Immunolabelling, histochemistry and in situ hybridisation in human skeletal muscle fibres to detect myosin heavy chain expression at the protein and mRNA level

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ABSTRACT

The distribution of muscle fibres classified on the basis of their content of different myosin heavy chain (MHC) isoforms was analysed in vastus lateralis muscle biopsies of 15 young men (with an average age of 22 y) by correlating immunohistochemistry with specific anti-MHC monoclonal antibodies, myofibrillar ATPase (mATPase) histochemistry and in situ hybridisation with probes specific for MHC β-slow, MHC-IIA and MHC-IIX. The characterisation of a large number of individual fibres was compared and correlated on a fibre-to-fibre basis. The panel of monoclonal antibodies used in the study allowed classification of human skeletal muscle fibres into 5 categories according to the MHC isoform they express at the protein level, types I, I+IIA, IIA, IIAX and IIX. Hybrid fibres coexpressing two isoforms represented a considerable proportion of the fibre composition (about 14%) and were clearly underestimated by mATPase histochemistry. For a very high percentage of fibres there was a precise correspondence between the MHC protein isoforms and mRNA transcripts. The integrated methods used demonstrate a high degree of precision of the immunohistochemical procedure used for the identification and quantification of human skeletal muscle fibre types. The monoclonal antibody S5-8H2 is particularly useful for identifying hybrid IIAX fibres. This protocol offers new prospects for muscle fibre classification in human experimental studies.

Key words: Immunohistochemistry; myofibrillar ATPase; vastus lateralis muscle; muscle biopsies; in situ hybridisation.

INTRODUCTION

Skeletal muscle cells fall into several specialised classes, termed fibre types, which show differences in morphological, contractile and metabolic properties. Furthermore, skeletal muscle fibres are characterised by a considerable potential for adaptation. Thus muscle fibres are versatile elements, which are capable of modifying their structure and properties (Pette & Staron, 1990). Myosin is a major structural protein of skeletal muscle filaments associated with power generation during muscle contraction. A myosin molecule comprises 2 identical heavy chains and 2 pairs of light chains. Myosin heavy chain (MHC) isoforms represent the best markers of muscle fibre diversity and adaptive changes (Schiaffino & Reggiani, 1996). In fact the MHC isoform expressed in a fibre underlies the phenotypic differences in contractile and metabolic properties observed among fibre types (Rivero et al. 1999).

In adult human leg muscle, 3 different MHC isoforms (types β-slow, IIA and IIX or IID) are expressed in types I, IIA and IIX or IID (henceforth IIX) fibres, respectively (Smerdu et al. 1994; Ennion et al. 1995; Sant'Ana Pereira et al. 1997). In addition to these 3 'pure' fibres expressing an individual MHC isoform, the coexpression of 2 (Klitgaard et al. 1990; Andersen and Schiaffino 1997) or even the 3 (Sant'Ana

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Pereira et al. 1995; Andersen et al. 1999) isoforms also occurs under physiological conditions. These hybrid fibres have been demonstrated to be particularly numerous in highly trained athletes (Klitgaard et al. 1990) and in very old individuals (Andersen et al. 1999). In man, as in other mammals, the cellular expression of MHC isoforms has been routinely characterised upon the basis of the myofibrillar actomyosin adenosine triphosphatase (mATPase) histochemical reaction proposed by Brooke & Kaiser (1970). Although refined mATPase protocols have been developed to delineate the full diversity of MHC expression in human skeletal muscle fibres (Staron 1991; Sant'Ana Pereira et al. 1995), mATPase histochemistry, when used qualitatively, is still not able to detect the coexistence of several MHC isoforms in many individual fibres (Andersen et al. 1994*a*, *b*; Rivero et al. 1996). The immunohistochemical technique of using monoclonal antibodies against specific MHC isoforms, although still qualitative, seems to be a more objective method for categorising muscle fibres (Linnane et al. 1999). Some attempts have been made in human skeletal muscle to delineate fibre types by immunohistochemical staining with anti-MHC monoclonal antibodies (Sant'Ana Pereira et al. 1997; Andersen & Schiaffino, 1997; Korfage & van Eijden, 1999), but the complete range of muscle fibre phenotypes was not obtained. The major problem occurs with the identification of 'hybrid' fibres containing 2 or 3 MHC isoforms. Here we present a novel approach based on the combined immunohistochemical analysis of MHC isoforms in serial cryostat sections of human skeletal muscle that allows identification of the 5 main muscle fibre type phenotypes (I, I+IIA, IIA, IIAX and IIX). The major focus was on the characterisation of the immunohistochemical reaction of the monoclonal antibody designated S5-8H2 to identify hybrid fibres coexpressing the 2 fast MHC isoforms. Antibodies used in the study were characterised in tissue homogenates by immunoblotting. We also used in situ hybridisation with specific probes for the 3 major human MHC transcripts (β-slow, IIA and IIX) to study the intracellular distribution of the various MHC isoforms at the mRNA level and its degree of correspondence with the protein content.

MATERIALS AND METHODS

Subjects and muscle biopsies

The study population comprised 15 male students with normal but sedentary lifestyles (mean age ± S.D., 22 ± 5 years; bodyweight, 73 ± 8 kg; height, 176 ± 7 cm). All subjects were considered to be reasonably active, but were not subject to any rigorous training for sporting activities etc. Subjects underwent prior physical examination to ensure that they were healthy. The subjects were fully informed of possible risks and discomfort associated with the muscle biopsy procedure before giving their informed written consent to participate. The study conformed to the code of ethics of the World Medical Association (Declaration of Helsinki) and the guidelines of the Universidad Complutense de Madrid (Spain).

Needle biopsies were taken from the middle portion of the vastus lateralis muscle (2 cm of depth) under topical anaesthesia (lidocaine 2%) following the technique of Bergström (1975). This muscle was selected because it is the most frequently sampled to investigate locomotor muscle characteristics in humans. The biopsy specimens were frozen by immersion in isopentane (5–10 s), kept at freezing point in liquid nitrogen, and then stored at −80 °C until analysis.

Table 1. *Specificity*[1] *of monoclonal antibodies against adult rat myosin heavy chain (MHC) isoforms used in the study*

	Myosin heavy chain isoforms			
Monoclonal antibodies	β-slow	IIA	IIX	IIB
Slow	+	−	−	−
Fast	−	+	+	+
SC-71	−	+	−	−
BF-35	+	+	−	+
S5-8H2	+	−	+	+

[1]According to Schiaffino et al. (1989), except S5-8H2 (Barrey et al. 1998); +, positive reaction for specific monoclonal antibody specific that muscle fibre; −, no reaction between monoclonal antibody and fibre type.

Immunohistochemistry, histochemistry and in situ hybridisation

Frozen biopsy samples were warmed to −20 °C in a cryostat and serially sectioned (10 μm) for immunohistochemistry, histochemistry and in situ hybridisation. Serial sections were reacted with a panel of different monoclonal antibodies (MAbs) specific to MHC isoforms (Table 1; Barrey et al. 1998; Schiaffino et al. 1989). The avidin-biotin peroxidase complex (ABC) immunohistochemical procedure was used for the localisation of primary antibody binding as previously described (Rivero et al. 1996). In brief, sections were preincubated in a blocking solution of

stock goat serum. The primary MAb was then applied and allowed to incubate overnight in a humid chamber at 4 °C. An additional section was incubated without specific primary MAb and used as blank tissue to demonstrate nonspecific reactivity. On the second day the sections were washed and then reacted with a secondary antibody (biotinylated goat antimouse IgG). Sections were again washed and reacted in ABC reagent. Diaminobenzidine tetrahydrochloride was used as a chromogen to localise peroxidase.

Additional serial cross-sections were stained for qualitative demonstration of mATPase activity after alkaline (pH 10.3) and acid (pH 4.2 and pH 4.5) preincubations using a modification (Nwoye et al. 1982) of the method described by Brooke & Kaiser (1970). Fibre types were distinguished on the basis of their staining intensities.

In situ hybridisation was performed as described by Smerdu et al. (1994). The final concentration of ^{35}S-labelled cRNA probes was 25000–50000 counts $\cdot$ min^{-1} $\cdot$ µl^{-1}. Slides were processed for autoradiography using Kodak NBT-2 emulsion and exposed for 8–14 d. Three MHC probes, corresponding to the 3′-untranslated regions of different MHC transcripts (Smerdu et al. 1994) were used in this study.

Image analysis

To characterise individual fibre types according to their MHC content expressed at the protein level, and to determine their relative proportions, a region of each cross-section containing 125 fibres was selected for further analyses. The sections stained for histochemistry, immunohistochemistry and in situ hybridisation were surveyed to find regions free of artifact. Serial sections were visualised and analysed using a Leica DMLS microscope (Leica Microsistemas, Barcelona, Spain), a high-resolution colour charge-coupled device Leica ICC A camera (Leica Microsistemas, Barcelona, Spain), an 8-bit Matrox Meteor II frame-grabber (Matrox Electronic Systems Ltd, Barcelona, Spain), combined with image-analysing software (Visiolog 5, Noemi, Microptic, Barcelona, Spain). The same fields of these serial sections were correlated under the microscope and systematically digitised. The fibres were numbered and classified at random with immunohistochemical, histochemical and in situ hybridisation methods. Five fibre types (I, I+IIA, IIA, IIAX and IIX) were distinguished on the basis of (1) their MHC isoprotein content as revealed by reactivity against MAbs (immunohistochemistry), (2) their mATPase staining intensities (histochemistry), and (3) their MHC mRNA content (in situ hybridisation). The fibre type distribution of each muscle biopsy was established with the three methodologies by counting the relative frequency of the various fibre types.

Measurements of the cross-sectional areas (CSA) of fibre types were made on sections stained for the MAb Slow, specific to MHC β-slow (immunohistochemistry), in accord with morphometric procedures previously published (López-Rivero et al. 1992). The fibre CSA was averaged in each muscle biopsy for each fibre type and the relative CSA that a fibre type occupied in a muscle biopsy was calculated by dividing the product of the fibre type percentage and the mean CSA of the fibre type by the sum of these products for all fibre types (Sullivan and Armstrong, 1978). In order to compare and correlate quantitative data from immunohistochemistry, electrophoresis and immunoblots, the relative areas of the 5 immunohistochemically delineated fibre types were collapsed into the 3 major types (I, IIA and IIX) using the following formulae: $I + 1/2(I + IIA) = I$; $1/2(I + IIA) + IIA + 1/2IIAX = IIA$; and $1/2(IIAX) + IIX = IIX$ (Staron et al. 2000).

SDS-PAGE and immunoblotting analyses

In order to characterise the specificity of the MAb S5-8H2, used for the first time on human skeletal muscle, MHC electrophoresis was performed following the protocol for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS) described by Talmadge & Roy (1993). Briefly, the original method was modified by: (1) boiling the myofibrillar protein at 60 °C for 19 min, (2) increasing the glycerol content of the separating and stacking gels to 30 %, (3) increasing the gel thickness to 1 mm, and (4) decreasing the voltage to 130 V for 24 h. Aliquots of diluted myofibrillar protein were electrophoresed in a large-gel apparatus and separating gels were stained with Coomassie blue. Three bands clearly separated in the gels were identified as MHC-I, MHC-IIA and MHC-IIX (Biral et al. 1988; Sant'Ana-Pereira et al. 1997). Gels were then scanned with a videoscanning densitometric system and a quantification of each MHC isoform was obtained in relative terms of each muscle biopsy.

In separate unstained gels, the MHC isoforms were electrophoretically transferred to nitrocellulose sheets for immunoblot analyses (Rossini et al. 1995). Nitrocellulose filters were stained for specific MHCs

using primary antibodies listed in Table 1 and the immunohistochemical procedure as previously described for immunohistochemistry at the cellular level.

Statistical analysis

The variability of data was expressed by standard deviation (S.D.). A one-way analysis of variance (ANOVA) was used to test the differences among different methods for the quantification of MHC isoform/transcript content employed in the study. Correlation analyses were also performed to compare immunohistochemical, SDS-PAGE and immunoblots information.

RESULTS

Immunohistochemistry, histochemistry and in situ hybridisation

Immunohistochemistry allowed subdivision of three different muscle fibre populations containing a individual MHC, one slow (type I) and 2 fast (IIA and IIX), and 2 hybrid populations, one containing MHCs I+IIA and another with both fast MHCs (IIAX) (Fig. 1*A–D*). The fibres that reacted with the MAb Slow (Fig. 1*A*) and not with MAb Fast (Fig. 1*B*) were identified as 'pure' type I fibres (e.g. fibre labelled 1 in Fig. 1). These fibres also reacted with MAbs BF-35 and S5-8H2 (Fig. 1*C*, *D*). A few fibres reacted positively with all MAbs used in the present study (e.g. fibres 2 and 2′ in Fig. 1). These fibres contain both MHC β-slow and MHC-IIA and were identified as I+IIA. The lower alkaline stability of mATPase in the fibre labelled as 2′ compared to the fibre 2 in Figure 1*E*, may be attributed to a very low content of MHC-IIA and a high expression of MHC β-slow in the fibre labelled as 2′. Thus these fibres may well correspond to the histochemical phenotype IC described by Staron & Hikida (1992), whereas the fibre labelled 2 in Figure 1 is a clear type IIC fibre. Those fibres that reacted positively with MAbs Fast (Fig. 1*B*) and BF-35 (Fig. 1*C*) and negatively with MAbs Slow (Fig. 1*A*) and S5-8H2 (Fig. 1*D*) were considered as containing only MHC-IIA and categorised as 'pure' type IIA fibres (e.g. fibre labelled 3 in Fig. 1). Fibres that stained positively to all MAbs anti-fast MHCs (Fig. 1*B-D*) and negatively with the MAb anti MHC β-slow (Fig. 1*A*) were demonstrated to coexpress the 2 fast MHCs and were 'hybrid' fibres identified as type IIAX (e.g. fibre 4 in Fig. 1). A very low proportion of fibres unreactive with MAbs Slow (Fig. 1*A*) and BF-35 (Fig. 1*C*) reacted positively with MAbs Fast (Fig. 1*B*) and S5-8H2 (Fig. 1*D*) and were identified as 'pure' type IIX fibres because they contained a MHC isoform other than MHC-IIA (e.g. fibre 5 in Fig. 1).

Based on the mATPase reaction after preincubation at pH 4.5, the muscle fibres could be objectively divided into 3 categories: I (black), IIA (white) and IIX (grey) (e.g. fibres labelled 1, 3 and 5, respectively, in Fig. 1*G*). A continuum in the staining intensity was observed between type IIA and type IIX mATPase-classified fibres. These fibres were identified as IIAX (e.g. fibre 4 in Fig. 1). The presence of type IIC fibres (i.e. fibres containing MHC-I and MHC-IIA) could be objectively delineated by histochemistry (e.g. fibre 2 in Fig. 1). These fibres show moderate-to-high reaction in mATPase after alkaline (Fig. 1*E*) and acid (Fig. 1*F*, *G*) preincubations. These fibres were named type I+IIA.

In situ hybridisation allowed subdivision of 3 muscle fibre phenotypes with an individual mRNA of MHCs β-slow, IIA and IIX, and 2 hybrid phenotypes, one containing mRNAs of MHCs β-slow and IIA, and the other coexpressing transcripts of MHC-IIA and MHC-IIX (Fig. 2*A–C*). Pure fibres with mRNA of the MHC β-slow were reactive with the MHC β-slow probe (e.g. fibre 1 in Fig. 2*A*), but unreactive with the other 2 probes in in situ hybridisation (Fig. 2*B*, *C*). Fibres containing exclusively mRNA of MHC-IIA were reactive exclusively with the MHC-IIA probe (e.g. fibre 3 in Fig. 2*B*). Fibres expressing solely mRNA of MHC-IIX only reacted with the MHC-IIX probe (e.g. fibre 5 in Fig. 2*C*). Fibres containing mRNAs of both MHC β-slow and MHC-IIA were reactive to MHC β-slow and MHC-IIA probes (e.g. fibre 2 in Fig. 2*A*, *B*), but unreactive with the MHC-IIX probe (Fig. 2*C*). Hybrid fibres with mRNAs of MHC-IIA and MHC-IIX were positive with probes specific for MHC-IIA and MHC-IIX transcripts (e.g. fibre 4 in Fig. 2*B*, *C*). No fibres containing either mRNAs of the three MHCs or of MHC β-slow plus MHC-IIX were observed.

Combined immunohistochemical, histochemical and in situ hybridisation analyses

Fibres were examined on a fibre-to-fibre basis and the percentages of each fibre type were established independently for the 3 methodologies (Table 2). The mean percentages of types I, I+IIA and IIA fibres were not statistically different ($P > 0.05$). However, the number of hybrid fibres coexpressing MHC-IIA and MHC-IIX was significantly lower when it was delineated by histochemistry than by immunohistochemistry and in situ hybridisation ($P < 0.05$). By

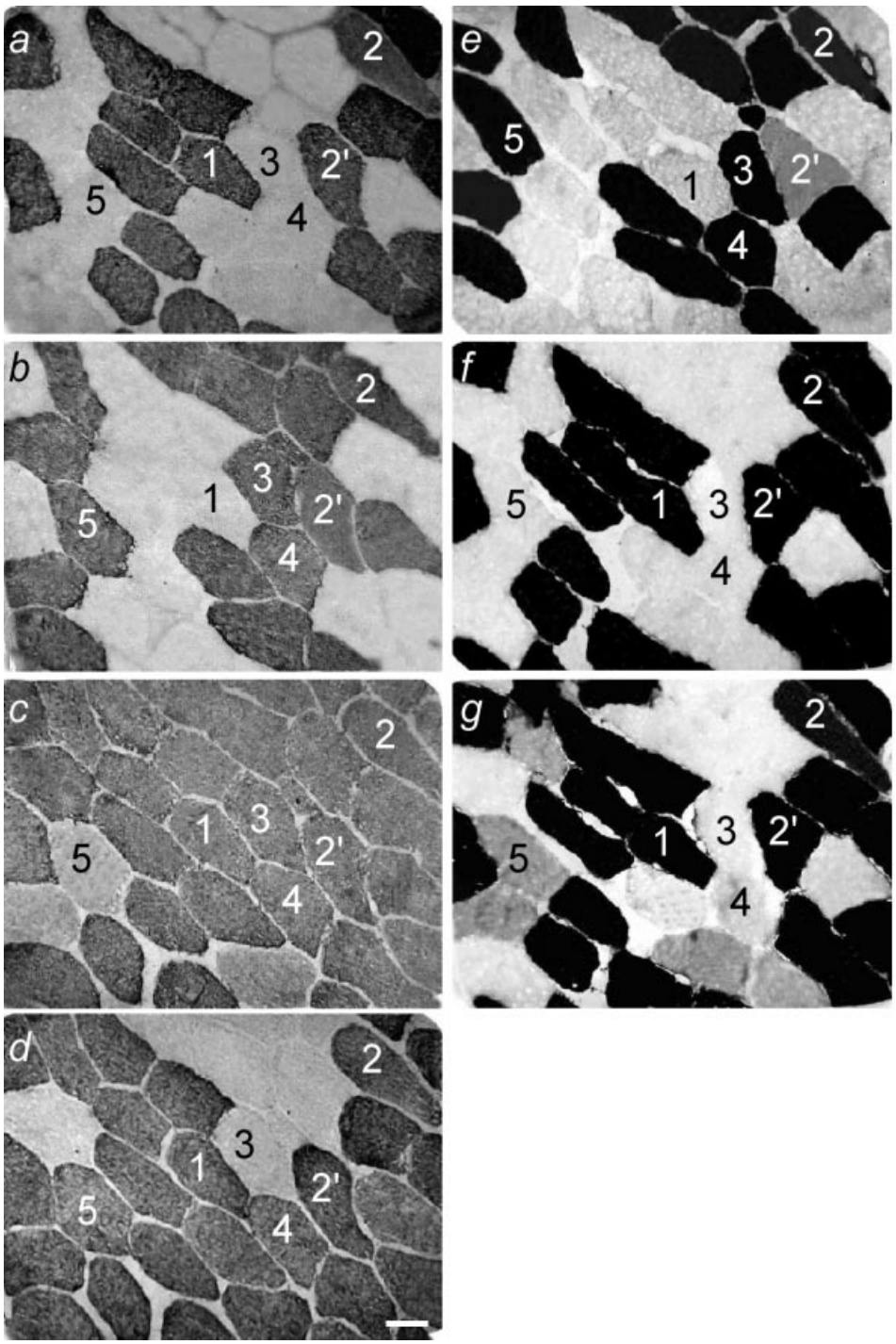

Fig. 1. Serial cross-sections of a human vastus lateralis muscle biopsy stained with a number of monoclonal antibodies against specific myosin heavy chain (MHC) isoforms (*a–d*; see Table 1 for specificities) and by myofibrillar ATPase histochemistry (*e–g*). (*a*) Slow (anti MHC β-slow) monoclonal antibody; (*b*) Fast (anti MHCs IIA + IIX) monoclonal antibody; (*c*) BF-35 (anti MHCs β-slow + IIA) monoclonal antibodies; (*d*) S5-8H2 (anti MHCs β-slow + IIX) monoclonal antibodies. (*e–g*) Myofibrillar ATPase activity after preincubations at pH 10.3 (*e*), pH 4.2 (*f*) and pH 4.5 (*g*). The fibres labelled 1, 3 and 5 are types I, IIA and IIX fibres; fibre 2 is a hybrid fibre I + IIA; fibre 2′ is a hybrid fibre I + IIA with a dominance of MHC-I; fibre 4 is a hybrid fibre IIAX. Bar, 50 μm.

contrast, the percentage of IIX fibres was significantly higher by histochemical analysis in comparison with immunolabelling and in situ hybridisation of MHCs ($P < 0.05$).

In general, fibres expressing total or partial MHC β-slow showed a correct matching between mATPase and immunolabelling determinants. The same was true for 'pure' IIA fibres. Mismatched phenotypes were, by contrast, frequently observed within the IIAX and IIX fibres. Many fibres judged as type IIX by mATPase techniques stained as hybrid type IIAX by immunolabelling.

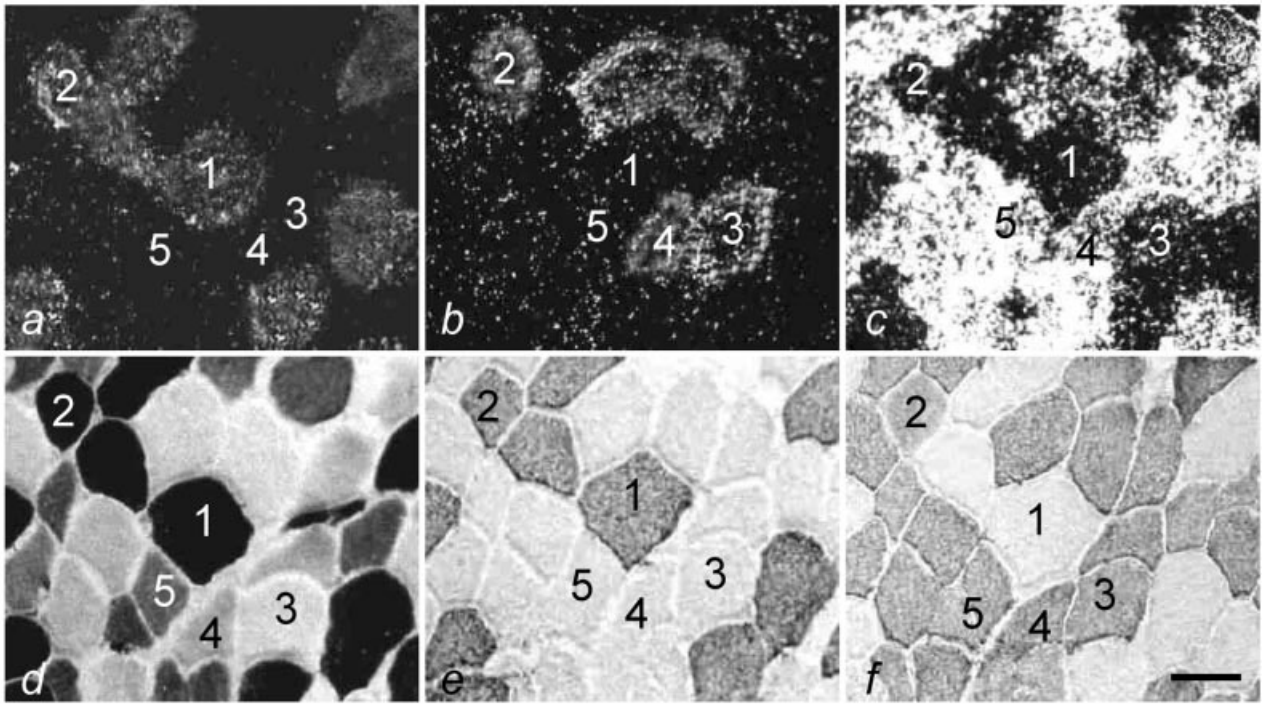

Fig. 2. Combined analyses of myosin heavy chain (MHC) isoforms and transcripts in serial sections of a sedentary human vastus lateralis muscle biopsy by in situ hybridisation (*a–c*), myofibrillar ATPase activity (*d*) and immunohistochemistry (*e*, *f*). (*a–c*) Sections hybridised with ^{35}S-labelled probes specific for MHC β-slow (*a*), MHC-IIA (*b*) and MHC-IIX (*c*) transcripts, processed for autoradiography, and visualised by dark-field microscopy. (*d*) Section processed for myofibrillar ATPase histochemistry after preincubation at pH 4.5. (*e*, *f*) Sections incubated with a monoclonal antibody specific for MHC β-slow (*e*) and an antibody reactive with all type II MHC isoforms (*f*). Fibres 1, 3 and 5 are pure phenotypes containing exclusively mRNAs of MHCs β-slow, IIA, and IIX, respectively. Fibres 2 and 4 are hybrid phenotypes coexpressing mRNAs of MHCs β-slow plus IIA, and IIA plus IIX, respectively. Bar, 50 μm.

Table 2. *Muscle fibre type composition* (%) *of the vastus lateralis muscle identified by immunohistochemistry*, *myofibrillar ATPase histochemistry and in situ hybridisation*[1]

	Fibre types				
	I	I+IIA	IIA	IIAX	IIX
Immunohistochemistry	45.5±9.3	3.9±1.9	29.5±7.2	13.9±5.1	7.4±2.9
Histochemistry	43.4±8.0	4.0±2.3	29.7±5.6	4.5±3.7*	18.4±2.9*
In situ hybridisation	46.5±9.1	5.5±2.4	28.0±3.5	13.9±4.7	6.1±2.5

[1]Values are means±S.D. of 15 biopsies. *$P < 0.05$ compared with immunohistochemistry and in situ hybridisation.

The majority (96%) of the fibres examined by in situ hybridisation matched the MHC content at the protein level by immunohistochemistry (Fig. 2). Furthermore, a small number of fibres (4% of those fibres examined) showed a mismatch between MHC expression at the mRNA and protein levels. Mismatched fibres were rarely found within the C fibre population. Some fibres with a MHC β-slow by histochemistry/immunohistochemistry were not reactive with MHC β-slow probe in in situ hybridisation, but reacted with the MHC-IIA probe. Some type IIAX fibres at the mRNA level by in situ hybridisation were identified as pure type IIA or IIX at the protein level by immunohistochemistry and histochemistry. Some fibres designated hybrid type IIAX by immunohistochemistry were only reactive with the MHC-IIX probe by in situ hybridisation.

Electrophoresis and immunoblotting

Figure 3 shows the characterisation of MAb S5-8H2 on blots after SDS-PAGE of human vastus lateralis muscle. Immunolabelling with all other MAbs used in the study in both species is also shown. The MAb S5-8H2 binds the slowest (IIX) and the fastest (β-slow) migrating protein bands (Fig. 3, *lane* 7).

The relative content of the three MHC isoforms (I, IIA and IIX) derived immunohistochemically par-

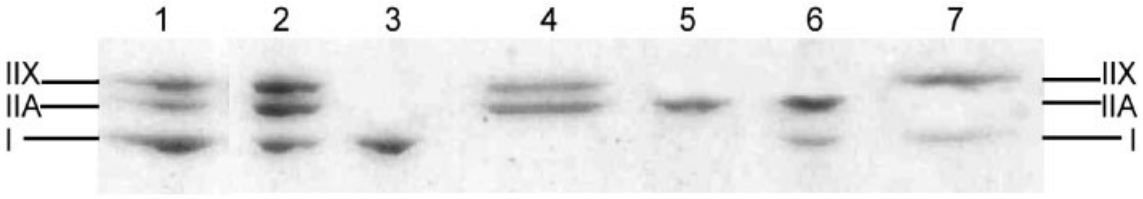

Fig. 3. Electrophoretic and immunoblotting analyses of myosin heavy chain (MHC) isoforms in human vastus lateralis muscle. Lanes 1 and 2, Coomassie blue staining to show MHC composition of whole-muscle extracts on 8 % sodium dodecyl polyacrylamide gel electrophoresis; MHC isoforms, by decreasing electrophoretic mobility, correspond to types IIX, IIA and β-slow in human muscle (Biral et al. 1988). Lanes 3–7, blots stained with monoclonal antibodies: Slow (3), Fast (4), SC-71 (5), BF-35 (6) and S5-8H2 (7). The MAb S5-8H2, used for the first time in human muscle, binds MHC β-slow and MHC-IIX (see Table 1 for specificities).

Table 3. *Myosin heavy chain* (*MHC*) *composition* (%) *based on immunohistochemistry, electrophoresis and immunoblot analyses*[1]

	MHC β-slow	MHC-IIA	MHC-IIX
Immunohistochemistry	49.3 ± 14.5	37.6 ± 12.2	13.1 ± 7.7
Electrophoresis	45.9 ± 9.1	41.4 ± 7.8	12.7 ± 4.7
Immunoblots	48.7 ± 12.5	39.1 ± 8.3	12.2 ± 5.3

[1]Values are means ± S.D. 15 biopsies. No statistically significant differences were observed over the different methods.

Table 4. *Correlation analyses for the myosin heavy chain* (*MHC*) *composition* (%) *based on immunohistochemistry, electrophoresis and immunoblot analyses*[1]

	MHC β-slow	MHC-IIA	MHC-IIX
Immunohistochemistry vs electrophoresis	0.861	0.881	0.758
Immunohistochemistry vs immunoblots	0.838	0.631	0.825
Electrophoresis vs immunoblots	0.964	0.868	0.874

[1]All correlation coefficients are statistically significant.

alleled the results obtained by SDS-PAGE and immunoblots (Table 3). The MHC content estimated in each biopsy sample by immunohistochemistry was again correlated with data obtained by electrophoresis and immunoblots. These analyses revealed significant correlations for the three MHC isoforms (Table 4).

DISCUSSION

The primary focus of this study was the accurate characterisation of human skeletal muscle fibres according to the MHC isoform they express at the protein and mRNA level. This goal was achieved by combining traditional techniques of mATPase histochemistry with updated methodologies of immunohistochemistry using monoclonal antibodies and in situ hybridisation using probes specific for human MHC transcripts. The application of the anti MHCs monoclonal antibody panel used in the study resulted in a sensitive method which enables human skeletal muscle fibres to be objectively classified on cryostat sections on the basis of the MHC isoforms they express. The protocol was particularly useful to identify and differentiate hybrid fibres. The results also showed evidence that, at least in young men who had not undergone athletic training, protein and mRNA determinants of MHC isoforms which are contained in individual fibres are identical in the vast majority of muscle fibres examined.

Some recent studies have used immunohistochemical techniques to characterise human skeletal muscle fibre types in cryostat sections according to the MHC isoprotein expressed (Andersen & Schiaffino, 1997; Sant'Ana Pereira et al. 1997; Korfage & van Eijden, 1999). However, none of them were able accurately to delineate the 3 major phenotypes of fast fibres, i.e. IIA, IIAX and IIX. With the panel of MAbs against MHC isoforms used in the present study, immunohistochemistry allowed subdivision of human skeletal muscle fibres into 5 major phenotypes on the basis of their MHC composition. The MAb S5-8H2 (Barrey et al. 1998), reactive against all adult MHC isoforms except the MHC-IIA, was used for the first time in human muscle, proving particularly useful to delineate fibres expressing or coexpressing the MHC-IIX isoform. Nevertheless, as immunohistochemistry (as used in this study) is a qualitative method, it is still possible that some hybrid fibres with very small amounts of one MHC and large amounts of another cannot be distinguished from pure fibres using this procedure. The main advantage of the immunohistochemical identification of muscle fibre is to permit studies to be undertaken at the cellular level (size, capillaries and metabolic properties of muscle fibres), whereas this is not possible with the quantitative electrophoretical analysis of single fibres.

Our data confirm a high correlation between mATPase activity and MHC content of myofibres solely when the MHC β-slow isoprotein is expressed, but not when MHC-IIX or both fast MHCs are expressed (Staron 1991; Staron & Hikida, 1992; Rivero et al. 1996). Some fibres delineated histochemically as pure IIX types were hybrid fibres coexpressing both isoforms. Recent studies have reported a mean percentage of fast hybrid fibres (named IIAB by the authors) by mATPase histochemistry below 7% in the vastus lateralis muscle of control and athletically trained men and women (Staron et al. 1994, 2000). This was also confirmed with our histochemical results, but not by the immunohistochemical procedure applied (see Table 2). In agreement with our data, other previous studies in human athletes reported that a great proportion of histochemical type IIX fibres (termed type IIB in these reports) coexpress MHC-IIA and MHC-IIX (e.g. Andersen et al. 1994*a*, *b*). As this mismatch between mATPase activity and MHC protein is not only restricted to the trained musculature but it is also evident in untrained subjects, it can be assumed that human type II fibres have been misclassified in previous publications. The reason for the mismatch between mATPase and antigenic determinants of fast fibres is unclear. A study of individual human muscle fibres demonstrated quantitatively a high correlation between the mATPase staining and MHC expression for hybrid fast fibres (Sant'Ana Pereira et al. 1995). Some other quantitative studies, however, have shown that fibres with the same unique MHC isoform vary greatly with respect to their mATPase activity (Botinelli et al. 1994; Rivero et al. 1999).

Results from the present study also showed evidence that the majority of the human skeletal muscle fibre types show good correspondence between the expression of MHC at the protein and mRNA level. Similar observations were reported in young subjects before a 3-mo training period (Andersen & Schiaffino, 1997). A minor proportion ($\sim 4\%$) of fibres showed a mismatch in the relative proportion of MHC isoforms at the protein and mRNA level. It has already been proposed that these fibres represent transitional fibres at various stages of the transformation process and that the particular phenotype of the mismatch can give a clue as to the direction in which the fibres are changing (Andersen & Schiaffino, 1997). This scheme was based on the general concept that mRNA changes are the first to occur during any transitional process, while protein changes follow with some delay. Thus, during the early phase of transformation, one would expect to detect the newly induced mRNA before the corresponding new protein together with both mRNA and protein of the original fibre type.

Hybrid fibres coexpressing 2 MHC isoproteins were particularly frequent. Thus, fibres coexpressing MHC-IIA and MHC-IIX represented $\sim 14\%$ of the fibre pool, whereas fibres coexpressing MHC β-slow and MHC-IIA represented $\sim 5\%$ of the total population. Previous reports have also shown a high degree of coexpression of MHC isoforms in human skeletal muscle fibres of sedentary (Biral et al. 1988), highly trained (Klitgaard et al. 1990; Andersen et al. 1994*a*, *b*) and old (Larsson et al. 1997; Andersen et al. 1999) subjects. Hybrid fibres have traditionally been interpreted as transitional fibres resulting from transformation of one fibre type into another. Current results, however, confirm that a considerable proportion of these fibres exists in skeletal muscles of young mature individuals who had not undergone athletic training. Thus these fibres are not necessarily the expression of transitional status, but they are presumably in a very dynamic equilibrium and may easily undergo transitions from type IIA to type IIX, and viceversa, in response to changes of muscle activity.

In conclusion, with the panel of monoclonal antibodies used in the present study it was possible to delineate in adult leg human skeletal muscle 5 muscle fibre types according to the MHC isoform they expressed (I, I+IIA, IIA, IIAX and IIX). The MAb S5-8H2 is particularly useful for the delineation of hybrid fibres co-expressing MHC-IIA and MHC-IIX, which are a more common phenotype than the pure fibres expressing MHC-IIX.

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