Satellite cell content is specifically reduced in type II skeletal muscle fibers in the elderly

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The gradual loss of skeletal muscle mass with aging, or sarcopenia, represents a major factor determining the decline in functional capacity, mobility, and general health in the elderly. Muscle mass represents a main determinant of muscle strength and has been strongly associated with performance in activities of daily living and the level of independence in the elderly (16, 36, 49). Much work has been devoted to describing the alterations in skeletal muscle that occur with aging. The most consistent findings have been the decline in the total number of muscle fibers and the specific atrophy of the type II fibers, both contributing to the loss of skeletal muscle mass (18, 28, 30). In accordance, type II muscle fiber atrophy and necrosis have been shown to result in a reduced (area) proportion of type II muscle fibers in skeletal muscle tissue in the elderly (15, 28, 29).

Although the exact mechanisms that cause sarcopenia remain to be elucidated, the hypothesis that satellite cells could play an important role in this age-related loss of skeletal muscle mass has recently gained much interest. Satellite cells (SC), or “muscle stem cells,” are the sole source for the generation of new myonuclei in vivo in skeletal muscle tissue (2, 34, 35). As such, SC seem to be essential for the repair and hypertrophy of skeletal muscle tissue. Normally, SC lie quiescent between the basal lamina and the plasma membrane of muscle fibers (32). On activation and subsequent proliferation, the majority of these SC differentiate to form new myonuclei that either fuse with existing fibers or generate new fibers, while a small proportion returns to quiescence (24, 50). Because SC are responsible for the maintenance of skeletal muscle mass, a decrease in the number of SC or in their ability to become activated and proliferate in response to anabolic stimuli is likely to result in impaired skeletal muscle structure and function (44). Although the latter has been speculated to occur in sarcopenia, studies investigating the effects of aging on skeletal muscle SC content in humans have been inconclusive. Whereas some studies reported both a reduced number of SC per muscle fiber and a smaller proportion of SC relative to the number of myonuclei in the elderly (22, 38), other studies have failed to observe such differences (15, 20, 40). Although it has been reported that no differences exist in the number of SC between type I and II muscle fibers in young adults (23), it could be speculated that the specific type II muscle fiber atrophy with aging is associated with a fiber type-specific reduction in SC content. Such a fiber type-specific reduction in SC content could represent an important factor in the etiology of sarcopenia and might explain the apparent discrepancy in the literature regarding the effects of aging on muscle fiber SC content.

In the present study, we assessed skeletal muscle fiber type-specific characteristics and fiber type-specific satellite cell content in young and elderly men. Therefore, skeletal muscle biopsy samples were taken from the vastus lateralis in both the left and right legs of eight young (20 ± 1 yr) and eight elderly (76 ± 1 yr) lean males. Immunohistological staining of muscle cross sections was performed to assess muscle fiber type-specific SC content and muscle fiber characteristics.

METHODS

Subjects. Eight elderly (76 ± 1 yr) and eight young (20 ± 1 yr) body weight-matched, male volunteers with no history of participating in any regular exercise program were recruited to participate in the present study. Characteristics of subjects are shown in Table 1. All
subjects were informed on the nature and possible risks of the experimental procedures before their written informed consent was obtained. This study was approved by the Medical Ethics Committee of the Academic Hospital, Maastricht.

Pretesting. The medical histories of subjects were evaluated, and an oral glucose tolerance test was performed at least 2 wk before the start of the trials (1, 31). Subjects with orthopedic and/or cardiovascular abnormalities or type 2 diabetes were excluded from participation. After selection for this study, maximal strength was assessed in two one-repetition maximum (1-RM) strength tests on a leg press and leg extension machine, respectively. First, a familiarization trial was performed. Proper lifting technique was demonstrated and practiced for each of the two lower limb exercises. Thereafter, maximum strength was estimated using the multiple repetitions testing procedure (33). In an additional session, at least 10 days before muscle biopsy collection, each subject’s 1-RM was determined. After warming up, the load was set at 90–95% of the estimated maximum strength and increased after each successful lift until failure. A 3-min resting period between subsequent attempts was allowed. A repetition was valid if the subject was able to complete the entire lift in a controlled manner without assistance.

The weight and height of subjects were measured, and leg volume was determined as described previously (21). In short, leg circumference and height were measured at seven different sites, dividing the leg into six segments. The volume of each segment was calculated by treating each segment as a truncated cone, using the anthropometric measurements for determining the height and the surface areas of each cone. All subjects were instructed to refrain from strenuous physical activity for 3 days before muscle biopsy collection and to maintain their normal habitual dietary intake. On the day before biopsy collection, a standardized meal was provided, and from 9:00 PM, subjects remained fasted.

Muscle biopsies. After an overnight fast, subjects arrived at the laboratory by car or public transportation at 8:00 AM. After 30 min of supine rest, a basal venous blood sample was collected. After local anesthesia, percutaneous needle biopsies (50–80 mg) were taken from the vastus lateralis muscle ~15 cm above the patella in both the left and right legs of each subject (3). Any visible nonmuscle tissue was removed from the biopsy samples, which were then frozen in liquid nitrogen-cooled isopentane, embedded in Tissue-Tek (Sakura Finetek, Zoeterwoude, The Netherlands), and stored at −80°C until further analyses.

Immunohistochemistry. From all biopsies, 5-μm-thick cryosections were cut at −20°C and samples from the left and right leg of each subject were mounted together on uncoated glass slides. Care was taken to properly align the samples for cross-sectional fiber analyses. Slides from elderly and young subjects were stained simultaneously to ensure identical staining efficiency.

An ATPase staining was performed after acid preincubation to determine muscle fiber type as described previously (5, 8, 9). Instead of dehydration of the slides at the end of the ATPase staining procedure (5), sections were stained with anti-laminin (polyclonal rabbit anti-laminin; Sigma, Zwijndrecht, The Netherlands) and thereafter with the appropriate secondary antibody goat anti-rabbit IgG AlexaFluor488 (Molecular Probes, Invitrogen, Breda, The Netherlands). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes), similar to the procedures described below. At a pH of 4.45, we were able to differentiate between black (type I), white (type Ia), and intermediate (type Ila/Ix) fibers (Fig. 1). The assessment of muscle fiber typing at this pH was validated by staining serial muscle cross sections of three young and three elderly subjects with myosin heavy chain (MHC)-I and MHC-Ila antibodies [A4.840 and N2.261, respectively; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA]. Because too few type Ila/Ix fibers were present for reliable SC counts in some of the biopsies, SC content and fiber characteristics were assessed in type I and type II muscle fibers only.

Immunostaining for the detection of myocellular SC content was performed on serial cross sections with both pax7 (mouse anti-pax7, DSHB), which marks both quiescent and activated SC (43, 50), and

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Table 1. Subject characteristics

<table>
<thead>
<tr>
<th>Age, yr</th>
<th>Young (n = 8)</th>
<th>Elderly (n = 8)</th>
</tr>
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<tbody>
<tr>
<td>20 ± 1</td>
<td>76 ± 1*</td>
<td></td>
</tr>
<tr>
<td>Weight, kg</td>
<td>73.7 ± 3.2</td>
<td>75.5 ± 2.1</td>
</tr>
<tr>
<td>Height, m</td>
<td>1.81 ± 0.03</td>
<td>1.71 ± 0.01*</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>22.5 ± 1.1</td>
<td>25.7 ± 0.7*</td>
</tr>
<tr>
<td>Leg volume, liters</td>
<td>9.4 ± 0.4</td>
<td>8.2 ± 0.3*</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>5.0 ± 0.2</td>
<td>5.7 ± 0.1*</td>
</tr>
<tr>
<td>Basal glucose, mmol/l</td>
<td>5.3 ± 0.1</td>
<td>5.4 ± 0.2</td>
</tr>
<tr>
<td>Basal insulin, mIU/l</td>
<td>12.8 ± 1.1</td>
<td>10.2 ± 1.5</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>3.0 ± 0.3</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td>1-RM leg extension, kg</td>
<td>108 ± 4</td>
<td>78 ± 4.3*</td>
</tr>
<tr>
<td>1-RM leg press, kg</td>
<td>204 ± 7</td>
<td>151 ± 7*</td>
</tr>
</tbody>
</table>

Values are means ± SE. BMI, body mass index; HOMA-IR, homeostasis model assessment of insulin resistance (31); 1-RM, 1-repetition maximum.

*Significantly different compared with the young (P < 0.05).

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Fig. 1. Muscle fiber type analyses performed on muscle tissue collected from elderly (A) and young (B) males by ATPase staining of muscle cross sections; type I fibers are black, and type II fibers are white (Ila) or show intermediate staining (Ilb/Ix). Type II fibers are significantly smaller in the elderly vs. the young.

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CD56 (mouse anti-CD56; BD Biosciences, San Jose, CA), which has been extensively used for SC determination in human muscle tissue (10, 15, 22, 23). Because of superior staining efficiency when using pax7, this marker was used for the quantification of SC in the present study. After fixation for 5 min with acetone, slides were air-dried and incubated for 60 min at room temperature with primary antibodies directed against laminin (dilution 1:50) and pax7 (dilution 1:20), diluted in 0.05% Tween-PBS. Slides were then washed for 3 × 5 min in PBS before incubation for 60 min at room temperature with the appropriate secondary antibodies, purchased from Molecular Probes. Goat anti-rabbit IgG-AlexaFluor555 (dilution 1:200) and goat anti-mouse IgG1-AlexaFluor488 (dilution 1:500) were diluted together with DAPI (0.238 μM) in 0.05% Tween-PBS to, respectively, stain laminin in red, pax7 in green, and myonuclei in blue (Fig. 2). After incubation, slides were again washed for 3 × 5 min in PBS and then covered with cover glasses using Mowiol (Calbiochem, Amsterdam, The Netherlands).

Although pax7 is essential for the specification of SC, it has been reported that pax7 expression declines when SC ontogeny proceeds into terminal differentiation (43, 50). As such, the use of pax7 staining as a marker for SC content could theoretically lead to an underestimation of total SC count. Therefore, we validated the use of pax7 by simultaneously staining cross sections of three young and three elderly subjects with pax7, CD56, and DAPI. In agreement with earlier findings (37), >96% of CD56-positive SC were also pax7 positive, with no differences between age groups. In the present study, the term “satellite cells” (SC) refers to the number of SC that stained positive for pax7.

Data analyses. All images were digitally captured, using both light microscopy (ATPase) and fluorescence microscopy (laminin, pax7, and DAPI) with a Nikon E800 combined light/fluorescence microscope (Nikon Instruments Europe, Badhoevedorp, The Netherlands) coupled to a Basler A113 C progressive scan color CCD camera with a Bayer color filter. Epifluorescence signal was recorded using a Texas Red excitation filter (540–580 nm) for laminin, an FITC excitation filter (465–495 nm) for pax7, and a DAPI UV excitation filter (340–380 nm) for the nuclei. Image processing and quantitative analyses were done using Lucia 4.81 software package (Nikon). All image recordings and analyses were performed by an investigator blinded to the subject coding.

From the ATPase-stained slides, images were captured at ×120 magnification. Laminin was used to determine cell borders, and for all fibers within each image, type I (black) and type II (white and intermediate) fibers were identified (Fig. 1). Within each image, the number of fibers, the mean fiber cross-sectional area (CSA), the number of myonuclei per fiber, and the mean fiber area per nucleus (fiber CSA/no. of myonuclei) were measured for the type I and type II fibers separately. As a measure of fiber circularity, form factors were calculated using the formula (4π·CSA)/(perimeter)². Fiber circularity was not different between the muscle cross sections from the elderly vs. the young for any fiber type.

From the pax7-stained slides, images were captured at ×240 magnification to allow clear determination of SC localization. Laminin was used to visualize the cell borders. Fiber typing was determined by matching the serial ATPase-stained cross sections. SC were determined in each fiber between the basal lamina and the plasma membrane and stained positive for both DNA (DAPI) and pax7 (see Fig. 2 for example). The data were used to calculate the number of SC per muscle fiber, the proportion of SC [no. of SC/(no. of SC + no. of...
myonuclei), and the number of SC per fiber area (in mm²) for the type I and type II muscle fibers separately.

Because there were no differences in muscle fiber type composition and CSA of the type I and II fibers between muscle samples taken from the left vs. the right leg, muscle fiber data were pooled from both biopsies in each subject, resulting in a mean number of 401 ± 32 and 507 ± 69 individual muscle fibers being analyzed in the young and elderly subjects, respectively. For SC count, 287 ± 26 and 291 ± 29 fibers were analyzed in the muscle samples collected in the young and elderly subjects, respectively.

Statistics. All values are expressed as means ± SE. Muscle characteristics in muscle biopsies collected from the left and right legs were analyzed using paired t-tests. General differences between elderly and young subjects were evaluated by independent t-tests. In addition, fiber type-specific variables were analyzed using mixed-model analyses with "age group" (young and elderly) as the between-subjects factor and "fiber type" (type I and II) as the within-subjects factor. In the case of significant interaction, between-group differences were analyzed for the different fiber types separately, and within-group differences were analyzed for the young and elderly separately. A P level of 0.05 was used to determine statistical significance. All analyses were performed using SPSS version 11.5 (Chicago, IL).

RESULTS

Subjects. Subject characteristics are provided in Table 1. Basal blood glucose and insulin levels and whole body insulin sensitivity as assessed by the homeostasis model assessment of insulin resistance (HOMA-IR) index (31) did not differ between groups. Blood Hb A1c concentrations were within a normal range for both groups but were significantly greater in the elderly. Muscle strength as assessed by 1-RM for both leg press and leg extension was significantly lower in the elderly (151 ± 7 and 78 ± 4 kg, respectively) vs. the young (204 ± 7 and 108 ± 4 kg, respectively; P < 0.001). The latter was accompanied by a lower leg volume in the elderly compared with the young. In accordance, leg volume was significantly correlated with muscle strength (r = 0.53; P < 0.05).

Fiber characteristics. Fiber type composition was significantly different between groups (Table 2). Type II muscle fiber content was significantly lower in the elderly (47 ± 3%) vs. the young (57 ± 3%; P < 0.05). In addition, the percentage of total muscle area occupied by type II fibers was also significantly smaller in the elderly (42 ± 4%) vs. the young (59 ± 3%; P < 0.01). Type II, but not type I, muscle fiber CSA differed significantly between groups, with mean type II muscle fiber CSA being smaller in the elderly vs. the young (4,451 ± 396 vs. 6,126 ± 393 μm², respectively; P < 0.05) (Table 2, Fig. 1). There was a significant interaction between age group and fiber type CSA (P < 0.05). In the young, fiber CSA did not differ between fiber types. In contrast, in the elderly muscle fiber CSA was significantly smaller in the type II vs. the type I fibers (P < 0.05; Table 2).

Myonuclei. In both the young and elderly, the number of myonuclei per fiber was significantly higher in the type I muscle fibers compared with the type II muscle fibers (Table 3). In accordance, the fiber area per myonucleus was smaller for the type I vs. the type II muscle fibers. The number of myonuclei per muscle fiber differed significantly between groups for both the type I and II muscle fibers (Table 3). More myonuclei were present per type I and II muscle fibers in the elderly compared with the young (type I, 3.5 ± 0.2 vs. 3.0 ± 0.2, and type II, 2.6 ± 0.1 vs. 2.3 ± 0.1, respectively; P < 0.01). For the fiber area per myonucleus, there was significant interaction between age group and fiber type. Separate analysis showed significant differences between groups, with the elderly showing a significantly smaller fiber area per myonucleus in both the type I and II muscle fibers when compared with the young, the latter difference being more pronounced in the type II muscle fibers (Table 3; P < 0.05).

SC. In type I muscle fibers, no differences were observed in SC content, neither as the number of SC per fiber nor as the number of SC per millimeter squared (mm²), between the young and the elderly (Table 3). In addition, SC proportion in type I fibers did not differ between the young and the elderly. In contrast, in the type II muscle fibers, SC content was significantly lower in the elderly vs. the young. The number of SC per type II muscle fiber was significantly reduced in the elderly when compared with the young (0.044 ± 0.003 vs. 0.080 ± 0.007, respectively, P < 0.01; Fig. 3). The latter was also evident when correcting for CSA, as the number of SC per mm² of type II muscle fiber was also significantly reduced in the elderly compared with the young (9.7 ± 1.0 vs. 12.6 ± 0.9, respectively; P < 0.05). In accordance, SC proportion was significantly different between groups, with SC representing 1.5 ± 0.2% of nuclei in the elderly type II muscle fibers compared with 2.9 ± 0.4% in type II muscle fibers of the young (P < 0.01). For all SC variables, there was a significant interaction between age group and fiber type. In the young, no significant differences were observed for any of the SC characteristics between the type I and II muscle fibers. In the elderly, the number of SC per muscle fiber, the number of SC per mm² of muscle fiber, and the proportion of SC were substantially lower in the type II vs. the type I muscle fibers (Table 3 and Fig. 3).

DISCUSSION

In the present study, we confirm that both the proportion and CSA of the type II skeletal muscle fibers are substantially reduced in skeletal muscle tissue in the elderly. As far as we know, this is the first report that shows that type II muscle fiber atrophy in the elderly is associated with a specific reduction in the number of SC in these fibers. The latter is evident when expressed as number of SC per muscle fiber, as proportion of SC relative to the total number of nuclei, or as number of SC per muscle fiber area. This muscle fiber type-specific decline in SC content might represent an important factor in the etiology of sarcopenia.

Numerous studies have demonstrated that the age-related loss of skeletal muscle mass is characterized by a reduction in...
the total number of muscle fibers and specific atrophy of the type II muscle fibers (18, 29, 30, 45). The decline in maximal strength with age has been attributed to this gradual loss of skeletal muscle tissue, but other intrinsic changes, e.g., fiber type shifts and reduced concentrations of contractile material, could also contribute to this phenomenon (6, 12). Our data seem to confirm earlier reports (26, 27, 48) showing a reduced leg volume and muscle strength in the elderly vs. the young (Table 1). Furthermore, at the muscle level, we observed a significantly higher percentage of type I muscle fibers and the specific atrophy of the type II muscle fibers in the elderly (Table 2). These findings are consistent with previous reports (15, 28, 45) and are likely responsible for the reduced muscle strength in the elderly (Table 1).

Even though the changes in muscle fiber type composition and morphology with aging have been described rather consistently in the literature, there is still considerable debate on the underlying mechanisms responsible for these changes. It has been suggested that SC content in muscle plays an important role in the age-related changes in skeletal muscle structure and function (44). However, although this topic has received much attention over the past 10 years (10, 15, 19, 20, 22, 38, 40, 41, 47), the exact changes in the SC pool that occur with aging still remain unresolved. Two recent studies have reported a decline in SC content with aging in the tibialis anterior (22) and in the biceps and masseter muscle (38) in humans. In contrast, other studies failed to observe such differences in either SC proportion or the number of SC in muscle fibers taken from the vastus lateralis muscle in young vs. elderly subjects (15, 20, 40). The apparent discrepancy between these findings might be attributed to differences in study design, e.g., selection of subject age, the number of fibers analyzed, and the specific muscle group studied.

So far, studies investigating age-related differences in SC content in human skeletal muscle have been limited to mixed muscle fiber analyses. In contrast to the fiber type-specific differences in SC content that have been consistently shown in rodent muscle (17, 42, 44), Kadi et al. (23) recently reported no differences in SC content between type I and II muscle fibers in young adults. However, because sarcopenia is associated with the specific atrophy of type II muscle fibers, we hypothesized that fiber type differences in SC content might exist at an older age. Therefore, in the present study, we determined type I and II muscle fiber type-specific SC content in both young and elderly men. In accordance with Kadi et al. (23), we observed ~0.075 SC per muscle fiber, with no differences between the type I and II muscle fibers in the young subjects (Table 3). Extending their findings and consistent with the specific atrophy of type II muscle fibers with aging, we observed a 44 ± 6% lower SC content in the type II vs. type I muscle fibers in the elderly. Furthermore, SC content in the type II muscle fibers in the elderly was significantly lower when compared with type II muscle fiber SC content in the young (Table 3, Fig. 3). Notably, even when correcting for fiber CSA, type II muscle fiber SC content was still significantly lower in the elderly vs. the young (Table 3). These data support the idea that SC content is strongly associated with myofiber maintenance and imply that the age-related reduction in type II muscle fiber SC content might represent an important factor in the specific loss and atrophy of type II muscle fibers with aging.

As stated in METHODS, we used pax7 staining to assess muscle fiber type-specific SC content in skeletal muscle cross sections. It should be noted that a slight proportion of SC may be pax7 negative in vivo, i.e., those SC that are progressing into terminal differentiation (43, 50). Therefore, we cross-validated our data by double staining with anti-CD56 in a subset of muscle samples. The vast majority of SC were both CD56 and pax7 positive, with no differences between the young and elderly (94.9 vs. 96.5%, respectively). As such, the latter could not be responsible for the substantial differences in skeletal muscle fiber SC content between groups.

Findings from several rodent (7, 39, 44) as well as human studies (10, 25, 46) suggest that the abundance of SC within skeletal muscle fibers represents an important factor determining the capacity for myofiber maintenance. On the other hand, type II myofiber atrophy and a reduction in SC content might also be caused by one and the same phenomenon, e.g., decreased neuronal input (13). In addition, Bonavaud et al. (4) recently reported that myotubes formed by the fusion of human SC from either fast or slow muscle fibers in vitro express both fast and slow MHC isoforms. This finding could argue against a direct causal link between type II fiber SC loss and atrophy, as the loss of SC could theoretically be compensated for by

<table>
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<tr>
<th>Table 3. Myonuclei and satellite cell characteristics</th>
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<tbody>
<tr>
<td><strong>Type</strong></td>
</tr>
<tr>
<td>Nuclei/fiber</td>
</tr>
<tr>
<td>Fiber area/nucleus, µm²</td>
</tr>
<tr>
<td>SC/fiber</td>
</tr>
<tr>
<td>SC/mm² Type I</td>
</tr>
<tr>
<td>%SC Type I</td>
</tr>
<tr>
<td>%SC Type II</td>
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Values are means ± SE. SC, satellite cells; %SC, SC proportion (no. of SC/no. of SC + no. of myonuclei). *Significantly different compared with the young (P < 0.05). †Significantly different compared with the type I muscle fibers (within groups, P < 0.05).
incorporating SC progeny from type I fibers. However, the latter has yet to be established, and one could argue that, in an in vivo situation, where SC progeny generally fuse with existing fibers, expression of fast MHC isoforms by SC originating from slow fibers might not occur.

Our data support the idea that SC abundance is an important factor regulating myofiber size. In addition, the reduced SC content in type II fibers in the elderly might attenuate their response to anabolic stimuli, stressing the need for potent interventions for reversing the loss of skeletal muscle mass and function with aging. Nevertheless, a direct causal link between SC loss and myofiber atrophy remains to be established. Furthermore, qualitative changes (e.g., blunted SC activation) likely play another important role in the impaired regenerative capacity of skeletal muscle tissue with aging. As such, endocrine changes as well as changes in the local SC environment with aging might also contribute to the loss of SC due to the prevention of SC self-renewal (11, 14, 44). At this moment, we can only speculate on the potential causes and clinical relevance of qualitative and quantitative changes in skeletal muscle fiber SC content with aging, and more research is warranted.

In conclusion, this study is the first to show that SC content is specifically reduced in the type II muscle fibers in elderly men. The latter is shown to be evident when SC content is expressed as number of SC per fiber as well as number of SC per fiber area. The fiber type-specific reduction in SC content might represent an important regulatory factor in the loss of skeletal muscle mass, structure, and function with aging.

REFERENCES


