Short communication

THE EXPRESSION OF MYOGENIC REGULATORY FACTORS AND MUSCLE GROWTH FACTORS IN THE MASTICATORY MUSCLES OF DYSTROPHIN-DEFICIENT (MDX) MICE

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Abstract: The activities of myogenic regulatory factors (MRF) and muscle growth factors increase in muscle that is undergoing regeneration, and may correspond to some specific changes. Little is known about the role of MRFs in masticatory muscles in mdx mice (the model of Duchenne muscular dystrophy) and particularly about their mRNA expression during the process of muscle regeneration. Using Taqman RT-PCR, we examined the mRNA expression of the MRFs myogenin and MyoD1 (myogenic differentiation 1), and of the muscle growth factors myostatin, IGF1 (insulin-like growth factor) and MGF (mechano-growth factor) in the masseter, temporal and tongue masticatory muscles of mdx mice (n = 6 to 10 per group). The myogenin mRNA expression in the mdx masseter and temporal muscle was found to have increased (P < 0.05), whereas the myostatin mRNA expressions in the mdx masseter (P < 0.005) and tongue (P < 0.05) were found to have diminished compared to those for the controls. The IGF and MGF mRNA amounts in the mdx mice remained unchanged. Inside the mdx animal group, gender-related differences in the mRNA expressions were also found. A higher mRNA expression of myogenin and MyoD1 in the mdx massterer and temporal muscles was found in females in

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comparison to males, and the level of myostatin was higher in the masseter and tongue muscle (P < 0.001 for all comparisons). Similar gender-related differences were also found within the control groups. This study reveals the intermuscular differences in the mRNA expression pattern of myogenin and myostatin in mdx mice. The existence of these differences implies that dystrophinopathy affects the skeletal muscles differentially. The finding of gender-related differences in the mRNA expression of the examined factors may indicate the importance of hormonal influences on muscle regeneration.

Key words: Mdx mice, Dystrophy, Masticatory muscles, MyoD1, Myogenin

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a progressive and fatal disease involving muscle degeneration caused by mutations in the gene encoding for the cytoskeletal protein dystrophin [1]. The muscles of DMD patients have been demonstrated to undergo massive pathological changes resulting in muscle weakness and the impairment of general mobility. Malocclusions that are probably due to decreased masticatory muscle function occur in later stages of muscular dystrophy [2]. In DMD patients, masticatory muscle dysfunctions and a high prevalence of malocclusions associated with feeding difficulties and weight loss are seen as the results of dystrophic changes in the orofacial region [3-5]. There is an urgent need for a thorough investigation of the pathological events occurring during masticatory muscle atrophy, especially the changes in the expression of myogenic regulatory factors, in order to reach a more profound understanding of the disease and develop therapeutic strategies for DMD patients.

In the animal model of DMD, the degeneration and regeneration processes in mdx muscles are associated with histopathological features, such as centronucleated fibres, increased collagen expression and fibrosis, variations in the size of the muscle fibres, and the presence of phagocyte cells [6-8], as well as an apparent stability with ageing [9].

The regeneration process in post-natal muscle fibres requires the activation of satellite cells to restore damage or loss of myofibres. A number of myogenic regulatory factors (MRFs) are involved during the process of regeneration. The MRFs are members of a super-family of basic helix-loop-helix transcription factors that induce the differentiation of pluripotent and satellite cells into muscle cells [10]. In vertebrates, they consist of MyoD1 (myogenic differentiation 1) and Myf-5 (myogenic factor 5), which are expressed before and after muscle differentiation, and myogenin and MRF4 (myogenic factor 4), which are expressed during muscle differentiation [11]. While myogenin plays an essential role in the terminal differentiation of myoblasts into myotubes, MyoD1 is essential for the differentiation potential of skeletal myoblasts [12].

The further division and differentiation of regenerated muscle cells is influenced by growth factors such as myostatin, which inhibits muscle development, and insulin-like growth factor (IGF1) and its splice variant, mechano-growth factor (MGF), which stimulates muscle growth [13-14]. Interestingly, it has been reported that oestrogen may also influence the degree of disruption and post-damage inflammatory response in the skeletal muscles [15-17]. Moreover, female homozygous dystrophic canines (CXMD) appeared less affected than their male counterparts [18]. It seems that MRF activity and growth factors accompany various processes, and they could even be considered as markers for the underlying pathophysiological disturbances of degenerative or regenerative processes. Unfortunately, in comparison to the limb and trunk muscles, the mdx masticatory muscles have been less investigated with regards to the influence of MRFs and growth factors.

Furthermore, mdx skeletal muscles often present varying patterns of histopathology [19]. In a recent preliminary study from our laboratory, we also confirmed such intermuscular differences for the masticatory muscles of 100-day old mdx mice [20]. Thus, mdx masseter and temporal muscles contained mostly regenerated fibres, some inflammatory foci and increased collagen content, whereas the mdx tongue muscles seemed almost to have been spared by dystrophinophinopathy. These intermuscular differences were also seen in the expression of the MyHC isoforms. Mdx tongue and temporal muscles revealed decreased MyHC-2b and -2x mRNA MyHC expression, whereas the mdx masseter showed no changes. However, the mdx soleus muscle revealed a shift from fast to slow type fibres [21]. In mdx mice, degeneration occurs at the age of 4 weeks followed by muscle fibre regeneration [1, 8], and at the age of 100 days, almost all of the fibres have regenerated and express characteristic centrally situated nuclei [20]. Therefore, the objective of this study was to evaluate the expression of mRNA for all MRFs and for three of the growth factors (myostatin, IGF and MGF) in regenerated mdx orofacial muscles, as well as to evaluate whether the intermuscular differences in mdx masticatory muscles are associated with differences in the expression of the mRNA of MRFs and growth factors. An additional objective was to see whether these potential changes are gender related. The findings of this study will contribute to a more complete picture of regeneration processes in dystrophic orofacial muscles.

MATERIAL AND METHODS

Animals

Mice of the inbred strains C57Bl/10ScSn (control and C57/Bl10ScSn-*Dmd*y (mdx) were originally obtained from Harlan Winkelmann (Borchen, Germany) and Charles River (Sulzfeld, Germany). The female mdx mice were homozygotic (mdx/mdx). Both strains were bred in the animal care section of the Department of Orthodontics of the Medical Faculty at the University of Greifswald. Age-matched adult mice (100 days old, each group n = 6-10) of both

genders were killed using an ether overdose by inhalation in a manner approved by the state committee for animal protection. Muscle tissue samples were taken from the superficial part of the masseter, the middle of the temporal muscle and the superior longitudinal tongue muscle, and frozen in liquid nitrogen.

RNA extraction and reverse transcription
Total RNA was isolated using guanidinium-isothiocyanate (RNeasy Fibrious Mini Kit, Qiagen, Hilden, Germany) and the RNA concentration was determined via UV absorbance measurements. An amount of 200 ng of total RNA was reverse transcribed using random hexamer primers and TaqMan Reverse Transcription Reagents (PE Applied Biosystems, Weiterstadt, Germany).

TaqMan RT-PCR
To quantify the expression of different mouse genes, we purchased gene-specific TaqMan PCR primers and probes from PE Applied Biosystems (Weiterstadt, Germany) and TIBMOLBIOL (Berlin, Germany; Tab. 1). The procedure was performed as described previously [22-23] using a real-time PCR cycler (StepOne Plus, Applied Biosystems). The gene-specific mRNA concentration in the mdx mice relative to the controls was calculated using the $2^{-\Delta\Delta CT}$ method [24], and is given in relation to those of the 18S cDNA. A "no-template control" with water was performed in parallel in all experiments. Each series of experiments was performed twice.

Tab. 1. The gene-specific TaqMan PCR primers and probes.

A – Primers and probes for real-time RT-PCR obtained from PE Applied Biosystems

Gene	Accession number	Assay-on-demand
Myf4 (myogenin)	NM_031189	Mm00446194_m1
MyoD1	NM_010866	Mm00440387_m1
IGF1	NM_184052	Mm01233960_m1
MSTN (myostatin)	NM_010834	Mm03024050_m1

B – The primers and probes for real-time RT-PCR obtained from TIBMOLBIOL

Gene	Accession number	Sequence 5'-3'
MGF	AK081019	for: AAATGTGACATTGCTCTAACATCTCC rev: GCTGATTTTCCCCATCGCTT probe: TTTTCGCCTCATTATCCCTGCCCA

Abbreviations: Myf4 – myogenic factor 4 (myogenin); MyoD1 – myogenic differentiation factor 1; IGF1 – insulin-like-growth factor 1; MSTN – myostatin; MGF – mechano-growth factor.

SDS polyacrylamide gel electrophoresis
Protein samples (30 μg) of the soluble fractions from mechanically homogenized murine tissue samples were applied to SDS (sodium dodecyl sulphate) gels and stained with Roti®-Blue (Carl Roth GmbH, Karlsruhe, Germany). Descriptive

analyses of all of the protein bands in the molecular weight range of 30 to 50 kDa were done using the GelScan 5.2 software (Serva, Heidelberg, Germany).

Statistical analysis
The statistical analysis was performed using the SigmaStat Software (Systat Software, Inc. San Jose, CA, USA). The obtained values for groups were compared using Student's unpaired t-test. Subgroup differences were compared using Two-Way ANOVA and Bonferroni post hoc test. The data is given as the means ± SEM. $P < 0.05$ was considered statistically significant.

RESULTS

In this study, we found no significant differences in the body weight between mdx and control mice (controls: 28.6 ± 4 g; mdx: 32.5 ± 3.9 g) nor between the male and female animals in the two groups (male vs. female; control 28.0 ± 3.9 g vs. 29.2 ± 4.2 g; mdx 32.0 ± 4.3 g vs. 33.0 ± 3.6 g). Analysis of Coomassie-stained polyacrylamide gels showed the protein expression of myogenin and MyoD1 in the molecular weight range of 30 to 50 kDa (descriptive data, not shown).

mRNA expression of MRF and growth factors in mouse orofacial muscles
The myogenin mRNA expression in mdx masseter and temporal muscles was higher than in normal mice ($P < 0.001$, both; Fig. 1 and Tab. 2). By contrast, the myostatin mRNA expression in mdx masseter and tongue muscles (Tab. 2) was diminished ($P < 0.001$ and $P < 0.05$, respectively) compared to the control masseter and temporal muscles. The mRNA expression of MyoD1, IGF-1 and MGF in all of the tested mdx orofacial muscles did not differ from that for the controls (Fig. 1 and Tab. 2)
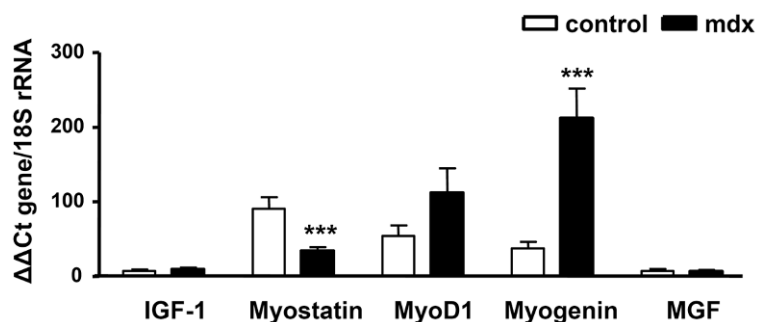

Fig. 1. The ratio of the levels of mRNA expression (relative to 18S rRNA) of the IGF-1 (insulin-like-growth factor 1), myostatin, MyoD1 (myogenic differentiation factor 1), myogenin (Myf4) and MGF (mechano-growth factor) genes between mdx and control mice in the masseter muscle. Filled boxes: mdx mice; open boxes: control mice. The results are the means ± SEM. Stars indicate significant differences: * $P < 0.05$, *** $P < 0.001$ (unpaired Student's t-test).

Tab. 2. The differences in expression of mRNA for growth factors in the masticatory muscles of mdx and control mice.

	Mdx	Control	P
Temporal			
Myogenin	445.28 ± 44.72	64.98 ± 29.77	
Myostatin	142.57 ± 52.10	217.68 ± 66.31	
MyoD1	275.02 ± 49.65	171.26 ± 35.90	< 0.001
IGF-1	41.77 ± 9.15	40.68 ± 15.83	
MGF	14.42 ± 3.92	12.31 ± 3.81	
Tongue			
Myogenin	459.04 ± 64.61	405.67 ± 47.60	
Myostatin	55.14 ± 10.64	98.19 ± 11.41	
MyoD1	42.66 ± 7.23	36.70 ± 4.26	< 0.05
IGF-1	11.89 ± 2.23	17.58 ± 2.65	
MGF	10.16 ± 1.23	12.98 ± 1.91	

The values are the mRNA expressions for each growth factor (myogenin, myostatin, MyoD1, IGF-1 or MGF) given as means ± SEM and relative to the 18S rRNA. n = 6-10; P = significance level of the difference between the mdx and controls. The obtained values for groups were compared using Student's unpaired t-test.

Tab. 3. Gender-based differences in the expressions of mRNA for the growth factors in the masticatory muscles of mdx and control mice.

	Mdx			Control		
	Female	Male	P	Female	Male	P
Masseter						
Myogenin	504.9 ± 126.1 (7)	88.04 ± 16.96 (7)	< 0.001	46.74 ± 7.06 (7)	8.34 ± 2.17 (7)	< 0.001
Myostatin	1355.58 ± 87.79 (7)	16.04 ± 3.81 (10)	< 0.001	2114.9 ± 298.47 (6)	114.59 ± 20.99(11)	< 0.001
MyoD1	130.31 ± 14.90 (7)	33.21 ± 4.1 (7)	< 0.001	71.03 ± 13.15 (7)	28.90 ± 5.33 (7)	< 0.001
Temporal						
Myogenin	317.71 ± 43.06 (7)	185.88 ± 34.88 (6)	< 0.001	71.267 ± 8.8 (6)	62.49 ± 6.13 (7)	NS
Myostatin	1732.51 ± 378.83 (10)	1160.88 ± 293.91 (11)	NS	3612.17 ± 450.33 (10)	12324 ± 2427.347 (10)	< 0.001
MyoD1	112.10 ± 13.61 (7)	41.98 ± 6.18 (6)	< 0.001	114.25 ± 12.51 (6)	128.67 ± 11.72 (7)	NS
Tongue						
Myogenin	291.99 ± 44.7 (7)	303.77 ± 84.85 (7)	NS	162.33 ± 12.29 (7)	182.37 ± 36.94 (7)	NS
Myostatin	943.18 ± 152.32 (10)	62.55 ± 17.66 (11)	< 0.001	1478.09 ± 159.13 (9)	61.31 ± 8.84 (11)	< 0.001
MyoD1	39.89 ± 5.4 (7)	35.7 ± 6.97 (7)	NS	25.41 ± 1.79 (7)	23.2 ± 3.58 (7)	NS

The values are the mRNA expressions for each growth factor (myogenin, myostatin or MyoD1) given as means ± SEM and as relative to the 18SrRNA. The number in brackets is the number of animals in the group. P = significance level of the difference between male and female, assessed using two-way ANOVA followed by the Bonferroni test; NS = not significant.

Gender differences in the mRNA expression pattern of MRFs
To find out if gender influences the mRNA expression pattern of myogenin, myostatin and MyoD1 in mdx orofacial muscles, we compared their amounts in male and female animals in the two groups (Tab. 3). The growth factors IGF-1 and MGF were not included because of their low and equal amounts in the mdx and control mice (Fig. 1).

An increased mRNA expression of myogenin and MyoD1 in females in comparison to males was found in mdx masseter and temporal muscle as compared to the controls (P < 0.001 for all comparisons). The myostatin mRNA expression was found to be increased in mdx masseter and mdx tongue muscle (P < 0.001 for all comparisons). Similar gender differences were found also between male and female control mice.

DISCUSSION

This study is a follow-up to a preliminary study on mdx orofacial muscles, which revealed that masseter and temporal muscles were affected by dystrophinopathy, while mdx tongue muscles were partially spared [20]. It was also found that these changes were accompanied by intensive regeneration. Normal skeletal muscles, where there is no intensive regeneration, do not show increases in growth regulatory factors [25-26]. However, these factors have been shown to increase during regeneration. In many recent studies, increased myogenin mRNA levels in mdx gastrocnemius muscles [25] or in the tibialis anterior [27], and increased myogenin expression on the protein level in soleus muscles [28] (90- to 100-day old mdx mice) were detected in the regeneration stages. Interestingly, the functional myogenic gene seems to be essential for survival. It was suggested that myogenin plays an essential role for the terminal differentiation of myoblasts into myotubes [11]. It has been shown that animals with a targeted mutation in the myogenin gene are immobile and die perinatally due to a deficiency in their skeletal muscle differentiation [29-30].

A novel finding of our study is that during regeneration, the mRNA expression of myogenin in mdx masseter and temporal muscles increased, and the mRNA expression of myostatin in mdx masseter and tongue muscles decreased in comparison to controls. This study also revealed differences in the mRNA expression of MRFs in the masticatory muscles of male and female mice. These findings, although new for the masticatory muscles, have already been demonstrated in other skeletal muscles at the transcriptional or protein levels.

The high expression levels of myogenin mRNA in young (100-day old) mdx temporal and masseter muscles seems closely related to muscle fibre regeneration. Contrary to the other masticatory muscles, we did not find changes in the level of mRNA for myogenin in the tongue muscles of mdx mice. It is likely that the unchanged mRNA level of myogenin in mdx tongue muscles could explain our earlier findings of only a few regenerated fibres in the mdx tongue muscles [20].

The MyoD1 mRNA expression in all the investigated mdx muscles was at a level close to that found in the control mice. These findings concur with previous studies, where the mRNA expression of MyoD1 showed basal levels in recently regenerated mdx hind-limb muscles [25, 27-28]. These studies, as well as our findings, permit the suggestion that the mRNA expression of MyoD1 may be elevated in the early stages of regeneration during myoblast determination [31], and during active regeneration, and that it decreases to its basal levels when regeneration is terminated.

Since dystrophinopathy is associated with muscle damage, which is followed by muscle fibre growth, we expected that the expression of myostatin, an inhibitor of muscle growth, would be reduced, thereby enabling hypertrophy. Indeed, decreased myostatin mRNA levels were also previously found in the mdx tibialis anterior muscle [32]. These findings could permit speculation that myostatin may be a therapeutic target that influences positive muscle regeneration in muscular dystrophy. However, myostatin mRNA expression, although reduced, was still found in all studied mdx orofacial muscles. It seems that other mechanisms in the process of myogenesis exist, and these require a minimal amount of myostatin [33].

The results of this study show that the mRNA expressions of MRFs and the growth factors of the different masticatory muscles vary significantly, although they have similar functions. MRFs are mainly expressed by activated satellite cells. It was recently suggested that satellite cells have different properties that may be activated for different tasks [34]. Thus, satellite cells, which undergo limited proliferation and therefore express limited concentrations of MRFs, may be geared to perform homeostatic functions. On the other hand, during muscle regeneration, the synchronic activation and expansion of the whole pool of satellite cells is required [34]. These satellite cells may express increased levels of MRFs in regenerating mdx muscles and contribute to the observed differences vis à vis control mice.

Furthermore, we suggest that the protein expression of myogenin and MyoD1 in mdx masticatory muscles had not differed from that found in control mice (descriptive data, not shown). This would be surprising and could be explained either by the lack of changes in the mRNA expression levels of MRFs and growth factors, or by the fact that gene expression is not immediately reflected in protein expression [40]. A complete analysis including a western blot of the expressions of all of the factors at the protein level would provide a complete picture of the events involved in mdx muscle regeneration.

Indeed, in the masseter and temporal muscles, the mdx females showed more pronounced myogenin and MyoD1 mRNA expression than the mdx males. This could imply female hormone influence and the possibility that regeneration processes could be more intense in female mdx mice. Recent studies support this suggestion, showing that female hormones such as oestrogen may promote muscle regeneration in the regions of lesions in otherwise normal skeletal muscles or in mdx mice [16, 17, 35]. Interestingly, it is reported that in canine

muscular dystrophy, homozygous females are less affected than their male counterparts [18]. Another study provided direct evidence that oestrogen induces MyoD1 mRNA expression in mouse skeletal muscles *in vivo* and in dividing myoblasts *in vitro* [36]. All these mutually supportive results are in accordance with our findings that female sex hormones may have an important influence on myogenin and MyoD1 mRNA expression.

The importance of our results arises further from reports that in mouse masseter muscles, myosin heavy chain expression is also sexually dimorphic and driven by sexual hormones [37]. Because MyoD1 and myogenin are key factors in myogenesis, we suggest that they may contribute to the observed dimorphic expression of myosin heavy chains. Further analysis of the results revealed that the myostatin mRNA expression, although low, was found to be relatively higher in the female masseter and tongue of both strains, when compared to their male counterparts. Thus, these results lend additional support to the above suggestion that myostatin might be required for initiating myogenesis [38-39]. The higher relative myostatin mRNA content in female mdx mice may contribute, via an as yet unknown mechanism, to the milder dystrophic character in female mdx mice and canine muscular dystrophy of homozygous females [18, 36]. The gender-related differences in the expression patterns the mRNA of MRFs and muscle growth factors found in our study indicate therefore that female hormones may influence the regulation of these factors at the transcriptional level. To the best of our knowledge, this is the first study of the mRNA expression of MRFs in mdx orofacial muscles. The effects of these opposing but synergistically acting growth factors should be verified at the level of protein expression in the orofacial musculature of the mdx mice.

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