Myosin Heavy Chain Composition in Regenerating Skeletal Muscle Grafts

Jaanus Järva, Karin Alev, Teet Seene

Institute of Exercise Biology, University of Tartu, Estonia

Abstract

To study myosin heavy chain (MHC) composition during regeneration, nerve reimplanted autografting of *extensor digitorum longus* muscle (EDL) was performed in young Wistar rats. 5-8% gradient sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate MHC isoforms. Quantitative changes in MHC isoforms were determined densitometrically at the 10th, 30th and 60th day after autografting in grafted and nongrafted muscles. Ten days after grafting in control muscles MHCIIb represented ~64% and MHCIId/IIa ~36% from total MHC in the nongrafted EDL. In the grafted muscles MHCIIb decreased to -27% and MHCIIdMIa increased to -73% from total MHC presented. Thirty days after grafting MHCIIb corresponded -57% and MHCIIdMIa -43% from total MHC in the nongrafted EDL muscles. In the grafted muscles, MHCIIb decreased and represented -2% while MHCIIdMIa represented -98% from total MHC. Sixty days after grafting MHCIIb represented -42% and MHCIIdMIa -58% from total MHC in the nongrafted EDL muscles, but in the grafted muscles MHCIIb was not detected and thus, the regenerated EDL muscles contained only MHCIIdMIa isoforms. These data indicate, that MHC composition shifts from heterogeneous composition to homogenous slower type of MHC, which is regulated by the cycle of denervation and reinnervation in regenerating skeletal muscle fibers.

Key words: EDL, autografting, muscle regeneration.

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Skeletal muscle fibers have remarkable capacity to regenerate after various types of injuries [2, 3]. The number of satellite cells, which depends on the type of muscle fiber determines the regeneration capacity of muscle fibers [22]. The higher the oxidative capacity of muscle fiber the higher the number of satellite cells [23]. Autografting of skeletal muscles has been used as a model for muscle degeneration and regeneration [5, 28]. Though, using injection of different myotoxic agents and its combination with grafting is an alternative method to the autografting [9, 27]. The result of application of both methods is muscle degeneration and subsequent regeneration, but the time of these processes is different and therefore the extent of regeneration is different. Treatment with myotoxic agents causes rapid degeneration and regeneration of muscle fibers, and in rats, muscle is completely regenerated within a month after treatment [27]. After autografting on rats, muscles are regenerated about two months after surgery [5]. In addition, the amount of connective tissue is small in the muscles treated with myotoxins [1] compared with the muscle grafts. The recovery of fiber diameter is better in muscles treated with myotoxins than in grafted muscles [1, 19, 27]. Yet muscle autografting has an advantage, like application of different innervational patterns what cannot be used with myotoxins [28].

In adult muscles of mammals, regeneration reproduce the events of normal development [7, 9, 27]. However, between these two processes of fiber formation, difference exists with regard to the type of transition of MHC isoforms[10]. The disappearance of developmental MHC isoforms is more rapid in regenerating than in developing muscles [8]. It has been demonstrated that with respect to MHC expression muscle regeneration causes more homogeneous expression of MHC than during development [27]. For instance, in rats Soleus muscle the composition of MHC isoforms shifted from heterogeneous fast and slow type of isoforms to the homogeneous slow type of MHC isoform [13, 27]. In these experiments the authors suggested that the transition is caused mainly by the positive extrinsic influence of the nerve [27]. It is known that Soleus muscle fibers contain small amount of fast type MHC IIa isoform which decreases during aging [25]. In that case it can be presumed that the disappearance of fast MHC isoform may be due to the small amount of this isoform. EDL muscle contains only fast type of MHC isoforms. In 10 weeks old rats the most prominent MHC

isoforms are MHCIIb and MHCIId [25]. There is about 10% of MHCIIa and about 1% or less of MHC I in EDL muscle. Consequently the amount of slow MHC isoform is negligible[24, 25] which leads to assumption that the transition of MHC isoforms could take place only between fast MHC isoforms in regenerating EDL muscle. The purpose of the study was to elucidate, whether transition occurs only between fast MHC isoform or also involves slow MHC isoform in regenerating EDL fibers. To evoke regeneration process we used nerve implanted autografts of EDL. Nerve implantation was used to ensure reinnervation which in this case is more reliable than using standard grafts. Grafts have all components of the regeneration process, including revascularisation and reinnervation, which are not always possible when using myotoxic agents, because different agents have different impacts to these processes [1].

Materials and Methods

Animal Procedures

Fifteen female rats of the Wistar strain were purchased from National Laboratory Animal Centre, Kuopio, Finland. In the beginning of the experiment rats were 8 weeks old and weighed 176± 4.3 g. Rats were randomly assigned into three groups with follow up times: 10th day (n = 5), 30th day (n = 5) and 60th day (n = 5) after autografting. Throughout the experiment rats were housed in identical environmental conditions in Type 3 polycarbonate cages, four per cage 12/12 h light and dark cycle. Animals were maintained on the diet (Special Diet Services, Witham, Essex, England), food and water were given *ad libitum.*

Muscle degeneration and subsequent regeneration were induced by autografting of EDL muscle with reimplantation of the nerve [6]. Before autografting, rats were anaesthetized by intraperitoneal injection of ketamine (Calypsol, Gedeon Richter A.O., Budapest, Hungary) 2.5 mg/100 g body weight and diazepam (Lab. Renaudin, France) 2.5 mg/100 g body weight. EDL muscle from the contralateral limb was served as a control. Animals were euthanized by decapitation under the ether narcosis. Muscles were resected, weighed and frozen in liquid nitrogen.

Separation of MHC isoforms

Muscles were pulverised in liquid nitrogen. Crude extracts were prepared by homogenizing the muscle powder 1:10 (w/v) in the buffer containing: 0.3 M KC1, 0.1 M KH_2PO_4 , 50 mM K2HPO4, 10 mM EDTA, pH 6.5. After stirring for 15 min on ice, the homogenate was centrifuged at 11000 g for 10 min. The supernatant fraction was two-fold diluted with glycerol and stored at -20°C [4]. Protein concentration was determined by the method of Lowry et al. [16]. MHC isoforms were separated using 1mm thick 5-8% gradient sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Aliquots containing 0.6 μg of protein were loaded on the gel after having been incubated for 7 min at 65°C in the lysis buffer containing: 10% (w/v) glycerol, 5% (v/v) p -mercaptoethanol, 2.3% (w/v) SDS, 0.05% (w/v) bromphenol blue, 62.5 mM TRIS-HC1, pH. 6.8 [4] . Electrophoresis lasted 24 h at 120 V. Gels were silver stained according to Oakley et al. [18]. Protein bands were identified according to their apparent molecular masses. In addition, myosin preparations purified from crural diaphragm and soleus muscles from Wistar rats were used to aid identification of MHC. Protein bands were analyzed densitometrically using LKB 2202 Ultrascan Laser Densitometer.

The data were analysed for statistical differences by One Way Analyses of Variance. Results are given: Mean ± SEM and were considered statistically significant for a value of p 0.05.

Results

Muscle mass

Changes in the mass of studied muscles were taken as a criterion of muscle regeneration. Table 1 shows that the mass of the grafted muscles in all groups was significantly lower from the nongrafted contralateral muscles. One month after grafting the mass of the grafted muscles remained the same with the 10th day group. It indicates that regeneration process as well as reinnervation occurred within that time, since the progress of atrophy of muscles is stopped. Two months after grafting the mass of the grafted muscles was significantly increased compared with previous groups (Table 1). It is well known that using autografting, muscle fibers become completely reinnervated about one month after operation [6]. First month of regeneration after autografting is period for formation of new muscle fibers, while this process is independent on the innervation, but in the second month, hypertrophy of

Table 1. Time course of the changes in the mass of grafted and control muscles.

DAYS AFTER GRAFTING	MUSCLE MASS (mg) CONTROL	GRAFTED	% FROM CONTROL
10	71.4 ±3.88	50.6 ± 4.27 #	70.6 ± 2.87
30	80.6 ± 2.44	52.8 ± 3.99 #	55 ± 3.99 *
60	103 ±3.20	78.4 ± 3.69 #*	78.4 ± 3.69 *

# $p < 0.01$ compared with the control muscles; * $p < 0.01$ compared with the previous group.

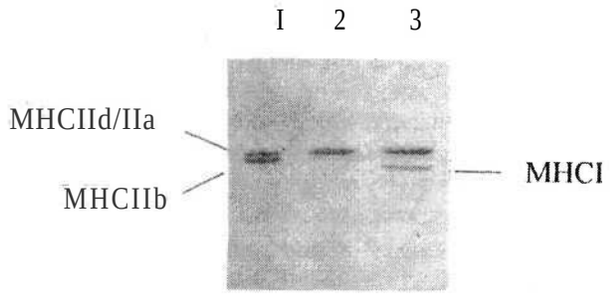


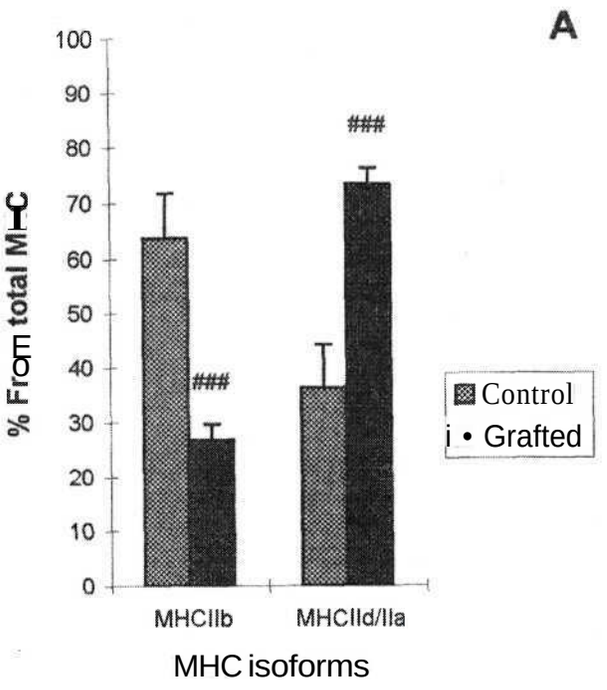
Figure 1. Separation of MHC isoforms in 5-8% SDS PAGE (110x 120x 1 mm). Gel was silver stained according to the method of Oakley et al. (1980). 1: MHC isoforms from the control EDL. 2: MHC isoforms from the grafted EDL from 30th day group. 3: Mixture of MHC isoforms from Soleus and costal diaphragm of 8 weeks old Wistar rats.

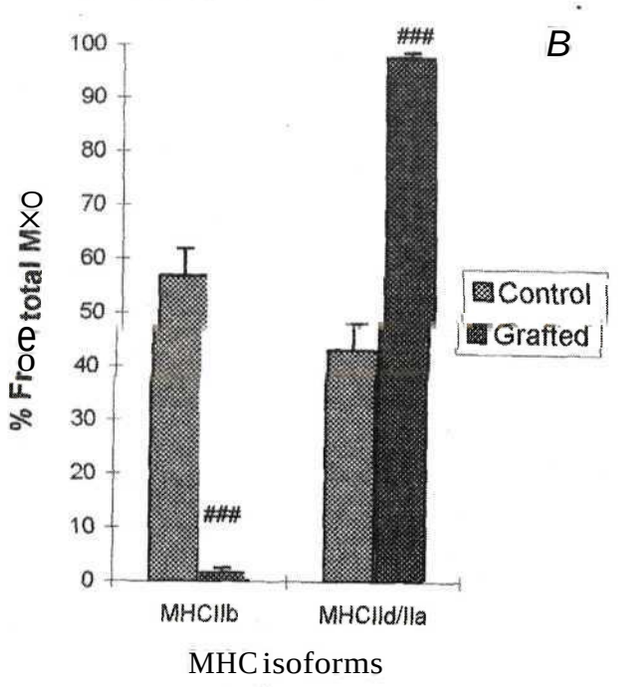
young muscle fibers occur in presence of innervation [5] which is an evidence for increased muscle weight during the second month in the present experiment.

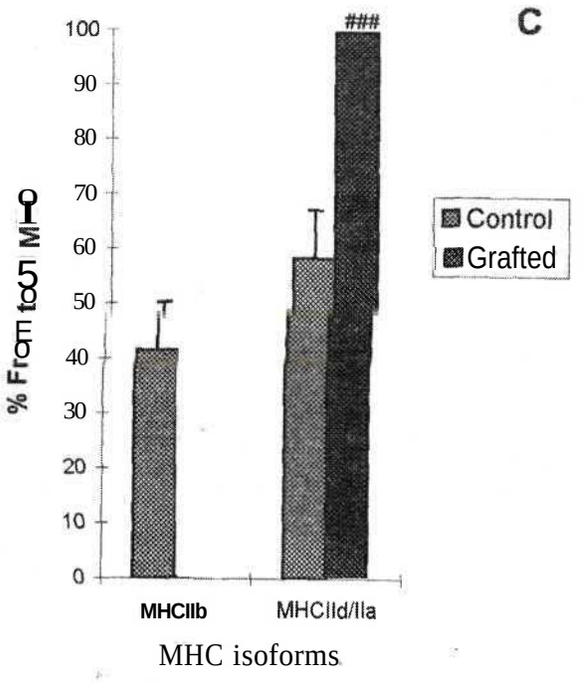
MHC isoforms

Using 5-8% SDS PAGE we could separate MHCIIb and MHCIId/IIa (Fig. 1). The reason for poor separation of MHCIId from MHCIIa is probably the thickness of the gel, which in our study was 1 mm. Therefore we consider it MHCIId/IIa. MHC of the control EDL muscle in thelOth day group contained ~64% of type IIb isoforms and ~36% type IId/IIa isoforms (Fig. 2A). Then IIb MHC isoforms were dominated in nongrafted EDL muscles. After grafting, changes in the proportion of MHC isoforms appeared: MHCIIb decreased and represented ~27% from total MHC while MHCIId/IIa increased and was ~73% from total MHC (Fig. 2A). Thirty days after autografting of EDL muscles decrease of MHCIIb and increase of MHCIId/IIa continued: MHCIIb from ~57% in the control muscles to ~2% in the grafted muscles and MHCIId/IIa from ~43% in the control muscles to ~98% in the grafted muscles (Fig 2B). Two months after grafting the composition of MHC isoforms in control EDL did not change compared with previous group. In the grafted muscles homogeneous com-

Figure 2. Time course of the changes in MHC isoforms during the regeneration of EDL (A 10 days, B 30 days, C 60 days). ### $p < 0.001$ compared with the control muscle.







position of MHCIId/IIa was detected (Fig. 2C).

Discussion

We used autografting of EDL muscles to investigate composition of MHC isoforms in regenerating skeletal muscle fibers. Autografting of skeletal muscles on small laboratory animals enables to apply several techniques regarding innervation. Mainly three different options can be used: 1) standard autografting, where nerve reinnervates spontaneously from the cut ends of the motor nerves; 2) nerve-implant autografting, where nerve stumps are reimplanted to the recipient site of muscle, and 3) nerve-intact autografting, where nerve branches leading to the muscle are not cut [28]. We used nerve-implant autografts to ensure the reinnervation. When using standard grafting method, the nerve stumps cut can move during the grafting procedure and reinnervation can be delayed. In the present study successful reinnervation became apparent after 30 days from the grafting since the mass of the grafts did not decrease compared with 10th day group, which indicates that atrophy of muscle fibers caused by the denervation is stopped. Furthermore, the mass of grafted muscles was increased in 60th day group compared with the 30th day group, which indicates the successful reinnervation of the grafts. In the end of the experiment the grafts were very well regenerated. Mass of the grafted muscles constituted ~76% from the control (Table 1) which is quite high. The high mass of the regenerated grafts is probably due to the age of animals used in present study. It is known that regeneration capacity of muscles from animals younger than 10 weeks is very good [20, 21]. Transition of MHC isoforms occurred in regenerating EDL muscles. MHC isoforms shifted from MHCIIb and MHCIId/IIa in control muscles to the MHCIId/IIa in regenerated muscles. The amount of MHCI was less than 1% in control and grafted muscles. Thus the transition occurred between the fast type of isoforms. Yet in this case we can suggest that the composition of MHC isoforms shifted towards the slower MHC isoforms. It confirms the findings of Davis and coworkers and Whalen and coworkers, that during the regeneration the composition of MHC isoforms changes to slow isoform in Soleus muscle [13, 27]. Since the resolution of our gels was not sufficient to separate MHCIId and MHCIIa we cannot be sure in what extent the ratio of these isoforms increased. However, we can suggest that the increase of these isoforms occurred mainly due to the increase of MHCIId but not MHCIIa, because the amount of MHCIIa is about 10% or even less in EDL muscles [24, 25]. What kind of factors can affect the composition of MHC isoforms in regenerating skeletal muscle fibers? One of the factors is innervation of muscle fibers. Jakubiec-Puka et al. [14], have shown that MHCIIb is susceptible to the lack of innervation. After denervation of EDL in adult rats the content MHCIIb isoform decreased and the content of MHCIIa increased, but when reinnervation occurred, the composition of MHC isoforms returned to the control level [14]. Muscle autografts became completely reinnervated within a month after grafting, which means that at least two or three weeks most of the fibers are denervated [5]. Thus one of the factors would be development of new muscle fibers during the regeneration process and most likely, its combination with denervation. It is shown that denervation caused in fast muscle fibers to transform MHC isoforms progressively to a slow phenotype and proposed that the disappearance of the fast-type MHC followed the sequence IIb $\rightarrow$ IId $\rightarrow$ IIa [12]. It has been determined that innervation appears to be predominant factor for differentiation into fast muscle in rabbits [11]. In the latter study MHC isoforms transformed to the pure MHCI, though in the control muscle the predominant isoform was MHCIId in Gastrocnemius muscle. In our study, the MHC composition changed only between the fast type of isoforms without no appearance or increase of MHCI. The discrepancy might be explained by the homogeneous constitution of type II fibers of the control EDL, while the control Gastrocnemius contained about 20% of slow-type myosin and fibers in the study of D'Albis et al. [11]. In conclusion we can suggest that transition of MHC isoforms towards slower type in regenerating EDL muscle is caused by the temporal denervation and its combination with the development and maturation of new muscle fibers.

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Address correspondence to:

Jaanus Järva, Institute of Exercise Biology, University of Tartu, Ülikooli 18, EE 2400, Tartu, Estonia, fax 372 7 465 440.

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