Dystrophin levels as low as 30% are sufficient to avoid muscular dystrophy in the human

Marcella Neri a, Silvia Torelli a, Sue Brown a, Isabella Ugo a, Patrizia Sabatelli b, Luciano Merlino b, Pietro Spitali b, Paola Rimessi b, Francesca Gualandi b, Caroline Sewry a,c, Alessandra Ferlini a,b, Francesco Muntoni a,*

a Dubowitz Neuromuscular Centre, Imperial College, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK
b Medical Genetics Section, Department of Experimental Diagnostic Medicine, University of Ferrara, Italy
c Wolfson Centre for Inherited Neuromuscular Disorders, Department of Musculoskeletal Pathology, Robert Jones and Agnes Hunt Orthopaedic Hospital, Oswestry, UK

Received 2 May 2007; received in revised form 9 July 2007; accepted 16 July 2007

Abstract

Mutations in the dystrophin gene give rise to Duchenne and Becker muscular dystrophies (DMD and BMD), in which both skeletal and cardiac muscles are affected, but also to X-linked dilated cardiomyopathy (XLDC), a condition characterised by exclusive cardiac involvement. XLDC patients with mutations at the 5' end of the gene typically have a cardiac specific severe transcriptional pathology, with absent dystrophin in the heart, while reduced levels of virtually normal dystrophin transcript and protein are present in the skeletal muscle. We now report the identification of a new XLDC family and the detailed characterisation of the levels of dystrophin protein present in skeletal muscle of this family, and of three previously studied XLDC families. We found that dystrophin levels comprised between 29% and 57% were sufficient to avoid muscle weakness in these XLDC families. This information will be of help for the development of therapeutic approaches aimed at restoring dystrophin levels sufficient to prevent the muscle pathology in DMD.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Dystrophin; X-linked dilated cardiomyopathy; Therapy; Duchenne muscular dystrophy

1. Introduction

Duchenne muscular dystrophy (DMD, OMIM #310200) is a fatal X-linked disorder caused by the absence of dystrophin, a protein expressed at the sarcolemma of all muscle types [1]. At the moment there is no effective treatment for this condition which affects 1 in 3500 male newborns. Affected individuals develop muscle weakness in the first few years of life, lose the ability to walk by their 13th birthday and typically die by their late teens unless respiratory and cardiac treatments are initiated. Mutations in the dystrophin gene comprise deletions (60–70% of cases), duplications (10–15%) and point mutations or other rare rearrangements (10–20%) (www.dmd.nl). In at least one-third of all cases, mutations occur de-novo and as a result new cases continue to be diagnosed despite the availability of genetic testing and carrier detection. Different therapeutic strategies are being developed to avoid the progressive muscle degeneration which characterises these patients [2]. A “direct” approach is aimed at directly restoring the protein expression in muscle, either improving the endogenous synthesis (using for example antisense oligonucleotides or read-through stop codon strategies, see below) or introducing an intact gene from outside using viral vectors or cellular systems; in contrast an “indirect” approach is aimed at tackling some of the consequences that the lack of dystrophin has in muscle (such as for example the increased inflammatory activity).
The direct approach is the most effective and attractive but also the most challenging, and has so far been refined using a natural genetic model of DMD, the mdx mouse, harbouring a nonsense mutation in exon 23. Despite the absence of dystrophin, the mdx mouse shows only limited signs of the dystrophic process in the late adult life [3,4].

One of the direct approaches is the gene delivery based on different viral vectors [5]. Either adenovirus or adeno-associated viral (AAV) vectors carrying truncated dystrophin cDNA have successfully been delivered in the mdx mouse [5–8], restoring the expression in >50% of myofibers. However currently there are both technical challenges and some concern regarding the vector induced immune response that complicates the systemic use of these viral vectors [9]. Cell-based therapy is another promising direct approach and very recently the interest in this field was boosted by the identification of a subset of mesangioblastic stem cells which can target skeletal muscle after intra-arterial delivery [10]. Cells with similar characteristics have now also been identified in the human (pericytes) and their properties are currently being characterised further in preparation for clinical trials [11]. Improving the endogenous synthesis of dystrophin has been approached acting at the mRNA level. A read-through strategy based on the administration of aminoglycosides has been used in the mdx mouse and also in DMD boys carrying point mutations leading to stop codons. While the studies in mice were encouraging, showing that protein levels equivalent to 10–20% of normal could be induced and were able to confer significant protection from muscle fibre degeneration [12], the studies in the human have been disappointing [13]. More recently, novel and more efficient compounds such as PTC124 which also induce read-through stop codons have been devised and shown to be effective in mdx mice; this latter drug is now in phase II studies in DMD boys [14–16]. Another approach aimed at improving the endogenous synthesis of dystrophin takes advantage of the administration of antisense oligonucleotides (AOs) to induce selective exon skipping and restoration of the reading frame, mimicking both the physiological phenomenon observed in the revertant fibres of DMD boys [17,18], and the well known occurrence of in-frame deletions in BMD patients, including very mild or asymptomatic cases [19]. Intramuscular and more recently repeated intravenous injections of AOs in mdx mouse resulted in restoration of dystrophin expression in a significant number (20–50%) of muscle fibres and significant protection from muscle damage [20,21]. A further technical improvement was achieved injecting an Adeno Associated Virus (AAV) constitutively expressing AOs, although the limitations of this approach are similar to those discussed above for gene therapy [22].

Following the preclinical studies, a number of these experimental therapies are now being exploited in phase I/II clinical trials and during the course of 2007 some of the preliminary results from these studies are expected. One key question that is relevant for all these studies relates to how much dystrophin is necessary to protect the muscle of DMD boys from further degeneration. Transgenic experiments have been performed in the mdx mouse, but there are concerns on how much this animal model is capable of recapitulating the pathological process observed in the human.

In order to provide an indirect answer to this question, we have taken advantage from the characterisation of dystrophin production in the skeletal muscles of four individuals affected by X-linked dilated cardiomyopathy (XLDC, OMIM 302045), an allelic disorder to DMD [23]. We have previously reported that the molecular basis for XLDC is often a mutation inducing a loss of protein production exclusively in the heart [24,25]. Most of these patients carry unusual mutations at the 5′ end of the gene, which result in the expression of normal dystrophin, although in reduced quantity, in skeletal muscle, in contrast to the complete absence of this protein in the cardiac muscle. Although these patients transcribe dystrophin evenly in all muscle fibres and therefore do not necessarily represent a perfect model of restoration strategies as often this results in uneven dystrophin expression in muscle, nevertheless these XLDC families represent a unique opportunity to study the levels of dystrophin necessary to avoid the development of symptomatic skeletal muscle degeneration.

2. Materials and methods

2.1. Patients

The genotypic characterisation in three of these four families has been previously reported. Family 1 has a splice site mutation in the first intron of the M isoform pre-mRNA [26]. Family 2 has a deletion removing the muscle promoter and first muscle exon [23]. Family 3 has a rearrangement in intron 11 of the gene, with activation of a cryptic splice site in the cardiac muscle and production of an aberrant transcript [27]. The same mutation previously identified in family 1 was found in a patient from an unrelated family from Northern Italy (family 4), which has not been reported before. The proband in family 4 presented at the age of 13 with sudden cardiac failure following a flu-like illness. His ejectional fraction (EF) was 14% and a diagnosis of dilated cardiomyopathy was made. He was asymptomatic from a skeletal muscle perspective, although reported myalgia after strenuous exercise (football), but never experienced urine discoloration. His serum CK was elevated at 2000 U/l on several occasions. Neurological examination by one of the authors (L.M.) showed no muscle weakness, nor any pseudohypertrophy or contractures. His DC worsened and he eventually underwent cardiac transplantation at the age of 15. At the age of 22 he continued to have a normal clinical event. At the age of 22 he continued to have a normal clinical
skeletal muscle and in heart in patients from families 1, 2 and 4. The production of a normal full length dystrophin in skeletal muscle of these patients was driven by the up regulation of B and P promoters (not shown).

The amplification of M (muscle), B (brain) and P (Purkinje) isoforms from the cardiac muscle of the propositus of family 3 showed that all the three isoforms were expressed; instead the skeletal muscle lacked B isoform expression and upregulated M and P isoforms (not shown). As family 3 has a mutation that does not abolish the M isoform transcription, the isoforms expression profile is different from the one observed in families 1, 2 and 4. The clinical features of the patients in these four families are summarized in Table 1.

2.2. Genomic analysis in patient from family 4

DNA was extracted from leukocytes by standard methods. Multiplex PCR DNA analysis of dystrophin exons was carried out according to the methods of Chamberlain et al. [28] and Beggs et al. [29]. Amplification of the full length muscle isoform (Dp427m) first exon was obtained using a forward oligonucleotide (5’-CAGTTACCTGTTTGACTCAGT-3’) located 235 nucleotides upstream of the ATG codon and a reverse oligonucleotide (5’-CTACCTAATTAGTGAGCTTGTCACC-3’) 76 nucleotides downstream. PCR reaction (25 l) utilized 2.5 U ExTaq (Takara), 0.5 mM each oligonucleotide, 200 mM each dNTP, 10× ExTaq buffer MgCl2 plus. Amplification conditions were: 94 °C initial denaturation (5 min), 94 °C denaturation (45 s), 63 °C (45 s) annealing, 68 °C (45 s) extension for five cycles followed by 94 °C denaturation (45 s), 62 °C (45 s) annealing, 68 °C (45 s) extension for 30 cycles, 68 °C (5 min) final extension. PCR product (342 bp) was purified (Nucleospin Extract – Macherey-Nagel) and sequenced on ABI Prism 3100.

2.3. Reverse transcription and PCR

Total RNA was isolated from frozen skeletal muscle biopsies of the patients. The cDNA synthesis was performed using random hexanucleotide primers and SscIII enzyme (Invitrogen) following the protocol supplied. PCR was performed in a reaction volume of 25 µl containing the cDNA template (2 µg) and oligonucleotide primers designed to amplify the mid-rod domain of dystrophin, a region not involved in the mutations identified in the three families. In particular we amplified exons 30–31 (oligonucleotides 30: 5’-GAGGCTTAGAGGAGCAAGATTTG-3'; 31: 5’-ATCCAATGAGTTGAC AAGTCAT-3'; respectively). Oligonucleotides for amplifying the laminin α2 chain gene were (a) forward oligonucleotide 5’-CTGTGTTATCAGTGCTGT TCAGGGATT-3' and (b) reverse oligonucleotide 5’-TGAGCATGTTTCTCATCCTTGAC-3', position 1350–1326.

PCR reactions (25 µl) utilized 0.5 units Taq polymerase, 0.25 mM each primer, 200 mM each dNTP, 10× ExTaq buffer MgCl2 plus. Amplification conditions were 94 °C denaturation for 10 min, followed by 94 °C (30 s), 58 °C annealing (60 s), and 72 °C extension (120 s), for 26 cycles. Nine microliter of the reactions was analyzed on 2.5% agarose gels containing 0.2 g/ml of ethidium bromide prior to photography.

2.4. Histology and immunocytochemistry

Patients were respectively 23, 32, 20 and 13 years old when the muscle biopsies were obtained from the quadriceps using a needle technique. Muscle biopsies were rapidly frozen in isopentane cooled in liquid nitrogen according to standard techniques. In addition to a routine panel of histological and histochemical stains [30] unfixed frozen sections (7 µm) were immunostained using a panel of

### Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at diagnosis</th>
<th>EF</th>
<th>Cardiac involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>12%</td>
<td>HT at 24, Patient died from post surgical complications</td>
</tr>
<tr>
<td>2</td>
<td>13 but asymptomatic at 23</td>
<td>14%</td>
<td>Patient died in cardiac failure before HT</td>
</tr>
<tr>
<td>3</td>
<td>16; episode congestive heart failure at 17</td>
<td>17%</td>
<td>HT at 28</td>
</tr>
<tr>
<td>4</td>
<td>13 with sudden cardiac failure</td>
<td>14%</td>
<td>HT at 15</td>
</tr>
</tbody>
</table>

EF, ejectional fraction; HT, heart transplant.

### Table 2

<table>
<thead>
<tr>
<th>Patient</th>
<th>Genomic mutation</th>
<th>CK (U/l)</th>
<th>Neurological examination</th>
<th>Muscle biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dp427m splice donor site</td>
<td>normal</td>
<td>normal</td>
<td>Minimal histological changes. Reduced intensity of sarcolemmal staining of dystrophin</td>
</tr>
<tr>
<td>2</td>
<td>Deletion of muscle promoter and first muscle exon</td>
<td>3362</td>
<td>normal</td>
<td>Mild variability in fibre size. Reduced intensity of sarcolemmal staining of dystrophin</td>
</tr>
<tr>
<td>3</td>
<td>Intron 11 deletion activating a cryptic splice site</td>
<td>1970</td>
<td>normal</td>
<td>Mild myopathic changes with increased number of internal nuclei. Reduced intensity of sarcolemmal staining of dystrophin</td>
</tr>
<tr>
<td>4</td>
<td>Mutated Dp427m splice donor site</td>
<td>2000</td>
<td>normal</td>
<td>Mild myopathic picture with mild–moderate fibre size variation, some internal nuclei, no necrosis regeneration or fibrosis. Reduced intensity of sarcolemmal staining of dystrophin</td>
</tr>
</tbody>
</table>

Please cite this article in press as: Neri M et al., Dystrophin levels as low as 30% are sufficient to avoid ..., Neuromuscul Disord (2007), doi:10.1016/j.nmd.2007.07.005
antibodies against dystrophin (DYS1, DY2, DYS3; Novo- 
castra Laboratories Newcastle), β-spectrin (SPEC-1 Novo-
castra Laboratories Newcastle) and laminin α2 (MAB 1924 Chemicon) [23,26,27]. Age matched control muscles 
used for both immunohistochemical and Western blotting 
analysis were obtained from patients who were biopsied to 
exclude a neuromuscular disorder and were eventually 
deemed to be free from a neuromuscular condition (Table 2).

2.5. Electrophoresis and Western blotting

Western blot analysis was performed using a 6% 
resolving/4% stacking polyacrylamide gels and DYS1 
(Novocastra, Newcastle, UK) monoclonal antibody to 
dystrophin. Blots were incubated with appropriate 
biotinylated secondary antibodies (Amersham Little 
Chalfont, UK), followed by streptavidin conjugated to 
horseradish peroxidase (Dako). Proteins were visualised 
using the ECL plus system (Amersham Little Chalfont, 
UK). Muscle protein loading was verified by measuring 
the density of the myosin heavy chain band on the dried, 
Coomassie stained gel. Each blot contained 30 μg of 
patient’s sample and one control loaded at different protein 
concentrations. Measurements of the integrated density 
value of the bands were carried out using an Alpha Imag-
ing system. Antibody dilutions and exposure times were set 
using a control muscle sample to ensure that the ECL sig-
nal was linear for protein loading between 5 μg and 30 μg.

3. Results

Multiplex PCRs DNA analysis of dystrophin exons in 
patient 4 did not reveal deletion of the examined exons. 
Sequence analysis of the DNA fragment corresponding to 
the first muscle exon–intron junction demonstrated the 
ocurrence of a G to T transversion at position +1 of the 
5’ splice site consensus sequence. Dystrophin isoforms pro-
filed demonstrated that patient 4, similar to the previously 
reported patient 1, upregulates the B isoform in the skeletal 
muscle but not in the heart with complete absence of the M 
isoform in both tissues (Fig. 1).

Semi-quantitative RT-PCR analysis with co-amplifica-
tion of dystrophin and laminin α2 chain identified levels 
of dystrophin transcript corresponding to 32% of control 
in family 1; to 22% in family 2; to 44% in family 3 (data 
not shown; and previously reported in [31]). We were not 
able to perform this analysis in patient 4 because we did 
not have sufficient sample for these transcription studies. 
Indirect immunohistochemistry showed continuous expres-
sion of dystrophin on all fibres analyzed in the propositus 
of each family, with some variability in staining between 
adjacent fibres and with a reduced intensity compared to 
the control (Fig. 2). Immunolabelling of sarcolemmal β-
spectrin and laminin α2 was normal on all muscle fibres 
(not shown); utrophin was moderately upregulated in most 
fibres in the muscle of patient 2, as previously reported [31].

The result of the Western blot on the muscle samples 
from these four patients indicated that the dystrophin con-
tent was equivalent to 29% of that seen in normal controls 
in patient 2, to 37% in patient 1 and to 40% in patient 3. 
The highest level, equivalent to 57% of normal controls, 
was present in patient 4 (Fig. 3).

4. Discussion

Although the molecular defect of DMD and BMD has 
been identified in 1986, the development of successful ther-
apeutic strategies is still an unsolved challenge. In the early
phases of the disease, regenerative processes may reduce the effect of the absence of dystrophin and pharmacological interventions aimed at reducing muscle necrosis might prove to be helpful in this early phase. However, restoration of dystrophin production is the ultimate goal to achieve permanent benefit and technical difficulties will likely result in incomplete protein restoration/replacement at least for the foreseeable future.

An increase in sarcolemmal utrophin in BMD and DMD patients has been reported and its level of expression has been related to the progression of the disease [32]. The specific role of utrophin upregulation in the context of the dystrophic muscle is not defined but the amount of the protein product in DMD patients is not sufficient to protect the muscle from the degeneration [33]. In the only family we could study utrophin expression, this was not significantly upregulated compared to what was found in symptomatic BMD patients, so we assume that the protection of muscle symptoms in this patient is due to his ability to produce normal dystrophin.

There is likely to be a threshold level of protein that it is sufficient to maintain proper membrane organization and function, and prevent the degenerative process. XLDC provides a unique opportunity to explore what levels of normal dystrophin are sufficient to avoid the muscle weakness and degeneration of muscular dystrophy. XLDC patients typically have a mild subclinical myopathy, as indicated by the elevated serum CK and mild histological muscle changes but the levels of dystrophin produced are clearly sufficient to avoid progressive muscle degeneration and muscle weakness until at least the fourth decade of life. Previous work on the mdx mouse suggested that levels of 30% or more are protective for mice [34,35], but there is no information on how much dystrophin is required to avoid progressive muscle degeneration in humans. All the four XLDC patients described here produce reduced levels of a normal full length dystrophin transcript in the skeletal muscle, as a result of the upregulation of the Brain and Purkinje isoforms in skeletal muscle in the families 1, 2 and 4, and from the correct splicing of exons 11 and 12 in the muscle isoform in family 3 [24]. Detailed protein quantification using Western blot analysis indicated levels of dystrophin ranging from 29% to 57% of normal amount in these four families.

On the basis of all these observations we can conclude that dystrophin mRNA and protein levels comprised between 29% and 57% of control muscle are sufficient to avoid muscular dystrophy in the human, when the protein is uniformly present in all muscle fibres. It is interesting to note that the lowest level of dystrophin present in these patients (29%) is similar to the one obtained with transgenic experiments in which dystrophin has been introduced on the mdx background [34,35], and therefore indirectly validate the previous figure provided for the mdx mouse.

**Acknowledgements**

The Department of Health grant to F.M. is gratefully acknowledged. Thanks are also due to the TREAT-NMD EU grant (to F.M. and A.F.), to the Telethon-Italy Grant GGP05115 (to A.F.) and to the ICE-Italy grant (to A.F.).
References