

## Effects of downhill running incorporated into long-term endurance training on skeletal muscle fiber-type switching and fatigue resistance

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**Abstract** The effects of the intermittent incorporation of high-intensity downhill running sessions into long-term endurance training were assessed by examination of the plantaris muscle of rats. First, the intrinsic effects of a single session were evaluated in otherwise sedentary rats. The experimental group showed histological injuries in 2–3 days after the session. In addition, compared with the sedentary control, the experimental group showed a sevenfold increase in the fraction of type IIc fibers, and decreases of 74 and 88% in tetanic force evoked indirectly and directly, respectively, by electric stimulation. The injured muscle fibers showed regeneration within 21 days as evidenced by centrally located nuclei. Next, the effects of intermittently incorporated downhill-running sessions into a 9-week endurance training regimen were tested using two experimental groups: Training and Training + Downhill. On the first day of the 1st, 3rd, 5th, and 7th weeks of the training period, the rats in the Training + Downhill group experienced downhill-running sessions. After the endurance training period, the plantaris muscle of the two experimental groups demonstrated higher fatigue resistance with an increase in the type IIa fiber fraction, at an expense of the type IIb fiber fraction. Compared with the Training group, the Training + Downhill group had a higher type IIa fiber fraction, with clusters of 70–100 type IIa fibers. These results indicate that intermittent high-intensity sessions promote the fiber type transition induced by daily endurance training. However, the potentially adverse effect of fiber type cluster formation suggests that there is an optimum intensity and frequency of the high-intensity exercise sessions for better enhancing the effects of long-term endurance training.

**Keywords** : eccentric exercise, endurance training, skeletal muscle

### Introduction

In the practical training scene, occasional high-intensity exercise is generally expected to benefit the effects of daily training. For instance, it has been reported that endurance exercise capacity is improved by occasional high-intensity exercise<sup>1,2</sup>.

There are several putative triggers for the physiological processes linking occasional high-intensity exercise to the beneficial outcomes of long-term daily training. Mechanical stress associated with high-intensity exercise is considered to play a key role as a trigger for muscle fiber hypertrophy<sup>3</sup> by suppressing catabolism and promoting anabolism<sup>4</sup>. Intense mechanical stress, encountered especially in eccentric exercise of high-intensity exercise, induces muscle injury as evidenced by delayed-onset muscle soreness, and a temporal, apparent loss of muscle

power<sup>5,6</sup>. This suggests a possibility that, as a mechanism of the effect of high-intensity exercise, exercise-induced muscle injury serves to promote fiber type transition as follows: Since the innervating motor neuron governs the muscle fiber type<sup>7</sup>, the triggers for the adaptive fiber type transition would be (1) temporal denervation due to mechanical injuries at the neuromuscular junctions<sup>8,9</sup> and (2) necrotic fiber damage leading to replacement and recruitment of muscle fibers from the fusion of satellite cells<sup>10</sup>. Although minor damage to neuromuscular junctions reinnervated by original motor neurons would have little effect, massive denervation followed by multiple innervations<sup>11</sup>, regeneration, and de novo formation of muscle fibers would effectively accelerate fiber type transition to adapt to the amount of muscle activity. We therefore hypothesize that the muscle fibers regenerating from injury would be more susceptible to the effect of endurance training than ordinary muscle fibers.

With these mechanisms in mind, we examined the ef-

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fects of eccentric exercise sessions that may induce muscle injuries in rats. We first examined the extent and the time course of the effect of a single downhill running session, and then examined the effects of the incorporation of repetitive downhill running sessions into long-term daily endurance training in plantaris muscle that is one of the major muscles of plantar flexion exercise adopted in the present study.

## Methods

**Animal care.** Female Fischer 344/Jcl rats (CLEA Japan, Tokyo, Japan) were housed two to three per cage under a 12 h/12 h light–dark cycle at room temperature ( $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ),  $55\% \pm 5\%$  humidity, and provided with food (CE-2, CLEA Japan) and water ad libitum. All animal studies were performed in accordance with the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences*, published by the Physiological Society of Japan. Although institutional approval was not available or mandatory at the time of the studies (from April 1998 to June 2004), institutional approval was issued by the Animal Care and Use Committee of Aichi University of Education in 2004.

### Exercise protocols.

1. A single session of downhill running of sedentary rats (Experiment A)

We first examined the extent and the time course of the effects of a single downhill running session. For this purpose, we used 14 to 15-week-old rats that were otherwise sedentary weighing approximately 200g. The experimental group were forced to run on a motor-driven treadmill at a constant velocity of 20 m/min with  $16^{\circ}$  head-down tilt for 2 h. The effects of this single session on the plantaris muscle were examined just before the session (Pre), and 1, 2, 3, 5, 7, 14, and 21 days after the session ( $n = 6$  for each day). The muscles on the right side of the body were used to examine contractile properties and those on the left were used to examine histological and histochemical properties.

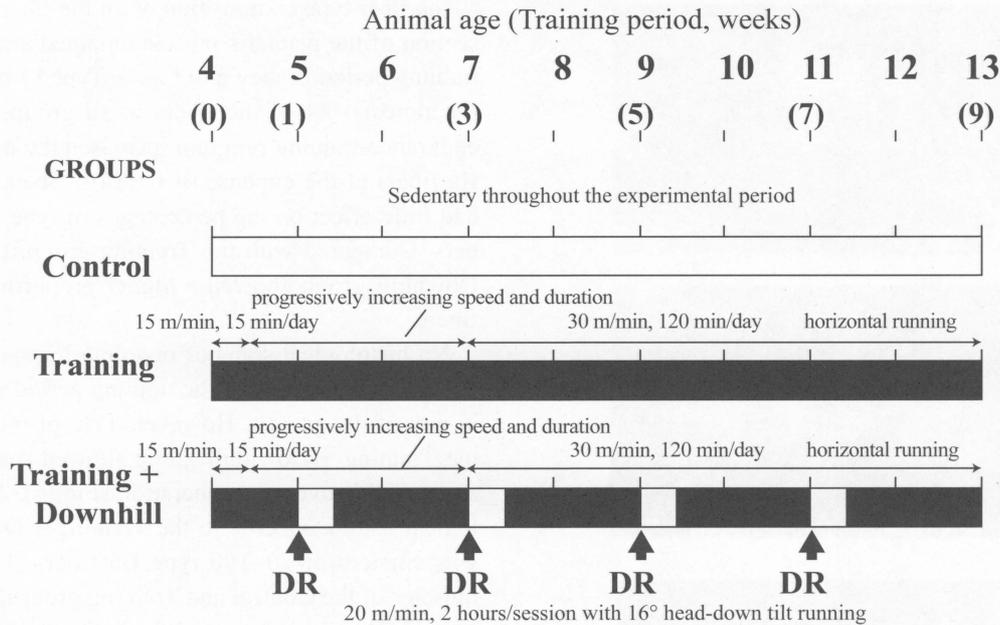
2. Intermittent downhill running sessions during daily endurance training (Experiment B)

Next, we examined the effect of intermittent downhill-running sessions incorporated into long-term endurance training. Three-week old rats weighing 40 to 45g were randomly separated into three groups: sedentary group (Control,  $n = 10$ ), training group (Training,  $n = 10$ ), and training plus intermittent downhill running group (Training + Downhill,  $n = 10$ ). The rats of the two experimental groups were trained for 9 weeks from the age of 4 weeks as follows. The rats were made to run on a motor-driven horizontal treadmill once a day for 6 days/week. The running velocity and duration were initially 15 m/min for 15 min/day during the first 3 days, and were gradually increased depending on the capacity of each rat to the fi-

nal values of 30 m/min and 120 min/day in 3 weeks. The final velocity and duration were maintained thereafter. A running velocity of 30 m/min is reported to require 55% of the maximal oxygen uptake ( $\dot{V}\text{O}_2\text{max}$ ) for Fischer 344 rats of the corresponding age (16 weeks)<sup>12</sup>, therefore this daily running exercise corresponds to endurance training. On the first days of the 1, 3, 5, and 7th weeks of the training period, the rats of the Training + Downhill group underwent downhill running sessions as described for Experiment A. The daily endurance training was suspended for 3 days after each downhill running session to ensure that the total exercise load of the Training + Downhill group did not substantially exceed that of the Training group. The plantaris muscle was examined since it contains numerous fiber types and is shown from biochemical studies to be one of the major muscles of plantar flexion exercise<sup>13</sup>. The contractile, histological, and histochemical properties of the plantaris muscle were examined at the end of the 9-week training programs at 13 weeks. Fig. 1 summarizes the experimental protocol of Experiment B.

**Examination of contractile properties.** Examination of contractile properties was performed as described by Close<sup>14</sup>. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbitone (50 mg/kg body weight) and placed in the prone position on an acrylic resin plate with  $30^{\circ}$  head-up tilt. The plate was heated to maintain body temperature. The distal tendon of the plantaris muscle of the right hind limb was attached to a force transducer (LTS-500GA, Kyowa, Tokyo, Japan). The sciatic nerve was then cut centrally at the right thigh, kept in a pool of Krebs–Ringer solution (in mM: 137 NaCl, 5 KCl, 1  $\text{NaHCO}_3$ , 1  $\text{NaH}_2\text{PO}_4$ , 1  $\text{MgCl}_2$ , 2  $\text{CaCl}_2$ , and 7.8 glucose; aerated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ , and maintained at  $36.0^{\circ}\text{C}$ – $36.5^{\circ}\text{C}$ ) embanked with the proximal hind limb muscles. A pair of chloride silver wire electrodes was attached to the distal end of the nerve leaving 10 mm of nerve segment proximal to the cut end. Supramaximal electrical square pulses of 0.1-ms duration were then applied to the sciatic nerve through the electrode pair for indirect stimulation of the muscle, and through another electrode pair placed on the belly of the muscles for direct stimulation. The muscle was set at an optimal length ( $L_0$ ) for maximal isometric twitch force. The main blood supply to the plantaris muscle was kept intact during the measurements. Intervals of  $>2$  min were allowed between any consecutive measurements. The amplitudes of twitch and tetanic force, and time to peak of twitch contraction were measured directly on the screen of a digital oscilloscope (COM7061, Kikusui, Kanagawa, Japan). The isometric tetanic force was maximal at 200-Hz stimulation.

Fatigability was evaluated by intermittent (1/s) 45-Hz tetanic stimulation lasting for 330 ms, each over a period of 300 s. The fatigue indices represent tetanic force expressed relative to the initial tetanic force in percentage (%).



**Fig. 1** Schematic diagram of the experimental design and exercise training protocol of Experiment B. Arrows labeled "DR" refer to the time point at which the downhill-running session was performed. Open boxes indicate the resting periods and closed boxes refer to the endurance-training periods. Running speed and duration of each session are indicated.

**Histological evaluation.** All the rats (in both Experiments A and B) were euthanized with an overdose of sodium pentobarbitone, prior to dissection of the left-side plantaris muscles. 10- $\mu$ m transverse serial sections of frozen tissue were stained for myofibrillar ATPase (mATPase) after alkaline and acid preincubation<sup>15</sup>. The fibers were classified according to the pH lability of the mATPase. The fibers that were acid (pH 4.6)-resistant, but alkaline (pH 10.3)-labile mATPase, were assigned as type I, those having mATPase of the opposite pH lability as type IIa, and those with intermediate ATPase properties as type IIb, IIc, and IId. Relative acid (pH 4.6) resistance of mATPase distinguished type IIc fibers from type IIb and IId. With the exception of slight differences in the acid and alkaline lability of mATPase, type IId fibers were almost indistinguishable from type IIb fibers with mATPase staining. When an adjacent serial cross section of the muscle was stained for succinate dehydrogenase (SDH) in a medium containing succinate for 30 min at pH 7.4 and 37 °C<sup>16</sup>, higher SDH activity distinguished type IId fibers from type IIb fibers<sup>17</sup>. For general histological assessment, another serial cross section of the muscle was also stained with hematoxylin and eosin. Muscle fiber types of all the 4000~4500 fibers in a cross-section of each muscle were identified and analyzed.

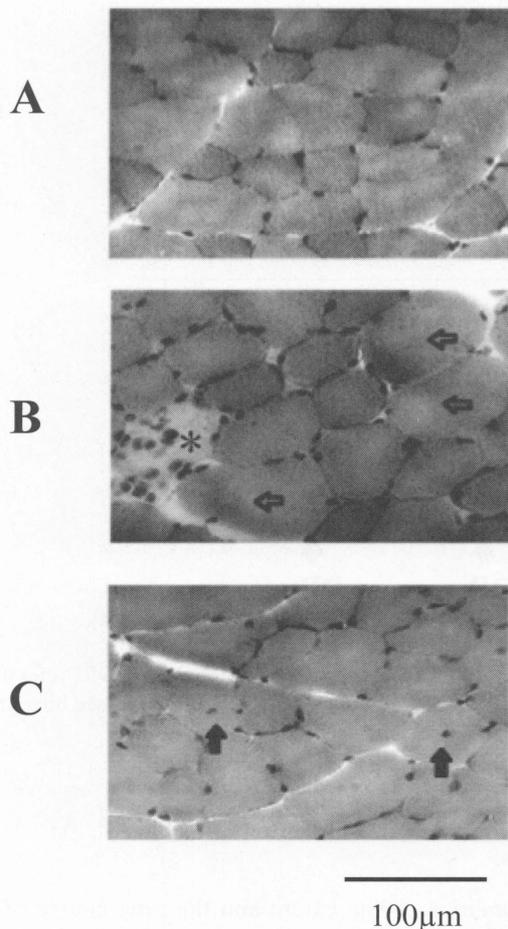
**Statistical Analysis.** All data are represented as mean  $\pm$  SD. Quantitative observations were evaluated using two-way analysis of variance. Scheffé's multiple-comparison test was used to evaluate significant differences between individual groups. Significance level was set at a p value of 0.05.

## Results

**Experiment A.** The extent and the time course of the effects of a single downhill-running session were evaluated in plantaris muscles of otherwise sedentary rats. Significant histological injuries evolved with time (Fig. 2). Three days after the session, swollen edematous muscle fibers, spreading of fibers and fasciculi, and mononuclear macrophage invasion into vacuolated fibers were evident. Twenty-one days after the session, most macrophage infiltration had resolved, probably because of the regeneration of muscle fibers as evidenced by centrally located nuclei found in 3% to 5% of fibers in the cross-section.

Changes in maximal tetanic force of the plantaris muscle, following the downhill-running session, were also observed (Fig. 3). Both indirectly and directly stimulated force levels before the session were almost identical (inset in Fig. 3). Both types of force decreased to a minimum within 2-3 days after the session, and recovered to the original level in a few weeks. Despite the similarity of the overall decremental and subsequent incremental patterns of the force changes, the loss of indirectly stimulated tetanic force, reaching 74% of the control at 2 days after the session, was more prominent than that of directly stimulated force, reaching 88% of the control at 2-3 days after the session. Indirectly stimulated force almost caught up with the directly stimulated force during 7 to 14 days of recovery. This suggests that neuromuscular transmission was impaired within a few days after the session, followed by gradual full recovery in a few weeks.

Changes in myofiber type composition of all the fibers in the cross-section of plantaris muscle after the downhill-



**Fig. 2** Light micrographs of the transverse sections of the rat plantaris muscle stained with hematoxylin and eosin (H&E). A: Sedentary control. B and C: Three and 21 days after a single bout of downhill running, respectively. In B, abnormally enlarged necrotic muscle fibers (open arrows), and disrupted cytoplasm of degenerated fiber invaded by mononuclear cells (open asterisk) are shown. In C, centrally located nuclei are shown by closed arrows.

running session was also observed (Fig. 4). The fraction of type IIc fibers markedly increased to approximately seven times the control in 2 days and decreased to the original level in 21 days (Fig. 4-A), paralleling the time course of the contractile property changes. Other fiber types showed no marked changes (Fig. 4-B).

**Experiment B.** The effects of intermittent downhill-running sessions incorporated into daily endurance training were examined in Experiment B.

The sedentary (Control) and trained (Training, Training + Downhill) groups showed no appreciable differences in body weight, tetanic force, twitch contraction time, and the weight of the plantaris muscle at the end of the 9-week training period (Table 1). The fatigue indices increased in both the Training and Training + Downhill groups. However, no significant difference was found between the Training and Training + Downhill groups.

The fiber type composition of all the fibers in the cross-section of the plantaris muscle obtained at the end of the training period is shown in Fig. 5. Type I fibers accounted for almost 10% of the fibers in all groups. The present endurance-training program increased the number of type IIa fibers at the expense of type IIb fibers. The training had little effect on the percentages of type IIc and IId fibers. Compared with the Training group, the Training + Downhill group showed a higher proportion of type IIa fibers.

No histological signs of ongoing degeneration and regeneration at the end of the training period were observed in any of the groups. However, 12% of muscle fibers in the Training + Downhill group showed centrally located nuclei indicative of regeneration (Fig. 6). Another histological feature specific to the Training + Downhill group was clusters of 70–100 type IIa fibers (Fig. 7). In the muscles of the Control and Training groups, no more than 10 type IIa fibers were tightly contiguous. This specific clustering of type IIa fibers was not observed in the plantaris muscle of Experiment A, which underwent only a single session of downhill running.

## Discussion

**Temporal muscle injuries induced by the present downhill-running session.** A single intensive training session of downhill running resulted in edematous swelling leading to necrotic deterioration of muscle fibers (Fig. 2-B) and the impairment of directly stimulated force by 12%, i.e. 100 to 88 (Fig. 3). Moreover, indirectly stimulated force showed an additional 14%, i.e. 88 to 74, decrease, indicating injured neural function of muscle fibers with preserved excitation-contraction capacity<sup>18</sup>.

Similar morphological changes and concurrent functional impairments have been observed as signs of muscle injury in previous studies<sup>6,19-21</sup>, which were repaired with fiber regeneration leaving histologically evident central nuclei<sup>22-24</sup>. In the present study, the signs of fiber degeneration diminished by 21 days after the downhill running session, leaving central nuclei in approximately 4% of muscle fibers on a single cross-section. These findings suggest that the intensity of the downhill running session was substantially high enough to induce histological degeneration of a considerable proportion of the fibers.

The loss of the directly stimulated force developing capacity indicated that 8%, i.e. 12 to 4, of the fibers lost excitability or contractility without showing any histological signs of fiber regeneration, and 14% of the fibers lost transmission of motor signals without losing intact excitability that leads to contraction, suggesting that a considerable proportion of fibers are subjected to a functional loss of innervation (“temporal denervation”). Temporal denervation and following reinnervation induced by eccentric exercise has been documented to cause histological and functional changes at neuromuscular junctions<sup>8,9</sup>.

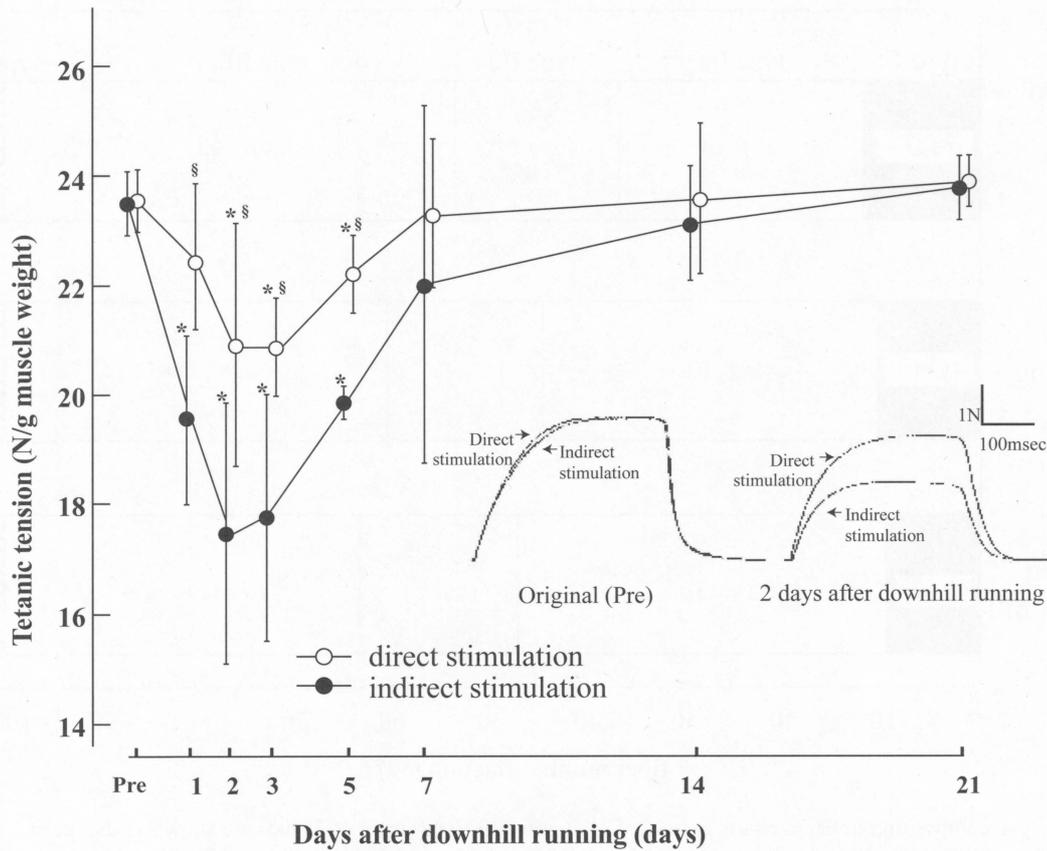


Fig. 3 Changes in tetanic tension developed by direct stimulation (open circles) and by indirect stimulation through a sciatic nerve (closed circles) of the plantaris muscle following a single session of downhill running. Values are shown as the mean  $\pm$  SD. \* $p < 0.05$ , significant difference from original (Pre), and † $p < 0.05$ , significant difference between direct and indirect stimulation. Insets show representative traces of force development induced by direct and indirect stimulation in rats before (Pre) and 2 days after the downhill session.

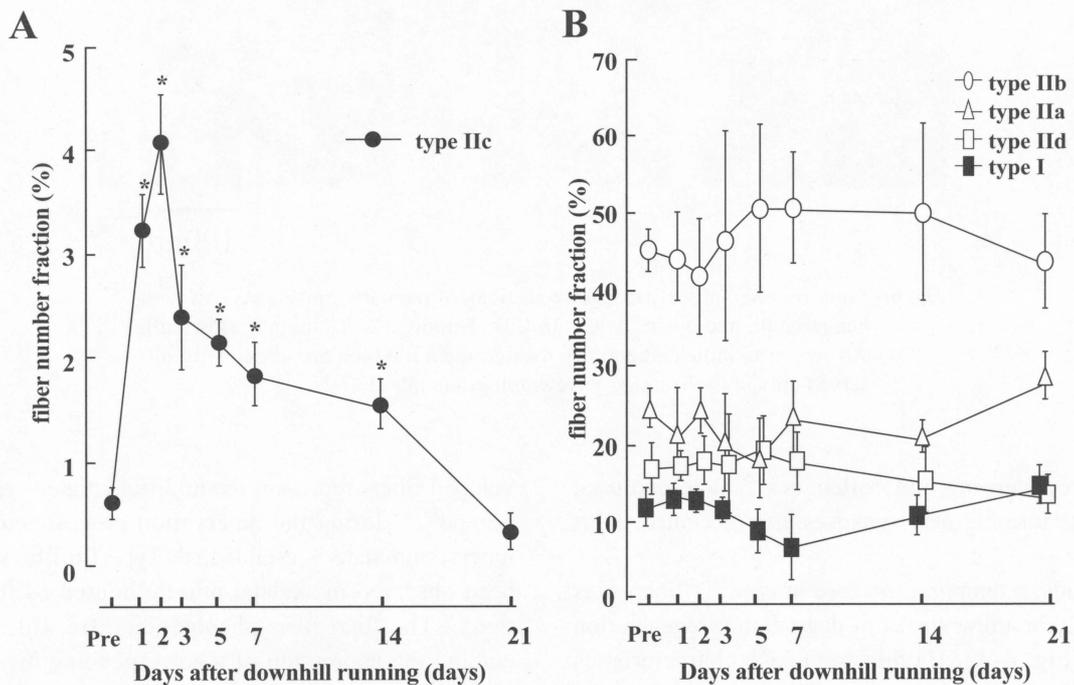
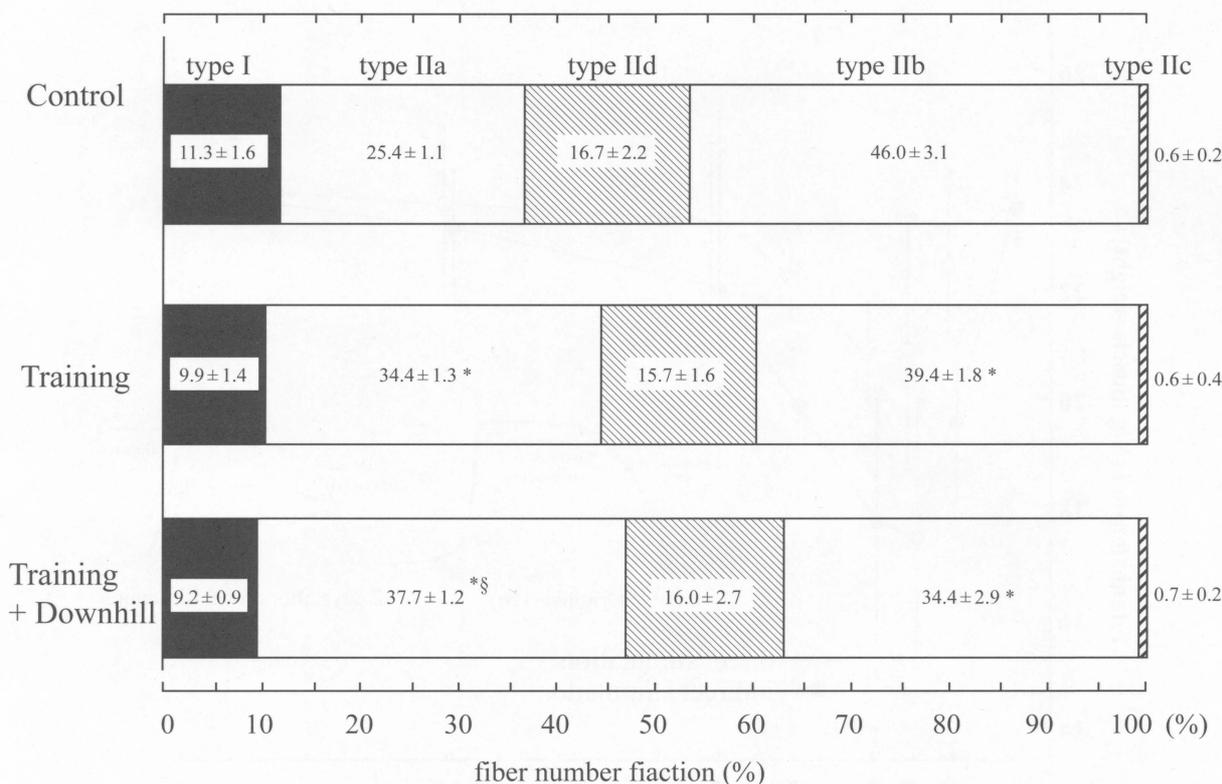
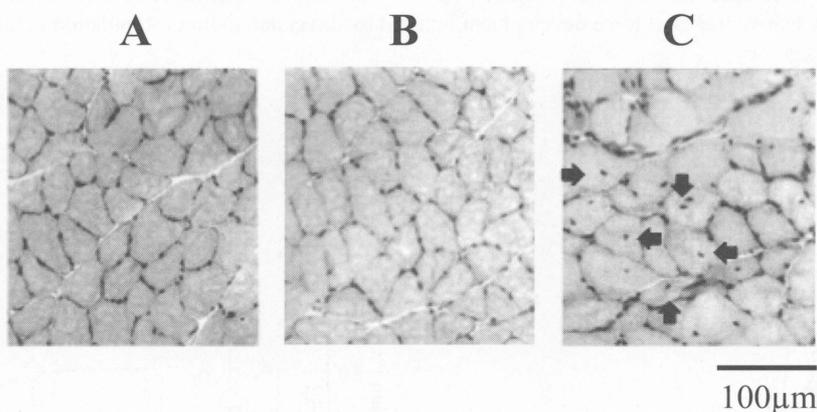


Fig. 4 Changes in muscle fiber type composition in the plantaris muscle following a single session of downhill running. A: type IIc fiber; B: type IIa, IIb, IIc and type I fibers: Note the difference in the scales of the ordinates. Values are shown as the mean  $\pm$  SD. \* $p < 0.05$ , significant difference from original (Pre).



**Fig. 5** Fiber type composition in the plantaris muscle of each group in Experiment B. Values are shown as the mean  $\pm$  SD. \* $p < 0.05$ , significant difference from the Control group; § $p < 0.05$ , significant difference between the Training + Downhill group and Training group.



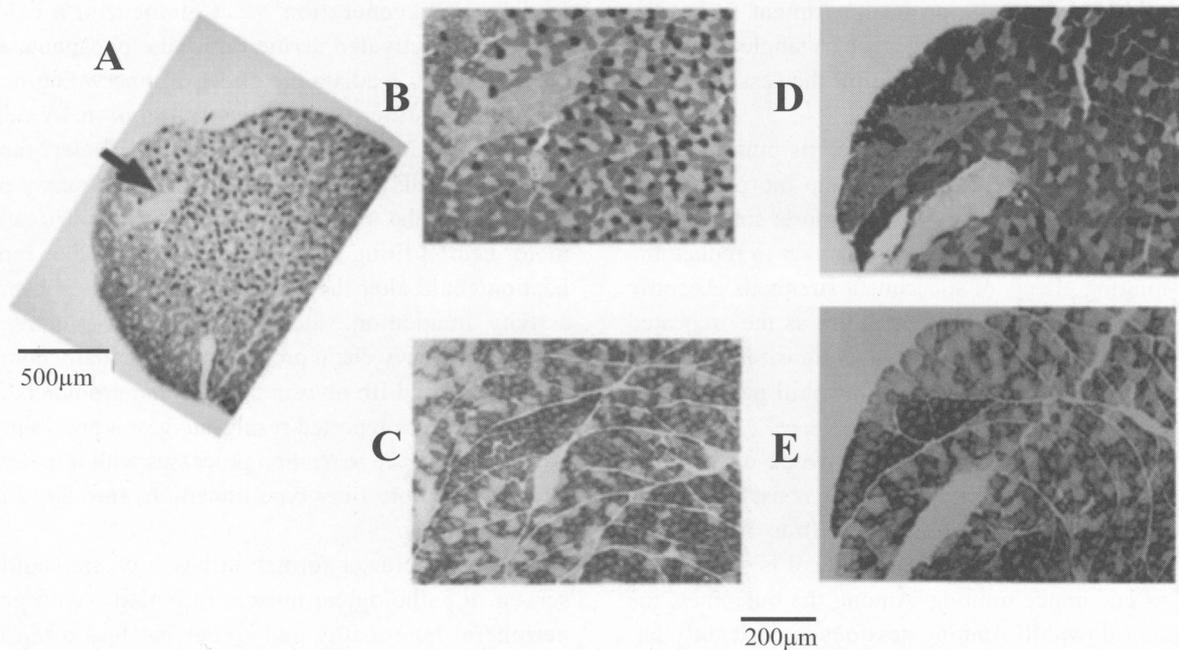
**Fig. 6** Light micrographs of transverse sections of plantaris muscle stained with hematoxylin and eosin. A: Control, B: Training, C: Training + Downhill. Arrows in C indicate centrally located nuclei, which are abundantly observed only in the Training + Downhill group muscle.

For instance, Werning and colleagues<sup>22)</sup> have captured axon sprouts forming new synapses after eccentric exercise.

In this study, a temporal increase in type IIc fibers was observed within a few weeks of degradation-regeneration processes (Fig. 4-A). Having mATPase characteristics of both type I and II fibers, type IIc may represent the mATPase characteristics of denervated and multi-innervated muscle fibers.

It is generally accepted that denervated and newly de-

veloped fibers represent the undifferentiated type IIc phenotype<sup>25,26)</sup> during the innervation process until a single innervation state is established. Type IIc fibers have also been observed in skeletal muscle injured by toxin injection<sup>27)</sup>. The final fiber phenotype (I, IIa, IIc, or IIb) including the expression of a corresponding myosin heavy chain isoform is known to be decided according to the type of the finally selected single motor neuron innervating the fiber<sup>7,28)</sup>. Therefore, it is highly probable that the fiber type transition can be propagated by denervation,



**Fig. 7** Light micrographs of transverse sections of plantaris muscle from Training + Downhill group. A, B, and D: mATPase staining (pre-incubation at pH 4.6); C and E: SDH staining. B and C, and D and E are pairs of serial cross-sections. D and E show a portion of A at a higher magnification. Specific cluster of type IIa fibers (arrow in A) were frequently observed. Note that type IIa fibers are lightly stained in mATPase staining after acidic pre-incubation (A, B and D) and densely stained for SDH (C and E).

**Table 1.** Effects of long-term (9 weeks total) endurance training on body weight, plantaris muscle weight and contractile properties.

	Control	Training	Training + Downhill
Body weight (g)	176.7 ± 8.2	182.2 ± 12.6	174.9 ± 24.0
Muscle weight (mg)	162.8 ± 10.5	168.3 ± 6.8	173.6 ± 20.9
Maximum tension (N/g muscle weight)	19.3 ± 3.04	21.3 ± 2.08	21.4 ± 2.00
Twitch contraction time (ms)	16.8 ± 2.3	17.0 ± 0.7	17.1 ± 1.9
Fatigue Index at 2min (%)	43.1 ± 6.7	59.7 ± 10.1 *	64.4 ± 10.5 *
Fatigue Index at 5min (%)	28.1 ± 6.0	35.4 ± 5.0	37.0 ± 3.4 *

\*p<0.05 significantly different from control group.

degradation, and generation of a considerable population of muscle fibers. Because the rats in Experiment A were kept sedentary except for the single downhill-running session, no fiber type transition stimulus was present. Therefore, it is unsurprising that no type transition was observed after the single session beyond the degradation-regeneration period (Fig. 4).

***Incorporation of intensive training sessions promotes fiber type transition.*** Abundant centrally located nuclei in the muscle of the Training + Downhill group, but not in others (Fig. 6), demonstrate that the earlier downhill running sessions had induced muscle injury as it did in the single downhill running session in Experiment A. The plantaris muscle was evaluated 2 weeks after the

last downhill-running session in Experiment B, but the intrinsic acute muscle injury effects of a single downhill-running session peaked 2–3 days after the session in Experiment A (Figs. 2–4).

Therefore, it is likely that the plantaris muscle of the Training + Downhill group showed no morphological (Fig. 6) signs of acute injury. Furthermore, since repetitive bouts of eccentric exercise are known to reduce the muscle-injuring effects of subsequent strenuous eccentric contractions, a phenomenon referred to as the “repeated bout effect”<sup>29–31</sup>, downhill-running sessions taken during the later phase of the Training + Downhill period probably caused less injurious effects.

Significant outcomes from the 9-week endurance-training protocol were increased fatigue resistance (Table 1) and fiber type transition from type IIb to IIa (Fig. 5). These outcomes indicate that the protocol is sufficiently intense as endurance training. Among the outcomes, the intermittent downhill running sessions significantly enhanced fiber type transition. Although the fatigue indices were not significantly different between the Training and Training + Downhill groups (Table 1), the direction of the fiber type transition indicates that the incorporation of the downhill running sessions also promoted oxidative capacity of the muscle.

***Intermittent intensive training sessions may cause pathological fiber type cluster formation.*** Although body weight, muscle weight, maximal tetanic force, and twitch contraction time were little affected by the daily endurance training even with the downhill running sessions, clusters of type IIa fibers were observed as a specific effect of the intermittent downhill running sessions (Fig. 7). Since a single downhill running session in Experiment A and endurance training in Experiment B induced no such effect, repetition of the intensive downhill running session, or incorporation of the downhill running session into the daily endurance training protocol is necessary for the emergence of the clusters.

One mechanism that could account for the emergence of fiber clusters of a specific fiber type is axonal sprouting with de novo synapse formation in regenerated muscle fibers<sup>32</sup>. In this case, the cluster formation in the Training + Downhill group would be a result of degradation-regeneration processes following a significant histological muscle injury induced by the earlier sessions. Similar cluster formation has been observed with obvious muscle atrophy induced by the denervation procedure<sup>39</sup>.

The cluster formation of type IIa fibers in the Training + Downhill group would be a result of degradation-regeneration processes under pressure for fiber type transition from the daily endurance-training. Recently, studies have been performed to identify the molecular pathways involved in the fiber type transition. Several studies have demonstrated that calcineurin plays an important role in expression of the slow-twitch muscle phenotype dur-

ing muscle regeneration<sup>33,34</sup>. Calcineurin, a calcium-calmodulin activated serine-threonine phosphatase, has been shown to mediate the effect of innervating neurons toward slow-fiber-type gene transcription by dephosphorylating transcription factors, such as nuclear factor of activated T-cells (NFAT)<sup>33</sup>. More recently, Launay et al.<sup>35</sup> reported that the increase or the decrease in activation of motor neuron firing during repeated exercise or immobilization could alter the peaks of calcineurin phosphatase activity. In addition, calcineurin is shown to activate the IIa myosin heavy chain promoter preferentially compared with the IIx and IIb myosin heavy chain promoters in cell culture<sup>36</sup>. These reported results suggest a possibility that the degradation-regeneration processes with reinnervation strongly promote fiber type transitions through this signaling pathway.

Fiber type cluster formation has been frequently observed in pathological muscle in patients with chronic peripheral neuropathy and somewhat less often in the advanced stage of motor neuron diseases<sup>37,38</sup>. No single fiber type forms an appreciable cluster in physiological skeletal muscle, which has been optimized with evolution. Fiber type cluster likely impacts muscle performance adversely: for instance, force development and shortening of locally clustered fibers of any specific type would cause local mechanical strain in the muscle and tendon.

***An optimized intensive training session protocol is necessary to balance the beneficial and adverse effects.*** When incorporated into a daily endurance-training protocol, intermittent downhill running sessions were shown to have a beneficial effect on promoting fiber type transition, which likely facilitates fatigue resistance. On the other hand, a huge cluster formation of type IIa fibers may lead to an adverse effect. Muscle fiber degeneration and regeneration occurring in a large muscle area would cause the cluster-like transition to type IIa fibers. An optimized level for the intensity and frequency of the high-intensity sessions is necessary to balance the benefits with the potential adverse effects. To some extent, the ‘repeated bout effect’ may serve to reduce the effective intensity of the high-intensity sessions to optimize the intensity level.

Although the effect of intermittent high-intensity downhill running sessions were studied only on daily endurance training in this study, the beneficial effects would likely be less evident when high-intensity sessions are incorporated into a daily resistance training protocol. This is because degradation and denervation of muscle fibers impedes the hypertrophy induced by resistance training<sup>40,41</sup>, at least until regeneration proceeds to recover the original mass and power of the muscle. It is of interest, however, whether or not the regeneration process would continue beyond the recovering point as a long-term effect of the high-intensity sessions.

Nonetheless, intense exercise sessions leading to the formation of fiber type-specific clusters should be avoid-

ed for better outcomes of any training. In search of better efficiency in training protocols, a possibility of the cluster formation should be taken into account. An effort to establish practical and convenient criteria for limiting the intensity is awaited.

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