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Changes in muscle fiber properties of murine digastric muscle before and after weaning

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Running title: Characteristics of the digastric muscle

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**Abstract**

The digastric muscle is one of the suprahyoid muscles, and consists of anterior and posterior bellies. Because muscle fiber alignments in these two bellies are different, the functional roles are said to be different. Since the digastric muscle relates mastication, its functions may change markedly before and after weaning, but many details remain unknown. The aim of this study was to clarify changes in muscle fiber properties of the anterior and posterior bellies of the digastric muscle before and after weaning. Expressions of myosin heavy chain (MyHC) isoforms were assessed at the protein and transcriptional levels. Expression of the MyHC-2b isoform, an isoform displaying fast, strong contraction, was greater in the anterior belly than in the posterior belly after weaning. This suggests that, in mice, the anterior belly of the digastric muscle needs to move rapidly anteroposteriorly for mastication, compared with the posterior belly.
Introduction

Different types of skeletal muscle fibers have been classified based on their properties and functions. Myosin is an important muscle protein that accounts for more than half of all proteins that make up muscle fibers. The myosin heavy chain (MyHC) is known to most closely reflect muscle function (Pette and Sarton, 1990). Several isoforms of MyHC exist, and are classified based on their contraction speed into fast (MyHC-2b, MyHC-2d, and MyHC-2a) and slow (MyHC-1) types (Schiaffino and Reggiani, 1996). It has been suggested that the composition ratio of MyHC isoforms may determine muscle properties (Brueckner et al., 1996; Hori et al., 1998). Therefore, the functional roles of muscles may seem to be clarified by ascertaining their MyHC isoform compositions (Table 1).

While most studies on MyHC isoform expression in muscle growth and development have examined muscles of the extremities, recent studies have analyzed head and neck muscles such as the masticatory and suprasyoid muscles (Negoro et al., 2001; Abe et al., 2002; Usami et al., 2003). During
development, these oral muscles undergo functional changes due to weaning. A series of studies on changes in muscle fiber properties of the masseter muscle before and after weaning have shown that the expression of MyHC-2b, an isoform with fast, strong contraction, increases after weaning, and that mastication brings about marked functional changes in the masseter muscle (Gojo et al., 2002; Doi et al., 2003).

A series of studies on the muscle fiber properties of masticatory and cervical muscles has been performed in the Department of Anatomy, Tokyo Dental College (Gojo K et al., 2002; Abe S et al., 2002; Usami A et al., 2003; Doi T et al., 2003; Shida T et al., 2005; Maejima M et al., 2005). However, the digastric muscle has not been studied in detail, even though the activity of this muscle becomes active in connection with the masticatory muscles during mastication (Thomas and Peyton, 1983). In relation to the hyoid bone, the digastric muscle is divided into anterior and posterior bellies, and differences in muscle fiber alignment suggest that these anterior and posterior bellies display different functions. Studies have been conducted on the digastric muscle, and both fast and slow muscles exist in the anterior and posterior bellies of the human digastric muscle, although the ratio of slow muscle fibers is slightly
greater for the posterior belly (Monemi et al., 2000; Korfage and Van Eijden, 2003). In rats, both the anterior and posterior bellies of the digastric muscle consist mostly of fast muscle fibers, and the ratio of fast muscle fibers is greater for the anterior belly than for the posterior belly (Cobos et al., 2001). A study on the digastric muscle in mice found no slow muscle isoform in the anterior belly (Hartmann et al., 2001). However, no studies have compared the anterior and posterior bellies of murine digastric muscle. To clarify the muscle fiber properties of the anterior and posterior bellies of the digastric muscle during development, including weaning, which brings about marked functional changes, expressions of MyHC-2a and -2b were assessed at the protein and transcriptional levels of mRNA.

**Materials and Methods**

1. **Materials**

   Since the mean weaning age of ICR mice (Sankyo Laboratory, Tokyo, Japan) is reportedly approximately 3 weeks, mice at 2-weeks-old (before weaning), 4-weeks-old (after weaning) and 9-weeks-old (adults) were analyzed in this study (Doi et al., 2003). After 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. A total of 30 mice were thus used. According to the animal study guidelines established by Tokyo Dental College, mice were sacrificed by injection of a lethal dose of pentobarbital. Anterior and posterior bellies of the digastric muscle were extracted (Fig. 1). These muscles were immediately frozen in liquid nitrogen and stored at -80 °C until testing.

2. Immunohistochemical analysis

Using a cryostat, each excised digastric muscle was serially sliced horizontally at a thickness of 10 μm orthogonal to the long axis of muscle fibers. Immunostaining was performed as follows: as primary antibodies, SC-71 (anti-MyHC-2a; American Type Tissue Culture, VA, USA) and BF-F3 (anti-MyHC-2b; American Type Tissue Culture, VA, USA) anti-mouse monoclonal antibodies extracted from hybridoma cells were used (Schiaffino et al., 1989; Eason et al., 2000). Hybridoma cells were cultured using Dulbecco’s modified Eagle’s medium (Sigma, MO, USA) with 10% fetal bovine serum (Sigma) at 37 °C with 5% CO₂ for 72 h, and then centrifuged. The supernatant
was used to provide primary antibodies. As secondary antibodies, FITC-labeled goat anti-mouse IgG antibody (Novocastora Laboratories, Newcastle, UK) was used to visualize SC-71, and RITC-labeled goat anti-mouse IgM antibody (Novocastora Laboratories, Newcastle, UK) 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.

To objectively assess immunohistochemical findings, the ratio of each muscle fiber within a square was determined based on the methods of Wakisaka et al., 1993 and Sartorius et al., 1998. Within a 200-µm square, numbers of MyHC-2a- and MyHC-2b-positive fibers were counted, and ratios of MyHC-2a- and MyHC-2b-positive fibers to the total number of muscle fibers in the square were calculated. For statistical analysis, t-tests were used to compare anterior and posterior bellies, with the level of significance set at p<0.05. Tukey’s q-tests were used to compare different age groups, with the level of significance again set at p<0.05.

3. 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 using a Quick Prep micro-mRNA Purification Kit (Amersham Pharmacia Biotech UK, Buckinghamshire, UK), and cDNA was prepared using Ready-To-Go (Amersham Pharmacia Biotech UK, Buckinghamshire, UK). 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 dilutions of a cDNA synthesis (4.0 ng/µl) were made, and $1/10^5$, $1/10^6$, $1/10^7$, $1/10^8$, and $1/10^9$ dilution ratios were used. The cDNA product contained 10.2 µl of sterile water and 5 µl of diluted control cDNA synthesis, 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). As a demonstration PCR mixture for the PCR product for each dilution, 1.6 µl of MgCl$_2$ (25 mM) and 2 µl of FastStart DNA Master SYBR Green I were added to 10.2 µl. In addition, 0.6 µl each of forward and reverse primers (10 pmol/µl) prepared using Oligo 5 primer design software (Biogene Ltd.) were added, followed by the addition of each PCR product dilution to a final reaction volume of 20 µl. MyHC-2a and -2b
primers were used and designed based on segments specific to the respective full DNA sequences. Base sequences for each primer are shown in Table 2.

As a PCR mixture, 1.6 µl of MgCl₂ (25mM), 2 µl of LC FastStart DNA Master SYBR Green I, and 0.6 µl each of forward primer (10 pmol/µl) and reverse primer (10 pmol/µl) were added to 14.2 µl of sterile water. Furthermore, 1 µl of target DNA was added to bring the final reaction volume to 20 µl. PCR mixtures (20 µl) for MyHC-2a and -2b that were prepared in the above manner were added to a capillary. PCR involved an initial period at 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 followed a melting program of 70 °C for 15 s, and during 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 using the above method was divided by the amount of GAPDH (a housekeeping gene) to calculate the final mRNA expression. The base sequence of GAPDH is also shown in Table 2. In the present study, t-tests were used to compare anterior and posterior bellies, 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.

**Statistical analysis**

Student’s t-test was used for statistical analysis in this study.

**Results**

1. Immunostaining

   At 2-weeks-old, no marked difference existed between MyHC-2a- and -2b-positive muscle fibers per area for both anterior and posterior bellies of the digastric muscle (MyHC-2a: anterior belly 26.2±6.7%, posterior belly 24.8±7.8%; MyHC-2b: anterior belly 18.4±5.3%, posterior belly 17.8±4.8%).

   At 4-weeks-old, the ratio of MyHC-2a-positive muscle fibers (anterior belly 19.7±5.5%, posterior belly 20.2±4.6%) tended to decrease in both anterior and posterior bellies compared to 2-weeks-old. On the other hand, the ratio of MyHC-2b-positive muscle fibers in the anterior belly (35.8±8.2%) was significantly higher compared to 2-weeks-old. But this significant increase was not statistically observed in the posterior belly (26.7±10.2%).

   At 9-weeks-old, the ratio of MyHC-2a-positive muscle fibers (anterior
belly 17.9±5.5%, posterior belly 18.8±6.2%) tended to be slightly decreased compared to 4-weeks-old, but no significant change was noted. The ratio of MyHC-2b-positive muscle fibers in anterior and posterior bellies at 9-weeks-old also tended to be greater than at 4-weeks-old, with no significant difference. At 9-weeks-old, the ratio of MyHC-2b-positive muscle fibers (anterior belly 43.6±7.9%, posterior belly 32.4±7.9%) tended to be greater in the anterior belly than in the posterior belly, but no significant difference was seen (Fig. 3).

Thus, no significant differences in the ratios of MyHC-2a-muscle fibers were seen between anterior and posterior bellies, and no marked changes occurred from 2- to 9-weeks-old (Fig. 4). The ratio of MyHC-2b-positive muscle fibers increased from 2- to 9-weeks-old in both anterior and posterior bellies. The ratio of MyHC-2b-positive muscle fibers in the anterior belly at 4-weeks-old was significantly greater than at 2-weeks-old (Fig. 5).

2. Analysis of mRNA expression using the LightCycler™

In the anterior and posterior bellies of the digastric muscle, expression of MyHC-2a mRNA tended to decrease slightly from 2- to 4- to 9-weeks-old, but no significant difference was observed (Fig. 6). Conversely, expression of MyHC-2b
mRNA increased with age from 2- to 4- to 9-weeks-old. Expression of MyHC-2b mRNA in the anterior belly at 4-weeks-old was about 4-fold higher than at 2-weeks-old, and the expression of MyHC-2b mRNA in the posterior belly at 4-weeks-old was about double that at 2-weeks-old. Between 4- and 9-weeks-old, MyHC-2b mRNA expression tended to increase slightly in the anterior and posterior bellies, but no significant differences were seen (Fig. 7).

**Discussion**

The digastric muscle is involved in mastication and swallowing, and is morphologically divided into anterior and posterior bellies. This muscle is a composite, derived both from the first and second pharyngeal arches, each belly being innervated by two different nerves, namely trigeminal nerve and facial nerve.

In this study, immunohistochemical analysis showed that the ratios of MyHC-2b- and MyHC-2a-positive muscle fibers at 2-weeks-old (before weaning) were mostly comparable between anterior and posterior bellies. This suggests that no functional changes occur in both the anterior and posterior bellies at 2-weeks-old.
At 4- and 9-weeks-old (after weaning), the ratio of MyHC-2b-positive muscle fibers in the anterior and posterior bellies increased with time. In other words, this increase represented prompt adaptation to changes in mastication due to weaning. Between 4-weeks-old (soon after weaning) and 2-weeks-old (before weaning), a significant increase was seen in the anterior belly, but not in the posterior belly. The masticatory system in rodents, as in mice, differs from that of other mammals in that mastication is based on rapid anteroposterior movements of the mandible (Hiiemae KM and Ardran GM, 1968; Okayasu I et al., 2003). Muscle fibers in the anterior belly, which attaches to the mandible, are aligned anteroposteriorly and are closely involved with masticatory movements (Kobayashi M et al., 2002). Therefore, the result of this study suggests that the anterior belly of the murine digastric muscle plays a dominant role in mastication, and it is consistent with the report of Kawata et al., which was documented that masticatory movements were closely involved in the development of the anterior belly of the digastric muscle. On the other hand, the ratio of MyHC-2b-positive muscle fibers in the posterior belly increased slightly with time in comparison with the anterior belly. This may be due to the fact that the posterior belly is involved in jaw opening, although it is not involved in the rapid
anteroposterior movement during mastication peculiar to the rodents.

The results of mRNA quantification using the LightCycler™ clarified that expressions of MyHC-2b and MyHC-2a mRNA were comparable in the anterior and posterior bellies at 2-weeks-old (before weaning). This suggests that, even at the transcription level, no functional changes occur in both the anterior and posterior bellies at 2-weeks-old (before weaning). At 4- and 9 weeks-old (after weaning), expression of MyHC-2b mRNA in the anterior and posterior bellies increased with age, and this tendency was greater in the anterior belly. In the anterior belly, a significant increase in MyHC-2b mRNA expression was seen between 2- and 4-weeks-old. This suggests that, again at the transcription level, the anterior belly is closely involved with rapid masticatory movements, like the masticatory muscles. The present results suggest that the digastric muscle, which comprises anterior and posterior bellies, acquires muscle fibers to match muscular roles.

Acknowledgements

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Technology, Japan and by Oral Health Science Center Grant HRC 7 (S.A.) from Tokyo Dental College.

References


masticatory muscles by immunohistochemistry and gel electrophoresis. J Histochem Cytochem 51: 113-119


Table 1. Myosin heavy chain isoforms identified in skeltal muscle

<table>
<thead>
<tr>
<th>Designation</th>
<th>Nomenclature</th>
<th>Distribution</th>
</tr>
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<tbody>
<tr>
<td>Embryonic</td>
<td>MyHCemb</td>
<td>Myobubes, intrafusal fibres, regenerating fibres</td>
</tr>
<tr>
<td>Neonatal</td>
<td>MyHCneo</td>
<td>Neonatal muscles, masseter, intrafusal fibres</td>
</tr>
<tr>
<td>Fast-twitch</td>
<td>MyHCeom</td>
<td>Super-fast fibres in extraocular muscles</td>
</tr>
<tr>
<td>Fast-twitch</td>
<td>MyHC-2m</td>
<td>Super-fast fibres in muscles derived from the first branchial arch</td>
</tr>
<tr>
<td>Fast-twitch</td>
<td>MyHC-2b</td>
<td>Fast-type isoforms in digastic muscle of mice contraction speed: 2b&gt;2d&gt;2a</td>
</tr>
<tr>
<td>Fast-twitch</td>
<td>MyHC-2d</td>
<td></td>
</tr>
<tr>
<td>Fast-twitch</td>
<td>MyHC-2a</td>
<td></td>
</tr>
<tr>
<td>Slow-twitch</td>
<td>MyHC-1</td>
<td>Type I fibres</td>
</tr>
</tbody>
</table>


Table 2. Base sequence of the primers

MyHC-2a

Forward: 5’-CGATGATCTTTGCCAGTAATG-3’
Reverse: 5’-TGATAACTGAGATACCAGCG-3’
Accession: NM_144961

MyHC-2b

Forward: 5’-ACAGACTAAAGTGAAGGCC-3’
Reverse: 5’-CTCTCAACAGAAAGATGGAT-3’
Accession: XM_126119

GAPDH

Forward: 5’-TGAACGGAAGCTCAGTGG-3’
Reverse: 5’-TCCACCACCTGTTGCTGTA-3’
Accession: NM_008084
Figure 1. Interior view of head and neck region

Location and alignment of anterior and posterior bellies of murine digastric muscle are shown.

AD: anterior belly of the digastric muscle

PD: posterior belly of the digastric muscle
Figure 2. Muscle fibers inside a square segment

Muscle fibers with black borders inside the square segment were counted and divided by the total number of muscle fibers inside the segment. Fibers touched by the upper and left lines were excluded.
Figure 3. Immunostaining of anterior and posterior bellies of the digastric muscle

AD: anterior belly of the digastric muscle; PD: posterior belly of the digastric muscle.

A: MyHC-2a-positive fibers
B: MyHC-2b-positive fibers

Bar, 50 µm.
Figure 4. Ratio of MyHC-2a-positive fibers to total number of muscle fibers per area

AD: anterior belly of the digastric muscle; PD: posterior belly of the digastric muscle.

Ratios of MyHC-2a-positive fibers in the anterior and posterior bellies were unchanged at 2-, 4- and 9-weeks-old.
**Figure 5.** Ratio of MyHC-2b-positive fibers to total number of muscle fibers per area

*p<0.05

The ratio of MyHC-2b-positive fibers in the anterior and posterior bellies increased with age from 2-, 4- to 9-weeks-old. A significant difference was identified in the anterior belly between 2- and 4-weeks-old.
Expression of MyHC-2a mRNA decreased slightly in the anterior and posterior bellies at 2-, 4- and 9-weeks-old, but no significant changes were seen.
Figure 7. Expression of MyHC-2b mRNA (LightCycler™)

*p<0.05

Expression of MyHC-2b mRNA in the anterior and posterior bellies increased with age from 2- to 4- to 9-years-old, revealing a significant increase in the anterior and posterior bellies from 2- to 4-weeks-old. At 9-weeks-old, expression of MyHC-2b mRNA was significantly greater in the anterior belly than in the posterior belly.