muscles, the utrophin distribution (Fig. 1a) was restricted to NMJ as shown by double staining with α-bungarotoxin (Fig. 1b). In EOM, there was neither any utrophin immunoreactivity (Fig. 1e) nor a positive staining of the AChR with α-bungarotoxin (Fig. 1f) along the sarcolemma of any muscle fiber.

On consecutive sections, we also used the method of Karnovsky for detecting NMJ. In leg muscles, the staining of AChE (Fig. 1c) showed the same pattern as staining with α-bungarotoxin. AChE staining of the EOM showed an occurrence of NMJ (Fig. 1g), in contrast to the staining with α-bungarotoxin. For checking our results about the distribution of NMJ, we did a double staining with α-bungarotoxin and a polyclonal

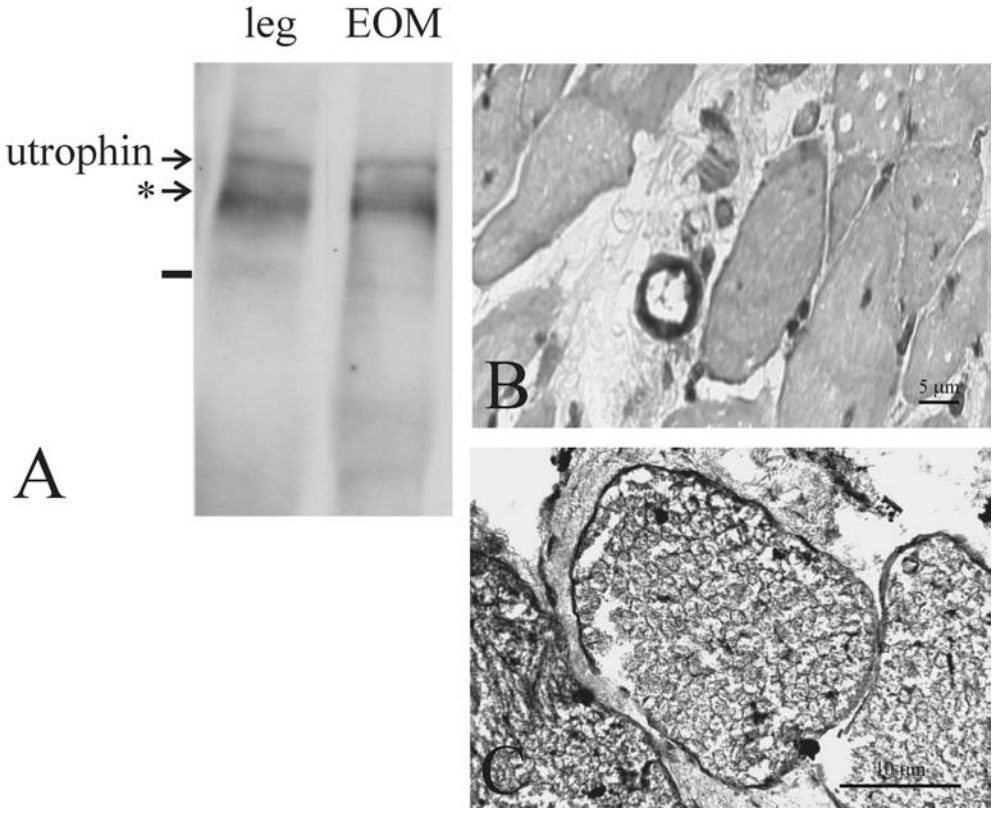

Fig. 2 Utrophin was detected with Western blot in leg muscles and extraocular muscles (EOM) (**a**). The double bands on both blots were interpreted as degradation products (*). Staining with indirect peroxidase immunohistochemistry in EOM showed a positive signal for utrophin in blood vessels (**b**) and nerves (**c**)

antibody against rapsyn. Slides from leg muscles (Fig. 1d) and the EOM (Fig. 1h) showed a positive staining of the AChR with rapsyn. The staining of the AChR with α-bungarotoxin showed again a positive result only in leg muscle but not in the EOM (data not shown). The Western blot analysis of both leg muscles and EOM showed a low level of expression for utrophin (Fig. 2a). The double band on both blots could be due to degradation products.

Nerves and blood vessels

Staining of utrophin with indirect peroxidase reaction showed in EOM a positive result in blood vessels (Fig. 2b) and nerves (Fig. 2c). The same was found in leg muscles (data not shown).

Discussion

Specific skeletal muscles are involved in a selective manner during muscular dystrophy. It is well known that in mdx mouse the small-caliber skeletal muscle fibers are less affected although dystrophin is deficient in all muscle fibers (Karpati et al. 1988). In DMD patients, EOM are structurally and functionally spared during the whole course of the disease. In hematoxylin and eosin staining, EOM lack the cardinal pathological manifestation of dystrophin deficiency such as hypertrophy, fiber size variation, fiber splitting, central nucleation, fatty degeneration and fibrotic scarring (Khurana et al. 1995). Also, clinical study of the ocular motility of DMD patients using infrared oculography showed the absence of altered function (Kaminski et al. 1992). The sparing of EOM in DMD may be linked to differences in morphology, cell and molecular biology of this muscle group compared with other skeletal muscles (Porter et al. 1997).

In this study, we evaluated the hypothesis that utrophin would also be expressed outside NMJ in normal EOM and thus could replace dystrophin in DMD. We used immunohistochemical methods to investigate distribution of utrophin along the sarcolemmal membrane in normal EOM and leg muscles of cats. For comparing the expression level of utrophin in these two muscles, we used Western blot. Our results clearly showed that in cat, there is a different expression of utrophin in EOM compared with other skeletal muscles. In leg muscles, the distribution of utrophin was restricted to NMJ while in EOM, we could not detect any immunoreactivity of utrophin. Another interesting finding was that by using α-bungarotoxin, we could detect NMJ in leg muscles but not in EOM while staining of AChE showed NMJ in both muscles. For checking these results, a double staining of AChR with α-bungarotoxin and an antibody against rapsyn was performed. In leg muscles, AChR were detected with both α-bungarotoxin and rapsyn. In EOM, there was again no signal for α-bungarotoxin although NMJ could be shown with rapsyn.

Our study shows two surprising findings in EOM of normal cat: the lack of utrophin at NMJ and the failure of staining AChR with α-bungarotoxin. To our knowledge, there is no study about the distribution of utrophin in EOM of cat. Further, we also could not find any data about staining with α-bungarotoxin NMJ in EOM of cat.

We are aware that our results contradict reports from other groups. In different studies, the expression of utrophin in EOM was examined (Khurana et al. 1995; Porter et al. 1998). In addition, various papers showed α-bungarotoxin staining in EOM of a variety of species (Briggs and Schachat 2002; Khanna et al. 2003; Kusner et al. 1999; Lukas et al. 2000). None of these treatises were done in EOM of cat. Nevertheless, there are different reasons which could let assume that our negative results are not true and were caused by processing artefacts:

1. The NMJ was missing in the serial sections chosen for the double staining of utrophin and α-bungarotoxin. To exclude this possibility, different samples from all three cats were cut along the tendon-to-tendon length of the muscle. The results presented in Fig. 1e–g are representative of our findings in all these sections.
2. Our protocol for the staining of NMJ with α-bungarotoxin was not suitable for EOM. To find out if this could be true, we applied the same protocol in EOM of a rat. Like in previous reports, we were able to detect NMJ with α-bungarotoxin in this species.

On account of all the controls performed, we believe that it is rather unlikely that our results were caused by a technical failure.

In normal mature striated muscle, utrophin expression is also detected in the wall of blood vessels and in the perineurium and Schwann cells of intramuscular nerves (Karpati et al. 1993). In EOM of cat, we did not find utrophin at the NMJ, but we were able to detect utrophin in blood vessels and nerves of this muscle group. This result explains why the Western blot of EOM showed a low expression level for utrophin although in the immunohistochemical analysis of this muscle group, no utrophin was found at NMJ.

The AChR are a large complex of four transmembrane glycoprotein subunits, which form an $\alpha_2\beta\gamma\delta$ pentameric complex (Raftery et al. 1980). α-bungarotoxin binds to AChR by interacting with the extracellular part of α-subunits (Harel et al. 2001; Moise et al. 2002; Young et al. 2003). Rapsyn interacts with the cytoplasmatic part of all subunits of AChR (Huebsch and Maimone 2003; Maimone and Merlie 1993; Willmann and Fuhrer 2002). Therefore, one possible explanation as to why in EOM of cats NMJ are detectable with rapsyn but not with α-bungarotoxin could be that in this species, α-subunits of AChR do not have the binding site for α-bungarotoxin.

At NMJ, utrophin is found at the crests of junctional folds whereas dystrophin occurs mainly in troughs (Bewick et al. 1992). AChR are also concentrated at the crests (Flucher and Daniels 1989). The clear functional role of utrophin and its interaction with AChR remain unclear. There are different explanations as to why we could not detect utrophin expression at NMJ in EOM of cat. One could be that in this species, utrophin is expressed at NMJ of EOM without the epitope that is recognized by the antibody we used in our study. Another interpretation of the outcome of our study would be that utrophin does not occur at NMJ of EOM because its expression depends on the structure of α-subunits of AChR.

We believe that the lack of utrophin at NMJ in EOM of cat is not an artefact but a true result. A possible explanation could be that in EOM of cat, NMJ are arranged differently from other species. Further studies are needed for a better characterization of AChR and their subunits in EOM of cat. Also, a more specific method than Western blot has to be used to better distinguish between expression of utrophin in muscle fibers of EOM and their blood vessels and nerves.

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