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Myofiber Properties of Mouse Mylohyoid Muscle in the Growth Period

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The mouse mylohyoid muscle belongs to the mastication-related suprahyoid muscle group. It shows a plate-like morphology and forms the mouth floor. There have been no reports on the characteristics of the mouse mylohyoid muscle fibers, and especially on their functional role during ingestion action, and many points remain unclear. We examined the mouse mylohyoid muscle at both the transcriptional and protein levels by RT-PCR, immunohistochemistry, and Western Blotting. MyHC-2b, which is expressed in almost all head and neck muscles and is thought to play a role in rapid mastication movement, was not detected in the mouse mylohyoid muscle. This result suggests that the mouse mylohyoid muscle has a special function and does not directly function during ingestion.

Key words: myosin heavy chain, muscle development, muscle, mRNA expression, mouse

INTRODUCTION

Myosin heavy chain (MyHC), a muscle contraction protein, consists of several isoforms classified into a fast-twitch fiber type (MyHC-2b, MyHC-2d, MyHC-2a) and a slow-twitch fiber type (MyHC-1) based on different muscle contraction speeds (Schiaffino et al., 1996). Moreover, the composition ratio of MyHC isoforms has been reported to demonstrate the characteristics of the muscle (Brueckner et al., 1996; Hori et al., 1998). These studies also reported that by observing the composition ratio of MyHC isoforms, the functional role of the muscle could be clarified (Table 1).

Research into the expression status of each MyHC isoform during growth and development has used mostly extremity muscles. However, masticatory muscles, as well as head and neck muscles, have recently been investigated (Negoro et al., 2001; Usami et al., 2003; Abe et al., 2007). These oral-region muscles show a functional change during the developmental period of weaning. To date, reports observing alteration of fibers of the masseter muscle during the weaning period have indicated that MyHC-2b, which is known to be involved in fast contraction of the muscle, is largely expressed after weaning, and masticatory movement results in a prominent functional change of the masseter muscle (Gojo et al., 2002; Doi et al., 2003). It has also been reported that differences in the composition ratio of MyHC isoforms leads to differences in muscle function (Pette et al., 1990). Following this report, various detailed studies on the relationship between the composition ratio of the fast-twitch fiber type and function in the muscle have been performed. Regarding muscles of the oral region, the adult mouse masseter muscle barely expresses MyHC-2a, and MyHC-2b has been reported to include most of the isoform types (Shida et al., 2005). Again, although the anterior belly of the digastric muscle of the adult mouse does not express MyHC-2a, a large amount of MyHC-2b has been observed, as in the masseter muscle (Okubo et al., 2006). The mouse, a rodent, has a special mastication-cycle ability to move the mandible rapidly in an antero-posterior direction (Hiiemae et al., 1968; Okayasu et al., 2003). To carry out this special mastication cycle, high expression of MyHC-2b, which is the fastest isoform of the muscle contraction proteins, has been indicated. The mouse, like other mammals, has a suckling period followed by a change to mastication through weaning for ingestion of food. To bear the burden during the transition from the suckling period to mastication, MyHC-2b expression gradually increases (Gojo et al., 2002; Doi et al., 2003).

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Table 1. Myosin heavy chain isoforms identified in skeletal muscle. Source: Brueckner et al. (1996).

Designation	Nomenclature	Distribution
Embryonic	MyHCemb	Myobubes, intrafusal fibres, regenerating fibres
Neonatal	MyHCneo	Neonatal muscles, masseter, intrafusal fibres
Fast-twitch	MyHCeom	Super-fast fibers in extraocular muscles
Fast-twitch	MyHC-2m	Super-fast fibers in muscles derived from the first branchial arch
Fast-twitch	MyHC-2b	Fast-type isoforms in digastric muscle of mice
Fast-twitch	MyHC-2d	contraction speed: 2b>2d>2a
Fast-twitch	MyHC-2a	
Slow-twitch	MyHC-1	Type I fibres

The mastication-related mouse mylohyoid muscle is one of the suprahyoid muscles and forms the mouth floor. Also, the rat mylohyoid muscle is composed mainly of a slow-twitch fiber type isoform (Cobos et al., 2001). However, many points still remain unclear about the characteristics and changes in mouse mylohyoid muscle fibers and their role in mastication. To clarify these points, we examined the mouse mylohyoid muscle at both the transcriptional and protein levels by RT-PCR, immunohistochemistry, and Western Blotting.

MATERIALS AND METHODS

Materials

Since the mean weaning age of ICR mice (Sankyo Laboratory, Tokyo, Japan) was reported to be approximately 3 weeks, 2-week-old (before weaning), 4-week-old (after weaning) and 9-week-old (adult) mice were analyzed in this study (Doi et al., 2003). At 3 weeks old, mice were placed in separate cages and fed a solid diet. Immunohistochemical investigation was conducted on 5 mice at each age, while mRNA expression was examined in another 5 mice at each age, so a total of 30 mice were used. According to the animal study guidelines established by Tokyo Dental College, mice were sacrificed by injection of a lethal dose of pentobarbital and the mylohyoid muscle was extracted (Fig.1). These muscles were immediately frozen in liquid nitrogen and stored at –80°C until testing.

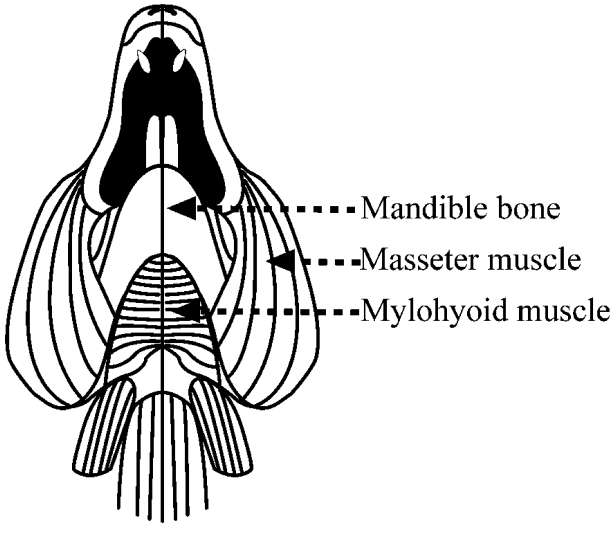

Fig. 1. Interior view of the head and neck region, showing the location and alignment of the murine mylohyoid muscle.

Immunohistochemical analysis

By using a cryostat, each excised mylohyoid muscle was serially sliced horizontally at a thickness of 8 μm orthogonal to the long axis of the muscle fibers. Immunostaining was performed as follows: as primary antibodies, SC-71 (anti-MyHC-2a; American Type Tissue Culture, Manassas, VA, USA) and BF-F3 (anti-MyHC-2b; American Type Tissue Culture) anti-mouse monoclonal antibodies extracted from hybridoma cells were used (Schiaffino et al., 1989; Eason et al., 2000). Hybridoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St Louis, MO, USA) containing 10% fetal bovine serum (Sigma-Aldrich.) at 37°C under 5% CO_2 for 72 h, and then the cells were harvested and centrifuged. The supernatant was used to provide primary antibodies. As secondary antibodies, a fluorescein isothiocyahate (FITC)-labeled goat anti-mouse IgG antibody (Novocastora Laboratories, Newcastle, UK) was used to visualize SC-71, and a rhodamine-labeled goat anti-mouse IgM antibody (Novocastora Laboratories) was used to visualize BF-F3. An MRC-1024/2P confocal laser microscope (Nippon Bio-Rad Lab, Tokyo, Japan) was used for observation and photography.

RNA extraction and mRNA expression analysis

A LightCycler® (Roche Diagnostics, Mannheim, Germany) was used to quantify the expressions of MyHC-2a and MyHC-2b at each age and location. Total RNA at each age and location was extracted by using a Quick Prep Micro-mRNA Purification Kit (GE Healthcare, Amersham, UK), and cDNA was prepared using Ready-To-Go (GE Healthcare). After determining optimal conditions for all primers, the study was conducted according the standard protocol for the LightCycler®. As a hot-start PCR solution for the LightCycler®, preadjusted LC FastStart DNA Master SYBR Green I (Roche Diagnostics) was used. A series of cDNA dilutions (4.0 ng/μl) were made, and $1/10^5$, $1/10^6$, $1/10^7$, $1/10^8$, and $1/10^9$ dilutions were used. PCR reactions for the diluted standards contained 10.2 μl of sterile

Table 2. Sequences of the primers used in this study.

MyHC-2a	
	Forward: 5'–CGATGATCTTGCCAGTAATG–3'
	Reverse: 5'–TGATAACTGAGATACCAGCG–3'
	Accession: NM_144961
MyHC–2b	
	Forward: 5'–ACAGACTAAAGTGAAAGCC–3'
	Reverse: 5'–CTCTCAACAGAAAGATGGAT–3'
	Accession: XM_126119
GAPDH	
	Forward: 5'–TGAACGGGAAGCTCACTGG–3'
	Reverse: 5'–TCCACCACCCTGTTGCTGTA–3'
	Accession: NM_008084

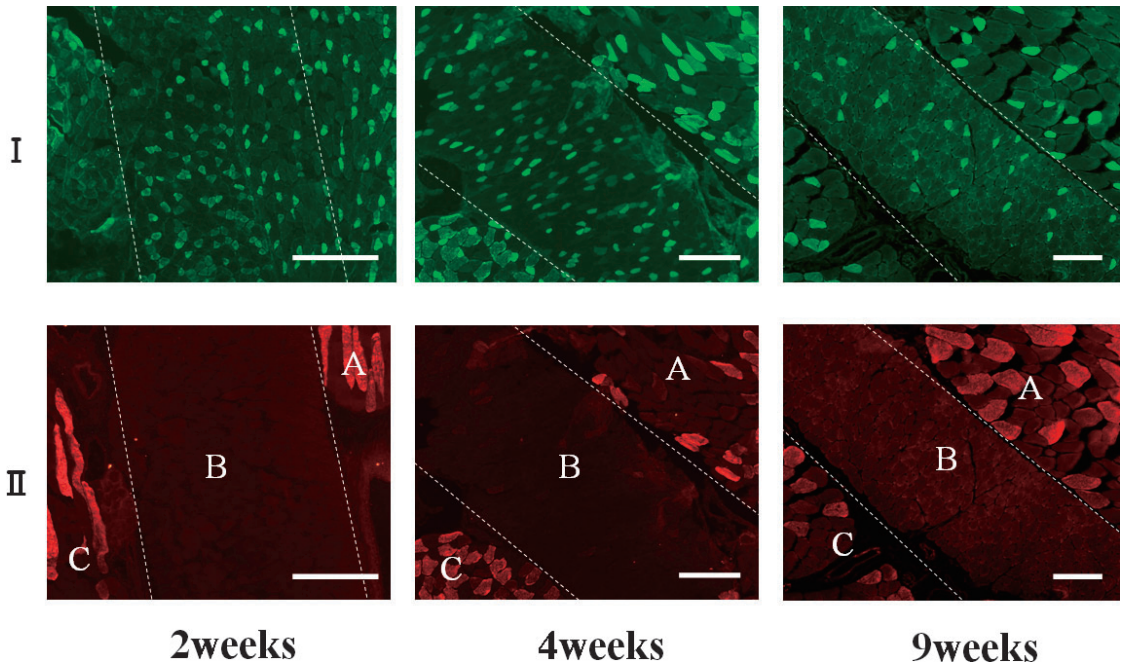

Fig. 2. Immunostaining of the mylohyoid muscle. (I) MyHC-2a-positive fibers; (II) MyHC-2b-positive fibers. The dotted line shows the approximate border between the mylohyoid muscle and other muscles. **(A)** Anterior belly of the digastric muscle. **(B)** Mylohyoid muscle. **(C)** Geniohyoid muscle. Scale bar, 50 μm.

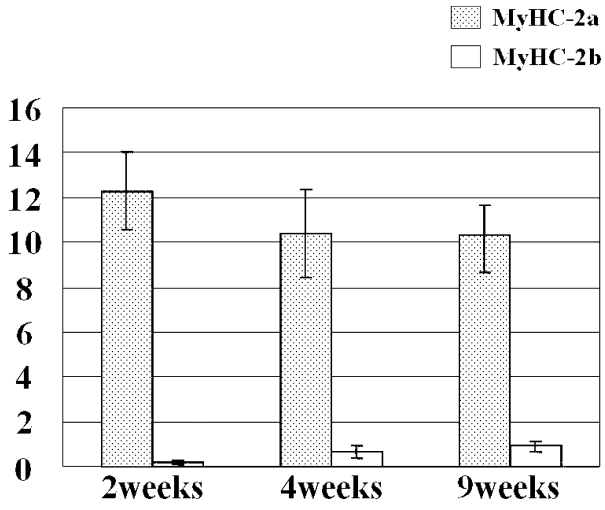

Fig. 3. Expression of MyHC-2a and MyHC-2b mRNA (LightCycler®). Expression of MyHC-2a mRNA decreased slightly in the mylohyoid muscle in 2-, 4-, and 9-week-old mice, but no significant change was detected. Expression of MyHC-2b mRNA increased slightly in the mylohyoid muscle in 2-, 4-, and 9-week-old mice, but no significant change was detected.

water and 5 μl of diluted control cDNA product, 1.6 μl of $MgCl_2$ (25 mM), and 2 μl of LC FastStart DNA Master SYBR Green I containing SYBR Green I (1/60,000 dilution). In addition, 0.6 μl each of forward and reverse primers (10 pmol/μl) prepared using an Oligo 5 primer design (Nihon Gene Research Laboratories, Sendai, Japan) were added to reach a final reaction volume of 20 μl for each tube (Iwanuma et al., 2008). MyHC-2a and -2b primers were used and designed based on segments specific to the respective full-length cDNA sequences. Base sequences for each primer are shown in Table 2.

For the test PCR mixture, 1.6 μl of $MgCl_2$ (25 mM), 2 μl of LC FastStart DNA Master SYBR Green I, and 0.6 μl each of the forward primer (10 pmol/μl) and reverse primer (10 pmol/μl) were added to 14.2 μl of sterile water. Finally, 1 μl of target cDNA was added to bring the final reaction volume to 20 μl. PCR mixtures (20 μl) for MyHC-2a and -2b prepared in the above manner were added to the capillary of a real-time RT-PCR system. PCR cycling conditions were 95°C for 10 min, followed by 50 cycles of 95°C for 10 s, 62°C for 10 s, and 72°C for 7 s. Gene amplification was performed according to a melting program of 70°C for 15 s, and during a transition period from 70°C to 95°C, fluorescence was continuously monitored at a rate of 0.1°C/s. As the fluorescent channel, F1 (530 nm) was used, and gains for MyHC-2a and -2b were 88.2°C and 89.9°C, respectively. The amount of each MyHC isoform calculated by using the above method was divided by the amount of GAPDH

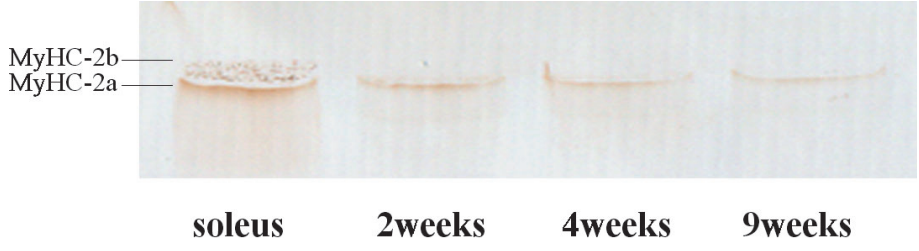

Fig. 4. Electrophoretic separation and Western-blot analysis of MyHC-2a and MyHC-2b from the mylohyoid muscle.

(a housekeeping gene) to calculate the final mRNA expression. The base sequence of GAPDH is also shown in Table 2.

Western-blotting analysis

Electrophoretic separation and analysis of protein bands by Western blotting was performed. Briefly, the mylohyoid muscles from 2-, 4-, and 9-week-old ICR mice were removed while the animals were anaesthetized. The muscles were weighed, frozen in liquid nitrogen, and stored at –80°C. Frozen muscles were minced with scissors in nine volumes of ice-cold homogenization buffer (250 mM sucrose, 100 mM KCl, 5 mM ethylenediaminetetraacetic acid, and 20 mM Tris hydroxymethyl aminomethane (Tris), pH 6.8). The minced muscle samples were subsequently sonicated in a Branson Sonifier 250D (Branson Ultrasonic Corporation, Danbury, CT, USA). The products were used for the preparation of washed myofibers, which were then boiled in sample buffer for 2 min at a final protein concentration of 0.125 mg/ml. Total proteins were determined by the Bradford technique using the Bio-Rad Protein Assay (Nippon Bio-Rad Laboratory) and a Gene Quantpro spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Western-blotting analyses were performed to detect MyHC-2a and -2b signals. Equal amounts of total protein (40 μg) for each group were separated on 7.5% SDS-polyacrylamide gel and transferred to Immobilon-P Transfer Membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% skimmed milk, incubated with each of the primary antibodies of Monoclonal Anti-Myosin (Skeletal, Fast) (1:1000) (Sigma-Aldrich) and detected with a horseradish peroxidase-conjugated secondary anti-mouse IgG antibody by using the Vectastain ABC system (Vector Laboratories, Burlingame, CA, USA).

Statistical analysis

To compare MyHC-2a and MyHC-2b, t-tests were used, with the level of significance set at $p<0.05$. Tukey's q-tests were used to compare different ages, with the level of significance again set at $p<0.05$.

RESULTS

Immunostaining

Muscle fibers immunopositive for MyHC-2a showed almost no difference between 2-week-old and 4-week-old mice. However, a tendency towards decreased MyHC-2a expression was detected in 9-week-old mice. MyHC-2b-expressing muscle fibers were barely observed in 2-, 4-, and 9-week-old mice (Fig. 2).

Analysis of mRNA expression using the LightCycler®

The ration of mRNA expression of MyHC-2a tended to decrease from 2-week-old to 4- and 9-week-old mouse muscles. However, mRNA expression of MyHC-2b was barely detected in any of the 2-, 4-, or 9-week-old mouse muscles. For mice of all ages, MyHC-2a was predominantly expressed compared to MyHC-2b (Fig. 3).

Western blotting

MyHC-2a protein expression was revealed in mice of all ages. However, MyHC-2b protein expression was not shown in mice at any age (Fig. 4).

DISCUSSION

The muscle examined in this study, the mylohyoid muscle, originates from the mylohyoid line that runs obliquely on the inner side of the mandible and widely inserts onto the hyoid bone in a fan-like shape. It separates the oral cavity from the neck and is also known as the diaphragm of the mouth. The characteristics of the mylohyoid muscle and its role during mastication are still being clarified, and many points remain unknown. Therefore, we aimed at clarifying the characteristics and functional role of the mouse mylohyoid muscle in the growth stage. Since the majority of mouse muscles with a mastication function have been reported to show characteristic prominent changes after weaning (Gojo et al., 2002; Shida et al., 2005), both pre-weaning (2-week-old) mice and post-weaning (4-week-old) mice, as well as adult (9-week-old) mice, were used in this study. Many studies of mouse head and neck muscle fibers have compared the ratio of MyHC-2a and MyHC-2b. These studies found that MyHC-2b exists as a high-contraction-speed type of fiber, and MyHC-2a as a low-contraction-speed type of fiber, and that these fiber types represent muscle function (Shida et al., 2005; Okubo et al., 2006; Suzuki et al., 2007). Therefore, we also analyzed these two isoforms.

When muscles of the mouth floor were extracted as one block, the upper and lower parts comprised the genihyoid muscle and the anterior belly of the digastric muscle, respectively (Fig. 1). These oral-floor muscles were extracted as one block and sections were prepared for immunohistochemistry. Thus, observation of three kinds of muscle in the same section was possible. The results showed that MyHC-2a-expressing muscle fibers exist both in the geniohyoid and anterior belly of the digastric muscle in mice of pre-weaning age (2 weeks old). However, MyHC-2b showed no expression in either muscle. In post-weaning mice (4 weeks old), although a decrease in the MyHC-2a level was seen compared to the 2-week-old mice, this isoform was still expressed in both muscles. However, no MyHC-2b-positive muscle fibers were detected in post-weaning mice (4 weeks old), similarly to pre-weaning mice (2 weeks old). Although the MyHC-2a level further decreased in 9-week-old mice compared with 4-week-old mice, its expression was still detected in the muscle fibers. On the other hand, MyHC-2b expression was not observed at a similar expression level in 2- and 4-week-old mice. Similar results were obtained at both the protein and transcription levels.

Research has clarified that MyHC-2b-expressing muscle fibers are prominently observed after weaning in the muscles bearing mastication, such as the masseter and temporal muscles. This change was considered to fit with the mouse-specific fast mastication action (Shida et al., 2005; Suzuki et al., 2007). However, in the current study, MyHC-2b expression was barely detected in the mouse mylohyoid muscle, even in the post-weaning period. Our results suggest that the mouse mylohyoid muscle does not directly function in mastication. MyHC-2a also continued to be expressed in the mylohyoid muscle even after weaning, differently from other muscles.

The geniohyoid muscle and the anterior belly of the digastric muscle were reported by Okubo et al. (2006) to form the floor of the mouth with the mylohyoid muscle. The locations of these muscles are approximately the same, but the characteristics of their muscle fibers are significantly different. The geniohyoid muscle plays an important role in the dynamic movement of the lingual muscle. It is considered that a change in masticatory function caused by weaning significantly affects the change in muscle fiber characteris-

tics. Similarly, the running direction of the anterior belly of the digastric muscle parallels the anteroposterior movement of the mandible, and therefore it is thought that a change in masticatory function brought about by weaning likewise significantly affects the change in muscle-fiber characteristics. However, the mylohyoid muscle originates from the bilateral mandible and stretches across the floor of the mouth. It is thought that this muscle has little influence on masticatory function. In this respect, the mylohyoid muscle has markedly unique muscle-fiber characteristics among the muscles in the head and neck of mice, in that little MyHC-2b is expressed even after weaning. Fig. 2 shows cells that are not stained with anti-MyHC-2a and -2b. These cells are considered to be MyHC-1 and -2d, respectively.

In the present study, a comparison was made only among cells stained with anti-MyHC-2a and -2b, as in the study by Okubo et al. (2006). These isoforms are considered to be necessary and appropriate for comparing muscle-fiber characteristics. However, further detailed investigations are necessary. This result suggested that continuous power is needed to form the mouth floor, and that this is supplied by expression of a slow-contraction-type protein, MyHC-2a. The current research showed that the mylohyoid muscle is very special among the muscles lining the oral cavity. Previous research and the current study have made it clear that various muscles with different roles cooperate of to execute the mastication function.

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