Myosin heavy chain composition of the human jaw muscles
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MYOSIN HEAVY CHAIN COMPOSITION IN HUMAN MASTICATORY MUSCLES BY IMMUNOHISTOCHEMISTRY AND GEL ELECTROPHORESIS

Abstract In the present study, we compared for the three major fibre types (I, IIA, and IIX) the immunohistochemically quantified fibre type area with the electrophoretically quantified myosin heavy chain (MyHC) contents. For this purpose, bundles of fibres (n = 42) were taken from the anterior and posterior belly of the human digastric muscle (n = 7). The relative MyHC contents determined electrophoretically (MyHC-I, -IIA, and -IIX: anterior belly: 32%, 35%, and 33%; posterior belly: 39%, 42%, and 19%) did not differ significantly from the immunohistochemically determined relative area (MyHC-I, -IIA, and -IIX: anterior belly: 32%, 31%, and 37%; posterior belly: 39%, 45%, and 15%). The correlation coefficient ranged between 0.71 and 0.96 and was higher for MyHC type I and MyHC type IIX than for MyHC type IIA. The MyHC contents of single fibres taken from the posterior belly indicated that many fibres in this belly co-expressed MyHC-IIA and MyHC-IIX. Despite the presence of these hybrid fibres, the correspondence between both methods was relatively large.

Introduction

Human muscle fibres can be divided into a few different fibre types depending on the myosin heavy chain (MyHC) isoform they express. MyHC type I is expressed in slow muscle fibres and MyHC types IIA and IIX are expressed in fast muscle fibres. The
latter type is said to have a faster contraction velocity than MyHC type II A. MyHC-IIB is not found in humans, although its gene is still present (Weiss et al., 1999a).

To describe the fibre type composition of various muscles, investigators calculated the relative number or the cross-sectional area that a particular fibre type occupies, as determined by either ATPase histochemistry (Johnson et al., 1973; Eriksson et al., 1982; Travnik et al., 1995; Monemi et al., 2000), or by immunohistochemistry (Schiaffino and Reggiani, 1994; Korfage et al., 2000), or by a combination of the two methods (Sciote et al., 1994; Zhou et al., 1995). Another technique is to separate the MyHC isoforms by sodium dodecyl polyacrylamide gel electrophoresis and to calculate the integrated density of the bands (Adams et al., 1993). This technique allows the determination of the MyHC contents of a muscle even in muscles that are difficult to cut, such as diseased muscles.

Some studies have made quantitative comparisons between the relative area, or the relative fibre type distribution, versus the MyHC contents determined by gel electrophoresis in human muscle biopsy specimens (Perrie and Bumford, 1986; Adams et al., 1993; Fry et al., 1994) or in single fibres (Staron, 1991; Staron and Hikida, 1992; Sant Ana Pereira et al., 1995b). In general, a good correlation between these two methods was found. These studies, however, investigated limb muscles (in particular the vastus lateralis muscle) and used ATPase histochemistry to determine the fibre types. ATPase histochemistry can lead to a mismatch of fibre types, especially among the fast fibre types (Andersen et al., 1994; Serrano et al., 2001). Furthermore, in most studies the fibre type composition was estimated on a small portion of the investigated biopsy, whereas the gel electrophoresis was performed on the total cross-section of the biopsy. A recent study included all fibres of a fibre bundle (human vastus lateralis muscle) and compared immunohistochemistry with gel electrophoresis (Serrano et al., 2001).

To our knowledge, a comparison of the fibre type area of all the fibres, as determined by immunohistochemistry, and the MyHC contents, as determined by gel electrophoresis, has not been performed on whole bundles of muscle fibres in human jaw muscles. The jaw muscles are different from limb and trunk muscles. Their fibres are four to five times smaller than those in limb and trunk muscles (Staron and Hikida, 1992), and often the type II fibres are smaller than the type I fibres, whereas in limb and trunk muscles the opposite is true. Furthermore, the jaw muscles contain many hybrid fibres (Korfage and Van Eijden 1999, 2000; Korfage et al., 2001).

The primary focus of the present study was to compare the cross-sectional
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area of a certain fibre type in a bundle of jaw muscle fibres, as determined by immunohistochemistry, with its MyHC contents, as determined by gel electrophoresis. We used the digastic muscle. Both bellies of this muscle were investigated separately because they differ in fibre type composition (Eriksson et al., 1982, Korfage et al., 2000).

Materials and Methods

In this study, the anterior and posterior bellies of the digastic muscle of seven Caucasian cadavers were used, four males and three females, mean age ± S.D. = 70.3 ± 15.6 years. The individuals were without known systemic disorders. None of the muscle specimens contained signs of muscle disease on the basis of histological examination. Of these seven cadavers, five had upper and lower dental prostheses, and two were partially dentate. The muscles were obtained within 12 to 36 hours post mortem, which was found not to influence the results greatly (Eriksson et al., 1980). After the muscles were exposed they were cut from their attachment sites. The muscles were rapidly frozen in liquid nitrogen-cooled isopentane. From each frozen belly, three bundles of fibres (approximately 5 mm long) were cut out and stored at -80°C until required for further processing. In total, 42 bundles (= 7 muscles x 2 bellies x 3 bundles) were investigated. The cross-sectional area of the bundles was 2.5 ± 0.8 mm² (mean ± S.D.). The number of fibres per bundle area ranged from approximately 700 to 3100. The data was grouped regardless of gender.

Immunohistochemistry

Serial transverse sections of 10 μm were cut in a cryomicrotome (Model HM 500 M, Adamas Instruments BV, Leersum, the Netherlands). The first and the last ten sections were collected for gel electrophoresis analysis. The 15 sections in between were mounted on coverslips and three of these sections were used for immunohistochemical analysis. After overnight fixation at -20°C in a mixture of methanol:acetone:acetic acid:water (35:35:5:25) (Wessels et al., 1988), the sections were incubated with monoclonal antibodies raised against purified myosin (Bredman et al., 1991), namely, antibody 219-1D1, which recognised MyHC-I. antibody 333-
7H1, which recognised MyHC-IIA, and antibody 332-3D4, which recognised MyHC-IIA and MyHC-IX. Since the human digastric muscle contains only a few fibres that express MyHC-fetal or MyHC-cardiac α (Korfage et al., 2000) these two antibodies were not included. The indirect unconjugated immunoperoxidase technique (PAP-technique) was applied to detect the specific binding of the different antibodies. Nickel-diaminobenzidine was used to visualise the staining (Hancock, 1982).

Sample Method and Fibre Cross-sectional Area Measurements

The three sections were photographed by means of a digital camera attached to a microscope and the fibre area cutlines were drawn on a sheet. The fibre areas were classified by means of the three sections. We calculated the cross-sectional area of the fibres by scanning the drawn sheets together with a grade mark for correction of enlargement into a personal computer via a flat-bed scanner (Hewlett-Packard, Scanjet 4c). A custom-made program (Korfage and Van Eijden, 1999) that converts the number of pixels into μm² was then used to determine the total cross-sectional area of each muscle fibre type in μm². The percental cross-sectional area (csa) of MyHC type I fibres was calculated as: csa type I = (csa type I + csa type IIA+IIX) x 100%, of MyHC type IIA fibres as: csa type IIA = (csa type I + csa type IIA+IIX) x 100%, and of MyHC type IIX fibres as: (csa type IIA+IIX - csa type IIA) / (csa type I + csa type IIA+IIX) x 100%.

Sodium Dodecyl-sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The collected frozen sections were washed and homogenised as described by Bamman and co-workers (1999) The washed myofibrils were diluted in sample mix [15% (w/v) glycerol, 2% (w/v) DTT, 0.01% (w/v) bromophenol blue, and 1% (w/v) SDS in 62.5 mM Tris/HCl buffer, pH 6.8]. The samples were lysed for 2 minutes at 100 °C and then stored frozen (-80 °C) until processed for protein separation.

Gel electrophoresis was performed in high glycerol containing (30%) sodium dodecyl-sulfate polyacrylamide gels (0.75 mm thickness) using an acrylamide to bis-acrylamide ratio of 67:1 in the separating gel (9% total acrylamide, pH 8.8) and of 50:1 in the stacking gel (4% total acrylamide, pH 6.8) (modified from Talmadge and Roy, 1993). The samples were run at constant current (13.5 mA) for 29 hours using a custom-built electronic timer device (pulse unit) connected to the power supply that
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switched the running current on and off (Sant’Ana Pereira et al., 2001). After this process the gels were silverstained. For a better quantification of the MyHC isoform bands, the gels were blue-toned according to Berson (1983). The gels were scanned with an LKB 2202 Ultrascan laser densitometer (LKB, Bromma, Sweden). The MyHC isoforms were identified on the basis of migration as MyHC types I, IIA, and IIX. The total integrated area of the three MyHC peaks was set to 100 and each individual area was expressed as a percentage of the total MyHC. The densitometric signal was linear over the range of MyHC concentrations loaded on the gels.

To estimate the quantification error of this scanning method of the MyHC isoform bands, 36 lanes were scanned for a second time on a different day. For each individual MyHC isoform, the mean and standard deviation values of the differences ($n = 36$) were determined and were, respectively. MyHC-I: $0.36 \pm 4.50\%$ (mean $\pm$ S.D.), MyHC-IIA: $-1.89 \pm 9.00\%$, MyHC-IIX: $1.52 \pm 9.94\%$.

Single Fibres

Our antibody panel cannot distinguish hybrid fibres that co-express MyHC-IIA and MyHC-IIX from fibres that express only MyHC-IIA. To get an indication whether the digastric muscle used in this study contains such hybrid fibres, we analysed the MyHC contents of a sample of single fibres by gel electrophoresis. Because the posterior belly contains more fibres denoted by immunohistochemistry as MyHC type IIA fibres, which could co-express more MyHC-IIX than the anterior belly (Korfage et al., 2000), a part of the posterior belly of three individuals was freeze-dried. The three individuals were chosen at random. Prior to dissection, the muscle parts were brought, under vacuum, to room temperature in 2 hours. From these bellies single fibres were microdissected (Essén et al., 1975). After dissection, each fibre (approximately 5 mm long) was split into two parts. One part was embedded in 15% gelatine and processed for immunohistochemistry to ascertain if the dissected fibre was indeed single. The other part was dissolved in sample mix and processed for gel electrophoresis using the same method as described above. The embedded single fibres were frozen in liquid nitrogen and cut in a cryomicrotome (10μm). The slides were incubated with the same three antibodies as used for the determination of the fibre areas but with a modified method (Sant’Ana Pereira et al., 1995b). In total, 61 single fibres were investigated.
Figure 6.1
Example of a cross-section of a fibre bundle from the anterior belly of the digastic muscle (A-D) and the electrophoresis results (E,F). (B-D) Magnifications of the indicated area in A after incubation with, respectively, antibodies against MyHC-I (B), MyHC-IIA (C), and MyHC-IIA+IIIX (D). Bar = 100 μm. (E) MyHC composition of this bundle as determined by gel electrophoresis. Only the MyHCs are shown. The plot (F) shows the integrated optical density of this particular fibre bundle.
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Statistical Analysis
In each individual the mean was calculated for each MyHC fibre type area and MyHC contents over the three bundles of each belly. Then the grand mean and standard deviation values were calculated over the means of the individuals (n = 7). The Wilcoxon ranking test for paired data was used to analyse differences between the mean fibre type area and its corresponding MyHC isoform contents within a belly, and between the anterior and posterior belly. Spearman’s rank correlation was used to examine the relation between MyHC contents, as determined by gel electrophoresis, versus fibre type area. The level of significance was set at $P<0.05$.

Results
In Fig. 6.1 an example of a cross-section of a fibre bundle from the anterior belly of the digastric muscle is shown together with the electrophoretic results. In Figure 6.2 the percentual fibre type area in the anterior and posterior belly of each individual is linked to its percentual MyHC isoform contents. There was a large interindividual variation of the proportion of each fibre type area and MyHC isoform contents.

For all MyHCs, no significant differences were found between the proportion of a MyHC fibre type area and its MyHC isoform contents (Table 6.1); the correlation ranged between 0.71 and 0.96. The fibre type areas of MyHC type I, MyHC type IIA, and MyHC type IIX differed significantly between the anterior and posterior belly. In the anterior belly, a larger MyHC type IIX fibre area and a smaller MyHC type IIA and MyHC type I fibre area were found than in the posterior belly.

Of the 61 investigated single muscle fibres, 37 were pure fibres, expressing only one MyHC isoform, and the other 24 fibres were hybrid fibres. The most frequent hybrid fibre type found in these three muscles were fibres that co-expressed MyHC-IIA and MyHC-IIX (Table 6.2).
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Discussion

Two methods can be used to quantify the fibre type composition of a muscle, namely to calculate either the percentage or the area that is occupied by a particular fibre type classified by ATPase histochemistry or immunohistochemistry, or to determine the MyHC contents by gel electrophoresis. An advantage of gel electrophoresis is that even in biopsies of muscles which are difficult to cut in a cryomicrotome, e.g., in
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<table>
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<th>Posterior</th>
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<td>%</td>
<td>S.D.</td>
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<tr>
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Table 6.1
Mean myosin heavy chain isoform percentages (n=7).

* Values denote statistical significance between the anterior and posterior bellies.

† Correlation between immunohistochemistry and gel electrophoresis.

diseased material, an MyHC profile can be established.

To justify the use of one of the two methods, the correlation between these methods has to be established. A comparison between fibre types as determined by ATPase histochemistry and gel electrophoresis (Perrie and Bumford, 1986; Adams et al., 1993; Fry et al., 1994), and as determined by immunohistochemistry and gel electrophoresis (Serrano et al., 2001), was made in human limb and trunk muscle biopsies and also in single fibres (Staron, 1991; Staron and Hikida, 1992; Sant Ana Pereira et al., 1995b). A comparison of fibre type composition determined by immunohistochemistry and gel electrophoresis in human jaw muscles has, to our knowledge, not been performed earlier. In most of the aforementioned studies the vastus lateralis muscle was investigated, in which the distribution of the fibre types in a biopsy was estimated by calculating the cross-sectional area for the different fibre types on approximately 50 fibres. The gel electrophoresis, however, was done on all fibres of the biopsy specimen. The study by Serrano and co-workers (2001) and the present study are, in this respect, more accurate because the total area of all fibres of a particular fibre type was measured.

The correlation coefficients for ATPase histochemistry versus gel electrophoresis in the vastus lateralis muscle found in literature ranged from 0.71 to 0.87 (Adams et al., 1993; Fry et al., 1994) and for immunohistochemistry versus gel
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Table 6.2
MyHC fibre type composition of single fibres (n = 61) from the posterior belly of the digastric muscle of three individuals, as determined by SDS-PAGE. For each fibre type the number (n) of fibres is given.

<table>
<thead>
<tr>
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<th>Subject H130296</th>
<th>H130696</th>
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<tr>
<td>I</td>
<td>5</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>IIA</td>
<td>5</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>IIX</td>
<td>1</td>
<td>3</td>
<td></td>
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<tr>
<td>IIA-IIX</td>
<td>5</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>I+IIX</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-IA+IIX</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>24</td>
<td>12</td>
</tr>
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</table>

Electrophoresis from 0.66 to 0.88 (Staron et al., 2000; Serrano et al., 2001). The correlation coefficients in the present study (range: 0.71 - 0.93) are comparable to the ones found in these studies.

The observed differences between the two methods might be explained as follows. Firstly, the concentration of myosin in a fibre differs between type I and type II fibres. In rabbit, it was found that type I fibres from the soleus contained approximately 22% less myosin than type IIX fibres from the psoas (Tikunov et al., 2001) and in chicken the red portion of the latissimus dorsi muscle contained 25% less myosin than its white portion (Everett et al., 1983). In general, a greater volume of a slow muscle fibre is occupied by mitochondria and sarcoplasmic reticulum than in a fast fibre (Rome and Lindstedt, 1998). Since there is no significant difference between the fibre cross-sectional area of type I and type II fibres in the digastric (Korfaege et al., 2000), we expected that the MyHC contents of type I fibres to be lower than its type I area proportion. However, this was not seen in the present study. Secondly, hybrid fibres that co-express more than one MyHC isoform, could contribute to the difference in results between the two methods. Indeed, the single fibre analysis showed that a significant part of the investigated fibres of the posterior belly were hybrid fibres. This was also noted in a study (Monemi et al., 2000) in which many fibres co-expressed both fast MyHC isoforms. With the antibody panel used in the present study it was not possible to detect hybrid fibres that co-express MyHC-IIA and MyHC-IIX. Many of these fibres were therefore misclassified as MyHC type IIA fibres by immunohistochemistry while they were actually hybrid fibres that co-express MyHC-IIA and MyHC-IIX. Antibodies that can distinguish this hybrid fibre type exist.
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(Schiaffino et al., 1989; Lucas et al., 2000; Serrano et al., 2001) but are not commercially available. Moreover, with immunohistochemistry the exact contents of the various MyHC isoforms in hybrid fibres cannot be determined. Whether hybrid fibres were the reason that the correlation coefficient for MyHC-IIA in the present study was lower than for MyHC-I and MyHC-IIX fibres remains to be determined.

In an ATPase histochemical study of the digastric muscle in young individuals (Eriksson et al., 1982) hardly any hybrid type II fibres were found. This may be explained either by the mismatch between ATPase histochemical fibre type estimation and its MyHC isoform contents or by the high age of the investigated subjects in the present study, because this particular hybrid fibre type increases with age (Klitgaard et al., 1990; Andersen et al., 1999b). In a study that compared the fibre type composition of the human digastric muscle in young and elderly individuals (Monemi et al., 2000), no significant differences were found in MyHC contents of muscle fibres between young adults and elderly.

In conclusion, both immunohistochemistry and gel electrophoresis give comparable results. In the human digastric muscle, hybrid fibres co-expressing MyHC-IIA and MyHC-IIX seem to be a common hybrid fibre type. Further studies of single fibres with gel electrophoresis could show the ratio of MyHC-IIA to MyHC-IIX within these hybrid fibres.