In vivo expression patterns of MyoD, p21, and Rb proteins in myonuclei and satellite cells of denervated rat skeletal muscle

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Ishido, Minenori, Katsuya Kami, and Mitsuhiko Masuhara. In vivo expression patterns of MyoD, p21, and Rb proteins in myonuclei and satellite cells of denervated rat skeletal muscle. Am J Physiol Cell Physiol 287: C484–C493, 2004. First published April 14, 2004; 10.1152/ajpcell.00080.2004.—MyoD, a myogenic regulatory factor, is rapidly expressed in adult skeletal muscles in response to denervation. However, the function(s) of MyoD expressed in denervated muscle has not been adequately elucidated. In vitro, it directly trans-activates cyclin-dependent kinase inhibitor p21 (p21) and retinoblastoma protein (Rb), a downstream target of p21. These factors then act to regulate cell cycle withdrawal and antia apoptotic cell death. Using immunohistochemical approaches, we characterized cell types expressing MyoD, p21, and Rb and the relationship among these factors in the myonucleus of denervated muscles. In addition, we quantitatively examined the time course changes and expression patterns among distinct myofiber types of MyoD, p21, and Rb during denervation. Denervation induced MyoD expression in myonuclei and satellite cell nuclei, whereas p21 and Rb were found only in myonuclei. Furthermore, coexpression of MyoD, p21, and Rb was induced in the myonucleus, and quantitative analysis of these factors determined that there was no difference among the three myofiber types. These observations suggest that MyoD may function in myonuclei in response to denervation to protect against denervation-induced apoptosis via perhaps the activation of p21 and Rb, and function of MyoD expressed in satellite cell nuclei may be negatively regulated. The present study provides a molecular basis to further understand the function of MyoD expressed in the myonuclei and satellite cell nuclei of denervated skeletal muscle.

denervation; protein expression; apoptotic cell death; immunohistochemistry

NEURAL ACTIVITY IS INVOLVED in altering the morphological and functional properties of adult skeletal muscles. Increased neural activity induces muscle hypertrophy (20, 37), whereas its absence, such as during denervation, results in muscle atrophy (18, 48). The physiological and biochemical alterations that occur in adult skeletal muscles due to denervation lead to reductions in muscle weight (18, 48) and cross-sectional area of the myofiber (7, 28), transition of myofiber phenotypes (21), changes in rates of protein synthesis and breakdown (12), and alterations in contractile properties (10). Also, long-term denervation induces apoptosis in myofibers (62) and activation and proliferation of satellite cells, which are myogenic precursors that reside in adult skeletal muscle (30, 42, 48). However, much of the molecular basis underlying denervation-induced changes that occur in adult skeletal muscles are not understood.

MyoD is a member of the basic-helix-loop-helix family of proteins and acts as a transcriptional factor in several skeletal muscle-specific genes (9). MyoD−/− myogenic cells are committed to myoblast and proliferation. However, delays in myotube formation, the continuation of DNA synthesis, incomplete cell cycle arrest, and a deficiency in fusion capacity have been observed during the differentiation stage (38, 60). On the other hand, MyoD in vivo is expressed in adult skeletal muscle in response to diverse stimuli, such as overload, injury, and exercise (19, 22, 60), suggesting that MyoD plays a role in the plasticity of skeletal muscles. In denervated adult muscle, the rapid expression of MyoD mRNA and protein also occurs during early postdenervation (18, 49, 52). Several studies suggested that the function of denervation-induced MyoD may be to prevent denervation-induced muscle atrophy (49, 52). However, there is no direct evidence that MyoD prevents muscle atrophy, and little is known about the function(s) of MyoD expressed in denervated muscles.

The target genes of MyoD contain an E-box in their enhancer or promoter regions. They include those for the myosin light chain (59), muscle creatine kinase (24), the acetylcholine receptor (34), troponin I (26), and desmin (25). In addition, cyclin-dependent kinase (cdk) inhibitor p21 (p21, also known as Waf1 or Cip1), an inhibitor of cdk and a downregulator of proliferation cell nuclear antigen (50, 61), also contains three E-boxes (11). Therefore, MyoD induces cell cycle arrest during differentiation of the myoblast by directly inducing the expression of p21 (14, 15). However, substantial cell cycle arrest is mediated by the dephosphorylation of retinoblastoma protein (Rb), which is a downstream target of p21. The p21 induces the dephosphorylation of Rb, which binds to members of the E2F family of transcriptional factors that prefer to activate S-phase genes. Rb-bonded E2F is then inactivated, and these sequential reactions contribute subsequently to permanent cell cycle withdrawal (58, 63). Mice lacking the p21 gene (p21−/−) fail to form myotubes and exhibit incomplete cell cycle arrest and increased apoptotic rates in myoblasts (51, 64). On the other hand, differentiated skeletal muscle cells lacking Rb (Rb−/−) show attenuated expression of myosin heavy chain and arrest during the S and G2 phases of the cell cycle (31). Furthermore, Rb−/− myoblasts enter incomplete permanent cell cycle withdrawal, and Rb−/− myotubes show an increased rate of apoptosis and abnormal DNA endo-reduplication (63). These findings indicated that p21 and Rb regulate the proliferation and differentiation of myoblasts and the survival (anti-apoptotic cell death) of myoblasts and/or myofibers, and that MyoD
directly induces the transactivation of the p21 gene. Consequently, p21 may regulate Rb-associated functions. Numerous observations have shown that MyoD expression is rapidly induced in denervated skeletal muscles (18, 49, 52). However, an examination has not been made on whether p21 and Rb, whose expression and function are regulated by MyoD, are expressed in adult skeletal muscles in response to denervation. Although MyoD expression is induced in myonuclei and satellite cell nuclei in hypertrophic and regenerating skeletal muscle (19, 22), the cell types that express p21 and Rb in denervated skeletal muscles have not been determined. Furthermore, the reduction in the cross-sectional area of myofibers in denervated skeletal muscles is greater for fast myofibers than slow myofibers (4, 8, 28, 52), and MyoD expressed in denervated muscles has been proposed to prevent muscle atrophy (49, 52). If MyoD prevents muscle atrophy, MyoD expression is expected to be more enhanced in slow myofibers than fast myofibers of denervated skeletal muscles. Therefore, we quantitatively compared the expression of MyoD, p21, and Rb among distinct myofiber types in denervated skeletal muscle. The aim of the present study is to provide a molecular basis to elucidate functions of denervation-induced MyoD in myonuclei and satellite cell nuclei in adult skeletal muscle.

MATERIALS AND METHODS

Experimental design and surgical procedures. Adult female Fischer 344 rats (8 wk of age) were used in this study (n = 3/group). They were housed in individual cages at 22°C under a 12:12-h light-dark cycle and were provided with food and water ad libitum. All experiments were carried out with the approval of the Osaka University of Health and Sport Sciences Animal Ethics Committee.

With the rats under pentobarbital sodium anesthesia (60 mg/kg ip), the right hindlimbs were denervated by cutting the sciatic nerve, and an approximate 3-mm segment of nerve was removed to prevent nerve regeneration. The contralateral hindlimbs were sham-operated. The rats were killed on days 1, 3, 5, and 7 postdenervation. In addition, age-matched rats in a sedentary group were killed at 8 wk of age. Because there was no significant difference between sham-operated and sedentary muscles (data not shown), the values for the sedentary muscles were taken to be those of the innervated muscles (inner). At death, the rats were anesthetized, and plantaris muscles were removed. Muscle samples were quickly frozen in liquid nitrogen and stored at −80°C until use.

Immunostaining. Primary antibodies used in the present study were as follows: mouse monoclonal anti-MyoD (1:100; DAKO, Carpinteria, CA), mouse monoclonal anti-cdk inhibitor p21 (1:100; BD PharMingen, San Diego, CA), mouse monoclonal anti-dystrophin (a marker molecule of the plasma membrane) (1:100; Sigma, St Louis, MO), mouse monoclonal anti-human Rb (1:100; BD PharMingen), mouse monoclonal anti-skeletal myosin (fast) (MY-32, 1:100; Novocastra Laboratories, Newcastle, UK), rabbit polyclonal anti-cdk inhibitor p21 (Ab-5, 1:100; Oncogene Research Products, Cambridge, MA), and goat polyclonal anti-M-cadherin (a marker molecule of satellite cells) (1:200; Santa Cruz Biotech, Santa Cruz, CA). M-cadherin is a molecular marker used to identify satellite cells in tissue sections (5, 16, 18). Triple immunostaining with M-cadherin, a rabbit polyclonal anti-laminin (a marker molecule for the basement membrane; 1:3,000; Sigma) antibody, and 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) showed that nuclei located inside the laminin-positive basement membranes of myofibers were marked with M-cadherin antibody (data not shown). Furthermore, by using triple immunostaining with the use of M-cadherin, dystrophin antibodies, and DAPI, M-cadherin immunoreactivity was detected in accordance with the dystrophin-positive plasma membranes of myofibers, and nuclei located outside of dystrophin-positive plasma membranes of myofibers could be marked with M-cadherin antibody (data not shown). These findings verified that satellite cells can be identified by using M-cadherin antibody, in accordance with previous observations (5, 16, 18).

For single, double, and triple immunostaining, serial cross sections (5- or 10-μm thickness) of midbelly muscle were cut by using a cryostat at −20°C and then fixed in 3% paraformaldehyde/0.1 M phosphate buffer (pH 7.4) for 15 min and washed with 0.1 M PBS (pH 7.4). To block any nonspecific reaction, the sections were incubated with 0.1 M PBS containing 10% normal serum and 1% Triton X-100 for 1 h. The primary antibodies were diluted as required with 0.1 M PBS containing 5% normal serum and 0.3% Triton X-100, and the secondary antibodies were diluted as required with 0.1 M PBS containing 5% normal serum and 0.1% Triton X-100.

Single immunohistochemical staining was performed by the avidin-biotin complex (ABC) method. The sections were incubated overnight at 4°C with the primary antibodies. They were then washed in 0.1 M PBS and incubated for 1 h with biotinylated anti-mouse IgG (1:200; Vector Laboratories). After washing with 0.1 M PBS, the immunoreactive products were visualized using a Vectastain Elite ABC kit (Vector Laboratories) and dianamobenzidin.

For double immunohistochemical staining, MyoD, p21, and Rb proteins were detected by using a Vectastain Elite ABC kit (Vector Laboratories) and dianamobenzidin. To visualize the plasma membrane of myofibers, dystrophin antibody was applied to the immunostained sections, and immunoreactive products were visualized by the ABC method by using a Vector VIP substrate kit (Vector Laboratories). By this method, the immunoreactive product of MyoD, p21, and Rb proteins were brown in color, whereas dystrophin-positive immunoreactivities were purples.

For triple immunofluorescence staining, the sections were simultaneously incubated for 16–48 h at 4°C with the primary antibodies. They were washed in 0.1 M PBS and then simultaneously incubated overnight at 4°C with secondary antibodies. The secondary antibodies used were fluorescein-labeled horse anti-mouse IgG (1:300; Vector Laboratories), rhodamine-labeled goat anti-rabbit IgG (1:300; Chemicon, Temecula, CA), or the Alexa Fluor 568-labeled donkey anti-goat IgG (1:300; Molecular Probes, Eugene, OR). The sections were washed in 0.1 M PBS and mounted in Vectashield mounting medium with DAPI (Vector Laboratories) to visualize the nuclei.

Quantitative analysis. A quantitative analysis of myofibers showing MyoD-, p21-, or Rb-positive immunoreactivity in the myonuclei was performed by using the method described elsewhere (19, 60). The percentage of myofibers showing positive immunoreactivity in the myonuclei was examined on each day postdenervation, and all myofibers in a cross section were analyzed. All myofibers possessing MyoD-, p21-, and Rb-positive myonuclei contained on a cross section were counted and represented as a percentage of the total myofiber number directly counted on the same cross section. Based on the results of immunohistochemical staining with fast-type myosin heavy chain (fMHC) and slow-type myosin heavy chain (sMHC) antibodies, all myofibers contained in a cross section were classified into three myofiber types: fast myofibers (fMHC positive/sMHC negative), slow myofibers (fMHC negative/sMHC positive), and hybrid myofibers (fMHC positive/sMHC positive). Using an adjacent serial cross section, which aided in clarifying the myofiber type, all myofibers belonging to a specific type showing MyoD-, p21-, or Rb-positive immunoreactivity contained in a cross section were counted and represented as a percentage of the total myofiber number directly counted on the same cross section.

Statistical analysis. All data are represented as means ± SD and were analyzed by using the StatView statistical analysis program (SAS institute, Cary, NC). Differences among the groups in which the
expression of MyoD (*days 1, 3, 5, and 7* postdenervation), p21 (*days 3, 5, and 7* postdenervation), and Rb (*inner, days 1, 3, 5, and 7* postdenervation) was detected in the myonuclei were tested by one-way ANOVA by using Scheffe’s S test. Also, differences in the percentages of distinct myofiber types expressing MyoD, p21, and Rb were estimated by one-way ANOVA by using Scheffe’s S test. Differences between denervated and innervated muscles were not tested, because the expression of MyoD and p21 was not detected in the myonuclei of innervated muscles. Differences were considered to be significant at the 0.05 confidence level.

RESULTS

One type of physiological alteration that occurs in skeletal muscles by denervation is severe muscle atrophy (6, 21). Therefore, we observed the time course changes in the plantaris muscle weight during denervation periods. After denervation, the relative weight of the plantaris muscle gradually declined and showed a significant decreased value compared with both innervated muscles and *day 1* postdenervated muscles on *day 7* postdenervation (*P < 0.05*) (Fig. 1). These observations were similar to previous studies (6, 21), indicating that denervation by the surgical procedure used in this study resulted in muscles lacking neural activity. Numerous studies have used denervation to induce MyoD expression in adult skeletal muscle (18, 49, 52). In addition to MyoD, we focused on p21 and Rb, which are involved in myogenesis, cell cycle arrest, and anti-apoptosis, and examined whether their expression is induced in myonuclei and/or satellite cell nuclei in response to denervation. Because, in our primary experiments, the greatest increase in the expression of MyoD, p21, and Rb was observed on *day 3* postdenervation, we identified the cell types that expressed these factors by using plantaris muscle sections prepared from *day 3* postdenervation.

**MyoD is expressed in both myonuclei and satellite cell nuclei in response to denervation.** To confirm whether MyoD expression was induced in adult skeletal muscle in response to denervation, we performed immunostaining by using MyoD antibody and DAPI on *day 3* on postdenervated muscles. As shown in Fig. 2, A–C, MyoD-positive immunoreactivity was detected in many DAPI-positive nuclei. We, therefore, further examined whether MyoD expression was induced in myonuclei by double immunohistochemical staining using MyoD and dystrophin antibodies. MyoD-positive immunoreactivity was detected in nuclei located inside dystrophin-positive plasma membranes, indicating that MyoD was expressed in myonuclei in response to denervation (Fig. 2D).

To clarify whether MyoD expression was induced in satellite cells in denervated muscle, we performed triple immunostaining using MyoD, M-cadherin antibodies, and DAPI (Fig. 2, E–H). MyoD expression was detected in the nuclei of M-cadherin-positive satellite cells. MyoD-positive satellite cells were observed on *days 1, 5,* and *7* postdenervated muscle but were not detected in innervated muscles (Table 1). These results show that denervation induced MyoD expression in myonuclei and satellite cell nuclei.

**Denervation induces the expression of p21 and Rb in myonuclei but not in satellite cells.** p21 blocks cell cycle progression by forming ternary complexes with various cyclin-cdk complexes (61). In addition, previous studies showed that p21 is expressed in differentiating myoblasts and that MyoD directly induces the transactivation of the p21 gene, thereby promoting the cell cycle withdrawal of myoblasts during myogenesis (14, 15). Because the induction of MyoD was detected in myonuclei and satellite cell nuclei in denervated muscle, we next examined cell types expressing p21 by immunostaining using p21 antibody and DAPI in *day 3* postdenervated muscles. The p21-positive immunoreactivity was observed in DAPI-positive nuclei (Fig. 3, A–C), and triple immunostaining with p21 and dystrophin antibodies and DAPI showed that p21-positive immunoreactivity occurred in nuclei located inside dystrophin-positive plasma membranes (Fig. 3, D–G). These results showed that p21 was expressed in myonuclei in response to denervation. However, p21 expression was not observed in innervated muscle and in *day 1* postdenervated muscle. On the other hand, unlike that for MyoD, triple immunostaining with p21 and M-cadherin antibodies and DAPI showed that p21-positive immunoreactivity was not present in nuclei of M-cadherin-positive satellite cells (Fig. 3, H–K), indicating that p21 was expressed only in myonuclei in response to denervation.

Dephosphorylated Rb negatively regulates members of the E2F family, resulting in permanent cell cycle withdrawal. Furthermore, Mal et al. (29) suggested that Rb functions downstream of p21 and that the activities of these two proteins may be functionally linked. Therefore, we examined Rb expression in denervated muscle. The Rb antibody used in this study was a monoclonal antibody, which recognizes the phosphorylated and dephosphorylated forms of Rb. After immunostaining using Rb antibody and DAPI, Rb expression was observed in DAPI-positive nuclei in *day 3* postdenervated muscles (Fig. 4, A–C). We, therefore, examined whether Rb was expressed in myonuclei and/or satellite cell nuclei. Double immunostaining using Rb and dystrophin antibodies and DAPI showed that Rb-positive immunoreactivity was present in nuclei located inside dystrophin-positive plasma membranes, indicating that Rb was expressed in myonuclei (Fig. 4D). Rb-positive myonuclei also were detected in innervated muscle (Table 1). Triple immunostaining with Rb and M-cadherin antibodies and DAPI showed that nuclei expressing Rb were not present in M-cadherin-positive satellite cells (Fig. 4, E–H). These results showed that, in response to denervation, Rb expression was exclusively induced in myonuclei.

Fig. 1. Changes in relative plantaris muscle weight during denervation. Relative plantaris muscle weights are represented as the plantaris muscle wet weight per 100 g body wt. Values indicate the means ± SD. *Significantly different compared with innervated plantaris muscle (inner), P < 0.05.* #Significantly different compared with *day 1* denervated plantaris muscle, *P < 0.05.*
Expression patterns of MyoD, p21, and Rb in the myonucleus. During denervation, MyoD, p21, and Rb expression was not observed in the nuclei of undefined cells localized in the interstitial space between myofibers (data not shown), indicating that they are the only myonuclei able to induce the expression of MyoD, p21, and Rb. Therefore, we examined whether MyoD, p21, and Rb are coexpressed in the myonucleus in day 3 postdenervated muscle. Triple immunostaining with MyoD and p21 antibodies and DAPI showed that MyoD expression was present in the p21-positive myonucleus (Fig. 5, A–D), and p21 immunoreactivity was observed in the Rb-positive myonucleus by triple immunostaining using p21 and Rb antibodies and DAPI (Fig. 5, E–H).

Furthermore, we examined whether the expression of MyoD, p21, and Rb was induced in the myonucleus in response to denervation. The length of an adult rat myonucleus is 11–15 μm (57). If serial sections are cut at a thickness of 5 μm, a myonucleus can be divided twice. Therefore, we prepared 5-μm-thickness serial cross sections, according to a previous study (22). One was produced by using triple immunostaining with MyoD and p21 antibodies and DAPI, and the adjacent serial section was produced by triple immunostaining with p21 and Rb antibodies and DAPI.

Table 1. Percentage of myofibers expressing MyoD, p21, or Rb proteins in myonuclei during denervation

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<tr>
<th>Innervated muscles</th>
<th>Days After Denervation</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>MyoD</td>
<td>ND</td>
</tr>
<tr>
<td>p21</td>
<td>ND</td>
</tr>
<tr>
<td>Rb</td>
<td>13.41±5.87</td>
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</table>

Values are means ± SD. ND, not detected. For MyoD and p21, there was no significant difference among 1, 3, 5 and 7 days postdenervation. For retinoblastoma protein (Rb) there was a significant difference compared with innervated muscle (*P < 0.05).
and Rb antibodies and DAPI. The results showed that coexpression of three proteins was present in the myonucleus of day 3 postdenervated muscle (Fig. 5, I–L). These observations indicated the existence of myonuclei expressing MyoD, p21, and Rb in denervated skeletal muscle.

Time course changes and myofiber-type specificity in MyoD, p21, and Rb expression. Because the expression of MyoD, p21, and Rb were induced in myonuclei in response to denervation, we observed the time course changes of these proteins (Table 1). The percentage of myofibers expressing MyoD in the myonuclei was increased on day 1 postdenervation, and this heightened expression was maintained until day 7 postdenervation. The expression of p21 in myonuclei was not detected in innervated and day 1 postdenervated muscle, but the percentage of myofibers exhibiting p21-positive immunoreactivity in the myonuclei was elevated in day 3 postdenervated muscle. The elevated immunoreactivity was maintained until day 7 postdenervation. Unlike MyoD and p21, myofibers showing
Rb expression in the myonuclei were observed in innervated muscle. However, denervation gradually increased the percentage of myofibers with Rb-positive myonuclei from day 1 postdenervation, and significantly elevated expression compared with innervated and day 1 postdenervated muscle was observed on day 3 postdenervation. The elevated expressions of Rb were maintained until day 7 postdenervation. We examined whether denervation-induced MyoD, p21, and Rb expressions in myonuclei occurred in a manner dependent on the myofiber type (Table 2). Myofiber types were distinguished as fast, hybrid, and slow types, as described in MATERIALS AND METHODS. Throughout denervation, significant differences among the types were not found for the expression of MyoD and p21 (Table 2). Also, significant differences in Rb expressions could not be detected among the three types during denervation, but significant denervation-induced upregulation of Rb compared with innervated muscles was seen (Table 2). These results showed that the expression of MyoD, p21, and Rb in myonuclei was induced irrespective of myofiber type in response to denervation.

DISCUSSION

MyoD is rapidly induced in denervated skeletal muscle (18, 49, 52). One possible role of MyoD expressed in denervated muscle is in preventing muscle atrophy (49, 52). MyoD in vitro directly induces the transactivation of the p21 gene, and p21 regulates Rb function. The present study characterized cell types expressing MyoD, p21, and Rb and the relationship among these factors in the myonucleus and quantitatively examined the expression of MyoD, p21, and Rb in distinct myofiber types in denervated skeletal muscle. Our observations provide an important molecular basis of the changes that occurred in myofibers and satellite cells in response to denervation.

The relationships among MyoD, p21, and Rb in myonuclei. The present study indicated that MyoD expression occurs in myonuclei and satellite cell nuclei on day 1 postdenervation. Recently, Hyatt et al. (18) reported on MyoD expression in myofibers and satellite cells in denervated muscle. Furthermore, our findings are consistent with previous studies, which showed that MyoD mRNA rapidly accumulates in skeletal muscle earlier (within ~48 h) after denervation (49, 52). However, in these studies, the downstream targets of MyoD were not investigated. MyoD is an upstream regulator of p21 (14, 15, 33), and Rb functions as a downstream effector of p21 (14, 29, 35, 54, 61). We determined that MyoD and p21, as well as p21 and Rb, were coexpressed in the myonuclei of denervated muscle. Moreover, the coexpression of MyoD, p21,
and Rb was also found to be induced in the myonucleus in response to denervation. These results suggest the possibility that the sequential MyoD/p21/Rb pathway may be activated in the myonuclei of adult skeletal muscle in response to denervation. However, the expression of MyoD in the present study was detected in myonuclei on day 1 postdenervation, whereas p21 expression was first detected on day 3 postdenervation. This time disagreement may be attributed to the delay originating from the transactivation of the p21 gene by MyoD and its translation into protein. In addition, the percentage of myofibers possessing Rb-positive myonuclei on day 1 postdenervation was not significantly higher compared with that of innervated muscle. This is supported by our observations that the expression of p21, which functions as a regulator of Rb, was not detected on day 1 postdenervation. On day 3 postdenervation, the percentage of myofibers possessing Rb-positive

![Fig. 5. Photomicrographs showing the relationships among MyoD, p21, and Rb in a myonucleus in day 3 postdenervated muscles. Triple immunostaining was performed to visualize the localization of MyoD (A), p21 (B), and nuclei (C), and the 3 images were merged (D). The arrows in A–D indicate a DAPI-positive nucleus expressing both MyoD and p21. Triple immunostaining was used to visualize the localization of Rb (E), p21 (F), and nuclei (G), and the 3 images were merged (H). The arrows in E–H indicate a DAPI-positive nucleus expressing both p21 and Rb. The expression of MyoD (J), p21 (K), and Rb (L) in a DAPI-positive myonucleus (I) was examined by using adjacent serial sections prepared with a 5-μm thickness. The arrows in I–L indicate a DAPI-positive nucleus showing MyoD-, p21-, and Rb-positive immunoreactivity. Bar = 30 μm.](image-url)
myonuclei was significantly elevated in parallel with the increase in the percentage of myofibers expressing p21 in their myonuclei. These findings suggest that the MyoD-mediated interaction between p21 and Rb may be activated in the myonuclei in denervated skeletal muscle.

**Possible roles of MyoD, p21, and Rb expressed in myonuclei.** MyoD, together with other members of the MyoD family, composed of myogenin, myf5, and MRF4, have a role in myogenesis in vivo and in vitro (22, 27, 38, 60). Several studies indicate that MyoD is expressed in the myonuclei in adult myofibers in response to stimulation, such as hypertrophy, injury, and exercise (19, 22, 27, 60). However, the function of MyoD expressed in myonuclei has not been elucidated. Several studies suggest that a possible function of MyoD expressed in denervated skeletal muscle is to prevent muscle atrophy that rapidly results from denervation (49, 52). We believe that this is possible, as it was suggested that MyoD in skeletal muscles may be contributing to the promotion of myofiber hypertrophy and regeneration (19, 22, 27, 60). However, not only did the present study show that denervation induces MyoD expression in myonuclei, but also the coexpression of MyoD, p21, and Rb was detected. Therefore, it suggests that the possible function of denervation-induced MyoD in myonuclei may be exhibited through interaction between p21 and Rb, which plays a role in regulating the permanent cell cycle withdrawal and differentiation in myoblasts (31, 51, 58, 63, 64). However, they may not be operated as cell cycle regulators in denervated myofibers, because myofibers that irreversibly exit the cell cycle at once cannot undergo mitosis (17, 32, 44, 45).

p21 and Rb have also been considered as effectors of anti-apoptosis (also called programmed cell death) (51, 55, 63, 64). For example, during myogenesis, the rate of apoptosis in mice myofibers lacking p21 or Rb genes is elevated before differentiation (51, 55, 63, 64). Moreover, when Rb is dephosphorylated by p21 and the dephosphorylated Rb-inhibited activation of E2F-1, which are involved in the induction of apoptosis (53, 56), apoptosis in myofibers was inhibited. These observations suggested that protection against apoptosis is regulated by the interaction between p21 and Rb. A number of studies reported that apoptosis can be induced in the skeletal muscles by several neuromuscular disorders (e.g., disease, denervation, and unloading), which suggests that apoptosis may contribute to myofiber atrophy and the loss of myonuclei (1, 2, 39, 46, 47, 62). It is unclear whether several events related to apoptosis are induced in skeletal muscle within 1 wk after denervation. However, there is evidence showing that the events related to apoptosis occur in skeletal muscles 2 wk after denervation (62) or 14 days after hindlimb unweighting (1). Although the critical functions attributed to interaction among MyoD, p21, and Rb in myonuclei may be involved in inhibiting apoptosis through, perhaps, the activation of p21 and Rb function rather than prevention of muscle atrophy in denervated skeletal muscle.

It has been suggested that fast myofibers rather than slow ones are highly sensitive to denervation and that the atrophy of myofibers is rapidly induced in fast myofibers (4, 8, 28, 52). We observed atrophy in fast myofiber compared with hybrid and slow myofiber on day 7 postdenervation (data not shown).

### Table 2. Myofiber type specificity for MyoD, p21, and Rb expression during denervation

<table>
<thead>
<tr>
<th>Protein</th>
<th>Myofiber Type</th>
<th>Innervated Muscles</th>
<th>Days After Denervation</th>
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<tbody>
<tr>
<td>MyoD</td>
<td>Fast</td>
<td>ND</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29.02±1.10</td>
<td>37.39±1.13</td>
</tr>
<tr>
<td>Hybrid</td>
<td>ND</td>
<td>33.64±4.60</td>
<td>27.99±2.01</td>
</tr>
<tr>
<td>Slow</td>
<td>ND</td>
<td>32.45±1.91</td>
<td>27.11±3.21</td>
</tr>
<tr>
<td>p21</td>
<td>Fast</td>
<td>ND</td>
<td>23.56±2.12</td>
</tr>
<tr>
<td>Hybrid</td>
<td>ND</td>
<td>23.23±4.12</td>
<td>20.61±5.82</td>
</tr>
<tr>
<td>Slow</td>
<td>ND</td>
<td>31.71±1.10</td>
<td>25.13±1.82</td>
</tr>
<tr>
<td>Rb</td>
<td>Fast</td>
<td>9.69±0.46</td>
<td>29.48±4.59*</td>
</tr>
<tr>
<td>Hybrid</td>
<td>5.46±1.71</td>
<td>24.85±4.67*</td>
<td>27.41±5.61*</td>
</tr>
<tr>
<td>Slow</td>
<td>2.51±0.61</td>
<td>28.58±2.70*</td>
<td>31.41±2.87*</td>
</tr>
</tbody>
</table>

Values are percentages and represent means ± SD. The definition of myofiber type and calculation methods are described in MATERIALS AND METHODS. For MyoD, p21, and Rb, there was no significant difference among specified myofiber types at 1, 3, 5, and 7 days postdenervation. *There was a significant difference compared with innervated muscle (P < 0.05).
Denervation transiently induces the activation and proliferation of satellite cells (30, 42, 48), and MyoD is expressed in activated and proliferating myoblasts (41). One function of MyoD is to transactivate target genes inhibited by the cyclin and cdk complex-mediated phosphorylation of MyoD in proliferating myoblasts (23, 36, 40, 43). In addition, the level of Id-1 transcripts, which bind to the DNA binding domain of MyoD, is elevated in denervated muscles (3, 13). Furthermore, Id-1 expression is upregulated in proliferating myoblasts and may inhibit the MyoD-mediated transactivation of target genes. These findings suggest that the function of MyoD expressed in satellite cell nuclei in response to denervation may be mediated by negative inhibitors, such as cyclin-cdk complex or Id-1 protein, and, as a consequence, p21 expression may be suppressed in satellite cells.

In conclusion, the present study showed that, during the first week postdenervation, MyoD, p21, and Rb are expressed in myonuclei, whereas satellite cells induce only MyoD expression in response to denervation. During the early stages of denervation, protection against apoptotic cell death and the proliferation of myoblasts in myofibers, which have an absence of neural activity, are important events in preventing muscle atrophy through the maintenance of myonuclear numbers. Therefore, we propose that a possible function of MyoD expressed in myonuclei may contribute to the protection of denervation-induced apoptotic cell death via perhaps the activation of p21 and Rb, and MyoD expressed in satellite cell nuclei may be maintained in an inactive state to initiate myoblast proliferation. Finally, in response to denervation, MyoD function may be controlled by different regulatory mechanisms in the myonuclei and satellite cell nuclei of adult skeletal muscle.

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REFERENCES


