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Expression of myogenic regulatory factors in rat skeletal muscles after denervation

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Abstract

Aim: *The aim of this study was to investigate expression of myogenic regulatory factors MyoD and myogenin during denervation in fast and slow rat skeletal muscles of different rat strains (Wistar and Sprague-Dawely).*

Material and Methods: *Immunohistochemical and Western blot analyses were performed on tibialis anterior and soleus muscles.*

Results: *Immunohistochemical analysis of tibialis anterior and soleus muscles during denervation demonstrated that both myonuclei and satellite cells were activated. Western blot analysis showed a significant upregulation of MyoD and myogenin proteins in m. tibialis anterior at all time points of denervation, except on 60th day for MyoD. In m. soleus during denervation Western blot analysis showed upregulation of MyoD and myogenin in first 14 days, and there was no expression after that period.*

Conclusion: *Our results indicate that MyoD and myogenin protein expression during denervation is muscle type specific. The role of MyoD and myogenin in adult muscle is potentially also important in orchestrating an adaptive response of existing muscle fibers during denervation.*

INTRODUCTION

The functional diversity of skeletal muscle fibers is related to their inervation pattern (*1*). Loss of inervation leads to general morphological and physiological deterioration of affected fibers (*2, 3*). Superficial position exposes skeletal muscle and their motoneurons to different types of injuries and they are damaged and repaired repeatedly throughout life. Denervation of adult muscle alerts within days the expression levels of numerous structural proteins and trofic factors, resulting in muscle fibers that show many characteristics of embryonic fibers. For example, the levels of neural cell adhesion molecule (NCAM) and nAChR subunit mRNAs, normally expressed at the neuromuscular junction of adult muscles, increase throughout the muscle fiber (*4*). Morphologic alterations such as the swelling and subsequent atrophy of muscle fibers are also observed (*5*), as well as the proliferation of satellite cells (*6, 7*). Muscle regeneration maintains locomotor function during aging and delays the appearance of clinical symptoms in neuromuscular diseases, such as Duchenne muscular dystrophy (*8*). This capacity for tissue repair is conferred by satellite cells located between the basal lamina and the sarcolema of mature myofibers (*9*). Upon injury, satellite cells reenter the cell cycle, proliferate, and then exit the cell cycle either to renew the quiescent satellite cell pool or to differentiate

into mature myofibers (*10*). Both the cell proliferation and differentiation programs are essential for myogenesis.

Denervation also alerts the expression levels of skeletal muscle specific basic helix-loop-helix (bHLH) proteins. The family of muscle specific bHLH regulatory factors consists of MyoD, Myf-5, myogenin and MFF4. These factors contribute to the transcriptional activation of muscle genes during commitment and differentiation (*11*). A distinct role for the bHLH myogenic factors initially was suggested by their different temporal expression patterns in the somites and limbs during embryonic development (*12*). Gene knockout experiments provided evidence for the role of MyoD and Myf-5 in myoblast commitment (*13,14*) whereas myogenin is required for terminal myoblast differentiation (*15*).

This study was undertaken to identify the changes at the cell and tissue level that correspond to the already demonstrated reduction in the restorative capacity of long-term denervated muscles. To our knowledge this is the first report about expression of MyoD and myogenin at protein level during denervation in fast and slow rat skeletal muscles of different rat strains.

MATERIALS AND METHODS

Animals and surgical procedures

Fifty adult male Wistar and Sprague-Dawely rats, 200–250g in weight (8 weeks old), were used and assigned randomly to different experimental groups. The study was approved by the Ethics Committee of the Medical Faculty, University of Rijeka.

The animals were anesthetized with ketaminhydrochloride (0.1 mg/g body weight, i.p.) and xylazinhydrochloride (0.02 mg/g body weight, i.p.). Denervation involved removing a 5-mm segment of the right sciatic nerve in the thigh region. The served proximal end was ligaded and then sutured into surrounding denervated muscles to prevent reinervation of the distal musculature.

Rats from Wistar and Sprague-Dawely strains were examined at 3, 14, 30, 45 and 60 days post surgery. We considered these to be reasonable time points to gain insight into the role of myogenic regulatory factors in the adaptation process.

The denervated tibialis anterior (TA) and soleus (SOL) muscles were removed and quickly frozen in isopentane cooled by liquid nitrogen. Unoperated rats were killed with ether to obtain normal TA and SOL muscles. The muscles were stored at –80 °C until further analysis.

For histological analysis, cross sections were collected and stained with hematoxylin and eosin (HE).

Protein extraction and western blotting procedures

For MyoD and myogenin immunoblotting, total muscle protein containing cytoplasmatic and nuclear fractions was extracted from SOL and TA muscles by pulverization of frozen samples. The samples were immersed in a homogenizing buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS. 0.1mM phenylmethylsulfonylfluoride was added at the time of use at 4 °C. The samples were incubated for 30 min at 4 °C. Each homogenate was transferred to microfuge tubes and centrifuged at 13000 rpm for 20 min at 4 °C, and the supernatant was obtained. An aliquot of the supernatant was used for determining protein concentration using Bredford method, and remainder of the supernatant was stored at –80 °C for subsequent Western analysis. Optimal loading for immunoblotting was determined to be 85 μg and 75 μg per sample for MyoD and myogenin, respectively. To verify MyoD and myogenin protein bands. two control standards were run simultaneously with each gel: a molecular weight marker (Precision Protein Standards); and protein isolated from neonatal rat muscles (highly expressing both myogenic regulatory factors). Proteins were denaturated by boiling for 3 min after adding 5% solution for denaturation (500μl Leammli, 25 μl 2-mercaptoethanol). Proteins were separated in either a SDS-10% (MyoD) or 12.5% (myogenin) polyacrylamide gel. The proteins were transferred to nitrocellulose membranes for 3 h at 70V. The membranes were immersed in a blocking solution containing 0.5% nonfat dry milk (Bio-Rad) dissolved in TBS (pH 7.46) for 1 h. Then the membranes were incubated in either monoclonal mouse anti-MyoD (1:400) (DakoCytomation) or monoclonal mouse anti-myogenin (1:400) (DakoCytomation) diluted in blocking solution overnight at 4 °C. After that the membranes were washed 6x10 min in TBS with 0.1 Tween and incubated for 1 h at room temperature with a second antibody (rabbit anti mouse conjugated with peroxidase 1:2000) (DakoCytomation). Then the membranes was washed 5x with TBS with 0.1% Tween 20 for 1h. Antibody bindings were visualized with DAB (diaminobenzidin, 0.1mg/ml), 0.02% hydrogen peroxide and 0.03% CoCl. Band intensity for each blot was quantified by scanning densitometry.

Immunohistochemistry

Serial 10μm-thick transverse sections were cut from frozen muscles. For the MyoD and myogenin staining the slides were fixed in 2% paraformaldehyde for 10 min, washed in TBS, and treated with 3% H_2O_2 in TBS with 0.1% Tween 20 (TBST) for 5 min. Then the cryosections were washed in TBS for 5 min. After that the slides were immersed in blocking solution (10% normal swine serum in TBS with 0.2% bovine serum albumin – TBSTB) for 20 min. Then the slides were incubated for 60 min with the primary mouse monoclonal antibody anti-MyoD (DakoCytomation) or primary mouse monoclonal antibody anti-myogenin (DakoCytomation) diluted 1:50 in TBST. The sections were washed 2x 5 min in TBST, treated with secondary biotinylated antibody for 15 min, and streptavidin conjugated to peroxidase for 15 min (LSAB+Kit, DakoCytomation, ready-to-use). Finally, the sections were washed in TBS, visualized with AEC (DakoCytoma-

tion) for 10 min. After having beenrinsed in water, the sections were mounted in glycerol gelatine medium.

The slides for double immunohistochemical staining were first fixed in 0.3% H_2O_2, then primary monoclonal mouse antibody anti-dystrophin (Sigma)was applied for 1 h. After intervening washes in TBS, the slides were covered with secondary biotinylated antibody for 15 min, then with streptavidin conjugated to alkaline phosphatase for 15 min (LSAB+Kit, DakoCytomation, ready--to-use). The slides were washed in TBS and BCIP/NBT/INT chromogen substrate for 10 min. Dystrophin was visualized by this method as dark brown. The cryosections were fixed in 2% paraformaldehyde, and thereafter the method was continued as described above for MyoD or myogenin.

Statistical Analyses

All data are expressed as means ± SE. Within group and between groups comparisons were performed using a two-way analysis of variance and the Tukey-Kramer multiple comparisons post hoc test. The level of significance was set at $P<0.05$.

RESULTS

Muscle morphology and effect of denervation

Myofibers of control muscles displayed the characteristic polygonal shape of normal, innervated muscles in transverse sections. Atrophy of muscle fibers was prominent in the second month after denervation, but the degree of atrophy was not consistent among all muscle fibers. The period up to two months of denervation was characterized by continued gross muscle and muscle fiber atrophy. Both histochemical (HE staining) and immunohistochemical methods demonstrated that soleus muscles undergo a higher level of atrophy than tibialis anterior muscles. One of the most noticeable changes with increasing time of denervation was a steady increase in the amount of interstitial connective tissue. In all of the investigated muscles we found atrophied fibers either in groups, or interspersed among fibers which had almost normal morphology. There were no differences in muscle morphology during denervation in skeletal muscles of different rat strains.

MyoD and myogenin protein expression

Control muscles. We found that MyoD and myogenin immunoreactivity was not observed at detectable level in normal, innervated TA and SOL muscles using immunohistochemical and Western blot analyses.

Tibialis anterior muscles. Western blot analyses showed a progressive increase in MyoD and myogenin protein in the TA muscles of the denervated rats. Immunoreactive bands were detected for MyoD and myogenin on the 3rd, 14th, 30th and 45th day. On the 60th day immunoreactive band was detected for myogenin, but not for MyoD. The highest level of expression was observed on the 30th day

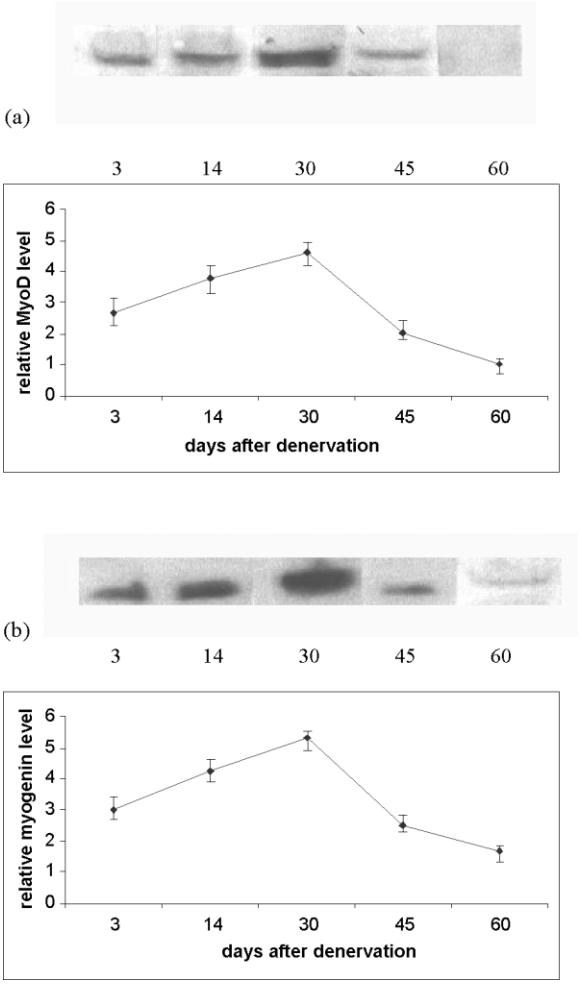

Figure 1. *MyoD and myogenin expressions in denervated m. tibialis anterior. (a) Levels of MyoD protein expression in the tibialis anterior muscles after 3, 14, 30, 45, and 60 days of denervation determined by Western immunoblot. An immunoreactive band of ~45 kDa corresponded to the predicted molecular mass of the rat MyoD protein. (b) Levels of myogenin protein expression in the tibialis anterior muscles after 3, 14, 30, 45, and 60 days of denervation determined by Western immunoblot. An immunoreactive band of ~34 kDa corresponded to the predicted molecular mass of the rat myogenin protein. The density of each band was expressed relative to the background density (considered to be 1.0).*

for both MyoD and myogenin (Figure 1). There were no significant changes in MyoD and myogenin protein in either muscle of the two different rat strains throughout the study.

Soleus muscles. Western blot analysis showed an increase in MyoD and myogenin protein in the SOL muscles of denervated rats. Immunoreactive bands were detected for MyoD and myogenin on the 3rd and 14th day with higher levels of expression on the 14th day than on the 3rd day. After the 14th day there was no immunoreactivity for MyoD or myogenin protein (Figure 2). There were no significant changes in MyoD or myogenin expression levels in SOL muscles of the two different rat strains.

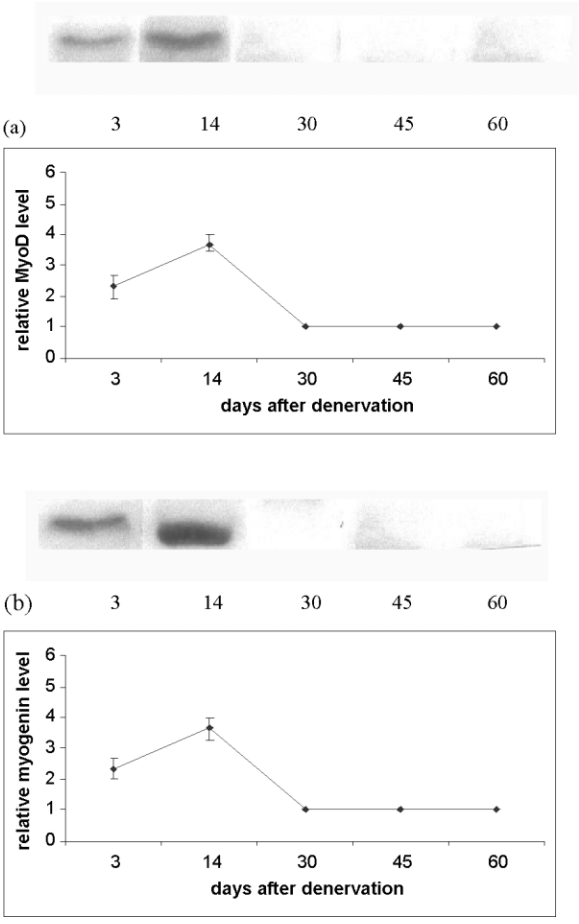

Figure 2. *MyoD and myogenin expressions in denervated m. soleus. (a) Levels of MyoD protein expression in the soleus muscles after 3, 14, 30, 45, and 60 days of denervation determined by Western immunoblot. An immunoreactive band of ~45 kDa corresponded to the predicted molecular mass of the rat MyoD protein. (b) Levels of myogenin protein expression in the soleus muscles after 3, 14, 30, 45, and 60 days of denervation determined by Western immunoblot. An immunoreactive band of ~34 kDa corresponded to the predicted molecular mass of the rat myogenin protein. The density of each band was expressed relative to the background density (considered to be 1.0).*

MyoD and myogenin proteins within myonuclei and satellite cells

By labeling the same section with dystrophin and MyoD or myogenin antibodies found that the nuclei containing MyoD or myogenin occurred predominantly in myonuclei, rather than satellite cells. Sarcolema was visualized using dystrophin antibody and all myonuclei were localizated inside, and satellite cells outside of sarcolema. In all examined muscles, intense MyoD and myogenin staining were higher in myonuclei than in satellite cells (Figure 3).

DISCUSSION

In our research, we studied gene expression of MyoD and myogenin in denervated m. tibialis anterior and m. soleus. Denervation causes changes of gene expression in skeletal muscles. These changes are not equal in the slow and fast muscle and therefore we wished to observe the way in which cutting off the nerve affects the expression of myogenic regulatory factors in fast and slow skeletal muscles. M. tibialis anterior belongs to a group of fast muscles, i.e. most of its fibers are intensively stained with antibodies specific for fast isoforms of myosin heavy chains. M. soleus is composed of numerous fibers stained with antibodies for slow isoforms of myosin heavy chains and therefore we classified it in the group of slow muscles. Earlier studies have led to conclude that satellite cells in fast and slow muscles differ from each other (*16*). That was an additional argument for us to further study gene expression of myogenic regulatory factors exactly in these muscles. Investigation of MyoD and myogenin expression in muscles was conducted in numerous research studies of fast muscles (*17*, *18, 19, 20*) and the obtained results were uneven. The expression of MyoD and myogenin in slow muscles after denervation has not been sufficiently studied to result in a reliable conclusion. At transcriptional level, it has been observed that fast muscles in denervation react with sudden increase in myogenin and MyoD while with the slow m. soleus there have been no significant increase of transcripts, not even after 48 hours (*21*).

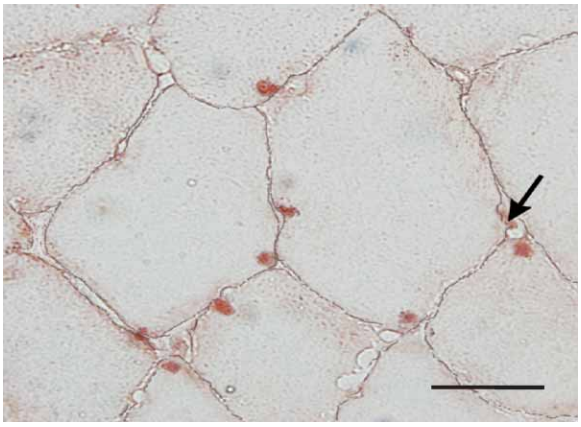

Figure 3. *Localization of myogenin protein. Cross section of tibialis anterior muscle was on the 3rd day after denervation immunohistochemically analyzed with antibodies against dystrophin and myogenin on the same section. Dystrophin antibody binding was visualized with a brown color and myogenin binding with red. Myogenin positive nuclei could be observed in myofibers as well as in satellite cells (arrow). Bar, 25μm.*

Several research studies pointed to myogenin as an important regulator of gene expression during muscle development, denervation and ageing (*22, 23, 24*). Increased level of myogenin expression has been observed in late phases of differentiation of myoblasts and during formation of myotubes. After the innervation of myotubes and their development, the level of myogenin returns to the low level of expression. Unlike the innervated muscle after the denervation of adult muscle, myogenin expression level strongly increases and seems to be pro-

voking gene expression that encodes for the αAChR subunit (*22*). Our study showed that denervation caused increased expression of myogenin protein from the 3rd day continuosly to the 30th day after the denervation of the fast m. tibialis anterior. After that, we observed decrease in myogenin expression up to the 60th day of postdenervation. These results obtained by immunohistochemistry were also confirmed by Western blot analysis. The results coincide with those obtained on protein level by Kostrominova (*18*). Also, these results coincide with the appearance of mRNA in postdenervation period (*18*). That study confirmed that mRNA for myogenin began to increase already on the 1st day after denervation, was strongly rising and achieved its highest level on the 3rd day after the provoked denervation. It remained at a such high level until the 30th day of postdenervation and then the mRNA level for myogenin began to decrease. However, although there is fundamental balance between the expression of mRNA and protein, there are certain differences in the appearance of those two molecules. For example, on the 3rd day of postdenervation period myogenin mRNA increased about 500 times while the protein increased only about 14 times in relation to control. This difference may be a reflection of different transcriptional and translational control mechanisms which in the end affect the protein level. Furthermore, the same study suggests that the level of myogenin protein reflects the level of mRNA but only in transitory denervated muscle for up to 30 days while with protracted denervation this was not the case. The level of myogenin mRNA expression between the 30th and 120th day of postdenervation decreased for about 80% whereas the level of protein decreased for only 30% in the same period (*18*). Our results show that on the 3rd day after denervation most myonuclei immunohistochemically reacted to myogenin antibodies. This coincides with present studies which proved that, after transitory denervation, most of the increased myogenin expression was localized in myonuclei (*25*). Previous studies showed that number of activated satellite cells on the 2nd and 3rd day after denervation was very low and represented up the to 4% of total nuclei number (*26, 27*). Between the first and the second month after denervation the proportion of satellite cells increased by 8–12% and thereafter suddenly decreased and came to a level of 2% after seven months in postdenervation (*26, 27*).

Immediately after denervation in a rat, there is the increase in expression of MyoD, myogenin and nAChR (*25*). This increased expression remains present also in long-time denervated muscles and so Adams et al. found that the increased level of these myogenic factors, at the protein level as well as at the mRNA level, remained such during the period of seven months *(28)*. Furthermore, Dedkov *et al*. confirmed, using immunohistochemistry, the presence of MyoD and myogenin proteins in myonuclei of the newly formed muscle fibers and the same in myonuclei of persistent muscle fibers in rats denervated for 25 months (*29*). Our study differs from the previous one as we did not record the expression of MyoD protein after the 60th day of denervation in the fast muscle and the expression of MyoD and myogenin in the slow muscle not even after the 14th day.

As evident from the above, there are numerous authors that have confirm in their studies the expression of MyoD and myogenin at the levels of mRNA and protein. But there are other studies confirming the opposite. Sakuma recorded significant decrease in MyoD protein in denervated muscles from the 1st to 28th day after denervation *(17)*. It is not known why these differences in expression of myogenic regulatory factors exist during denervation. Hyatt and coworkers assumed the reason might be different rat species used in experiments *(20)*. With the intention to eliminate this dilemma, we conducted our research on the both rat species mentioned. Our results confirmed equal expression of myogenic regulatory factors in both species and so it remains unclear as to why Sakuma did not obtain the expression of MyoD during denervation. Further studies are necessary in order to explain our result that m. soleus expresses MyoD and myogenin in a weaker manner after denervation. The reason may be that after the denervation rat's leg is hanging in a way that m. tibialis anterior is stretched out and so receives stimuli as with stretching exercise and then the expression of MRF occurred *(30)*. As a difference to m. tibialis anterior, m. soleus is not stretched in denervation.

Furthermore, we were interested to find out where the expression of MyoD and myogenin is localized in skeletal muscles. By immunohistochemical double staining with antibodies against dystrophin in combination with antibodies against MyoD or myogenin, we obtained MyoD and myogenin expression localized in a large number of myonuclei but the expression was also detected in the nuclei that with their location correspond to satellite cells. This result was obtained in fast as well as in slow muscles.

Using immunohistochemical analysis, our most important result obtained is that satellite cells and also myonuclei to an even larger extent were activated in the denervated muscle.

As far as proteins MyoD and myogenin are concerned, one could assume that their location is related to satellite cells since there are studies confirming the increased activity of satellite cells in denervated skeletal muscles *(7, 31)*. Although all these facts remain as such, some authors showed, using immunohistochemical investigations, that proteins of myogenic regulatory factors accumulate predominantly in myonuclei and less in satellite cells *(20)*. Results quoted by Hyatt *et al*. suggest that MyoD and myogenin at protein level appeared at all investigated time intervals (3rd, 14th, 28th day) predominantly in myonuclei and then in satellite cells *(20)*. These results coincide with the results of this study.

Most important conclusion of our study is that denervation differently affects the expression of proteins MyoD and myogenin in fast and slow skeletal muscles and that proteins MyoD and myogenin are expressed in myonuclei and less in satellite cells in the denervated muscles.

Additionally we confirmed the difference in protein MyoD and myogenin expression does not exist between skeletal muscles of two rat strains.

Differences that appear in the study of expression of myogenic regulatory factors during muscle denervation may evolve because of the different detection methods used, different muscle injuries, injuries of not always the same intensity, etc.

As far as denervation of skeletal musculature is concerned, the study needs to be directed toward the isolation of stem population of satellite cells and factors that would accelerate their proliferation and differentiation in vivo. This will be of immeasurable value for treatment of different myopathies as well as sport injuries, injuries during muscle transplantations, and prevention of skeletal muscle atrophy due to ageing.

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