Molecular Markers of Muscle Plasticity, Damage, Regeneration and Repair

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Abstract

The motor units of skeletal muscles differ in their ability to sustain power without fatigue and stress. They may therefore be damaged by different levels of exercise, as well as by events involving ischaemia and reperfusion, or administration of myotoxic agents. Quantitation of such damage is often performed morphometrically, but such an approach is vulnerable to the artifacts that can appear in cryostat sections taken from seriously disrupted tissue. Paraffin and acrylic embedding for light and electron microscopy are laborious procedures, and require substantial biopsies whose quantitation can be time-consuming.

We will describe scaled-down procedures, based on protein and nucleic acid chemistry, that are sensitive enough to allow several markers of plasticity, damage, regeneration, and repair to be determined on a muscle sample of just a few milligrams. A suitable source of material is a small biopsy specimen removed from the muscle for fibre typing and histopathology; a few additional serial cryostat sections cut from the block constitute a sample that is adequate for biochemical analysis. Since needle biopsies can be taken sequentially, it becomes feasible to construct a time course for the response of skeletal muscle to different demands in either experimental or clinical settings.

Key words: damage, molecular markers, plasticity, skeletal muscle, regeneration, repair.

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We have validated micromethods for protein and collagen content myosin:actin ratio, and myosin heavy chain (MHC) composition. Total protein and collagen may be used as indices of muscle plasticity, regeneration, and repair; the myosin:actin ratio is a reliable index of the onset of muscle atrophy; MHC composition is an indicator of regenerative events and of adaptation to changing demands.

Where indicated, these protocols may be combined with other analyses, such as the identification of proteins with antibodies or in situ hybridisation with specific nucleic acid markers. A variety of highly sensitive detection methods may be added in this way: for example, chemiluminescence and polymerase chain reaction techniques are capable of detection sensitivities that extend almost to the level of a single molecule [6]. The procedures can be adapted to the characterisation of any tissue by selecting the appropriate molecular markers. In the case of muscle, information obtained from needle

biopsy material can be supplemented by determinations of myoglobin and creatine kinase (CK) in the serum.

We have developed a number of standard protocols for the molecular characterisation of muscle biopsies in different scientific or clinical contexts. Examples are: dystrophin in Duchenne muscular dystrophy [4, 20]; inflammatory cell markers in chronic myosites [29]; mutant MHC in familial hypertrophic cardiomyopathy [18] mitochondrial or glycolytic enzymes in mitochondrial myopathies or glycogenoses; fragmented DNA from apoptotic myonuclei [36, 37, 42-44, 46] protein or isotopic markers in exercise-induced muscle damage; isomyosin transitions in skeletal muscle-powered circulatory assist devices [10, 11, 26, 48] or in cardiac failure [54, 55]; MyoD and others muscle transcription factors or LCemb and MHCemb in myotubes and young fibers to identify early muscle regeneration [8, 19].

To obtain the best correspondence between conventional histopathological methods and the molecular characterisation based on these analyses, the latter needs to be performed on serial sections cut from the same frozen muscle samples. This poses the problem of distortion and shrinkage of the sections as they adhere to and dry on glass slides, which would of course affect calculations of the absolute content of the various components. A simple solution to this problem is described, and this allows us to reproduce the results that are obtained when standard biochemical approaches are applied to larger samples of the same tissue.

Morphometry

Determination of the area and volume of cryostat sections

When the entire muscle is available for analysis, it is held at resting length, frozen quickly in liquid nitrogen, and stored at -80°C until needed. The sample for analysis is taken from midbelly. Serial sections are cut from these samples, or from samples obtained by biopsy, in a cryostat. For histology or immunohistochemistry, sections of 10 µm thickness are collected on glass slides; for biochemical analysis, sections of 20 gm thickness are transferred to conical glass test tubes. The slides and test tubes are usually stored at -80°C to await analysis. After the sections have been taken, the face of the muscle block still mounted in the cryostat is photographed alongside a standard microgrid. At a later stage the slides can be projected, and from a knowledge of the. grid size the cross-sectional area can be determined. Since the cryostat sections are of known thickness, their volume is readily calculated.

For whole muscles, the area of the sectioned face of the cryostat-mounted block corresponds to the actual cross-section of the muscle belly in vivo, and provides a good measure of muscle mass. This is not true of the area of cryostat sections that are cut from the block and mounted on glass slides, because of shrinkage during adhesion and drying. For example, the mean section area of cryostat blocks obtained from the normal extensor digitorum longus (EDL) muscles of large rats was found to be 6.5+0.3 mm^2 whereas the area of the sections mounted on slides had decreased to 4.76±0.16 mm^2 (mean+SEM, n = 12 muscles, $p < 0.0001$).

Provided that the overall shape of the muscles is the same, the ratio of section area to muscle weight may be used in comparisons as an index of muscle hypotrophy or hypertrophy. Where, as in the case of muscle biopsies, it is not possible to freeze the whole muscle at resting length, the parameter is of less value as an index of muscle girth. Section area is still worth determining, however, as it allows the concentration of a given muscle component to be calculated.

The use of these techniques, and techniques to be described below, will be illustrated by reference to the following experimental material. Table 1 summarises data on normal rat muscles, taken from two groups of animals in which the average body weights were 250 g and 350 g. Table 2 presents data from the control and experimental sides of rats in which EDL and soleus muscles were removed, treated with bupivacaine (a myotoxic agent), and transplanted as free grafts, each muscle into the bed of the other. Values from normal and contralateral control muscles were unimodally distributed, and data are therefore presented as mean±SEM. Results from the transplanted muscles are presented individually because of the large variation they showed in the extent of fibre regeneration and reinnervation.

Fibre typing and interstitial tissue content

Serial sections (10 µm) are stained by the conventional techniques for haematoxylin and eosin (H & E) and haematoxylin-Van Gieson, and for myofibrillar ATPase according to the method of [7]. The ATPase sections are preincubated at alkaline or acid pH and used to measure the size of type 1 and type 2 fibres. Morphometric analyses are performed on glass slides or photographs with an image analyser (Kontron IBAS 2000, Zeiss). The section profile is imaged by a television scanner and gray level detector, which facilitates identification, selection, and measurement of single features [33]. Fibre counts are based on all the fibres identified in H & E-stained sections. The percentage content of type 1 fibres is determined in randomly selected areas of ATPase-stained sections. In experimental muscles it may not be easy to classify type 2 fibres into the traditional subgroups, because some fibres are stained with intermediate intensity after acid and alkaline preincubation (see, for example, Fig. 3). These intermediate fibres are often encountered in muscles undergoing transformation, for example during long-term electrical stimulation of either innervated muscles [2,

10, 28, 30, 41] or denervated muscles [2, 15]. They are hybrid fibre types that contain more than one isoform of myosin [14, 34, 35].

In the normal and contralateral control soleus and EDL muscles there were about 3000 fibres (Table 1). The muscles contained the expected complement of type 1 fibres: about 3% in EDL and 90% in soleus. Type-grouping was evident in the transplanted muscles, indicating that most of the fibres had been reinnervated. The total area of connective tissue was less than 1% in the normal and contralateral control EDL and soleus muscles; in the cross-transplanted muscles, it had increased as much as 10-fold (Table 1).

Molecular Markers

Total protein

Two cryostat sections are collected in cylindrical glass test tubes and are dissolved in 0.5% deoxycholic acid by sonication at room temperature (Biosonik III, Bronwili Scientific). The protein content is determined by the method of [27].

Skeletal muscle normally contains about 20% of total protein by wet weight, a result of the high content of contractile proteins in the fibres [50]. The value obtained by cryostat section analysis of normal and contralateral muscles in the experimental material was 21.93±0.76% (mean±SEM), with minor variation between the EDL and soleus muscle groups (Table 2).

In determining total protein from sections a problem arises if the medium used to embed the frozen tissue specimen is included with the sections. This material interferes with the assay, whether by Lowry [27] or Bradford [5] methods, and such contamination must therefore be carefully avoided. An additional problem is encountered in experimental or pathological conditions that involve an increase in the collagen content of the muscle, since collagen does not resister in the Lowry method [27] and is underestimated with the Bradford method; this is because it contains very low amounts of the aromatic amino acids that are the basis for the colorimetric reaction [57].

Collagen

Two serial sections are collected in a pluggable Pyrex test tube and analysed for total collagen by a modification of the method described in [58] and [21], as follows. The sections are hydrolysed for 20 h in 1 ml of 6N HCl. After hydrolysis the samples are dried either in a rotary evaporator or in a multi-sample bench-top centrifuge connected to a water-driven vacuum pump (Electrofor, Rovigo, Italy).

Dried samples are dissolved in 1 ml of distilled water. Aliquots of 0.4 ml are added to 0.5 ml of chloramine-T solution; this is prepared by dissolving 1.41 g sodium N-chloro-p-toluene sulfonamide in 10 ml n-propanol, 10 ml water, and 80 ml of a pH 6 buffer. The pH 6 buffer is prepared by dissolving 25 g citric acid monohydrate, 60 g sodium acetate trihydrate, 17 g NAOH, and 6 ml glacial acetic acid in distilled water to a final volume of 500 ml. The sample is mixed vigorously in the chloramine-T solution and incubated at room temperature (20-25°C). After 20 min, 0.77 ml of a freshly prepared aldehyde/perchloric acid solution is added; this is made up by solubilising 15 g p-dimethylaminobenzaldehyde in 62 ml n-propanol to which 26 ml 60% perchloric acid are slowly added, followed by distilled water to a total volume of 100 ml. The samples are vortexed and incubated at 65°C for 20 min. Full color development occurs in 20 min. The samples are read at 550 nm, usually straight away, although this is not essential as the color is stable for several hours. Hydroxyproline content is determined by interpolation from a hydroxyproline standard curve made on each occasion. Under the conditions described, the curve is linear from 0.5 to 20 µg of hydroxyproline. Since the mean content of hydroxyproline in collagen is 13.4%, collagen is easily calculated, due account being taken of the lower molecular weight of the amino acid in polypeptides, in which it is linked by peptide bonds.

The concentration of collagen usually increases in muscles undergoing tissue repair when necrosis is due to physical trauma, and when ischaemic and/or toxic necrosis is complicated by infection. Collagen content may also change with muscular activity: for example, morphological studies have demonstrated a considerable increase in the proportion of connective tissue in muscle immobilised in a lengthened position, and a decrease during remobilization, indicative that the biosynthesis of collagen in muscle adapts to stretch or activity [47]. The collagen:protein ratio varies even among normal muscles, because variation in total protein content is amplified by the failure of the Lowry [27] method for protein determination to include collagen. Nevertheless, the collagen:protein ratio, like the total protein content, provides a useful index of functional integrity.

In our experiments, normal and contralateral muscles had collagen contents close to the expected value of 0.7% for rat muscles [47]. The collagen concentration had increased in cross-transplanted soleus muscles, but the figures for cross-transplanted EDL muscles were not significantly different from controls. Notwithstanding this last observation, the percentage area occupied by interstitial tissue had increased more than IO-fold in the cross-transplanted muscles (Table 1). Because the cross-transplanted muscles had a low content of contractile proteins, the collagen: protein ratio increased substantially- up to ten-fold in cross-transplanted soleus muscles (Table 2), indicative of a very limited functional recovery of the transplanted muscles. This is an important conclusion to derive from such small biopsy sam-

ples. Further evidence of atrophy in the cross-transplanted muscles came from their low myosin:actin ratio (see below).

Contractile proteins

Two serial sections are solubilised at 100 mg/ml in 2.3% sodium dodecyl sulphate (SDS), 10% (by volume) glycerol, 0.5% 2- mercaptoethanol, 62.5 mM Tris HCl, pH 6.8, based on their protein content determined by the Lowry method [27]. Test tubes are closed with aluminium foil, and incubated for 3 min in boiling water to denature the proteins.

Myosin: actin ratio

Analytical SDS polyacrylamide gel electrophoresis (SDS-PAGE) is performed essentially according to Laemmii (1970) in a discontinuous gel gradient system within a 0.75 x 130 x 130 mm polyacrylamide gel slab: this comprises a 10-mm stacking gel and a separating gel consisting of 90 mm of 12.5% polyacrylamide and 30 mm of 7% polyacrylamide. A major modification is that 37.5% (by volume) glycerol is included in both separating and stacking gels [12, 17]. The electrophoresis buffer consists of 25 mM Tris, 192 mM glycine pH 8.3, and 0.1% SDS. Overnight separation is achieved in constant-current mode at a current of about 5 mA per slab, the actual current being set to a level that gives an initial voltage of 35 V. Gel electrophoresis is started late in the afternoon and the run is terminated after about 18 h when the dye front reaches the end of the slab. The voltage usually rises to 140-150 V by the end of the run. Normally 5 µg of total protein are loaded per well. MHC and actin bands are easily detected after shaking for lh with 200 ml of 0.1% Coomassie Brilliant Blue in 5% acetic acid, 40% methanol and destaining in 6 changes (every 15 min) of 200 ml 40% methanol, 7% acetic acid. To avoid variability in the stain-destain procedure the MHC:actin ratio is checked against a standard muscle homogenate that is run with each gel. MHC and actin content are determined by gel densitometry, as described later.

Myosin and actin have different rates of metabolic turnover, and the myosin:actin ratio changes during muscle atrophy and regrowth, for example, after denervation-reinnervation or tenotomy-repair [23]. Figure 4 shows the patterns of contractile proteins on the discontinuous 7/12.5% polyacrylamide gradient. The discontinuous gradient gel is needed to resolve MHC and actin from proteins that migrate very closely under normal gel electrophoresis conditions, particularly in the region of the actin band in the case of soleus muscle. An electrophoretic approach similar to that used to calculate the myosin:actin ratio may be used to determine the myoglobin content of muscle, which provides an index of the oxidative metabolic capacity of the tissue. In the illustrative experiments, the myosin:actin ratio obtained by densitometry of the gel slabs had the expected mean value of 2.2 in normal and contralateral control muscles. In agreement with histological evidence of hypotrophy (Fig. 3), the ratio decreased to values typical of atrophying muscle in the cross-transplanted muscle grafts. These muscle grafts contained many small angular fibres. By analogy with changes in the size and myosin composition of regenerated muscle fibres in innervated or permanently denervated limbs [13, 33] these could be interpreted as fibres that initially underwent regeneration but then atrophied because they were not reinnervated. Small fibres that could be type-grouped by ATPase staining were probably reinnervated fibres, their small size reflecting either an early stage of growth or ongoing disuse atrophy.

Myosin heavy chain isoforms

MHC are separated by analytical SDS-PAGE on 7% polyacrylamide gel slabs measuring 0.75x130x130 mm [12]. The slabs are prepared according to [25], except that 37.5% (by volume) glycerol is present in both separating and stacking [17]. The stacking gel is composed of 37.5% glycerol, 4% T acrylamide-Bis (36.5:1), 125 mM Tris HCI (pH 6.8), and 0.1% SDS. The separating gel is composed of 37.5% glycerol, 7% T acrylamide-Bis (36.5:1), 375 mM Tris HCI (pH 8.8), and 0.1% SDS. The running buffer consists of 50 mM Tris, 384 mM glycine pH 8.3, and 0.2% SDS (without correcting the pH with HCI). Separation of MHC is achieved in the constant-current mode at 4 mA per slab, corresponding to a voltage of about 40 V. After 4-6 h the buffer is changed and electrophoresis is restarted with the same parameters. Usually the run is started in the morning; after 24 h the voltage rises to 130-160 V and the run is then stopped. Gels containing 0.2 µg of protein per band are stained with 0.1% Coomassie Brilliant Blue in 5% acetic acid, 40% methanol and destained in 40% methanol, 7% acetic acid. Gels with less than 0.1 µg of protein per band are stained by the silver method [32].

For Western blotting, MHC are transferred in 25 mM Tris, 193 mM glycine, 20% methanol for 5 h at 40°C at a constant voltage (80 V). Ponceau Red staining is used after blotting as a cheek on the amount and position of MHCs transferred. Immunostaining is then performed according to [9] with an antibody to MHC 2B (G6 clone), a generous gift from Professor Stefano Schiaffino.

Figure 5 shows the MHC patterns of our normal and experimental muscles. Quantitative densitometry of MHC separated on 7% polyacrylamide (Table 7.2) revealed in normal soleus of 250-g rats the expected values of 91.6+1.2% for MHC 1 and 8.4 + 1.2% for MHC2A (mean+SEM). The figures for normal EDL muscles of 35.5+3.2% for MHC 2A+2X, 64.0+3.2% for MHC2B, and 0.5+0.3% for MHC 1 were also in good

agreement with published data [3, 12, 16, 22, 24, 39, 40, 45, 49, 51, 52].

Earlier cross-innervation studies [53] would have led us to expect complete transformation of fibre type in the cross- transplanted muscles. The MHC patterns fell short of this predicted out come. In the EDL muscle that had been transplanted to the soleus bed, a large to very large proportion of the MHC consisted of MHC2A or 2X. This muscle never attained the MHC 1 content typical of the normal soleus, although the slow isoform was present in much larger amounts than in the normal EDL. A substantial MHC2B component was still seen in two out of the three experimental muscles. The soleus muscle transplanted to the EDL bed had lost almost all of its complement of MHC 1 (3-6%), and showed an increase in the content of MHC2A+2X (74-75%), particularly MHC2X. The MHC2B content (19-24%) was much lower than in normal EDL muscles. Reference has already been made to the type-grouping found when the regenerated fibres in the cross-transplanted muscles were examined morphologically. The same observation could have been made by biochemical analysis of single fibres, but only at the expense of a good deal of tedious and time-consuming work [34]. This illustrates the value of the combined approach.

Silver staining of electrophoretograms prepared from transplanted muscles revealed trace amounts of the embryonic MHC isoform, MHCemb [40]. Quantitation of this component could have been improved by chemiluminescent detection, for which commercial products are available [6, 31, 56].

MHCemb can be used as a marker of skeletal muscle regeneration in adult muscle, if the timing is appropriate. The immunochemical analysis can be performed routinely on muscle biopsies, although the results can be compromised by cross-reactivity of the antibodies between MHC emb and adult fast MHC isoforms. In the next section we describe a peptide mapping approach to MHC emb identification, based on SDS-PAGE of hydroxylamine-cleaved contractile proteins, that may overcome problems of cross-reactivity.

Gel densitometry

In this work, gel electrophoresis was followed by densitometric scanning of gel slabs with a GS-300 Transmittance-Reflectance Scanning Densitometer (Hoefer Scientific Instruments) connected to an Apple Macintosh computer. Data were processed by the GS-370 Data System for the Hoefer GS-300 Scanning Densitometer (Macintosh version). Slabs were scanned first along the direction of electrophoretic migration and then along an axis perpendicular to this. Densitometric values were linear between 0.25 and 5 mg of protein after staining with Coomassie Blue [45].

Apoptosis

We have recently extended the analysis of cryosections to the demonstration of apoptotic myonuclei and DNA fragmentation in normal and dystrophin- deficient muscles of mice after spontaneous running [36, 37, 42-44, 46]. As this could shed fresh light on the pathogenesis of muscle dystrophy and exercise-induced muscle damage, the method will be described briefly here.

Cryostat sections are fixed in 10% neutral buffered formalin for 10 min at room temperature. After the slides have been washed in phosphate- buffered saline, the sections are post-fixed in ethanol:acetic acid (2:1) for 5 min at room temperature. Proteins in the tissue sections are digested by applying proteinase K (20 mg/ml) for 15 min at room temperature. The slides are then washed in distilled water. Endogenous peroxidase activity is blocked by exposing the slides to 2% hydrogen peroxide in phosphate-buffered saline for 5 min at room temperature. In situ end-labelling of fragmented DNA is performed by terminal deoxynucleotidyl transferase with digoxigenin-conjugated nucleotides, followed by immunodetection of incorporated nucleotide with the use of the ApopTag in situ Apoptosis Detection Kit (distributed by Oncor).

Conclusions

The assay of molecular markers from cryostat sections can be carried out simply and reliably. The values obtained with this approach for a larger series of normal EDL and soleus muscles in adult rats, expressed as mean+SEM, are as follows:

protein 21.9+5.2%;
collagen 0.7+0.4%;
myosin:actin ratio 2.1+0.3.

These results are either similar or identical to those obtained with the use of standard methods on whole muscles [38].

The data obtained for the MHC profiles of normal whole EDL and soleus muscles in adult rats are shown in Table 1.

Table 1. MHC profiles of EDL and soleus muscles of adult rats obtained with standard methods on whole muscles.

	MHC profile (%)	
	EDL	Soleus
MHC 1	0.5+1.0	79.9+9.0
MHC2A	-	20.1±9.0
MHC2A+X	36.1+4.3*	†
MUC2B	63.3±5.9	†

* When the bands are well separated, MHC2A:MI-1C2X MHC2X = 0.5
† MHC2X and MHC2B are not present.

MHCemb, a marker of ongoing muscle regeneration, was used as an example to show how the analysis can be extended when suitable antibodies are available for immunoblotting. In this way molecular markers of inflammation or of any other normal or pathological event can be included in a procedure that can be carried out on a few cryostat sections. Clearly the approach is not confined to skeletal muscle tissue.

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