Tongue muscle is protected from dystrophic changes in the Duchenne Muscular Dystrophy Rat

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Abstract

Background: Duchenne muscular dystrophy (DMD) is an X-linked muscle disease caused by a complete lack of dystrophin, which stabilizes the plasma membrane of myofibers. The orofacial function is affected in an advanced stage of DMD and this often leads to an eating disorder such as dysphagia. Dysphagia is caused by multiple etiologies including decreased mastication and swallowing. Therefore, preventing the functional declines of mastication and swallowing in DMD is important to improve the patient's quality of life. In the present study, using a rat model of DMD we generated previously, we performed analyses on masseter and tongue muscles, both are required for proper eating function.

Methods: Age-related changes of masseter and tongue muscle of DMD rats were analyzed morphometrically, histologically, and immunohistochemically. Also, gene expressions of p16, a marker of cellular senescence, and utrophin, a functional analog of dystrophin, were examined.

Results: The masseter muscle of DMD rats showed progressive dystrophic changes as observed in their hindlimb muscle accompanied by increased expression of p16. On the other hand, the tongue of DMD rats showed macroglossia due to hypertrophy of myofibers without degenerative/necrotic changes. Proliferative activity was preserved in the satellite cells from the tongue muscle but was perturbed severely in those from the masseter muscle. While utrophin expression was increased in the masseter muscle of DMD rats compared to WT rats, probably due to compensatory mechanism, its expression level in the tongue muscle was comparable between WT and DMD rats and was similar to that in the masseter muscle of DMD rats.

Conclusions: The tongue muscle of the rat is protected from degenerative/necrotic dystrophic changes despite the absence of dystrophin.

Background

Duchenne muscular dystrophy (DMD) is an X-linked muscle disease caused by out-of-frame mutations in the dystrophin gene that results in the complete loss of dystrophin protein [5]. Dystrophin is a structural protein that stabilizes the plasma membrane of the myofibers, and the lack of dystrophin protein causes myofibers to become fragile [5]. Skeletal muscle myofibers normally have remarkable regenerative capacity [7]. Once myofibers are damaged, the myogenic stem/progenitor cells, satellite cells, are activated and proliferate, then eventually fuse each other to form multinucleated myotubes. The myotubes mature to constitute newly regenerated myofibers. DMD is characterized by repeated cycles of degeneration and regeneration of myofibers, which finally result in a loss of regenerative capacity due to an exhaustion of satellite cell pool [14]. The DMD patients show progressive weakness and premature death due to a loss of ambulation [5].

In an advanced stage of DMD, orofacial function is affected [1] and eating disorders such as dysphagia are often a problem [25]. Dysphagia is caused by multiple etiologies. Decreased mastication is one of the causes of dysphagia. Masticatory movement involves masseter, temporalis, medial and lateral pterygoid

muscles [10]. Masticated foods are then swallowed using tongue muscle in the oral cavity. Several reports demonstrated that dysphagia in DMD patients is associated with dystrophic changes in muscles related to mastication [24]. Macroglossia, which is common in DMD patients [19, 25] is defined as an enlarged tongue and is often associated with dysphagia [2]. Therefore, preventing the deterioration of mastication and swallowing functions in DMD is important to improve the patient's quality of life.

We previously reported the generation of a rat model of DMD (DMD rat) [15, 22]. DMD rats show a progressive muscle pathology in their hindlimb muscle and diaphragm like human DMD [15, 22]. The pathological changes seen in the muscle of DMD rats include decreased strength, increased fibrosis, and fatty infiltration [15, 22]. We further demonstrated the occurrence of premature cellular senescence in the muscle of DMD rats and suggested that this could be one of the causes of satellite cell depletion, which leads to a loss of regenerative capacity [22].

In the above our studies using DMD rats [15, 22], we analyzed only the muscle of hindlimbs and diaphragm. During breeding and maintenance of DMD rats, we had experienced a decreased food intake in accordance with the decrease in the body weight in their later life, and this prompted us to examine whether the muscles related to food intake are also affected. Thus, in this study, we focused on the masseter and tongue muscles as the muscles involved in mastication and swallowing and investigated their development and histological changes, as well as the dynamics of satellite cells.

Methods

Animals

We previously generated and characterized a strain of DMD rat [15, 22]. Adult $X^{Dmd}X$ female rats were mated with wild-type (WT) male rats to generate male WT (XY) and DMD ($X^{Dmd}Y$) rats. They were maintained under controlled environmental conditions, at 23°C with a light/dark cycle (lights on 0800–2000). Laboratory chow (Labo MR Standard, Nihon Nousan Co., Yokohama, Japan) and water were given ad libitum. All animal experiments performed in this study were in accordance with the Guide for the Care and Use of Laboratory Animals of the University of Tokyo and were approved by the Institutional Animal Care and Use Committee of the University of Tokyo (P18-125).

Measurement of food intake

At 3- or 7-month-old, the rats were transferred individually to a plastic cage and their amount of food intake was measured for two consecutive days. The measured values were divided by 2 and expressed as food intake per day.

Histological and immunohistochemical analyses

At indicated age, the rats were killed by inhalation of carbon dioxide gas. After removal of facial skin, the area corresponding to the masseter muscle was measured. The masseter muscle was considered as an ellipse (Fig. 2A), and its long and short diameters were measured with calipers to calculate the area. Then,

the lower half part of the masseter muscle was removed. Tongue was cut out at the root. Collected tissues were fixed in 4% paraformaldehyde (PFA) in phosphate buffered-saline (PBS) for paraffin-embedded sections or snap-frozen in liquid nitrogen-cooled isopentane for cryosections.

The paraffin-embedded sections were used for hematoxylin-eosin (HE) or Masson's trichrome staining. The sections were observed and photographed using a microscope (BX51, Olympus, Tokyo, Japan) equipped with a digital camera (DP73, Olympus). The HE-stained tongue sections were used for measurement of width and area of the tongue (Fig. 3). Masson's trichrome-stained sections were used for quantitative analysis of the fibrotic area. Two fields were randomly selected in the section using a 4× objective and the area occupied by fibrotic tissues stained blue and the total area of sections were calculated using ImageJ software (v1.47; National Institutes of Health, Bethesda, MD, USA).

The cryosections (7 µm) were used for immunohistochemistry. The sections were fixed with 4% PFA in PBS for 15 min, followed by blocking with 5% normal donkey serum (NDS) in PBS. The sections were incubated with primary antibody(ies) overnight at 4°C, then secondary antibody(ies) for 1 h. For identification of satellite cells, anti-Pax7 (1:200 with 5% NDS in PBS, mouse, clone P3U1; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA) and AlexaFluor-labeled donkey anti-mouse IgG (1:500 with 5% NDS in PBS, Jackson ImmunoResearch, West Grove, PA, USA) were used as primary and secondary antibodies, respectively. For identification of necrotic myofibers, anti-laminin (1:400 with NDS in PBS, rabbit, L9393 Sigma, St Louis, MO, USA) was used as primary antibody, and AlexaFluor-labeled goat anti-rat IgG and donkey anti-rabbit IgG (1:500 with 2.5% NDS/2.5% normal goat serum (NGS) in PBS, Jackson ImmunoResearch, West Grove) were used as secondary antibodies. For identification of newly formed regenerated myofibers, anti-embryonic myosin heavy chain (eMHC) (1:400 with with NDS in PBS, mouse, clone F1.652, DSHB) and anti-laminin were used as primary antibodies, and AlexaFluor-labeled donkey anti-mouse IgG and donkey anti-rabbit IgG (1:500 with 5% NDS in PBS, Jackson ImmunoResearch) were used as secondary antibodies. After the reaction with secondary antibodies, nuclei were counterstained with Hoechst 33258. The sections were observed and photographed using a microscope equipped with a digital camera. For quantification of Pax7-positive cells, IgG-positive necrotic myofibers embryonic myosin heavy chain (eMHC)-positive myofibers, several numbers of the field were photographed using x 4 or x 10 objectives, and the number of positive cells or myofibers were counted. Myofibers were identified by double-staining with anti-laminin antibody. Data were expressed as percent positive myofibers or number of positive cells per area.

For quantification of myofiber size, the sections were immunostained with anti-laminin, followed by labeling with AlexaFluor-labeled secondary antibody. The sections were photographed as described above. The myofibers were automatically identified by Cellpose Google Colab script (<https://colab.research.google.com/drive/1958UQIH-XAYogKvbxnaUHALYvR73KLj2>) [26], and their diameters (minimum Feret diameters) were calculated using ImageJ software.

Cell culture and immunocytochemistry

Mononuclear cells were isolated from skeletal muscle as described previously [27]. In brief, the rats were killed by inhalation of carbon dioxide gas, and their tongue and masseter muscles were extirpated. As described above, the lower half part of the masseter muscle and tongue cut out at the root were used for cell isolation. They were minced with scissors and digested with 1.25 mg/mL protease (from *Streptomyces griseus*, type XIV; Sigma) at 37°C for 1 h. Cells were separated from myofiber fragments through differential centrifugation and plated onto poly-L-lysine- and fibronectin-coated 48-well plates. Cells from each muscle piece were divided into two equal portions (one for immunocytochemistry of Pax7 and another for MyoD) and plated. Cells were cultured in Dulbecco's modified Eagle medium (Gibco, Life Technologies, Palo Alto, CA, USA) containing 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 µg/mL gentamicin for 4 days.

The cells were fixed with 4% PFA/PBS for 15 min, then blocked with 5% NGS/PBS containing 0.1% Triton X-100 for 10 min. The cells were incubated with anti-Pax7 (1:200 with 5% NGS/PBS) or anti-MyoD (1:200, with 5% NGS/PBS, mouse, clone 5.8A, Novocastra, Newcastle upon Tyne, UK) overnight at 4°C. After washing, they were incubated with AlexaFluor-labeled secondary antibody for 1 h. Nuclei were counterstained with Hoechst 33258. The Pax7- and MyoD-positive cells were counted in randomly selected five fields at 20 x objective of a fluorescence microscope (BX50, Olympus). Total numbers of nuclei were also counted. The data were expressed as percent positive cells.

Quantitative real-time PCR

Total RNA was extracted from 100 cryosections (7 µm) with FastGene RNA Basic kit (Nippon Genetics Co., Ltd., Tokyo, Japan), and reverse transcribed to cDNA using Super Script II kit (Invitrogen). Quantitative real-time PCR was performed on a Light Cycler 2.0 (Roche Diagnostics, Roche, Basel, Switzerland) with the Thunderbird SYBR qPCR Mix (TOYOBO, Osaka, Japan). The following primer sets were used: p16: forward, 5'-TTC ACC AAA CGC CCC GAA CA-3'; reverse, 5'-CAG GAG AGC TGC CAC TTT GAC-3'; Utrophin: forward, 5'-TAG AGC AAT ACG CCA CAC GA-3'; reverse, 5'-ACG CTC TTC CTT CTC CAC AG-3', and hypoxanthine-guanine phosphoribosyltransferase (HPRT): forward, 5'-GAC CGG TTC TGT CAT GTC G-3'; reverse, 5'-ACC TGG TTC ATC ATC ACT AAT CAC-3'. The expression of each gene was analyzed using the crossing-point method and expressed after normalization with that of HPRT.

Statistical analyses

Except for an analysis of myofiber diameters, graphed data are expressed as means ± SE. Unpaired Student's t-test (between two groups) and one-way analysis of variance (ANOVA) followed by a Tukey–Kramer test (for multiple group comparison) were used to evaluate statistical differences. For the experiment to assess the difference of myofiber diameters, the p-value was determined using the Wilcoxon rank sum test. p-values less than 0.05 were considered statistically significant.

Results

We previously reported that the body weight of DMD rats decreased progressively after the age of 6 months [22]. Suspecting that eating disorder occurs in DMD rats, we measured the amount of food intake

at the ages of 3 months (before the onset of body weight decrease) and 7 months (after the onset of bodyweight decrease). The amount of food intake at 3-month-old was comparable between WT and DMD rats (Fig. 1). However, at 7-month-old, significantly decreased food intake was observed in DMD rats, indicating the occurrence of eating disorder (Fig. 1).

The above results suggest that muscles involved in food intake are affected by the progress of the disease. We then performed morphometric analyses of muscles involved in food intake. We chose masseter and tongue muscles because these two muscles are to be approached easily and distinguishable from other muscles. As shown in Fig. 1A, the area corresponding to the masseter muscle was considered as an ellipse, and its long and short diameters were measured with calipers to calculate the area. The area of masseter muscle was measured from 1-month to 8-month-old. At 1-month-old and after 6-month-old, the area of masseter muscle was significantly reduced in DMD rats compared to WT rats. Especially, the greater reduction was observed at 6- and 8-month-old (Fig. 2B).

The appearance of the tongue at 8-month-old is shown in Fig. 3A. The cross-section of the posterior part of the tongue was larger in DMD rats than in WT rats (Fig. 3A). Then, we measured the area and width of the upper half of the tongue sections. Quantitative analysis showed that the width of the tongue of DMD rats was significantly larger than that of WT rats after 3-month-old (Fig. 3B). In addition, the area of the tongue was significantly larger in DMD rats at the age of 8 months than in WT rats (Fig. 3C). These results indicate that macroglossia is occurring in DMD rats.

To further gain insight into the age-related changes of masseter and tongue muscles of DMD rats, histological analyses were performed. The masseter muscle of DMD rats showed myofiber degeneration and necrosis with infiltration of neutrophils and macrophages at 1-month-old (Fig. 4B), and regenerative myofibers with enlarged centralized nuclei and basophilic cytoplasm at 3-month-old (Fig. 4D). At 8-month-old, in addition to inflammatory and degenerative changes, severe fibrosis and fatty infiltration were observed (Fig. 4F and H). No significant pathological changes were observed in the masseter muscle of WT rats at all ages (Fig. 4A, C, E, and G).

In contrast, in the tongue muscle of DMD rats, regenerative myofibers with enlarged centralized nuclei and basophilic cytoplasm were observed at 1-month-old (Fig. 5B), and these regenerative changes were accompanied by mild fibrosis at 3-month-old (Fig. 5D). At 8-month-old, mild fibrosis, also confirmed by Masson's trichrome stain (Fig. 5H), was persisted (Fig. 5F). No significant pathological changes were observed in the tongue muscle of WT rats at all ages (Fig. 5A, C, E, and G).

To further examine whether myofiber necrosis and muscle regeneration are ongoing at 6-month-old when macroglossia is occurring in the tongue muscle of DMD rats, immunohistochemical analyses of endogenous IgG (myofiber necrosis) and embryonic MHC (muscle regeneration) were performed. The masseter muscle of DMD rats, though there is variation among individuals, showed marked myofiber necrosis (Fig. 6A and E) and muscle regeneration (Fig. 6C and F), while the tongue muscle showed none of these, (Fig. 6B, D, E and F). These results indicate that, although the masseter and tongue muscles of

DMD rats undergo pathological changes that are not seen in WT rats, the changes seen in the tongue muscle are much milder than in the masseter muscle of DMD rats.

To investigate the factors that are responsible for macroglossia seen in DMD rats, we measured the myofiber diameters of the tongue muscle of WT and DMD rats at 6-month-old. The myofiber diameters of DMD rat were larger than those of WT rats (Fig. 7A and B; $p < 0.01$ by the Wilcoxon rank sum test). The ratio of myofibers with central nuclei was significantly higher in DMD rats than in WT rats, indicating that muscle regeneration had occurred in the past (Fig. 7C). In addition, quantitative analysis of Masson's trichrome stained sections as shown in Fig. 5 revealed that the fibrotic area in the tongue muscle of DMD rats is about twice that of WT rats (Fig. 7D). Thus, these results suggest that both the hypertrophic response of myofibers and the accumulation of collagen between myofibers were combined to produce macroglossia.

We reported previously that cellular senescence with increased expression of p16 occurs in the hindlimb muscles of DMD rats, and that the proliferation of muscle satellite cells is markedly suppressed in culture [22]. Therefore, we examined the expression of the p16 gene in masseter and tongue muscles at 9-month-old and found that it was markedly up-regulated in the masseter muscle of DMD rats compared to WT rats, whereas it was only slightly up-regulated in the tongue muscle (Fig. 8A). There was no significant difference in the number of Pax7-positive satellite cells in the sections, although there was a large variation between individuals (Fig. 8B). On the other hand, when cultured in vitro for 4 days, the number of Pax7- or MyoD-positive satellite cells derived from the masseter muscle of DMD rats was significantly lower than that of WT rats (Fig. 8C and D, Supplementary Fig. 1A and B), while the number of satellite cells derived from the tongue muscle of DMD rats was only slightly lower than that of WT rats (Fig. 8C and D, Supplementary Fig. 1A and B). These results indicate that cellular senescence occurs in the masseter muscle of DMD rats as was seen in their hindlimb muscles [22], while it is significantly less severe in the tongue muscle.

The above results suggest that the tongue muscle of DMD rats is protected from degenerative changes, which occur in other muscles due to the absence of dystrophin. Utrophin, known as dystrophin-related protein, had been shown to compensate for the function of dystrophin [4, 8, 13, 17]. In a mouse model of DMD, mdx mouse, the skeletal muscle phenotype is milder than that seen in DMD patients [13], and this had been once thought to be due to an up-regulation of utrophin [13]. Thus, we examined the expression of utrophin in the masseter and tongue muscles of WT and DMD rats at 9-month-old. In masseter muscle, utrophin expression was significantly upregulated by the absence of dystrophin (Fig. 8E). Interestingly, in tongue muscle, utrophin expression was also upregulated but its basal expression in the presence of dystrophin (WT rats) was comparable to that in the masseter muscle of DMD rats (Fig. 8E). This result suggests that the tongue muscle is intrinsically expressing a higher level of utrophin even in the presence of dystrophin, and this might be related to the resistance of this tissue against degenerative changes in the absence of dystrophin.

Discussion

In the present study, we demonstrated that the tongue of DMD rats shows macroglossia and degenerative changes were observed transiently only in the early period of life. On the other hand, chronic degenerative and necrotic changes were seen in the masseter muscle of DMD rats.

Cellular senescence is caused by oxidative stress due to chronic inflammation [3]. We previously reported progressive myofiber degeneration and necrosis, along with cellular senescence with increased expression of p16, in the tibialis anterior (TA) and diaphragm of DMD rats [22]. p16 was mainly expressed in satellite cells and mesenchymal progenitor cells, and their proliferative activity was severely suppressed in vitro. The phenotype of the masseter muscle of DMD rats seen in this study is similar to our previous reports; the masseter muscle showed progressive myofiber degeneration and necrosis, and accordingly, p16 expression was upregulated. In contrast, the expression of p16 in the tongue muscle of DMD rats was much lower than in the masseter muscle, and proliferative activity of the satellite cells was relatively well-preserved in vitro. This is in agreement with the histological observation that myofiber necrosis and inflammation were not observed in the tongue muscle of DMD rats, suggesting the absence of oxidative stress that could induce cellular senescence.

A similar phenotype to the tongue muscle of DMD rats had been observed in the hindlimb muscle of mdx mice [6, 12, 23]. In the tongue muscle of DMD rats, regeneration of myofibers was transiently observed at 1-month-old, and at later periods of their life, myofibers with central nuclei persisted without any degenerative changes. In the skeletal muscle of mdx mice, necrotic regeneration is observed only up to 1 to 2 months of age, and regenerated myofibers with central nuclei remain for a long time [6, 12, 23]. At present, it is not clear why myofiber necrosis occurs only transiently in mdx mice, but it has been suggested that one reason is that compensatory increases in utrophin, a functional homolog of dystrophin, may replace the function of dystrophin [13]. In the present study, the expression of utrophin was higher in the tongue muscle of WT rats than in their masseter muscle. Therefore, one of the possibilities is that some mechanism may be responsible for the increased expression of utrophin in the tongue muscle of the rat, and myofiber necrosis may not occur even under dystrophin deficiency.

The degree of skeletal muscle regeneration after injury varies depending on the body part; it has been reported that the diameter of myofibers and the degree of fibrosis in masticatory muscles of 100-day-old mdx mice vary according to the muscles [21]. In addition, Yoshioka et al. reported that when injury and subsequent regeneration of skeletal muscle were induced by cardiotoxin-injection in WT and mdx mice, there were differences in the rate of regeneration and the degree of increase in muscle weight after regeneration [28]. In particular, masseter muscle had a longer time to recover its muscle weight after injury than TA muscle, and no hypertrophy of regenerated myofibers occurred [28]. They suggested that these differences were due to a distinct nature of the satellite cells present in each skeletal muscle. Regarding this, the origin of the satellite cells varies among different muscles [18]. The tongue muscle is composed of intrinsic and extrinsic muscles, and the satellite cells of the former originate from the first occipital somite, whereas the latter originates from the cranial paraxial mesoderm [16, 18]. On the other hand, the satellite cells of the masseter muscle are derived from the first and second pharyngeal arches of the cranial paraxial mesoderm with contributions from the splanchnic mesoderm [18]. At present, it is

unclear how the different origins of these satellite cells ultimately affect the nature of the skeletal muscle from which they arise. However, in the multiple types of myopathies, including DMD, it has been suggested that differences in the origin of satellite cells may contribute to the distinct pathological susceptibility among different muscles [18]. Thus, the phenotypic difference between the masseter and tongue muscles observed in DMD rats may be due to the difference in the nature of the satellite cells.

The tongue muscle has a unique structural feature among skeletal muscles in that it is covered by epithelium, and its development is indirectly influenced by epithelium [9, 11]. During development, the epithelium-derived sonic hedgehog (shh) acts on cranial neural crest cells (CNCC), which constitute the mesenchyme [9]. When the hedgehog signal in CNCC is defective, migration of myoblasts from the occipital somite is perturbed, resulting in dysplasia of the tongue. Although the involvement of the epithelium in the adult tongue has not been reported, it is possible that factors derived from the epithelium act directly or indirectly via mesenchyme on the tongue muscle, which may be protective against degenerative changes of tongue muscle myofibers caused by dystrophin deficiency. This point awaits further investigation.

Macroglossia has been reported to occur not only in human DMD but also in canine X-linked muscular dystrophy (CXMD) [20]. In human DMD, it is known that macroglossia interferes with tongue function [24, 25], but no detailed histological study has been performed. On the other hand, it has been suggested that hypertrophy of muscle fibers is the cause of macroglossia in CXMD [20]. This is consistent with the results of the present analysis in DMD rats. However, the limitation of this study is that it is unclear whether the macroglossia in DMD rats influences tongue function, and it is unknown whether the decreased food intake seen in DMD rats is attributed to macroglossia. Regarding this point, it is necessary to evaluate the tongue function experimentally in the future.

Conclusions

We demonstrated that, unlike the other muscles, degenerative/necrotic changes of myofibers, decreased satellite cell proliferative activity, and increased p16 expression were not observed in the tongue muscle of DMD rats. Furthermore, the tongue muscle of the rat showed higher utrophin expression than the masseter muscle, suggesting that this compensatory utrophin expression might protect the tongue myofibers from degenerative/necrotic changes under the lack of dystrophin. Unraveling the mechanism protecting the tongue muscle in DMD rats would benefit to establish the effective treatment for DMD.

Abbreviations

ANOVA

analysis of variance

CNCC

cranial neural crest cells

CXMD

canine X-linked muscular dystrophy
DMD
Duchenne muscular dystrophy
DMD rat
a rat model of DMD
DSHB
Developmental Studies Hybridoma Bank
eMHC
embryonic myosin heavy chain
HE
hematoxylin-eosin
HPRT
hypoxanthine-guanine phosphoribosyltransferase
NICHD
National Institute of Child Health and Human Development
NDS
normal donkey serum
NGS
normal goat serum
PBS
phosphate buffered-saline
PFA
paraformaldehyde
Shh
sonic hedgehog
TA
tibialis anterior
WT
wild-type
XY
male WT
 χ^{DmdY}
male DMD

Declarations

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Authors' contributions

KY conducted the experiments, analyzed the data, prepared the figures, and wrote the manuscript; YT and MI conducted the experiments; SK prepared tissue sections and conducted the experiments; JC, TM and KU edited the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors have no competing financial interests related to the conduct of the work presented in this manuscript.

Ethics approval

All animal experiments performed in this study were in accordance with the Guide for the Care and Use of Laboratory Animals of the University of Tokyo and were approved by the Institutional Animal Care and Use Committee of the University of Tokyo (P18-125). All studies were conducted in compliance with the Helsinki Declaration.

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Figures

Figure 1

Food intake in WT and DMD rats. The amount of food intake per day was measured at 3- and 7-month-old. The data are expressed as mean±SE. **, p<0.01 by unpaired Student's t-test

A **B** **Figure 2**

Area of the masseter muscle of WT and DMD rats. (A) The masseter muscle was considered as an ellipse (dotted white circle) and its long and short diameters were measured with calipers to calculate the area. (B) Changes in the area of masseter muscle at 1-, 3-, 6- and 8-month-old. The data are expressed as mean $\pm$ SE. **, $p < 0.01$ by unpaired Student's t-test

Fig. 3

Figure 3

Width and cross-sectional area of the tongue of WT and DMD rats. (A) The tongue was cut at the root (dotted black line) (left) and the width (dotted black line) and area (dotted red circle) were measured. Scale bar=1 cm (B and C) Changes in the width (B) and cross-sectional area (C) of tongue at 1-, 3- and 8-month-old. The data are expressed as mean±SE. **, p<0.01 by unpaired Student's t-test

Fig. 4

Figure 4

(A-F) HE- and (G and H) Masson's trichrome-stained sections of the masseter muscle of WT and DMD rats. In the masseter muscle of DMD rats, myofiber degeneration and necrosis with infiltration of neutrophils and macrophages at 1-month-old, regenerative myofibers with enlarged centralized nuclei and basophilic cytoplasm at 3-month-old, and severe fibrosis, confirmed by Masson's trichrome staining, at 8-

month-old were observed, respectively. No significant pathological changes were observed in the masseter muscle of WT rats at all ages.

Fig. 5

Figure 5

(A-F) HE- and (G and H) Masson's trichrome-stained sections of the tongue muscle of WT and DMD rats. In the tongue muscle of DMD rats, regenerative myofibers with enlarged centralized nuclei and basophilic

cytoplasm at 1-month-old, the regenerative changes accompanied by mild fibrosis at 3-month-old, and persistence of mild fibrosis, confirmed by Masson's trichrome staining, at 8-month-old were observed, respectively. No significant pathological changes were observed in the tongue muscle of WT rats at all ages.

Fig. 6

Figure 6

Analyses of necrotic and regenerating myofibers in masseter and tongue muscle of WT and DMD rats. (A and B) Representative immunohistochemistry of endogenous IgG (red) and laminin (green) in the masseter (A) and tongue (B) muscle of DMD rats at 6-month-old. Nuclei were stained with Hoechst 33258 (blue). Arrowheads (white) indicate necrotic myofibers. (C and D) Representative immunohistochemistry of embryonic myosin heavy chain (eMHC) (red) and laminin (green) in the masseter (A) and tongue (B) muscle of DMD rats at 6-month-old. Nuclei were stained with Hoechst 33258 (blue). Arrowheads (white) indicate regenerating myofibers. (E) Quantitative analysis of necrotic and regenerating myofibers in in masseter and tongue muscle of WT (n=5) and DMD (n=6) rats at 6-month-old. The data are expressed as mean $\pm$ SE. *, p<0.05 vs others by Tukey–Kramer test

Fig. 7

Figure 7

Morphometric analyses of tongue muscle of WT and DMD rats. (A and B) Distribution of myofiber diameters at 6-month-old. The left panel (A) depicts the relative distribution of myofibers with certain diameters. The right panel (B) depicts relative cumulative plots based on the data shown in the left panel. The myofiber diameters of DMD rat (n=6) were larger than those of WT rats (n=5). $p < 0.01$ by Wilcoxon rank sum test (number of myofibers analyzed, WT=3112 and DMD=2662) (C) Ratio of myofibers with

central nuclei at 6-month-old. The data are expressed as mean±SE. **, p<0.01 by unpaired Student's t-test
(D) Relative fibrotic area at 6-month-old. The data are expressed as mean±SE. **, p<0.01 by unpaired Student's t-test

Fig. 8

Figure 8

Quantitative analyses of gene expression and satellite cells in muscles of WT and DMD rats. (A) p16 gene expression in the masseter and tongue muscle at 9-month-old. The data are expressed as mean $\pm$ SE. Different letters indicate statistical difference. $p < 0.01$ by Tukey–Kramer test (WT, $n=5$; DMD, $n=6$) (B) Number of Pax7-positive satellite cells in the sections at 9-month-old. The data are expressed as mean $\pm$ SE. There was no significant difference amongst all groups by Tukey–Kramer test (WT, $n=5$; DMD, $n=6$) (C and D) Relative number of Pax7- (C) and MyoD-positive (D) satellite cells from at 9-month-old rats after 4 day-culture in vitro. The data are expressed as mean $\pm$ SE. Different letters indicate statistical difference. $p < 0.05$ by Tukey–Kramer test (WT, $n=3$; DMD, $n=3$) (E) Utrophin gene expression in the masseter and tongue muscle at 9-month-old. The data are expressed as mean $\pm$ SE. Different letters indicate statistical difference. $p < 0.05$ by Tukey–Kramer test (WT, $n=5$; DMD, $n=6$)

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