Natural Occurrence of Myofiber Cytoplasmic Enlargement Accompanied by Decrease in Myonuclear Number

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Abstract: It has been shown that changes in the nuclear number in myofibers are synchronized with myofiber size. Therefore, under some conditions, the myonuclear number is thought to be a determinant factor of myofiber size. However, we have clearly shown that denervation-induced fiber atrophy occurs without any decrease in myonuclear number, indicating that the myonuclear number is not always an important determinant factor of myofiber size. However, this was an event found under experimental conditions. In the present study, we examined the morphological features of single myofibers under normal conditions throughout the lifespan of normal

mice. We discovered that the C/N ratio (cell volume/nucleus) greatly increases during the growth period and clearly decreases during the aging period. From 5 weeks to 6 months old, the myofibers undergo fiber hypertrophy accompanied by a decrease in myonuclear number. In muscle at 18 months, we found no correlation between myonuclear number and fiber cross-sectional area. These results suggest that, even under normal physiological conditions, the myonuclear number is not always a determinant factor of the myofiber size. [The Japanese Journal of Physiology 53: 145–150, 2003]

Key words: myofiber size, myonuclear number, lifespan, normal muscle.

Skeletal muscle can show change in its tissue size in response to functional demand, mediated by changes in myofiber size. Although the mechanism underlying this change is still unclear, the concept of DNA unit as proposed by Cheek [1] may help to illustrate the subcellular regulatory mechanism. According to this concept, the DNA unit (protein/DNA) is constant; therefore, increments in DNA owing to the fusion of myoblast or satellite cells into myofibers drive myofiber cytoplasmic enlargement. Indeed, many studies have shown that, in various situations, changes in myofiber size appear to be driven by changes in DNA content (i.e., changes in myonuclear number) within the myofiber.

In overload-induced fiber hypertrophy [2] or during recovery from fiber atrophy [3], the myonuclear number increases proportionately. Further, X-ray irradiation of the muscle prevents fiber hypertrophy owing to the blockage of satellite cell incorporation into the

myofiber [4, 5]. These studies strongly suggest that the increase in myonuclear number is essential for fiber hypertrophy. Myofibers also undergo fiber atrophy accompanied by a decrease in myonuclear number under a range of conditions, including hind limb suspension [6], spinal cord isolation [2], and spaceflight [7, 8]. In particular, long-term denervation induces both marked fiber atrophy and a decrease in myonuclear number [9, 10]. However, we have clearly shown that mature mice undergo marked fiber atrophy without any decrease in myonuclear number, even after 120-d denervation [11], indicating that a reduction in myonuclear number is not always essential for fiber atrophy.

Our new finding that no reduction in myonuclear number occurs after long-term denervation was made partly due to the methodological advantages of our myofiber isolation technique. In a previous study, we developed a novel fiber isolation method and observed

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that the single myofibers isolated by conventional methods have a smaller number of myonuclei than the single myofibers isolated using our method [11]. This suggests that conventional methods carry the risk of artificial myonuclear loss; however, in our method, this risk is minimal. It is thus possible that some previous studies recorded incorrect values for the myonuclear number. Our fiber isolation method has an additional advantage: even very thin or structurally weak myofibers, such as those in newborn animals or embryos, can be easily isolated. We were therefore able to follow the morphology of single fibers through the animal's entire lifespan.

In the present study, we isolated single myofibers by means of alkali maceration and examined the changes in morphological features of myofibers throughout the mouse lifespan (from newborn to 18 months old). Myofibers undergo fiber hypertrophy and atrophy in the process of growth and aging, respectively, under normal physiological conditions. Therefore, our examination provides important information on the relationship between myonuclear number and myofiber size.

MATERIALS AND METHODS

Animals. The present study was approved by the University of Tsukuba's Animal Experiment Committee. Healthy ICR mice were used, bred, and aged in an air and humidity-controlled room with animal feed and water provided *ad libitum*. Mice were sacrificed by ether overdose or cervical dislocation before dissection of the tibialis anterior (TA) muscle. Mice that were new-born mice and 1, 3, and 5 weeks, and 6 and 18 months old were used to examine the changes in the morphological features of single myofibers. Mice at the age of 1 year and above were used to evaluate calculated fiber cross-sectional area (CSA).

Preparation of single myofibers and cross-sections. We isolated single myofibers using a previously described method [11]. To prevent myofiber contraction, hindlimb muscles still attached to the bone were fixed in 4% paraformaldehyde diluted in relaxing solution (137 mM NaCl, 5.4 mM KCl, 5 mM MgCl₂, 4 mM EGTA, 5 mM HEPES, pH 7.0), and the ankle was kept at a constant angle with no plantar or dorsal flexion. After more than 2 d of fixation, the TA muscles were dissected and divided into several bundles. At least five bundles from different regions of the muscle were used to isolate single myofibers. Non-muscle tissues were removed to the maximum degree possible using fine tweezers under a binocular microscope. Muscle bundles were macerated in a 40%

NaOH solution for 3 h at room temperature and then shaken for 8 min in a 20% NaOH solution. These processes separated the muscle tissue into single myofibers. Macerated, but still loosely bundled myofibers (seen in newborn and 1 week-old muscle) were divided into single myofibers using fine tweezers under a binocular microscope. Isolated single myofibers were then rinsed with phosphate-buffered saline (pH 7.2) and mounted on a gelatin-coated glass slide. After air-drying, the single myofibers were stained with hematoxylin to visualize their myonuclei.

Muscle cross-sections were prepared in the following way. The TA muscles were dissected following sacrifice, and then the mid-region of the muscle was cross-sectioned in 10 μ m-thick slices after being rapidly frozen in liquid nitrogen-cooled isopentane. During the process of preparation, muscles were not soaked in any saline as saline will cause the muscles to contract. After air-drying, the thin sections were stained with hematoxylin and eosin without any fixation.

Morphometry and statistics. We counted all the myonuclei in each fiber segment, carrying out observations at various focal depths, and measured the length and width of single myofibers on microscopic images using computer software (NIH Image, National Institutes of Health, USA). Fiber CSA and cell volume were calculated using the following formulas: fiber CSA = $c\pi(w/2)^2$, cell volume = fiber CSA $\times$ l , where w and l are the width and length, respectively, of the myofiber measured on the image, and c and π are the coefficients for correction and circular constant, respectively. In the present study, we established c as being 1.47 (see below and RESULTS). The C/N ratio (cell volume/nucleus) of each myofiber was determined with the nuclear number and cell volume from the same region of myofiber.

In order to determine the value of c , we compared calculated fiber CSAs with empirical ones within the same mouse. The right and left legs were used to measure fiber CSA of cross-sections and single-fiber samples, respectively. The means of fiber CSAs estimated from >1,000 randomly selected myofibers in each cross section were used as the standard. The calculated myofiber CSAs were then compared with the standard.

Differences among each group were determined using one-way ANOVA followed by Scheffe's Post Hoc test, with $p < 0.01$ regarded as significant for individual comparisons.

RESULTS

Morphology of single myofibers

Isolated myofibers possessed obvious cross-striations and multinuclei with nucleoli, and showed no structural damage. All nuclei in the single myofibers were elliptical, with their long axis in line with the direction of the myofiber (Fig. 1). We confirmed that capillaries, the most abundant nonmuscle cells around the myofiber, have spindle-shaped nuclei that are distinct from myonuclei. Further, when myofibers were mechanically isolated from muscle bundles using fine tweezers without alkali maceration, many of them contain spindle-shaped nuclei with their long axis out of line with the myofiber. Therefore, we believe that our alkali maceration procedure successfully separates single myofibers without leaving non-muscle cells attached.

Evaluation of calculated fiber CSA

Since myofibers shrunk during the process of fixation and air-drying, the calculated fiber CSAs needed to be corrected. The calculated fiber CSAs showed the closest coincidence with the standards when c was 1.47. The relative value of calculated fiber CSAs as compared to the standard is presented in Fig. 2. In three of five cases, the difference of the mean calculated fiber CSA was within 10% (Fig. 2, Nos. 1, 4, 5), and even in the most inaccurate case, the difference

was 13.2% (Fig. 2, No. 2), indicating that the calculated fiber CSA is closely consistent with the actual value.

Changes in fiber CSA, myonuclear number, and C/N ratio

We followed the morphological changes in myofibers throughout the mouse lifespan, using a total of 1,770 myofibers (Table 1). The change in fiber CSA, myonuclear number per 1-mm length of myofiber, and C/N ratio from newborn mice to mice at 18 months are presented in Fig. 3. Myofibers showed different morphological characteristics during the growth, maturation, and aging processes. During the growth period (from newborn to 5 weeks old), both the fiber CSA and myonuclear number increased significantly. However, the degree of increase in fiber CSA exceeded that of the myonuclear number. Mean fiber CSA grew 36.1-fold, but that of myonuclear number increased by only 2.8-fold during the same period.

In the maturation process (from 5 weeks to 6 months old), surprisingly, myofibers underwent fiber hypertrophy accompanied by a decrease in myonuclear number. However, this observation was the result of examining a small number of animals (5-week-old mice: $n=3$, and 6-month-old mice: $n=2$, Table 1). To

Fig. 1. Photomicrographs of single myofibers isolated from alkali-macerated TA muscle. Single myofibers isolated from the TA muscles of newborn and 1-, 3-, and 5-week-old mice, respectively. Isolated single myofibers contain no non-muscle cells. In thick myofibers, some myonuclei are out of focus. Bar: 50 μ m.

Fig. 2. Relative values of calculated fiber CSA. Relative values of calculated fiber CSAs compared with the standard are presented as means (open circles) $\pm$ SE. Nos. 1–5 represent each animal examined, and numbers in parentheses described below represent the number of single myofibers examined.

Table 1. Number of mice examined and single myofibers.

	Age					
	0 d	1 w	3 w	5 w	6 m	18 m
Sex	n.d.	n.d.	Male	Male	Male	Male
Animal	>5	>5	2	3	2	3
Single fiber	127	355	305	428	177	378

n.d.: not distinguished.

Fig. 3. Change in fiber CSA, myonuclear number, and C/N ratio of TA muscle throughout the mouse lifespan.

Fiber CSA (open bars), myonuclear number (black bars), and C/N ratio (gray bars) of TA muscle at various ages. The numbers of analyzed single myofibers are shown in Table 1. Between any age, a significant difference was observed except for the difference in myonuclear number between 6 and 18 months. Values are presented as mean $\pm$ SE. n.s., not significant ($p < 0.01$).

re-evaluate the myofiber morphological changes occurring in the maturation process, we carried out another set of morphological observations on both young (5 weeks old) and mature (from 5 to 7 months old) mice ($n=5$, for both young and mature mice). The comparison between young and mature mice was carried out using 30 myofibers from each animal. The results of re-evaluation also show fiber hypertrophy accompanied by a decrease in myonuclear number; the mean fiber CSA and myonuclear number of mature mice were 126 and 82%, respectively, compared with that of young mice (data not shown).

In the process of aging (from 6 to 18 months old), although fiber CSA decreased, no decrease in myonuclear number was observed, resulting in a significant decrease in the C/N ratio.

Relationship between fiber CSA and myonuclear number

The relationships between myonuclear number and fiber CSA in newborn mice and mice at 1, 3, and 5 weeks, and 6 and 18 months of age are presented in Fig. 4. There was a significant correlation between fiber CSA and myonuclear number at all stages ($r > 0.5$) except in mice 18 months old ($r = 0.21$). In 18-month old mice, even when each muscle was individually examined, no significant correlations were observed (data not shown).

DISCUSSION

The soleus muscle is, perhaps because of its slow-type dominant character, more sensitive to environmental alterations than fast-type muscles [3, 12]. Hence, many previous studies have examined fiber hypertrophy or atrophy using the soleus muscle. However, slow-type myofibers have a greater number of myonuclei than fast-type myofibers [13, 14], indicating that using a uniform-type muscle is more suitable than a mixed-type muscle for examination of the relationship between myonuclear number and myofiber size. The soleus muscle and other major muscles in the ICR mouse never show a completely slow-type phenotype, whereas on the other hand, some fast-type muscles such as the TA, extensor digitorum longus, and plantaris muscle show a uni-phenotype. Notably, the TA muscle is large and does not act as an anti-gravity muscle; therefore, this muscle is suitable for examining the relationship between myonuclear number and myofiber size under normal physiological conditions. For these reasons, we examined the TA muscle in the present study.

Single myofibers have been isolated using several methods, including mechanical isolation from glycerol-extracted muscle, nitric acid maceration, and enzymatic digestion. The isolated myofibers are then investigated by counting the number of myofibers comprising the muscle [15, 16], morphology using scanning electron microscopy [17], and enzyme activity [18], and utilized for *in vitro* culturing [19]. However, these methods have several disadvantages with respect to morphometric and statistical analysis. First, it takes a great deal of time to obtain a large number of single myofibers, leading to a limitation in the number of samples that can be collected. Second, it is difficult to

Fig. 4. Relationships between myonuclear number and fiber CSA of the TA muscle. Every solid circle and line represents the properties of a single myofiber and the linear regression between myonuclear number and fiber CSA, respectively. Note that there is little correlation between myonuclear number and fiber CSA in 18-month-old mice ($r=0.21$).

isolate thin or connective tissue-adhered myofibers from muscle tissue, thus inducing a bias in the collection of single myofibers and the inability to isolate fibers from regenerated, pathologic, or very juvenile muscle. Third, some of the isolated single myofibers are structurally incomplete; isolated single myofibers are often attached to non-muscle cells or are in the contracted state. In some cases, some myonuclei are lost during the isolation process [11]. In our series of studies, single myofibers were isolated by means of alkali maceration (see MATERIALS AND METHODS). In the isolated single myofibers, artificially induced myonuclear loss and damage are likely to be minimal [11], and calculated fiber CSA was closely consistent with that measured from cross-sectioned tissues (Fig. 2). Therefore, we were able to carry out reliable morphometric and statistical analyses on isolated single myofibers.

The finding that myofibers undergo fiber hypertrophy accompanied by a reduction in myonuclear number under physiological conditions (Fig. 3) is a critical counter-example to the hypothesis that the myonuclear number is a determinant factor of myofiber size.

Therefore, the transcriptional activity level in each myonucleus, rather than the myonuclear number, may be more influential in the modification of myofiber size. Indeed, it has been clearly shown that myonuclei in the same myofiber have different levels of transcriptional activity [20].

Although the reason for maturation-associated reduction in myonuclear number is unclear, it may be due to the natural occurrence of myonuclear apoptosis. Both the proliferation of myogenic stem cells and myonuclear apoptosis occur in postnatal growing muscle [21, 22]. If myonuclear apoptosis continues even after the proliferation of myogenic stem cells ceases, the total myonuclear number is likely to decrease thereafter.

Although we have no direct evidence that the maturation-associated reduction in myonuclear number is not due to artificial myonuclear loss, we have shown that the plantaris muscle also undergoes maturation-associated fiber hypertrophy accompanied by a decrease in myonuclear number. From 3 weeks to 4 months of age, the plantaris muscle undergoes fiber hypertrophy. However, on average, 3-week- and 4-

month-old muscles have respectively 77.1 and 61.2 myonuclei per 1 mm length of fiber segment [11]. Moreover, our single myofiber preparation procedure is unlikely to induce artificial myonuclear loss. Thus, we believe that this discrepancy occurs as part of the process of maturation.

In summary, reliable morphometric and statistical analyses of single myofibers were carried out throughout the lifespan of normal mice, and the following results were obtained: (1) Myofibers undergo fiber hypertrophy accompanied by a reduction in myonuclear number even under normal physiological conditions. (2) Myofibers show significant changes in their C/N ratio throughout the mouse lifespan. (3) In aged muscle, there is no significant correlation between myonuclear number and fiber CSA. Our findings suggest that the myonuclear number is not always a determinant factor in the modification of myofiber size, even under normal physiological conditions.

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