GENETIC MUSCLE DISEASES: NEW INSIGHT INTO THE BASIS OF THE MYOTONIC DYSTROPHIES

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Introduction

Neuromuscular diorders can be divided into 1) myopathies, primary disease of the muscle fiber, 2) myasthenias, diseases caused by defects of the neuromuscular junction and 3) neurogenic muscular atrophies, caused by the defects of the motor nerve. Muscular dystrophies are a heterogeneous group of myopathies. They are genetic disorders caused by muscle fiber degeneration often causing progressive weakness and wasting and they can be further divided into the following

Myotonic dystrophies – DM1 andDM2
Dystrophinopathies - DMD, BMD
Facioscapulohumeral - FSHD
Limb-girdle – LGMD subtypes
Distal muscular dystrophies
Congenital dystrophies - CMD
Oculofaryngeal - OPM, OPDM
Emery-Dreifuss - X-EMD, AD-EMD
Other and unclassified muscular dystrophies

Myotonic dystrophy (Dystrophia myotonica, DM) is the most common inherited muscular dystrophy in adults. Two different types of myotonic dystrophy have been identified. Both myotonic dystrophy type 1 (DM1, Steinert´s disease [OMIM #160900]) and type 2 (DM2, [OMIM #602668]) are dominantly inherited disorders caused by repeat expansion mutations. The estimated prevalence of DM1 is 1/8000 *(*Harper 2001*)*, while in DM2 the prevalence has not been established, but is considered to be even as common as DM1 in many European populations *(*Udd 2006*).*

DM1 was described one hundred years ago and the (CTG)n trinucleotide repeat expansion mutation in the 3´untranslated region (UTR) of the *DMPK* gene was identified in 1992. The gene is located in chromosome 19q13.3 *(*Brook; Fu; Mahadevan 1992*).* The mutation underlying DM2 disease is a (CCTG)n tetranucleotide expansion located in the first intron of *ZNF9* gene on chromosome 3q21 *(*Liquori 2001*).* The repeat expansion size in DM1 may vary from more than 50 to more than 3000 repeats and there is a gross correlation between repeat length and disease severity. The number of repeats in the expansion mutation causing DM2 varies from 75 to 11000. No correlation in the disease severity and the size of the expansion mutation has been shown in DM2. There is no clear evidence for anticipation in DM2 as there is in DM1, in which successive generations inherit increasing disease severity with decreasing age of onset due to increased size of the repeat expansion *(*Day 2003*).* In both DM1 and DM2 the molecular pathomechanism is based on RNA gain-of-function. Transcription of the repeats into mutant $(CUG)_{DM1}$/$(CCUG)_{DM2}$-containing RNAs is both necessary and sufficient to cause disease by formation of ribonuclear foci and interference of the splicing of downstream "effector" genes through *trans*-acting splicing factors, namely muscleblind 1 (MBLN1) *(*Osborne 2006, Ho 2005*)* and CUG binding protein 1 (CUGBP1) *(*Timchenko

2006*)* . Several 'effector' genes including, *CLCN1* (chloride channel-1), *INSR* (insulin receptor), *TNNT2* (cardiac troponin T), *TNNT3* (skeletal fast troponin T), *ZASP*, *ATP2A1* (SERCA1) and *MAPT* (microtubule-associated protein tau) show aberrant splicing in DM1, and in DM2. *(*Charlet-B N 2002, Mankodi 2002, Maurage 2005, Savkur 2004*)*

DM1 and DM2 mutations

The DM1 mutation was identified in 1992 as an expanded trinucleotide (CTG)n repeat track in the 3´ untranslated (UTR) region of the dystrophia myotonica protein kinase coding gene (*DMPK*; OMIM *605377) *(*Brook; Fu; Mahadevan 1992*)*In DM1 patients the repeat sizes range from 50 – 4000 (150 – 12,000 bp), whereas normal individuals have 5 – 37 repeats. Repeat lengths 38 – 49 are considered a premutation allele pool, showing decreased stability. The DM1 mutation length predicts the clinical outcome so some extent: classical DM1 100 – 1,000 repeats; congenital >2,000 repeats.

The mutation causing DM2 was identified in 2001 in the first intron of the zinc finger 9 (ZNF9) protein coding gene *CNBP* (*ZNF9*; OMIM *116955) (Liquori 2001). This means that both DM1 and DM2 mutations consist of long uninterrupted repeat tracks, which are thermodynamically less stable than the normal alleles, which show a more complex pattern with interrupting sequences.

Symptoms

Both DM1 and DM2 are progressive multisystemic disorders with both shared and distinct clinical features and pathomechanisms. The core symptoms expressed at various degrees in DM2 include proximal muscle weakness, muscle pain, myotonia, posterior subcapsular iridescent cataracts, tremors, cardiac conduction defects, and endocrinological disturbances such as increased insulin resistance and male hypogonadism.Laboratory findings include elevated creatine kinmase (CK), hypo IgG, positive autoimmune serology (Tieleman 2009) and elevated liver enzyme γ-glutamyl transferase (Udd 2003, Day 2003). Many of the clinicamanifestations are similar in classical adult onset DM1, although with also clearly distinct features: more severe weakness of distal and facial muscles, including ptosis leading to dysphagia and respiratory deficiency. The congenital DM1 form and the early childhood onset form with mental retardation in DM1 are not observed in DM2 (Harper 2001, Turner 2010).

Muscle histopathology in myotonic dystrophies

Muscle histopathology in DM1 and DM2 share some general common chronic myopathic features, such as high number of central nuclei and fiber size variation which were initially reported to be quite similar (Ricker 1994, Thornton 1994). The muscle pathology is progressive, with end-stage pathology showing severe reduction in fiber number and size accompanied with fatty and connective tissue replacement in the most severely affected muscles (Harper 2001). In DM1, ring fibers and sarcoplasmic masses are more frequent, and type 1 fibers are generally more affected (Harper 2001).

In general, muscle wasting in DM2 is less prominent, and in accordance with this, severe muscle fiber loss is not usually encountered. The biopsies show usually mild to moderate myopathic changes, and more severe fatty replacement and fibrosis, typically starting in the interfascicular space, is observed only in proximal muscles of older patients with considerable muscle weakness. The number of internalized nuclei is greater in type 2 fibers in DM2, and in type 1 fibers in DM1, further reinforcing the observation that the distinct fiber populations are differentially affected in DM1 and DM2 (Bassez 2008, Pisani 2008). Fiber atrophy is seen in most biopsies; small angulated fibers and atrophic nuclear clump fibers, similar to those in neurogenic atrophy, are a frequent finding, (Day 2003, Vihola 2003, Schoser 2004), whereas small group atrophy is less common and fiber type grouping is

not observed (Vihola 2003, Schoser 2004). In addition, there is a number of rather infrequent findings in DM2 muscle, including occasional moth-eaten fibers, rimmed vacuolar and ragged-red fibers (Vihola 2003, Schoser 2004). Necrotic fibers, ring fibers, targetoid fibers, and subsarcolemmal or sarcoplasmic masses are rarely seen in DM2.

However, it was only after using the immunohistochemical method for fiber type differentiation in the muscle sections, that the preferential fiber type 2 atrophy was fully recognized in DM2 (Vihola 2003), even though the possibility of it had been suggested previously (Bassez,G. 2001). Schoser et al. later confirmed the finding with a larger set of samples (Schoser 2004). It should be noted, that there is hypertrophy of both fiber types in DM2 (Vihola 2003), and also atrophy of type 1 fibers occurs in DM2, however, to lesser extent: percentage of atrophic type 1 fibers is lower than that of type 2 fibers (Vihola 2003, Schoser 2004), and practically all very atrophic fibers (< 5um) are of type 2 in DM2; whereas in DM1, the very atrophic fibers occur later in muscle histopathology, together with other signs of advanced pathology, and typically express both fast and slow myosin isoforms.

No clear correlation has been observed between the pattern of muscle histopathological findings and the clinical muscle phenotype, regarding myotonia, cramps, weakness and myalgia, or the order of onset of symptoms (Schoser 2004). Internalized nuclei and scattered nuclear clump fibers may be prominent even in clinically asymptomatic muscle.

Pathomechanisms in myotonic dystrophies

The main molecular pathomechanism underlying the myotonic dystrophies is dominant RNA gain-of-function (Mankodi 2000, Liquori 2001, Lee 2009). The expansion mutation, (*CCTG*)n in DM2 and (*CTG*)n in DM1, is transcribed but not translated, and the mutant RNA itself is necessary and sufficient to induce the molecular events leading to clinical symptoms. The mutant repeat RNA species are retained in the nucleus, where they aggregate and sequester RNA-binding proteins, forming the ribonuclear inclusions typical of DM1 and DM2 (Taneja 1995, Liquori 2001). According to the present knowledge, the mutant RNA exerts its toxic effect via disturbing the distribution and function, and hence the equilibrium between the two antagonistic splicing factors, MBNL1 (muscleblind-like 1) and CUGBP1 (CUG-binding protein 1), resulting in aberrant mRNA splicing, translation, and turnover.

The MBNL and CUGBP1 act as antagonist splicing regulators, often/sometimes promoting opposite splicing events in their mutual target genes, and their binding sites are distinct (Ho 2004). Both of them regulate multiple alternative splicing events. The important downstream effect of the dysregulation of these two antagonistic splicing factors is the aberrant splicing in myotonic dystrophies, resulting in inappropriate embryonic splicing pattern, which is considered the main cause of direct symptoms. More than 20? aberrant splicing events have been identified in DMs, summarized in table x. Of these, at least CLC1 has been linked to myotonia (Charlet-B 2002, Mankodi 2002, Wheeler 2007); insulin receptor (IR) to insulin resistance (Savkur 2001); and cTNT (cardiac troponin T) to cardiac problems (Philips 1998).

The atrophy of type 2 fibers in DM2

In this thesis work the concept of very atrophic fibers is used referring to the pool of very small fibers with a diameter < 6 µm. They are characteristic for DM2 where they appear very early in the disease course, even in clinically unaffected muscles. Morphologically, they fall into at least three categories: (1) nuclear clump fibers, (2) extremely small fibers (< 6 µm) with very little cytoplasm and none to few nuclei (in the plane of sectioning), and (3) very thin, angulated fibers. Most of these fibers are round and scattered. They seem to represent a unique pool of fibers, because they form a distinct population apart from larger type 2A fibers.

They may be encountered in DM1, but only in later stages of pathology, less numerous compared to DM2 and in DM1 they may also express slow MyHC.

It is not clear if (1) and (2) represent distinct fiber populations. Examination of longitudinal sections suggests that they may represent a single population of fibers, which show differential morphology depending on the number of nuclei trapped inside the slim fibers at the section site.

The nuclear clump fibers in DM2 are morphologically indistinguishable from those seen in neurogenic atrophy, where they form as an end product of denervation atrophy in the absence of re-innervation. However, no primary neurogenic involvement has been shown in myotonic dystrophies, which is supported by the lack of fiber type grouping.

Protein expression in very atrophic fibers

We carried out further studies on protein expression in DM2 and DM1 muscles to identify possible differences between them, which could lead to better understanding of their differential muscle and fiber type involvement. In addition, due to the presence of nuclear clump fibers in myotonic dystrophies, similar to neurogenic atrophy, we also included a group of patients with various neurogenic disorders.

Altogether, we characterized the expression of 14 proteins, which are involved in muscle contraction and Ca2+ handling, regeneration, denervation, and apoptosis, by immunohistochemistry. A subset of the proteins show preferential expression in type 2 fibers as described below. Myosin heavy chain (MyHC)-encoding genes (*MYH1,2, -3, -7* and *-8*) are differentially regulated in fast and slow fibers, and during muscle development. The expression of MyHC-IIa isoforms in the highly atrophic fibers of DM2 was confirmed with three different monoclonal antibodies raised against fast type myosin heavy chains. As a new finding, we showed that the population of very small fibers in DM1 co-expressed myosins fast IIa and beta/slow, in contrast to DM2, where they expressed only type IIa. In neurogenic atrophies, virtually all small atrophic fibers expressed MyHC-IIa, showing moderate to high co-expression of MyHC-beta/slow. The MyHC-pn is widely used as a regeneration marker (Dubowitz 2007). There was high expression of the MyHC-pn isoform in very atrophic fibers in all groups. Since the majority of very atrophic fibers in progressive neurogenic atrophy without any regeneration activity express MyHC-pn, this may be turned on and expressed also by other mechanisms than regeneration. MyHC-emb positive fibers were absent in most biopsies, showing expression in a few DM fibers only in the biopsies with advanced muscle pathology.This observation is congruent with its reported expression mostly in small basophilic regenerating fibers having survived severe injury such as withmuscular dystrophies and other necrotic myopathies (Dubowitz 2007). A similar finding was observed with the transcription factor myogenin (*MYOG*), and the intermediate filament protein vimentin (*VIM*), which appear early during myogenesis and serve as regeneration markers in muscular dystrophies (Dubowitz 2007, Olive 1997).

Neural cell adhesion molecule (NCAM-1) has distinct roles in myogenesis, synaptogenesis, and synaptic maintenance (Covault 1986). It is transiently re-expressed after denervation and during re-innervation, decreasing progressively as regeneration proceeds (Winter 1999). Hence, it has been a candidate marker for recently occurred denervation atrophy. Interestingly, we found that one third of the atrophic fibers in DM2 abundantly expressed NCAM-1, outnumbering the small fibers in DM1, and even more intriquingly, the severely atrophic fibers in neurogenic atrophies. In fact, the pattern of NCAM-1 expression was different in the latter group, showing positive signal more frequently in intermediate-large fibers, which was not encountered in any specimens in myotonic dystrophies. The usage of NCAM expression as a marker of neurogenic atrophys thus seems to be very limited.

Because we were preoccupied with the question why the highly atrophic type 2 fibers persist in the DM2 muscle, we investigated the expression of the oncoprotein BCL2 (*BCL2*)

in the muscle biopsies. Apoptosis is required during normal tissue remodeling, development and regeneration. BCL2 inhibits cells from entering apoptosis, but it also has a distinct effect on cell cycle (Cory 2003). Apoptosis is not generally involved in muscular dystrophies as a pathomechanism (Dubowitz 2007). In order to verify this we used an Ab raised against activated Caspase-3 (*CASP3*), a cysteine protease playing a key role in executing apoptosis in many cell types, and a commonly used marker for apoptosis (viite). To our surprise, the majority of the very atrophic fibers in DM2 expressed BCL2. In contrast, in DM1 BCL2 immunoreactivity was present in only one biopsy, in which approximately 5% of the very small fibers were positive. In neurogenic atrophies, BCL2 positive fibers were moderately common, albeit with a lot of dispersion among the samples (range 1-100% positive cells). Caspase-3 was not detected in DM2 biopsies, supporting the prevailing concept of apoptosis not playing a major role. However, the combined high BCL2 expression and absent Caspase-3 could offer a partial explanation to the persistence of the very atrophic, non-functional type IIa fibers in DM2: the anti-apoptotic BCL2 might prevent the cells from entering apoptosis. Another interesting finding in DM2 was revealed when serial sections, stained for MHCpn and BCL2, were examined. Mutually exclusive expression of these markers was often observed. We do not have a specific explanation to this finding at the moment.

We observed reduced sarcolemmal labeling of dystrophin and neuronal nitric oxide synthase (nNOS) in the very atrophic fibers in DM2. However, these findings, although not reported with DM2 before, are likely not specific, as several sarcolemmal proteins are reported to show low immunoreactivity in MyH-pn positive, small atrophic fibers (Dubowitz 2007). We also studied two proteins preferentially expressed in, but not restricted to fast type 2 fibers: the fast skeletal muscle troponin T (fTnT/*TNNT3*), a sarcomeric thin filament component, and the Ca^{2+} ATPase SERCA1 (*ATP2A1*), both of which are involved in regulation of muscle contraction (Wu 1994, Brandl 1986). SERCA1 was expressed in majority of the atrophic fibers in all groups, whereas fTnT showed moderate to high expression in atrophic fibers in DM2 and DM1, and high expression in neurogenic atrophies. Unfortunately, even though not strictly following the fast-slow fiber distribution, neither of these proteins were differentially expressed in very atophic and regular type 2 fibers, thus giving no further insight into the question why a subpopulation of type IIa fibers are subject to severe atrophy in DM2.

It should be noted that the neurogenic atrophies was the most heterogeneous group, relative to MyHC-pn, NCAM-1 and BCL2 expression in the very small, atrophic fibers, showing more dispersion in results and greater standard deviation values. This is understandable because of their divergent etiologies.

Satellite cells

Defective myoblast-myotube differentiation in cultures from satellite cells have been reported with DM1. Similar findings have not been observed in DM2. However, repeatedly in our DM2 cell cultures myoblast proliferation after 4-5 passages has been limited even if differentiation seemed to be normal.

Pax7 is expressed in quiescent skeletal muscle satellite cells, and considered an appropriate protein marker (Relaix 2005). The number of satellite cells has been reported to decline with age (Sajko 2004). As a separate experiment, we assessed the number of skeletal muscle satellite cells in DM2 (n=4), DM1 (n=5), neurogenic atrophy (n=4), and healthy control (n=1), approximately 200 fibers were included per biopsy, counting all fibers in randomly selected fields. The number of Pax7 positive cells in satellite cell position was proportioned to the total number of fibers; the results are indicated as the number of Pax7+ cells (in satellite cell position) per 100 muscle fibers counted:

DM2	DM1	NA	C
25	20	20	7

NA, neurogenic atrophy; C, healthy control

There were other Pax7+ cells present in the interstitial space of the biopsies examined, which likely represent a distinct pool of progenitor cells and these were not included in the numbers of satellite cells. The results indicate that the number of spindle-shaped, Pax7+ positive cells in satellite cell position was increased in all diases groups, compared to control biopsy. We observed the highest number of Pax7+ cells in DM2. The reported proportion of satellite cells in human muscle normally varies between 3-6% of all muscle fiber nuclei (Sajko 2004), which is in accordance with our control muscle. The number of satellite cells is affected by age and muscle type. The increase of satellite cells in DM2, DM1 and neurogenic atrophy is probably caused by basic attempts to regenerate in response to muscle injury. The results are congruent with previous reports of DM2 and DM1 indicating unaffected satellite cell proliferation (Furling 2001, Pelletier 2009).

Conclusion

Defective control of gene expression which leads to abnormalities in a large number of proteins expressed in the muscle fibers is one component of the molecular pathology underlying the myotonic dystrophies. To a major extent these abnormalities are based on abnormal splicing events due to malfunction of MBLN1. According to the global impact of the primary mutations on RNA-metabolism also other machineries in the muscle cell such as translation and turnover handling are affected. Considering these large scale molecular changes, any therapy towards a limited sector of abnormal gene and protein expression may not be very successful, and approaches to directly eliminate the mutant transcripts generated by the primary repeat expansion mutations should be the target in the search for curative treatment.

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