European Journal of Orthodontics 33 (2011) 613–619
doi:10.1093/ejo/cjq113
Advance Access Publication 27 December 2010

Differential expression of myosin heavy chain isoforms in the masticatory muscles of dystrophin-deficient mice

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SUMMARY The dystrophin-deficient mouse (mdx) is a homologue animal model of Duchenne muscular dystrophy (DMD) and is characterized by slowly progressive muscle weakness accompanied by changes in myosin heavy chain (MyHC) composition. It is likely that the masticatory muscles undergo similar changes. The aim of this study was to examine the masticatory muscles (masseter, temporal, tongue, and soleus) of 100-day-old mdx and control mice (n = 8–10), and the fibre type distribution (by immunohistochemistry) as well as the expression of the corresponding MyHC messenger RNA (mRNA) (protein and mRNA expression, using Western blot or quantitative real-time polymerase chain reaction (RT-PCR)).

Immunohistochemistry and western blot analysis revealed that the masticatory muscles in the control and mdx mice consisted mainly of type 2 fibres, whereas soleus muscle consisted of both type 1 and 2 fibres. In the masseter muscle, the mRNA in mdx mice was not different from that found in the controls. However, the mRNA content of the MyHC-2b isoform in mdx mice was lower in comparison with the controls in the temporal muscle [11.9 versus 36.9 per cent; $P < 0.01$; mean ± standard error of the mean (SEM), Student's unpaired t-test], as well as in the tongue muscle (65.7 versus 73.8 per cent; $P < 0.05$). Similarly, the content of MyHC-2x isoforms in mdx tongue muscle was lower than in the controls (25.9 versus 30.8 per cent; $P < 0.05$).

The observed down-regulation of the MyHC-2x and MyHC-2b mRNA in the masticatory muscles of mdx mice may lead to changed fibre type composition. The different MyHC gene expression in mdx mice masticatory muscles may be seen as an adaptive mechanism to muscular dystrophy.

Introduction

The force–velocity properties of muscle fibres, responsible for differentiated muscle functions, are mainly dependent on their myosin heavy chain (MyHC) composition (Bottinelli *et al*., 1996). Myosin is a highly conserved, ubiquitous protein found in all eukaryotic cells, where it provides the motor function for diverse movements such as cytokinesis, phagocytosis, and muscle contraction. There are several MyHC isoforms of the skeletal muscle myosin that can be classified according to their contraction speed (Schiaffino and Reggiani, 1996; Sartorius *et al*., 1998) as fast (MyHC-2b, MyHC-2x, and MyHC-2a) and slow (MyHC-1). The genes coding for MyHC isoforms are both temporally and spatially regulated, and differential expression of MyHC isoforms contributes to the extensive diversity observed in skeletal muscle fibre types (Schiaffino and Reggiani, 1996).

The fibre type composition of different muscles varies during development, or in response to ageing or exercise, and is influenced by the geometry of the supporting anatomical structures (Gedrange and Harzer, 2004). Studies of the masticatory muscles of growing mice revealed that when there is a shift from sucking to mastication, the expression of MyHC-2b increases in tongue and masseter muscles (Gojo *et al*., 2002; Maejima *et al*., 2005). Furthermore, it is known that during muscle adaptation to the changes of the anatomical structure during development, the expression of messenger RNA (mRNA) for the MyHC of type 1 and 2 fibres may be changed. Animal studies indicate differential expression of MyHC mRNA in muscles of mastication during functional advancement of the mandible (Gedrange *et al*., 2001). Furthermore, changes in the MyHC mRNA amounts were found after orthognathic surgery in patients with malocclusions (Gedrange *et al*., 2006).

MyHC expression is also often influenced by diseases of the muscles. In human Duchenne muscular dystrophy (DMD), a reduced expression of MyHC-2b has been demonstrated (Webster *et al*., 1988) while in another study it was demonstrated that the masticatory muscles undergo significant pathological changes (Earnshaw *et al*., 2002). In DMD patients, as a result of dystrophic changes in the orofacial region and concomitant masticatory muscle dysfunction, a high prevalence of malocclusion has been

found. This disorder could be associated with feeding difficulties that would result in a significant weight loss (Pane *et al*., 2006; Manzur *et al*., 2008; Botteron *et al*., 2009).

An appropriate animal model to study MyHC expression in various dystrophic masticatory muscles is the dystrophin-deficient (mdx) mouse that is characterized by a loss of the protein dystrophin caused by a gene mutation (Bulfield *et al*., 1984; Koenig *et al*., 1989). As could be expected, changes in the MyHC composition have been found in the limb muscles of mdx mice (Bulfield *et al*., 1984). In a recent study, it was demonstrated that in 100-day-old mdx mice, the masseter, temporal, tongue, and soleus muscles contained an increased number of fibres with centralized nuclei, as compared with a control group (Spassov *et al*., 2010). Moreover, all muscles, except the tongue, displayed numerous inflammatory foci and accumulation of collagen, while a significantly increased mean fibre diameter was observed in masseter, temporal, and tongue muscles. These findings suggest that mdx muscles are unequally implicated in the development of the disease. Indeed, properties of muscles are mainly dependent on their fibre type composition and distribution. However, it is not clear whether histopathological changes in masticatory muscles of mdx mice found by Spassov *et al.* (2010) are accompanied by changes in MyHC composition.

Thus, the present study was undertaken to assess possible differences in the fibre type composition and mRNA expression of MyHC isoforms in masseter, temporal, tongue, and soleus muscles in mdx mice. Such changes could have impact on masticatory muscle function. At 100 days of age, in the mdx mice, regeneration processes overcome degeneration. However, there is a lack of data in the literature concerning the muscle fibre properties within newly regenerated fibres. Information from the present study may allow a more complete understanding of how masticatory muscles adapt to dystrophic changes.

Materials and methods

The protocol and all experimental procedures of the study were approved by the State Commission for Animal Protection of Mecklenburg–Vorpommern (LALLF M-V/TSD/7221.3-2.3-001/09).

Animals

Mice of the inbred strains C57Bl/10ScSn (control) and C57/Bl10ScSn-*Dmd*^{mdx}/J (mdx) were originally obtained from Harlan Winkelmann (Borchen, Germany) and Charles River (Sulzfeld, Germany). Both strains were bred in the section for animal care at the Department of Pathophysiology, Medical Faculty, University of Greifswald. The mice were kept under conventional conditions at a room temperature of 21–23°C, a humidity of 45–60 per cent, and a night–day frequency of 12 hours (Hogan *et al*., 1994).

Age-matched adult mice (100 days of age, each group n = 8–10) of both genders and the same approximate body weight (30 g) were killed by inhalation of overdose of ether. Muscle tissue samples were completely removed and immediately frozen in liquid nitrogen until use.

Immunohistochemistry

Serial cryosections (5 μm thick) from the middle of the masseter, temporal, tongue, and soleus were air dried, fixed with acetone for 10 minutes, and kept at −80°C until staining. Before staining, the thawed slices were fixed in acetone again for 10 minutes. Staining was performed following the manufacturer's instructions for the M.O.M. kit of Vectastain (Vector Laboratories, Burlingame, California, USA). The primary monoclonal anti-myosin (skeletal, fast) clone MY-32 (Sigma-Aldrich, Munich, Germany) was diluted 1:800, and the monoclonal anti-myosin (skeletal, slow) clone NOQ 7.5.4.D (Sigma) was used at a dilution of 1:3000. The bound antibodies were detected using ABC-AP (Vector Laboratories) and the new fuchsin alkaline phosphatase protocol with naphthol AS–BI phosphate as the substrate.

A 290 × 200 μm grid was randomly placed on each muscle section, and the number of MyHC-1 and MyHC-2 positive muscle fibres was counted to give a ratio of the total number of muscle fibres in the section. The test was repeated three times for each animal and the mean and ± standard error of the mean (SEM) for each group were calculated.

Western blot analysis

Murine tissue samples were mechanically homogenized during thawing in lysis buffer (5 per cent glycerol, 0.1 per cent Triton X-100) supplemented with a protease inhibitor cocktail (Sigma-Aldrich) using the SpeedMill P12 homogenizer (Analytikjena, Jena, Germany). The suspension was then centrifuged at ×11000 *g* for 1 hour at 4°C. The protein concentration of the supernatant (containing soluble proteins) was determined using the bicinchoninic acid method (Kruger *et al*., 2008). Protein samples (3–5 μg) of the soluble fractions were subjected to sodium dodecyl sulphate (SDS) gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) using a tank-blotting system (Invitrogen, Karlsruhe, Germany). Blots were incubated with monoclonal antibodies against MyHC proteins (dilution in phosphate-buffered saline containing 5 per cent powdered milk and 0.025 per cent NaN_3) at 4°C over night. Secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulins (Dako, Hamburg, Germany) were used at a 1:5000 dilution. Visualization and detection of bound antibodies were carried out using an enhanced chemiluminescence system (Perbio Science, Bonn, Germany). As the loading control of the gel, every membrane was stripped with Restore™ Plus Western blot stripping buffer (Perbio Science) and incubated with a monoclonal anti-α-actinin antibody (clone AT6/172,

Millipore, Billerica, Massachusetts, USA, dilution 1:1000, for 2 hours at room temperature). Quantitative analyses of protein bands from MyHC isoforms and α-actinin in the masticatory muscle of the 100-day-old C57Bl/10Sc and mdx mice were undertaken using GelScan 5.2 software (Serva, Heidelberg, Germany). Mean optical density ± SEM are given in all cases for $n = 4$ muscle samples and four independent Western blot analyses.

RNA extraction and reverse transcription

Total RNA was isolated using guanidinium isothiocyanate (RNeasy Fibrious Mini Kit, Qiagen, Hilden, Germany) and RNA concentration was determined by ultraviolet absorbance measurements. Two-hundred nanograms of total RNA was reverse transcribed using random hexamer primers and TaqMan Reverse Transcription Reagents (PE Applied Biosystems, Weiterstadt, Germany).

Preparation of DNA fragment standards

For real-time quantification by TaqMan, DNA fragments containing the target sequences of MyHC genes and those of 18S, ribosomal RNAs (rRNAs) were cloned in the pGEM-T-Easy cloning vector (Promega, Mannheim, Germany) according to the manufacturer's instructions and sequenced. Sequence identities were established by searching the databases (http://www.ncbi.nih.gov/BLAST/, 2010).

TaqMan real-time polymerase chain reaction (RT-PCR)

The methods used have been described previously (Kunert-Keil *et al*., 2006). To quantify expression of mouse MyHC genes, gene-specific TaqMan polymerase chain reaction (PCR) primers and probes (PE Applied Biosystems) were used (Table 1) with each probe having been synthesised with a fluorescent 5′-reporter dye (FAM: 6-carboxy-fluorescein) and a 3′-quencher dye (TAMRA: 6-carboxy-tetramethylrhodamine). Parallel TaqMan PCR assays for each gene target were performed with complementary DNA (cDNA) samples and genomic standards. Reaction mixtures contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 1× murine-specific primer and probe mixture (Table 1), or 1× eukaryotic 18S rRNA endogenous control (Applied Biosystems). To quantify MyHC gene expressions, 8 ng of reverse transcribed RNA was used in a 20 µl reaction volume. To amplify 18S rRNA (internal control), only 20 pg was used. PCR products were amplified (50°C, 2 minutes and 95°C, 10 minutes was followed by 40 cycles of 95°C, 15 seconds and 60°C, 1 minute) and analysed on an RT–PCR cycler (SDS 5700, Applied Biosystems). Absolute copy numbers of MyHC transcripts and 18S cDNA were determined using calibration curves generated with cloned PCR fragment standards. Copy numbers of individual MyHC transcripts are given in relation to those of 18S cDNA. A 'no-template control' with water was performed parallel in all experiments. Each series of experiments was carried out twice.

Table 1 Primers and probes for real-time polymerase chain reaction of myosin heavy chain (MyHC) transcripts.

Gene	Accession number	Assay on demand
Myh1 (MyHC-2x/d)	NM_030679	Mm01332489 m1
Myh2 (MyHC-2a)	NM_001039545	Mm00454982 m1
Myh4 (MyHC-2b)	NM_010855	Mm01332518 m1
Myh7 (MyHC-1)	NM_080728	Mm00600555 m1

Statistical analysis

Statistical analysis was performed using the SigmaPlot Software (Systat Software, San Jose, California, USA). The obtained values for the groups were compared using a Student's unpaired *t*-test. Data are given as the mean ± SEM. $P < 0.05$ was considered significant.

Results

Immunohistochemical analysis

The muscle fibre distribution into slow and fast types was investigated immunohistochemically using antibodies against slow (NOQ 7.5.4D) and fast MyHC (MY-32). The soleus muscle (Figure 1) of the controls contained 45.8 ± 4.4 per cent MyHC-1-positive fibres and 54.2 ± 4.4 per cent MyHC-2-positive fibres (total fibre amount = 549). In contrast, the masseter, temporal, and tongue muscles of the control mice (Figure 1) showed a predominance of MyHC-2-positive fibres. Staining with the antibody against the slow MyHC isoform in the masticatory muscles of the control mice was almost negative.

Stained tissue probes from mdx mice are presented in Figure 2. All three investigated mdx masticatory muscles revealed exclusively MyHC-2-positive fibres. In contrast to the normal mice, the mdx soleus showed an increased amount of MyHC-1-positive fibres and decreased expression of MyHC-2-positive fibres (56.6 ± 18.9 and 43.4 ± 14.5 per cent respectively; fibres = 576).

Western blot analysis

The masticatory muscles of the control and mdx mice consisted mainly of type 2 fibres (Figure 3A) and the soleus muscle type 1 and 2 fibres in both animal strains (Figure 3A and 3B). For the tongue and temporal muscle less type 1 protein was found as compared with the soleus muscle (Figure 3A).

Quantitative analysis of protein expression of MyHC-1 and MyHC-2 in mdx and control mice is shown in Figure 3B–3C. The protein expression of MyHC-2 in mdx

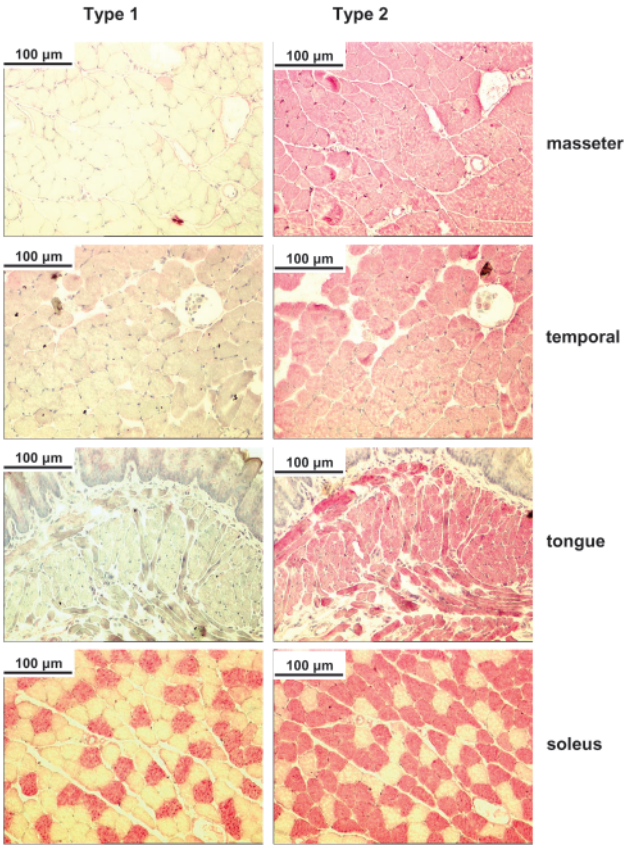

Figure 1 Distribution of slow (type 1) and fast (type 2) muscle fibres in the masseter, temporal, tongue, and soleus muscles of the control mice. It can be seen that the type 1 fibres (left column) are present only in the soleus muscle, while type 2 fibres (right column, type 2 fibres are also stained red) are present in all tested muscles.

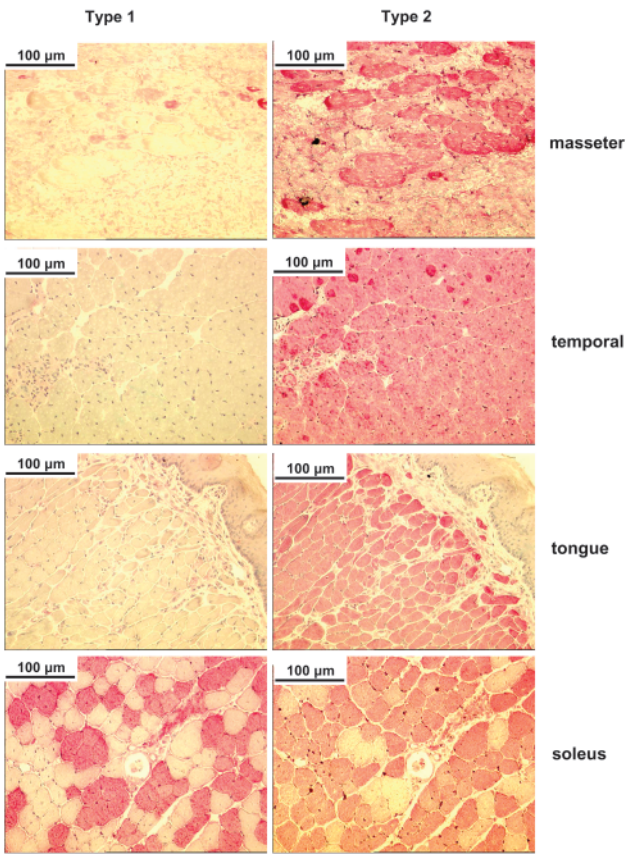

Figure 2 Distribution of slow (type 1) and fast (type 2) muscle fibres in masseter, temporal, tongue, and soleus muscles of mdx mice. Type 1 fibres (left column, coloured red) are present only in soleus muscle, while type 2 fibres (right column, fibres type 2 are also stained red) are present in all tested muscles.

tongue, temporal, and masseter muscles remained unchanged as compared with normal mice. The protein amounts of MyHC-2 in mdx tongue and temporal muscles were slightly increased in comparison with normal mice (Figure 3d). For the mdx soleus, the quantity of MyHC-1 proteins increased whereas the amounts of MyHC-2 remained unchanged as compared with the controls mice.

mRNA expression of MyHC isoforms

Since Western blot data did not distinguish between MyHC-2 isoforms (type 2a, 2x, and 2b), gene-specific TaqMan RT–PCR was performed to quantify the expression of the MyHC genes coding for MyHC-2b, MyHC-2x, and MyHC-2a and for MyHC-1 in the masseter, temporal, tongue, and soleus muscles. In the masseter, temporal, and tongue muscles of the control mice, a very low expression level of transcripts coding for MyHC-1 and MyHC-2a was found (Figure 4). The soleus muscle (Figure 4) in the control group consisted of equal amounts of MyHC-1 and MyHC-2 mRNAs.

In mdx mice, there was a significantly lower percentage of transcripts coding for MyHC-2b in both the temporal (80.5 per cent; $P < 0.01$) and tongue (56.3 per cent; $P < 0.01$) muscles, and of MyHC-2x in the tongue muscle (65.7 per cent; $P < 0.05$) as compared with normal mice (Figure 4). RT–PCR analysis performed on mdx masseter muscle revealed no significant differences in the mRNA expression of MyHC-isoforms between mdx and control mice (Figure 4). For the soleus muscle, however, the mdx strain showed a 2.5-fold ($P < 0.05$) elevated expression level of transcripts coding for MyHC-1 as compared with the controls (Figure 4).

Discussion

In the present study, changes in the gene expression of MyHC isoforms in mdx masticatory muscles, as compared with control mice, were demonstrated. At the transcriptional level in mdx masticatory muscles, a significantly lower percentage of transcripts coding for MyHC-2b was found for the temporal and tongue muscles. Decreased mRNA expression of MyHC-2b isoform in mdx temporal and tongue muscles has previously been observed in studies that estimated preferential degeneration of type 2 fibres in mdx limb and trunk muscles (Petrof *et al.*, 1993). In this context,

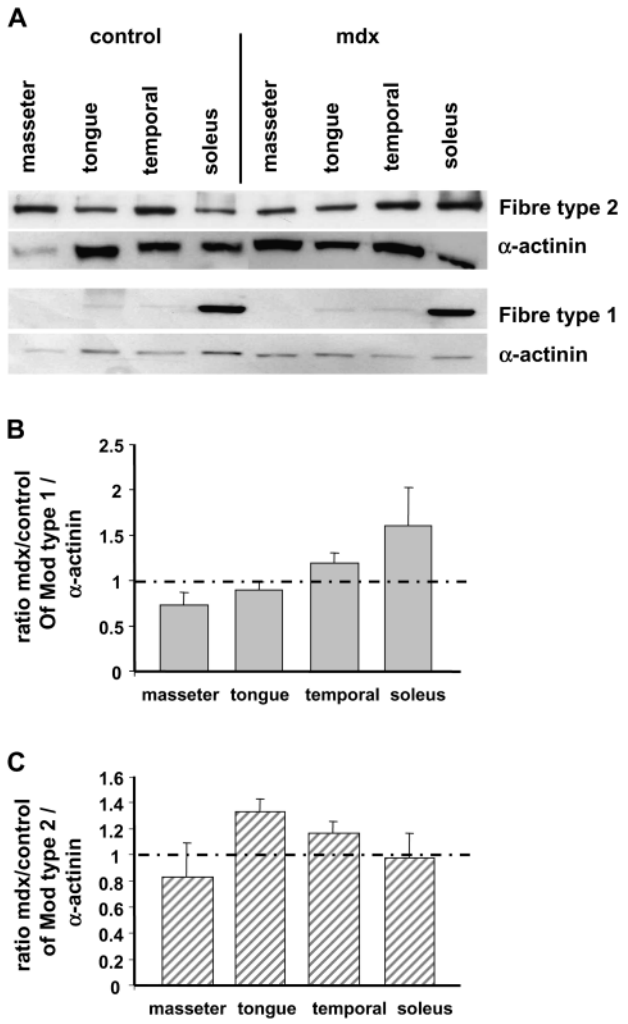

Figure 3 Skeletal muscle myosin heavy chain (MyHC) protein levels in different muscles of control and mdx mice. (A) Western blot analysis of slow and fast skeletal muscle myosin as compared with α-actinin in the soluble protein fraction of control and mdx mice masticatory muscles: masseter, temporal, tongue, and soleus muscles. Typical bands after western blot analysis for fast (type 2) and slow (type 1) MyHC protein. Ratios of the mean optical densities (Mod) of MyHC-1 as compared with α-actinin (mdx versus controls), for fibre type 1 (B) and fibre type 2 (C).

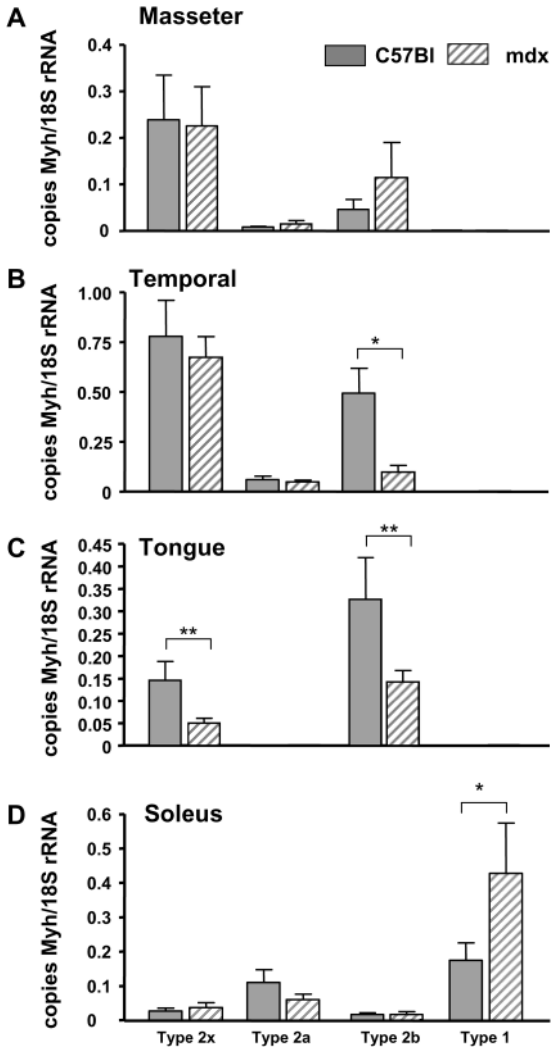

Figure 4 Ratio of the levels of Myh versus 18S ribosomal RNA (myosin heavy chain genes versus house keeping gene) from masseter, temporal, tongue, and soleus muscles in control and mdx mice. *$P < 0.05$, **$P < 0.01$.

it is reported that a subset of fast fibres, the 2b fibres, degenerate first in DMD patients (Webster *et al*., 1988). For the tongue muscle, however, the MyHC-2x expression was additionally diminished, in contrast to the temporal and masseter muscles, where the MyHC-2x expression remained stable. The presence of higher MyHC-2x isoforms in mdx massetter and temporal muscles, as compared with mdx tongue muscle, may correspond to some specific adaptive changes related to functional demands such as more precise modulation of mandibular position and occlusal force.

In contrast to the masseter, temporal, and tongue muscles of mdx mice, the mdx soleus muscle in the present study contained a higher percentage of type 1 fibres than those of the control mice. It appears that this muscle undergoes significant transition towards a larger proportion of slow fibres. These findings are confirmed by the results of the analysis of mRNA expression (Figure 4d), that are in agreement with the results of a gene expression study, suggesting that up-regulation of slow muscle genes specifically characterizes limb muscles in dystrophin and utrophin deficient mice (Baker *et al*., 2006). These findings from mdx soleus and masticatory muscles imply intermuscular differences in the progression of muscular dystrophy.

Interestingly, the results of the present research reveal discrepancies between the findings at the protein and transcription levels. In mdx muscles, Western blot analysis revealed higher type 2 fibre expression, as compared with the controls which is in contrast to the decreased levels of MyHC-2b and MyHC-2x expression found in the same muscle samples. This might be explained by the fact that changes in gene expression, of MyHC

during transcription may not be immediately translated into proteins (myofibrills). Gene regulation may involve different steps in gene expression, and changes in MyHC expression may therefore sometimes not occur at the protein level. Additionally, the anti-myosin (fast, type 2) antibodies used in the present study do not distinguish between MyHC-2 isoforms including foetal MyHC isoforms that may contribute to the observed difference between mRNA and protein expression of MyHC. Regarding type 1 fibres, Western blot data revealed a constant expression level for all investigated masticatory muscles, whereas RT–PCR data did not show any expression of MyHC-1. The expression of type 1 fibres at the protein level observed in mdx masticatory muscles probably results from the persistence of foetal and intermediate MyHC-2x that cannot be distinguished with the use of antibodies for type 1 and type 2. In this regard, it has been reported that functional correlation of the histochemical typing of mdx muscles is difficult because foetal MyHC may mask the characteristics of adult isoforms (Pastoret and Sebille, 1993).

In contrast to temporal and tongue muscles, the results of this study showed, at the transcription level, that the masseter muscle of 100-day-old mdx mice exhibited a similar MyHC expression pattern as the controls. These findings are in accordance with a study on masseter muscle in mdx mice of the same age, where some limited modifications in fibre type patterns were observed between mdx mice and controls (Muller *et al*., 2001).

Differences in the expression of MyHC isoforms in masseter muscle between mdx and control mice were found in a study, where the masseter muscle from 2- to 9-week-old mdx mice expressed low amounts of MyHC-2b in contrast to control mice (Lee *et al*., 2006). Weaning-associated MyHC isoform changes (Gojo *et al*., 2002) and the age difference of the examined animals cannot fully explain these contradictory results. It appears that after 10 weeks of age, muscle fibres of mdx mice seem to become stabilized and do not undergo further degeneration and regeneration (DiMario *et al*., 1991). There are certainly regional variations in fibre type distribution that are difficult to avoid when small samples are used (Widmer *et al*., 2002). In addition, the geometry of the masseter, which has an extreme pinnate form, may imply different loading along its cross-section that could also influence variation in muscle fibre type distribution. It was not possible to achieve the high precision required during sampling and this could have affected the results.

The above-mentioned studies together with the present results, indicate that mdx masseter muscle adapts to dystrophic processes from initially changing its MyHC composition towards reconstituting its characteristic fibre composition that may be seen as an attempt to maintain functional requirements.

Conclusions

The dystrophic disease progression in mice is not homogeneous because differential changes in MyHC expression were found in the masticatory muscles of 100-day-old mdx mice. Moreover, the findings suggest intermuscular differences in the response to dystrophy deficiency, i.e. the characteristic transition for mdx soleus muscle from MyHC-2 to MyHC-1 is not present in mdx masseter, temporal, and tongue muscles. Therefore, studies on the mdx mouse model are important and could permit elucidation of the influences of the dystrophin deficiency on fibre properties of the masticatory muscles.

Acknowledgements

We wish to thank I. Pieper for excellent technical assistance.

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