Age-associated decrease in muscle precursor cell differentiation

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Age-associated decrease in muscle precursor cell differentiation. Am J Physiol Cell Physiol 290: C609–C615, 2006. First published September 28, 2005; doi:10.1152/ajpcell.00408.2005.—Muscle precursor cells (MPCs) are required for the regrowth, regeneration, and/or hypertrophy of skeletal muscle, which are deficient in sarcopenia. In the present investigation, we have addressed the issue of age-associated changes in MPC differentiation. MPCs, including satellite cells, were isolated from both young and old rat skeletal muscle with a high degree of myogenic purity (>90% MyoD and desmin positive). MPCs isolated from skeletal muscle of 32-mo-old rats exhibited decreased differentiation into myotubes and demonstrated decreased myosin heavy chain (MHC) and muscle creatine kinase (CK-M) expression compared with MPCs isolated from 3-mo-old rats. p27Kip1 is a cyclin-dependent kinase inhibitor that has been shown to enhance muscle differentiation in culture. Herein we describe our finding that p27Kip1 protein was lower in differentiating MPCs from skeletal muscle of 32-mo-old rats than in 3-mo-old rat skeletal muscle. Although MHC and CK-M expression were ~50% lower in differentiating MPCs isolated from 32-mo-old rats, MyoD protein content was not different and myogenin protein concentration was twofold higher. These data suggest that there are inherent differences in cell signaling during the transition from cell cycle arrest to the formation of myotubes in MPCs isolated from sarcopenic muscle. Furthermore, there is an age-associated decrease in muscle-specific protein expression in differentiating MPCs despite normal MyoD and elevated myogenin levels.

Sarcopenia is an age-related loss of muscle mass and strength. This loss of muscle mass occurs at a rate of ~10% per decade after age 50 yr (21). Furthermore, skeletal muscle’s ability to repair, regrow, and/or hypertrophy diminishes or is absent in old age (4, 9, 33).

Skeletal muscle contains muscle precursor cells (MPCs) that exist as satellite cells wedged between the plasmalemma and the basal lamina and a stemlike cell population located outside the basal lamina (27). MPCs are required for growth (37), repair or regeneration (36), and hypertrophy (1) to maintain a constant myonuclear domain (2).

The mechanisms responsible for sarcopenia and dysfunctional repair, regrowth, and hypertrophy of aged skeletal muscle are largely unknown. Because MPCs are required for maintenance, repair, regrowth, and hypertrophy of skeletal muscle, however, it is logical to investigate MPCs in aged skeletal muscle. It has been reported that MPCs isolated from aged skeletal muscle exhibit impaired activation (6, 13) and proliferation (6, 24) compared with MPCs isolated from young skeletal muscle. However, some discrepancies in the literature have been published regarding the myogenic potential of MPCs isolated from aged skeletal muscle. Although MPCs isolated from aged skeletal muscle exhibited no apparent difference in myotube formation in one study (13), other investigators have reported decreased differentiation (12, 22). It is important to note that prior researchers who investigated differentiation of MPCs isolated from aged skeletal muscle did not report the expression of muscle-specific proteins.

The expression of cyclin-dependent kinase (cdk) inhibitors induces MPC cell cycle withdrawal. Furthermore, the expression of cdk inhibitors allows for MyoD stabilization and/or accumulation and myogenin expression (34). The Cip/Kip family of cdk inhibitors p1/Cip/Waf1, p27Kip1, and p57Kip2 are all upregulated coincidentally with myogenesis (16, 43, 44). In addition, ectopic expression of p27Kip1 was shown to enhance myoblast differentiation in culture (43). More recently, inhibition of p27Kip1 expression was found to attenuate the differentiation of C2C12 myoblasts, whereas overexpression of p27Kip1 induced differentiation of C2C12 cells cultured at low density, a condition that prohibits differentiation (29). The expression of p27Kip1 is regulated by the Forkhead box class O transcription factor (FOXO)1 (28). Interestingly, Bois and Grosveld (8) found that differentiation induced by serum starvation in mouse primary MPCs resulted in increased FOXO1 expression. Furthermore, these investigators reported that expression of a transcriptionally active FOXO1 mutant augmented primary MPC fusion during 48 h of differentiation and that a dominant-negative form of FOXO1 completely impaired the formation of myotubes.

The myogenic regulatory factors (MRFs) MyoD, Myf5, myogenin, and MRF4 (Myf6) have a well-defined role in myogenesis. Despite a vast amount of research on the role of MRFs in differentiation, limited data have been produced regarding the expression of MRFs in either aged skeletal muscle or MPCs isolated from aged skeletal muscle. mRNA levels for MyoD, myogenin, and Myf5 have been shown to be elevated in aged compared with young skeletal muscle (23, 26, 31). Also, immunohistochemical analysis of aged skeletal muscle revealed accumulated MyoD and myogenin in the nuclei of satellite cells (15). To our knowledge, no data quantifying the expression of MRF protein levels in differentiating MPCs isolated from aged skeletal muscle have been published to date.

The purposes of the present investigation were to test the following two hypotheses. 1) Differentiating MPCs isolated from 32-mo-old rats have decreased expression of myosin heavy chain (MHC) and creatine kinase muscle (CK-M) pro-
teins, which are markers of myogenic status, compared with MPCs isolated from young rats. In the present study, we have demonstrated the existence of decreased MHC and CK-M proteins in 32-mo-old MPCs. 2) Differentiating MPCs isolated from 32-mo-old rats express lower FOXO1 and p27Kip1 protein while expressing paradoxically high levels of MyoD and myogenin compared with MPCs isolated from 3-mo-old rats.

**MATERIALS AND METHODS**

**Animals.** All experimental animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Missouri-Columbia. Fischer 344 × Brown Norway F1 hybrid male rats (3 mo old, n = 4; 32 mo old, n = 5) were obtained from the National Institute on Aging. These two age groups were used because the purpose of the present study was to compare growing vs. sarcopenic muscle. Animals were housed at 21°C, maintained on a 12:12-h light-dark cycle, and allowed free access to food and water. They were killed by intraperitoneal injection of ketamine (80 mg·kg⁻¹), xylazine (10 mg·kg⁻¹), and acepromazine (4 mg·kg⁻¹).

**MPC isolation and cell culture.** MPC isolation was performed according to the method of Allen et al. (3) with some modifications. Briefly, cells isolated by pronase digestion were preplated first for 2 h and then for 24 h on tissue culture-treated 150-mm-diameter plates. After 24-h preplating, cells were seeded onto Matrigel (0.2 mg/ml; BD Biosciences, San Jose, CA)-coated, 150-mm-diameter plates for 60 min at 37°C and cultured for 3–4 days in growth medium (GM) composed of 20% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 40 µg/ml gentamicin in Ham’s F-10 medium until cells reached ~80% confluence. Cells were then passaged once and seeded onto appropriate Matrigel tissue culture plates. For MPC differentiation, cells were seeded in two different conditions. First, cells were seeded at high density (HD; 2 × 10⁵ cells/35-mm well), allowed to adhere overnight in GM, and then switched to differentiation medium (DM) composed of 2% horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 40 µg/ml gentamicin in DMEM, which was changed after 24 h. The HD method was used to minimize any potential effect of different rates of proliferation in our samples (3- vs. 32-mo-old rats) on differentiation. Second, cells were seeded at low density (LD; 3 × 10⁴ cells/100-mm plate), allowed to proliferate for 2 days in GM (cells proliferated to ~80% confluence), and then switched to DM, which was changed after 24 h (Fig. 1). The LD method was used because MPCs are commonly induced to differentiate in this manner. During the passage, cells were preplated for 20 min on tissue culture-treated 150-mm-diameter plates before being seeded onto Matrigel-coated plates.

**Sample preparation.** A high ionic strength buffer must be used to solubilize the MHC. After 2 days in DM, cells were lysed with Cell Lytic lysis buffer (Sigma-Aldrich, St. Louis, MO) containing 1.04 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 800 nM aprotinin, 20 µM leupeptin, 40 µM bestatin, 15 µM peptstatin A, and 14 µM E-64 (Sigma-Aldrich). Cell lysates were vortexed, and an aliquot was removed and added (1:1 dilution) to a 600 mM KCl solution and then stored at −80°C for MHC analysis. The remaining cell lysate was centrifuged at 12,000 g for 15 min at 4°C, and then the supernatant was removed and stored at −80°C for further analysis. MHC samples were prepared for Western blot analysis by being thawed and allowed to sit on ice for 30 min before being centrifuged at 12,000 g for 15 min at 4°C. The supernatant was collected, protein concentration was determined using the Bradford assay method, and samples were diluted 1:10 in SDS reducing buffer.

**Immunocytochemistry.** For MyoD and desmin staining, 10,000 cells were seeded onto Matrigel-coated, two-well Permanox Lab-Tek chamber slides (Nalg Nunc International, Rochester, NY). For MHC staining of myotubes, 40,000 cells were seeded onto Matrigel-coated, two-well Permanox chamber slides, allowed to proliferate for 2 days in GM, and then switched to DM for 2 days. Cells to be analyzed for MyoD (MoAb 5.8A; BD Biosciences) were fixed with 4% paraformaldehyde, and cells to be analyzed for desmin (D3; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and MHC (MF20; Developmental Studies Hybridoma Bank) were fixed with 100% ice-cold methanol. Alexa Fluor 488 anti-mouse secondary antibody (Molecular Probes, Carlsbad, CA) was used for all immunocytochemical experiments. Nuclei were counterstained using 4',6-diamidino-2-phenylindole (Molecular Probes).

**Western blot analysis.** Equal amounts of protein were loaded and separated using SDS-PAGE and transferred onto nitrocellulose membranes (Osmotics). Nitrocellulose membranes were stained with Ponceau S (Sigma-Aldrich) to ensure equal loading. We were able to detect Ponceau S-stained protein bands on the nitrocellulose membranes that exhibited no treatment differences between groups. MHC and myogenin (FD5) antibodies were purchased from the Develop-
mental Studies Hybridoma Bank, CK-M (C-14) and MyoD (M-318) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), FOXO1 and phospho-FOXO1 (Ser256) antibodies were purchased from Cell Signaling Technology (Beverly, MA), p27Kip1 antibody was purchased from Upstate (Lake Placid, NY), horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG antibodies were purchased from Amersham Biosciences (Little Chalfont, UK), and HRP-conjugated anti-goat IgG antibody was purchased from R&D Systems (Minneapolis, MN). Immunocomplexes were visualized using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Boston, MA) and exposed to Hyperfilm (Amersham Biosciences), with the exposure time adjusted to keep the integrated optical densities within a linear and nonsaturated range. The signal bands were scanned using a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) and quantified using ImageQuant software (Molecular Dynamics).

**Statistics**: Western blot analysis was performed on samples from 3- and 32-mo-old rats, and differences were compared using the unpaired t-test with SigmaStat software, version 3.1.

**RESULTS**

Animal body mass and absolute and relative muscle mass data for tissues used to isolate MPCs are presented in Table 1. Relative muscle mass was significantly lower in 32-mo-old rats for all muscles studied and ranged from 51% of values for lateral gastrocnemius muscle to 62% for soleus and plantaris muscles from 3-mo-old rats.

MPCs were immunostained for both MyoD and desmin after passage (Fig. 2). MPCs isolated from both 3- and 32-mo-old rats exhibited a high purity level of MyoD and desmin (>90% positive). Representative myotubes formed by MPCs isolated from 3-mo-old and 32-mo-old animals are shown in Fig. 3. After 48 h in DM, myotubes were immunostained for MHC (Fig. 3, A and B) and light microscopic images were captured (Fig. 3, C and D).

MHC expression in the HD samples was ~50% less in myotubes formed by MPCs isolated from 32-mo-old compared with 3-mo-old rats (Fig. 4A) when presented as either total MHC content per well or MHC content normalized to total protein per well. We hypothesized that the MPCs isolated from 3-mo-old rats would differentiate more robustly, thereby increasing the amount of contractile protein. Indeed, differentiating MPCs isolated from 3-mo-old animals tended to contain more insoluble protein (which contains the MHC) than those isolated from 32-mo animals (19.4%) (P = 0.056) (Fig. 4B). Therefore, we think that because insoluble protein tended to be

<table>
<thead>
<tr>
<th>Muscle Type</th>
<th>3-Mo-Old Rats</th>
<th>32-Mo-Old Rats</th>
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<tbody>
<tr>
<td>Soleus</td>
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<tr>
<td>Absolute, g</td>
<td>0.134±0.010</td>
<td>0.149±0.003</td>
</tr>
<tr>
<td>Relative, mg/g</td>
<td>0.456±0.011</td>
<td>0.280±0.012*</td>
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<tr>
<td>Plantaris</td>
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<tr>
<td>Absolute, g</td>
<td>0.318±0.019</td>
<td>0.351±0.011</td>
</tr>
<tr>
<td>Relative, mg/g</td>
<td>1.087±0.010</td>
<td>0.662±0.041*</td>
</tr>
<tr>
<td>Medial gastrocnemius</td>
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<tr>
<td>Absolute, g</td>
<td>0.782±0.037</td>
<td>0.742±0.021</td>
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<tr>
<td>Relative, mg/g</td>
<td>2.678±0.031</td>
<td>1.398±0.080*</td>
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<tr>
<td>Lateral gastrocnemius</td>
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<tr>
<td>Absolute, g</td>
<td>0.910±0.065</td>
<td>0.846±0.037</td>
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<tr>
<td>Relative, mg/g</td>
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<td>1.593±0.105*</td>
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<td>Quadriceps</td>
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<tr>
<td>Absolute, g</td>
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<td>3.065±0.156</td>
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<tr>
<td>Relative, mg/g</td>
<td>9.664±0.066</td>
<td>5.760±0.348*</td>
</tr>
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Values are means ± SE of wet muscle mass (mg)/body mass (g) ratio. For 3-mo-old rats, the mean body mass values are 292.4 ± 16.5 g (n = 4). For 32-mo-old rats, the mean body mass values are 534.3 ± 15.0 g (n = 5), significantly different from 3-mo-old rats. *P ≤ 0.05 vs. 3-mo-old rats.
increased in muscle tissue samples from 3-mo-old rats, the use of total protein (Fig. 4B) to normalize MHC content (Fig. 4A) might not produce data representative of differences between the 3- and 32-mo-old groups. Similar results were found for LD samples (data not shown).

FOXO1, which has been shown to regulate p27Kip1 expression, was measured using Western blot analysis (Fig. 6). Phospho-FOXO1 (Ser256) also was measured, because phosphorylation at Ser256 has been shown to disrupt transactivation by FOXO1 (17). FOXO1 protein expression was 23% lower, and the ratio of FOXO1 to phospho-FOXO1 (Ser256) was 41% lower, in myotubes formed by MPCs isolated from 32-mo-old rats compared with 3-mo-old rats (Fig. 6). These data suggest that transactivation by FOXO1 was lower in myotubes formed by MPCs isolated from 32-mo-old animals than that in 3-mo-old animals. However, it is important to note that FOXO1 and phospho-FOXO1 (Ser256) were measured only in LD samples and were not detected in HD samples. p27Kip1 protein expression in HD samples was 34% lower in myotubes formed by MPCs isolated from 32-mo-old rats than that in 3-mo-old rats (Fig. 7). Similar results for p27Kip1 were found in the LD samples (data not shown).

The MRFs MyoD and myogenin were measured using Western blot analysis (Fig. 8). MyoD protein expression in HD samples of myotubes formed by MPCs isolated from 32-mo-old animals was not different from that of 32-mo-old animals. However, myogenin protein expression in HD samples was twofold higher in myotubes formed by MPCs isolated from 32-mo-old animals than in 3-mo-old animals. Similar results were found in the LD samples (data not shown).
DISCUSSION

The mechanisms responsible for sarcopenia and dysfunctional repair, regrowth, and hypertrophy of aged skeletal muscle are largely unknown. However, the importance of MPCs for maintenance, repair, regrowth, and hypertrophy of skeletal muscle led us to hypothesize that MPCs isolated from aged skeletal muscle would not differentiate as well as MPCs from young skeletal muscle. Indeed, myotubes formed by MPCs isolated from 32-mo-old rats exhibited 50% less MHC and CK-M protein than those of 3-mo-old rats. There are some discrepancies in the literature with regard to the myogenic potential of MPCs isolated from aged skeletal muscle. Chargé et al. (12) and Lorenzon et al. (22) reported that MPCs isolated from aged skeletal muscle produced fewer myotubes than MPCs isolated from young skeletal muscle under differentiation conditions using mouse single-fiber ex vivo satellite cell culture and human primary myoblast culture, respectively. However, Conboy et al. (13) reported that although satellite cells isolated from aged skeletal muscle produced fewer myotubes than MPCs isolated from young skeletal muscle under differentiation conditions using mouse single-fiber ex vivo satellite cell culture and human primary myoblast culture, there was no apparent difference in myotube formation when MPCs were adherence purified, expanded, and plated at equal cell densities. For two reasons, it is somewhat difficult to account for the varied results in the literature. First, it is difficult to compare results from experiments in which different cell culture techniques as well as, in some cases, different species were used. Second, muscle-specific gene expression has not been quantified in any previous studies on age-associated impaired differentiation of MPCs.
Because we were interested in studying inherent differences in the MPC population due to aging, we thought it important to perform the experiments such that the tissue samples spent a minimal amount of time in culture. In other words, our intent was to study a MPC population in culture that best represented the MPC population present in vivo. Previously, we reported that increasing the time in culture to three passages caused a significant shift in the cell population as characterized by the cell markers MyoD, desmin, Pax7, CD34, and CD45 (25). Furthermore, Machida et al. (25) found that increased time in culture resulted in decreased differentiation. Therefore, in the present study, we limited the time in culture to a maximum of 8 days, including a single passage (Fig. 1).

Cell cycle withdrawal is a prerequisite for the expression of muscle-specific genes (20, 41). MPC cell cycle withdrawal is induced by cdk inhibitors, including p27Kip1. Furthermore, ectopic expression of p27Kip1 has been shown to enhance myoblast differentiation (43), whereas inhibition of p27Kip1 expression has been demonstrated to attenuate differentiation (29). In the present study, p27Kip1 expression was approximately one-third lower in MPCs isolated from 32-mo-old animals than it was in 3-mo-old animals in both the LD and HD samples. These data suggest that lower p27Kip1 protein expression could be involved in age-associated impaired differentiation of MPCs.

FOXO1 has been shown to trans-activate p27Kip1 expression (28). Also, phosphorylation of FOXO1 at Ser256 disrupts trans-activation by FOXO1 (17). It is interesting that we were able to detect both FOXO1 and phospho-FOXO1 in only the LD samples. These findings indicate that the density of the cells may be relevant to FOXO signaling during MPC fusion. This conclusion is based on the fact that the LD samples were subconfluent when they were induced to differentiate, whereas the HD samples were confluent when induced to differentiate. Therefore, the age-related differences in p27Kip1 expression during differentiation are not completely explained by FOXO1 expression or by the phosphorylation of FOXO1 at Ser256.

We measured the MRFs MyoD and myogenin because they have a well-defined role in myogenesis (32, 35) and hypothesized that they would be increased in MPCs isolated from old skeletal muscle. The MRFs MyoD, Myf5, myogenin, and Myf6 all share a conserved basic helix-loop-helix domain that enables them to bind DNA E-box sequences (CANNTG) as monomers and dimers or heterodimers with E-proteins. E-box sequences are found in many promoter regions of muscle-specific genes, including the upstream regions of the skeletal muscle MHC type IIb gene (39) and the CK-M gene (38). Furthermore, MyoD and myogenin expression cause contractile protein gene trans-activation (11, 42) and can bind a number of promoter regions associated with cytoskeletal and contractile genes during differentiation of C2C12 myoblasts (7).

In the present study, we found that despite ~50% lower protein expression of MHC and CK-M, MyoD protein was not different and myogenin protein was twofold higher in differentiating MPCs isolated from 32-mo-old rats than that in 3-mo-old rats. It has been reported previously that MyoD, myogenin, and Mrf-5 mRNA levels are elevated in aged compared with young skeletal muscle (23, 26, 31), which Musarò et al. (31) suggested was a compensatory mechanism to maintain muscle products. Alternatively, increased MRF transcription levels might be a result of aging-associated des-
REFERENCES


