Fetal myosin heavy chain increases in the human masseter muscle during aging

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Abstract Biochemical, immunohistochemical and molecular biological methods were used to detect fetal myosin heavy chain (MyHC) in the human masseter of elderly and young subjects. Samples from the elderly subjects contained larger amounts of fetal MyHC than those of young adults. Only a very small amount of embryonic MyHC could be detected in both age groups. Embryonic and fetal MyHCs were never detected in the control adult orofacial, limb and trunk muscles. Polymerase chain reaction (PCR) analysis revealed the presence of fetal mRNA sequences in elderly and young masseter muscles. We conclude that fetal MyHC is present in the human masseter throughout the life span and that there is an increase in the relative amount of this protein with age.

Key words: Aging; Human; Masseter; Muscle; Myosin; mRNA

1. Introduction

Embryonic and fetal myosin heavy chain (MyHC) isoforms are replaced after birth in limb and trunk muscles by adult slow and fast isoforms of MyHC [1,2]. However, in the human masseter muscle these myosin isoforms have been shown to persist during postnatal [3] and adult life, mainly within the population of fibers also containing fast myosin [4-8]. The role of embryonic and fetal MyHCs in masseter muscle contraction remains unclear. However, it has been suggested that their presence is related to the unique demands required of the human masseter muscle during the various jaw functions and/or to its cranial nerve supply [3,4,6-8].

Recently, we have shown a significant age-related increase of histochemically defined type II fibers, i.e. fibers containing fast MyHCs, in the human masseter of elderly subjects (Monemi et al., submitted). To further characterise these fibers and the expression of the embryonic and fetal MyHCs in the human masseter in relation to aging, we have examined muscle specimens from the masseters of elderly and young subjects, using biochemical, molecular biological and enzyme- and immunohistochemical techniques. For comparison the study included orofacial, limb and trunk muscles.

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2. Materials and methods

2.1. Muscle specimens

Muscle specimens from the anterior and posterior parts of the superficial portion and the deep portion of the masseter muscle and the biceps brachii muscle of six elderly subjects (mean age 74 years, 58–83 years) and two young adults (22 and 24 years) were obtained 1–3 days post mortem [9]. Additional samples from quadriceps at 16 weeks of development and young adult buccinator and diaphragm muscles were used for PCR analyses (see below). The investigation was approved by Socialstyrelsen, The National Board of Health and Welfare, Stockholm, Sweden.

2.2. Enzyme- and immunohistochemistry

The muscle specimens were frozen in liquid propane chilled with liquid nitrogen. In a cryostat microtome, 5–10 μm thick serial cross-sections, were cut at −20°C and processed for myofibrillar Ca^{2+}-activated adenosine triphosphatase (ATPase, EC 3.6.1.3) at pH 9.4 after alkaline (pH 10.3), or acid (pH 4.6, 4.3) preincubations [10]. Fiber type classification was based on the staining intensity for ATPase after alkaline and acid preincubations according to the following criteria (Monemi et al., submitted). At pH 10.3, weakly stained fibers were termed type I and moderately to strongly stained fibers type II. After acid preincubations, type II fibers were subdivided into IIA (unstained or weakly stained at pH 4.6 and unstained at pH 4.3), IIAB (moderately stained at pH 4.6 and unstained at pH 4.3), IIB (moderately to strongly stained at pH 4.6 and unstained at pH 4.3) and IIC (moderately stained at pH 4.6 and weakly stained at pH 4.3). Fibers with a moderate staining at alkaline pH and moderate to strong at acid pH were labelled type IM. Previously documented, antibodies (Ab) against fetal (NN5) [4-7,11] and embryonic (2B6) [6,7,12] MyHC isoforms, were used on cross-sections, serial to those used for enzyme histochemistry. Immunohistochemical detection of the primary antibody was performed with the indirect peroxidase-antiperoxidase (PAP) technique [13]. The PAP products were obtained from Dakopatts (Copenhagen, Denmark).

Muscle cross-sections of each muscle region from elderly subjects were photographed in 2–4 areas. Fiber type classification and evaluation of immunostaining intensity of fiber types were performed in serial sections at 300× magnification for the masseter and 160× for the biceps brachii. More than 10000 fibers were examined. Detailed analysis of immunoreactivity of fiber types was based on evaluation of 2016 ($\bar{x}$ = 351, S.D. = 101) fibers in the old masseter, 1909 fibers ($\bar{x}$ = 318, S.D.=136) in the old biceps and 638 ($\bar{x}$ = 319, S.D. = 28) fibers in the young adult masseter.

2.3. Immunoblotting analysis

Muscles were extracted as described previously for the native myosins [11], denatured with SDS and run on 12% polyacrylamide SDS slab gels. The proteins were electrophoretically transferred to nitrocellulose. The paper was incubated with the anti-fetal antibody (1/100) and specific binding was revealed with the indirect peroxidase-antiperoxidase technique.

2.4. Polymerase chain reaction analysis (PCR)

mRNAs from adult buccinator, diaphragm and elderly and young masseters as well as from quadriceps from 20 weeks of development were prepared according to [14]. Random cDNA was prepared from 2 μg of total mRNA, using random hexaprimers and M-MLV reverse

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transcriptase for 45 min at 42°C (Gibco). Amplification was performed with oligonucleotides specific for the perinatal myosin heavy chain sequence [15], using Taq-Pol (Gibco) according to the manufacturer's instructions. The specificity of the amplified fragment was determined by (i) the size of the band as compared to what is expected from the sequence, and (ii) restriction of the amplified material with *Hin*fI (Gibco) (data not shown). Possible DNA amplification was eliminated by choosing the oligonucleotides in different exons. Amplified fragments were separated on a 6% acrylamide gel.

3. Results

3.1. Enzyme- and immunohistochemistry

The anti-fetal reactive fibers were in general heterogeneously distributed over the masseter muscle cross-sections. In the young masseter, anti-fetal immunoreactivity was exclusively detected in the type IM+IIC fibers ($\bar{x}$=37%, S.D.=11%) and the type IIB fibers ($\bar{x}$=38%, S.D.=20%). In the old masseter, anti-fetal immunoreactivity was preferentially detected in the type IM+IIC fibers ($\bar{x}$=48%, S.D.=34%) and the type IIB fibers ($\bar{x}$=39%, S.D.=21%). Only minor populations of the type I fibers ($\bar{x}$=4%, S.D.=5.4%) and the type IIA+IIAB fibers ($\bar{x}$=1%, S.D.=2%) showed reactivity with the anti-fetal MyHC Ab (Fig. 1a–c). A very small proportion of the masseter fibers showed immunoreactivity with the anti-embryonic Ab (<1%). No immunoreactivity was detected in the biceps brachii muscle specimens.

3.2. Immunoblotting

In Fig. 2 it can be seen that fetal MyHC was present in both old and young masseter muscles. However, the fetal MyHC band was consistently stronger in all of the older masseter muscles. Reactivity for fetal MyHC was always absent in the biceps brachii muscle specimens (data not shown).

3.3. Polymerase chain reaction (PCR)

A specific band of 602 bp, was observed in the elderly masseter sample. A band of the same size was present in large amounts in fetal muscle, but was not detected in either adult buccinator or diaphragm (Fig. 3). The presence of fetal MyHC mRNA in the masseter sample is therefore confirmed by the detection of the corresponding mRNA.

Table 1
Percentage (%) of anti-fetal MyHC Ab reactive fibers in various fiber type groups of the old and young human masseter muscle

	Percentage			
	I	IM+IIC	IIA+IIAB	IIB
Elderly	4.0 (31.0)	48.0 (16.0)	1.0 (14.0)	39.0 (39.0)
Young adults	– (62.0)	37.0 (9.0)	– (2.0)	38.0 (27.0)

Values in parentheses show the relative frequency of fiber types in the elderly (Monemi et al., submitted) and young adult [18] masseter.

4. Discussion

In the present study we show by immunohistochemical and biochemical techniques that the fetal MyHC, which was initially characterized during muscle development, not only persists in the adult human masseter muscle but increases in relative amount with aging. The result of the PCR analysis confirms the presence of fetal MyHC mRNA in the elderly masseter.

The age-related increase in the amount of fetal MyHC in the masseter muscle could be related to a relative increase of this myosin isoform within single muscle fibers and/or an increase in the total number of fibers expressing fetal MyHC. In accordance with previous reports in young adults [5,7], the immunoreactivity with the anti-fetal MyHC antibody was mainly detected in the type IM, and type II fiber populations and in about the same proportions. Thus, the increased amount of fetal MyHC in the old masseter should be related to an increase in the total number of fibers containing fetal MyHC. In fact, in a recent study carried out on the human masseter of elderly subjects (Monemi et al., submitted) we show that there is an age-related shift in fiber type composition, towards a relatively higher proportion of type IM and type II fibers (Table 1). It has been hypothesized that since the masseter muscle is innervated by a cranial nerve and derived from the first branchial arch, this may be responsible for the different phenotypic expressions of MyHCs in this muscle [3,7]. However, this would not seem to be the reason since the anterior portion of the human digastric muscle is, like the masseter, evolved from the first branchial arch and supplied by the same branch of the trigeminal nerve but the fiber type

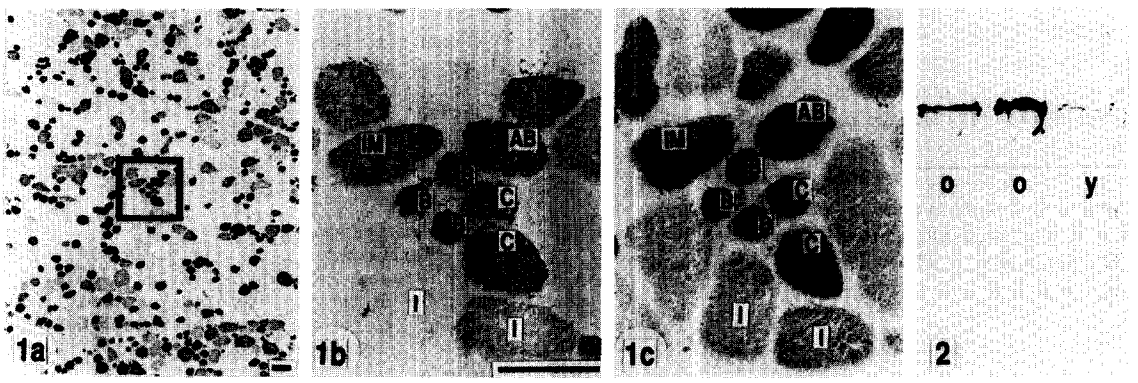

Fig. 1. Serial cross-sections of the masseter muscle from an elderly subject (78 years) labelled with anti-fetal MyHC antibody followed by PAP staining (a,b) and stained for myofibrillar ATPase activity at pH 10.3 (c). Fiber types I, IM, IIC (C), IIAB (AB) and IIB (B) have been labelled. Note immunostaining preferentially in the type IM and II fibers. Magnification: (a) ×50, (b,c) square in (a) ×300. Scale bars = 50 μm.

Fig. 2. Immunoblotting analysis of myosin extracted from old (o) and young (y) masseters reacted with antibody specific against fetal MyHC. Note thicker bands, i.e. larger amounts of fetal MyHC in the samples from the elderly subjects.

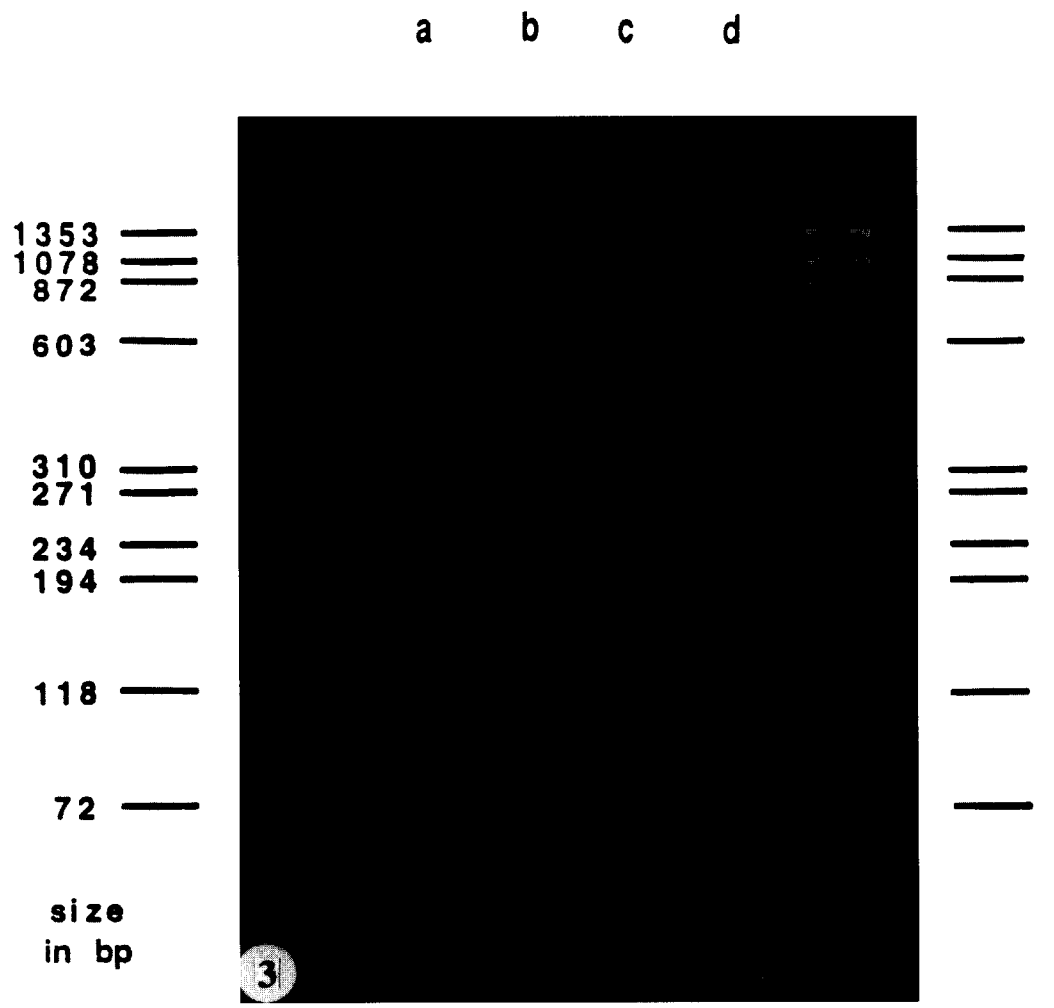

Fig. 3. Electrophoresis of PCR products from masseter muscle sample of an 73 year old man (d) after 29 cycles of amplification with oligonucleotides specific for the fetal MyHC. For comparison, the mRNA from buccinator (a), diaphragm (b) and quadriceps at 16 weeks of development (c) were used. The marker, is $\Phi \times 174$, digested by *Hae*III (Gibco).

composition of this muscle resembles more closely the limb muscles [16] and lacks fetal MyHC (in preparation). Functional specialization, related to genetic control of trigeminal motoneurones and nerve branches, may be important for the regulation of the muscle phenotype. The age-related shift in the fetal MyHC composition may reflect inherent changes in the central nervous system with age and/or in changes of functional requirements.

The expression of fetal MyHC demonstrated in the human masseter muscle both during development and in the adult obviously shows that this protein is a normal constituent of the adult human masseter muscle. The fact that fetal MyHC is present also in the adult human extraocular [17], and tensor tympani (Thornell et al., unpublished) muscles and in muscle spindle fibers [5,6], provides further evidence that fetal myosin is part of the adult myosin repertoire, related to specialisation of phenotype. Therefore, a revision of the present nomenclature of fetal myosin seems justified.

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