J Orthop Sci (2003) 8:75–78

Journal of
Orthopaedic Science

Changes of fiber type ratio and diameter in rabbit skeletal muscle during limb lengthening

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Abstract Changes in the fiber-type ratio and diameter during limb lengthening in 10 adult rabbits were studied using histochemical techniques. Changes in the ratio and diameter of muscle fibers (classified as type 1, 2A, or 2B fibers) in tibialis anterior muscles were examined after 20% gradual distraction of the tibia. There was an increase in the number of type 1 fibers and a decrease in type 2B fibers after tibial lengthening. Moreover, the average diameter of the type 1 fibers increased, whereas that of the type 2B fibers decreased. The diameters of muscle fibers measured immediately after completion of the lengthening showed a tendency to recover to normal levels within 1 month; however, the ratio profile of the muscle fibers changed both immediately and 1 month after lengthening. The above results demonstrate that stretched skeletal muscle adapts differently to bone lengthening according to the type of muscle fibers present, resulting in qualitative changes in the fiber-type profile.

Key words Limb lengthening · Skeletal muscle · Change of fiber

Introduction

Complications during limb lengthening such as joint contracture and muscle weakness after lengthening have been previously reported.[8] These complications are thought to be caused by dysfunctional stretching of the soft tissues. Skeletal muscle is believed to be a limiting factor in the process of limb lengthening. We believe that changes in muscle function are caused not only by quantitative changes such as muscle atrophy but also qualitative changes in the fiber-type ratio. Consequently, we have examined the ratio and diameter of fiber types in rabbit skeletal muscle to assess adaptive morphological changes that take place in response to limb lengthening.

Materials and methods

This study was performed according to the guidelines of the Institute for Laboratory Animal Research at Osaka Medical College and all procedures were approved by the Osaka Medical College Animal Research Committee.

The experimental study was carried out using 20 mature male Japanese white rabbits weighing 3.0 kg on average (range 2.7–3.4 kg). The 20 animals were divided into four groups. In group A (control group, $n = 5$), the animals did not undergo operation. In group B (sham operation group, $n = 5$), the animals underwent sham operations and were killed 27 days after the operation without limb lengthening. In group C (early-stage group, $n = 5$), the right tibia was lengthened by 0.5 mm twice daily (1 mm/day) for 20 days beginning day 7 after the operation. The animals were killed immediately following completion of the limb-lengthening procedure. In group D (late-stage group, $n = 5$), the animals were killed 1 month after the same limb-lengthening procedure that was performed in group C.

The surgery was done under pentobarbital (Nembutal, Abbott Laboratories, IL, USA) intravenous anesthesia following sedation with ketamine chloride. A unilateral external fixation device (modified Orthofix M-100, Orthofix Srl, Verona, Italy) was applied to the right tibia, and a transverse osteotomy was then performed just below the tibiofibular junction using a fine-toothed circular saw. Following the operation the animals were fed with standard pellets and water in a standard size cage, and limb lengthening for groups C and D was performed as described above.

We selected the tibialis anterior muscle for observation, as this muscle is not affected by the position of

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Received: February 1, 2002 / accepted: August 26, 2002

the knee joint. In a preliminary experiment, it was ensured that this muscle was not affected by pins inserted during the surgical procedure. After the animal was killed, the ankle joint was maintained in a neutral position, and the length of the tibialis anterior muscle was measured using a sliding caliper (accuracy ± 0.01 cm).[7]

The muscle was removed with tendons intact from their points of origin and insertion, and the length of the tendon and the wet weight of the muscle were measured. Samples were taken from the central portion of the muscle belly dissected from each animal. These specimens were frozen in isopentane, cooled to −160°C by immersion in liquid nitrogen, cut into 7-μm serial thick transverse sections using a cryostat, and stained with myofibrillar actomyosin adenosine triphosphatase (ATPase) and succinate dehydrogenase (SDH).

Enzyme histochemistry

ATPase staining. Modified ATPase staining of Dubowitz and Brooke[1] was used. Unfixed consecutive cryosections were processed after two pretreatments at room temperature: (a) incubation in 60 mM barbital-acetate acid buffer (pH 4.4) for 5 min; (b) incubation in 40 mM $CaCl_2$ in 20 mM barbital buffer (pH 9.4) for 5 min. Thereafter, the following consecutive steps were carried out. (1) Sections (preincubation a) were washed for 1 min in 20 mM barbital buffer (pH 10.0) containing 18 mM $CaCl_2$; (2) incubated for 10 min (preincubation a) and then incubated for 5 min (preincubation b) at 37°C in 20 mM barbital buffer containing 18 mM $CaCl_2$ and 4.5 mM ATP disodium salt; (3) washed nine times for 1 min each with 1% $CaCl_2$; (4) kept in 2% $CoCl_2$ for 3 min; (5) washed 10 times for 1 min each with 10 mM barbital buffer; (6) immersed in 1% ammonium sulfide for 1 min; (7) rinsed in distilled H_2O; (8) dehydrated with graded ethanol solutions; (9) immersed in xylene; and (10) mounted.

SDH staining. The SDH staining was carried out using the technique of Nachlas et al.[6] Unfixed sections were incubated for 30 min at 37°C in 100 mM Tris-HCl buffer(pH 7.6) containing 70 mM sodium succinate, 1.2 mM nitroblue tetrazolium, and 0.08 mM phenazine methosulfate. After incubation the sections were briefly washed in distilled H_2O and fixed for 30 min in 1% neutral buffered formalin. The sections were then mounted with 70% glycerol.

With ATPase staining, type 1 fibers from rabbits stained black at pH 4.4 and white at pH 9.4. Consistently, type 2B fibers stained gray at pH 4.4 but white with SDH staining, whereas type 2A fibers stained grayish white at pH 4.4 but gray when stained with SDH. Based on these characteristics 500 muscle fibers were selected from each muscle, and their type ratio was examined.[11] The diameters of 50 fibers from each muscle fiber were measured using an image analysis program (NTM image), which identified the smallest diameter of each muscle fiber.

Statistics

All data are expressed as means (SD) of the groups. An unpaired *t*-test was used for comparison between groups A and C in relation to the length of tibia and tibialis anterior muscle. A one-way layout ANOVA and a Scheffe-type multiple comparison test were used to compare wet weights of muscles, differences in the fiber ratio, and changes in fiber diameters between groups. Results were considered significant at $P < 0.05$.

Results

Length of the tibia and tibialis anterior muscles

The average length of the operated tibia in group C was 127.7 ± 4.1 mm, which was 18.7 ± 0.71 mm longer (17.2% ± 0.42%) than the average length of tibia in group A (109 ± 3.5 mm) ($P < 0001$). The average length of the tibialis anterior muscle was 63.8 ± 3.6 mm in group C, which was 10.6 mm longer (19.9% ± 2.0%) than that measured in group A (53.2 ± 3.8 mm) ($P = 0.002$).

Wet weight of the tibialis anterior muscles

The average wet weight of the tibialis anterior muscles was less in group B (90.2% ± 1.9%) than in group A ($P = 0.02$), but it was almost the same as that in group C (102.1% ± 4.0%) ($P = 0.9$) and much greater in group D (112.7% ± 7.5%) ($P = 0.003$).

Muscle fiber ratio

In group A, the tibialis anterior muscles from operated legs contained on average 4.1% ± 0.57% type 1 fibers, 32.6% ± 6.3% type 2A fibers, and 63.3% ± 6.1% type 2B fibers (Table 1). Compared with group A, muscles in group B had fewer type 2B fibers ($P = 0.048$). In group C, muscles had more type 2A fibers ($P = 0.008$) and fewer type 2B fibers ($P = 0.001$). In group D, muscles had more type 1 fibers ($P = 0.02$) and fewer type 2B fibers ($P = 0.024$) (Fig. 1).

Muscle fiber diameters

The average diameter of type 1 fibers in tibialis anterior muscles from group A was 62.0 ± 4.5 μm, and those of

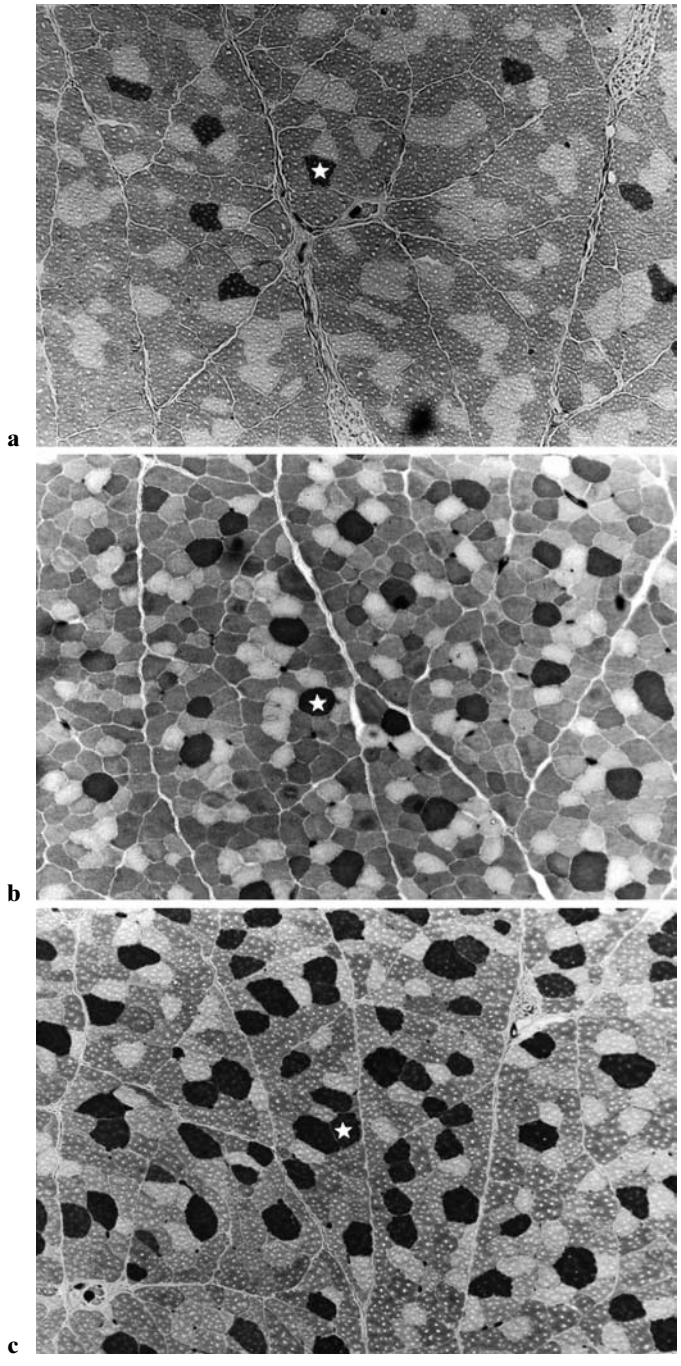

Fig. 1. Photomicrographs showing an increase in type 1 fibers (*stars*) in tibialis anterior muscle of groups C and D. **a** Group A. **b** Group C. **c** Group D. ATPase (pH 4.4) ×40

type 2A and 2B fibers were 60.6 ± 5.4 μm and 64.1 ± 3.6 μm, respectively (Table 2). The average diameter of type 1 fibers in muscles from group C increased compared with that of group B ($P = 0.03$), whereas the average diameter of type 2B fibers decreased compared with that of group A ($P = 0.03$) (Fig. 2).

Table 1. Muscle fiber ratio

Group	Type 1	Type 2A	Type 2B
A	4.1 ± 0.57	32.6 ± 6.3	63.3 ± 6.1
B	6.1 ± 1.4	41.3 ± 4.1	52.6 ± 4.0*
C	9.4 ± 2.9	44.6 ± 3.6*	46.0 ± 5.5*
D	13.9 ± 7.8*	34.8 ± 4.0**	51.3 ± 5.6*

Results are means ± SD (%)
* Significantly different from group A, $P < 0.05$. ** Significantly different from group C, $P < 0.05$

Table 2. Diameter of muscle fibers

Group	Type 1	Type 2A	Type 2B
A	62.0 ± 4.5	60.6 ± 5.4	64.1 ± 3.6
B	59.4 ± 6.7	58.5 ± 8.7	55.7 ± 7.1
C	72.7 ± 8.1*	59.8 ± 3.7	54.5 ± 3.2**
D	65.9 ± 4.4	57.5 ± 2.2	55.6 ± 2.2

Results are means ± SD (μm)
* Significantly different from group B, $P < 0.05$. ** Significantly different from group A, $P < 0.05$

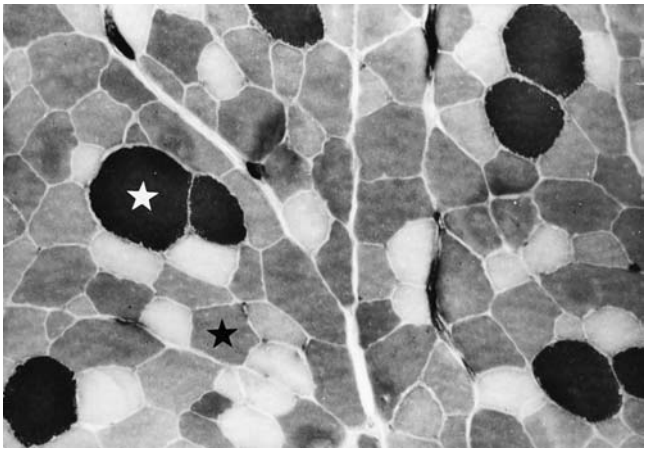

Fig. 2. Photomicrograph showing atrophy of type 2B fibers in tibialis anterior muscle (*black star*) and hypertrophy of type 1 fibers (*white star*) in group C. ATPase (pH 4.4) ×100

Discussion

It is important to investigate changes in the fiber-type ratio due to limb lengthening, as we believe they represent qualitative changes in muscle properties. The literature contains several reports on the effects of lengthened skeletal muscle on the muscle fiber-type ratio, but a consensus on the morphological changes that occur under these conditions has not been achieved. Correct determination of fiber types requires a comparison of at least two enzyme activity patterns.[11] Hence, we examined the ratios of three types of muscle fiber thoroughly in the present study by staining with both ATPase (pH 4.4) and SDH.

With regard to the muscle fiber ratio after limb lengthening, some studies have reported no change,[3,4] whereas others showed that the percentage of type 1 fibers increased and that of type 2 fibers decreased.[2,10] Our study shows an increase in the percentage of type 1 fibers and a decrease in type 2B fibers in the tibialis anterior muscle following lengthening of the tibial bone. Weight-bearing and low-frequency electrical stimulation have been shown to elicit a change from type 1 to type 2 fibers.[9] Likewise, kinetic loading and high-frequency electrical stimulation are believed to cause a change from type 2 to type 1 fibers.[5] We think that the reason for the increase in type 1 fibers in the tibialis anterior muscle in our study was because of the stretch stimulus resulting from gradual lengthening.

Regarding changes in fiber diameter with limb lengthening, several studies have reported that the diameter of type 2B fibers decreases after the lengthening procedure.[2–4,10] However, conflicting results have been reported changes in the diameter of type 1 fibers, including no change,[4] decreasing diameter,[3] and increasing diameter.[2,10] In the experiments reported herein, we observed three changes in muscle fiber diameters immediately after lengthening of the tibialis anterior muscle that were dependent on the fiber type: Type 1 fiber diameters increased; type 2A fiber diameters were unchanged; and type 2B fiber diameters decreased. We believe that gradual lengthening, a light load, and a sustainable stretch stimulus were conducive to the observed increase in diameter of type 1 fibers.

The change in the diameter (compared to controls) of the muscle fibers immediately after completion of the lengthening procedure had a tendency to recover to normal (control) levels within 1 month. However, the ratio of the muscle fiber types changed both immediately and 1 month after the lengthening procedure had been completed.

Conclusions

Mild, gradual lengthening gives rise to a qualitative change in the ratio of muscle fibers in skeletal muscle. In clinical sense, one must take into consideration the fact that a change in muscle function might be caused not only by muscle atrophy but also by qualitative changes in the fiber-type ratio of individual skeletal muscles.

Acknowledgments. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

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