High bone mass gained by exercise in growing male mice is increased by subsequent reduced exercise

Jian Wu,¹ Xin Xiang Wang,¹ Mitsuru Higuchi,²,³ Kazuhiko Yamada,¹ and Yoshiko Ishimi¹

¹Division of Applied Food Research and ²Division of Health Promotion, The National Institute of Health and Nutrition, Tokyo 162-8636; and ³School of Sport Sciences, Waseda University, Tokyo 359-1192, Japan

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THE INCIDENCE OF OSTEOPOROSIS increases with advancing age, and the risk of osteoporosis later in life is determined by the peak bone mass attained during childhood and adolescence (1, 4, 5, 16, 33). In humans and animals, exercise training during growth increases the peak bone mass (2, 13, 19, 36, 39, 40). However, several studies suggest that exercise-induced bone gains are lost if exercise ceases (11, 14, 17, 29, 42). Therefore, continued exercise training is necessary to maintain exercise-gained bone mass. However, these self-selected sports players are an elite group whose skeletal status is already above average, making it possible to gain and sustain increased bone mass within a period of intensive training followed by reduced exercise (25). Thus a controlled randomized trial to examine whether reduced exercise maintains bone mass is needed to clarify this issue. To our knowledge, the effect of reduced exercise on the maintenance of bone mass in animals has not been shown. In this study, we evaluated whether reduced exercise with half the frequency after 4 wk of moderate treadmill running in growing male mice results in the maintenance of high bone mass.

MATERIALS AND METHODS

Animals and interventions. Four-week-old ddY-strain male mice were purchased from the Shizuoka Laboratory Animal Center (Shizuoka, Japan) and fed an AIN-93G diet (Funabashi Farm, Chiba, Japan) (30). They were individually housed in 24 × 15 × 15 cm cages under a 12:12-h light-dark cycle at 22°C and allowed free access to water and diet. After a week of adapting to the environment, the mice were randomly divided into the following six body weight–matched groups with eight mice in each group: 1) baseline control; 2) 4 wk control (4C); 3) 4-wk exercise (4E); 4) 8-wk control (8C); 5) 4-wk exercise followed by 4-wk cessation of training and 6) 4-wk exercise followed by reduced exercise at half the frequency. The regimen consisted of exercise 6 days/wk, and the reduced exercise regimen consisted of running 3 days/wk on a treadmill for 30 min/day, at 12 m/min on a 10° uphill slope. Running exercise significantly increased bone mineral density of the femur, periosteal mineral apposition rate, bone formation rate, percent labeled perimeter at the midfemur, and osteogenic activity of bone marrow cells. However, these parameters declined to the age-matched sedentary control after cessation of training. In contrast, the reduced exercise group had significantly higher mineral apposition rate compared with those of the sedentary control and cessation of training groups. Furthermore, bone mineral density for the reduced exercise group was significantly higher than those for the other groups. These results suggest that the high bone formation gained through exercise can be maintained, and bone mass was further increased by subsequent exercise even if the exercise frequency is reduced.

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Bone mineral measurements. The bone mineral content (BMC) and BMD of the femur were measured by dual-energy X-ray absorptiometry by using a bone densitometer adapted for small animal research (model DCS-600R; Aloka, Tokyo, Japan). BMC of the mouse femur was closely correlated with the ash weight (r = 0.978) (23). BMD was calculated by using the BMC of the measured area.

Histomorphometric analysis. Histomorphometry was performed using a semiautomated image analyzing system (Osteoplan; Carl Zeiss, Thornwood, NY) (20) linked to a light microscope. An undecalcified section was obtained from the site of middiaphysis of the femur. The specimen was embedded in methylmethacrylate without staining to yield a 40-μm-thick cross-cut ground section. Dynamic

Address for reprint requests and other correspondence: Y. Ishimi, Division of Applied Food Research, National Institute of Health and Nutrition, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8636, Japan (E-mail: ishimi@nih.go.jp).

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parameters such as mineral apposition rate (MAR, interlabel width/day), percent labeled perimeter (single- and double-labeled perimeters/bone surface (BS)), and bone formation rate (BFR/BS, MAR × (double-labeled surface + single-labeled surface/2) × BS⁻¹ × 10⁻¹⁰⁻¹) at the periosteal and endocortical surface were measured by calcine double labeling.

Cell culture and assay. Marrow cultures were initiated according to the method of Maniatopoulos et al. (21). The proximal epiphyseal end and the distal-most third of the tibia cortex were cut away. Bone marrow was flushed out from the proximal cut end, a single-cell suspension was prepared by repeated aspiration, and then the bone marrow cells were counted by using a hemocytometer. To determine the number of alkaline phosphatase-positive (ALP⁺) colony-forming unit-fibroblasts (CFU-F) and ALP activity in the cultures, bone marrow cells (1 × 10⁶ cells) were cultured in 12-well plastic plates in α-MEM containing 10% FBS. To identify ALP⁺ CFU-F colonies, the cultures were maintained for 6 days, fixed with 10% formalin in PBS for 10 min, and washed with 0.2 mM of Tris-HCl buffer (pH 7.6) containing 0.1% Triton X-100 and 10 mM MgCl₂ on day 6 (34). Enzyme activity was measured colorimetrically using p-nitrophenylphosphate (pH 10) (Sigma) as a substrate and recoding the optical density at 405 nm. Also, the protein content of the cultures was measured for 20 min at room temperature. ALP⁺ colonies containing more than 50 cells were designated as CFU-F colonies. ALP activity was determined by extracting the cultures in 1 ml of 10 mM Tris-HCl (pH 7.6) containing 0.1% Triton X-100 and 10 mM MgCl₂ on day 6 (34). Enzyme activity was measured colorimetrically using p-nitrophenylphosphate (pH 10) (Sigma) as a substrate and recoding the optical density at 405 nm. Also, the protein content of the dishes was determined by the Lowry method, and ALP activity was expressed as units per milligram protein per minute.

Statistical analysis. Results were presented as means ± SD. One-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons was used to determine significant differences among the experimental groups. Statistical analyses were performed using the SAS program. Differences were considered significant for P < 0.05.

RESULTS

Body weight, BMD, and bone area. The body weight of the mice at baseline and after 4 and 8 wk is shown in Fig. 2. The initial body weight was the same for all groups (28.3 ± 0.3 g). The final body weight for the 4- and 8-wk groups was significantly greater than that of their baseline counterparts. It was significantly higher for the 8C group than for the 4C and 4E groups. The final body weight of the 4E4R group was also significantly greater than that of 4E group. However, the body weights did not significantly differ between the exercise and control groups after 4 wk. Furthermore, the body weight of the 4E4C and 4E4R groups did not significantly differ from that of the 8C group.

Figure 3 shows BMD and bone area of the femur at baseline and after 4 and 8 wk. The femoral BMD and bone area significantly increased in the 9-wk-old mice (4C) owing to the growth spurt, compared with the 5-wk-old mice (baseline). BMD also significantly increased after 4 wk of exercise training (4E), compared with the 4 wk control group (4C). There was no difference in BMD between the 4E4C and 8C groups. However, BMD was further increased by reduced exercise (4E4R), and the value was significantly higher than those of the other groups.
other groups. Bone area for the 4E4C and 4E4R groups was slightly higher than that for the other groups.

**Bone histomorphometry.** Figure 4 shows the histological parameters of bone formation in the cortical bone of the femoral diaphysis. On the periosteal surface, MAR and BFR/BS for the 4C group were higher than those for the 8C group. MAR, percent labeled perimeter, and BFR/BS in the 4E group were significantly higher than for the 4C group. However, these parameters in the 4E4C group declined to the same level as those in the 8C group. In contrast, in the 4E4R group, MAR and BFR/BS were maintained at a high level compared with those in 4E4C and 8C groups. However, percent labeled perimeter in the 4E4R was not significantly different from those in the 4E4C and 8C groups. On the endocortical surface, MAR and BFR/BS of the 4C and 4E groups were higher than those of the 8C, 4E4C, and 4E4R groups, although the differences were not statistically significant.

**Number of CFU-F colonies and ALP activity in bone marrow cultures.** The number of CFU-F colonies and ALP activity in bone marrow cultures in the 4E group were significantly higher than that for the 4C group (Fig. 5). The number of CFU-F colonies and ALP activity were also higher for the 4E4R group compared with those for the 8C and 4E4C groups, although they were not statistically significant.

**DISCUSSION**

The present study suggests that an exercise-induced high bone mass was further increased and that high MAR and BFR/BS can be maintained with a subsequent reduced exercise schedule in growing male mice.

Previous studies reported that exercise-induced bone gains are poorly maintained after cessation of the exercise. Yeh and Aloia (42) showed that bone mass gained through treadmill running exercise is lost during deconditioning as a result of a decline in bone formation (indicated by bone uptake of $^{45}$Ca) and an increase in bone resorption (indicated by urinary excretion of $[^{3}H]$tetraacycline) in young female rats after the growth spurt. In an extension of the study by Yeh and Aloia, Iwamoto et al. (11) reported that the 4 wk of deconditioning after 8 wk of exercise in growing female rat results in a decrease in bone formation on the periosteal surfaces and a decrease in bone mass of the femur. Furthermore, exercise-induced positive effects on femoral neck characteristics in rats that trained during adolescence gradually disappear after deconditioning, which can be seen in an almost lifelong follow-up (56 wk) later (29). Our findings are consistent with these reports. In the present study, we found that stopping exercise declined MAR and BFR/BS to the same level as those of sedentary controls. However, the parameters of bone for-
mation were maintained at a high level by the additional 4 wk of reduced exercise. Furthermore, the BMD was further increased by the reduced exercise in the growing mice (Fig. 3). Therefore, exercise during the growth spurt period enhances bone formation and results in a relatively high bone mass (8, 9, 13), which can be maintained or increased even when the exercise frequency is reduced by half.

Similar to animal studies, the effects of stopping exercise on bone mass have been seen in human studies. Brooke-Wavell et al. (3) reported that calcaneal BMD in postmenopausal women declines after walking exercise has stopped. Dalsky et al. (6) also showed that weight-bearing exercise leads to a significant increase in lumbar BMC above baseline, but BMC reverts to baseline after stopping exercise. In premenopausal women, Winters and Snow (37) found that the positive benefits of impact plus resistance training on BMD in the proximal femur reverse when training is withdrawn (37). Similar results are also seen in young women, in whom unilateral limb strength training increases BMD in the lower extremity, but BMD declines toward baseline after the training has stopped (34). According to these findings, continued exercise at a reduced frequency or intensity is required to maintain an increase in bone mass resulting from exercise.

Bone formation was augmented in the early stages of exercise in the cortical bones, which was suggested in previous studies (10, 24, 26, 27). In this study, the running exercise significantly increased the parameters governing periosteal bone formation in the midfemur. However, endocortical bone formation parameters were not affected by running exercise.

Thus the effects of running exercise on bone formation were site specific in the femoral cortex, supporting accelerated cortical drift by mechanical stimulation. This result is similar to those of Notomi et al. (28), who found that treadmill and climbing exercise did not affect bone formation at the endocortical surface. One explanation proposed by Frost and colleagues (7, 32) is that different mechanical thresholds exist on cortical envelopes.

Cell cultures were used to examine the association between mechanical stress on bone and the osteogenic potential of bone marrow cells. Osteogenic differentiation in cultures decreases after the removal of mechanical loads caused by paralysis in humans and animals subjected to spaceflight, immobilization, or tail suspension (12, 15, 22, 31, 38). In this study, we first reported that ALP activity and ALP+ CFU-F colonies in bone marrow cultures were significantly increased by exercise training but declined after cessation of training (Fig. 5). These results suggest that increase in osteoblast activity and number in bone marrow may associate with the increase in bone mass by running exercise in the growing mice. Furthermore, in vivo analysis, the periosteal MAR indicating osteoblast activity and periosteal-labeled perimeters indicating the change of osteoblastic recruitment in the 4E group were significantly higher than those in the 4C group (Fig. 4) (11). These findings are consistent with the data in bone marrow cell cultures. However, our study lacked the direct evidence of increase in osteoblast number analyzed by static histomorphometric measurements. This limits the interpretation regarding increase in osteoblast activity vs. osteoblast numbers by exercise training. On the other hand, although BMD was increased by reduced exercise, the periosteal-labeled perimeters and ALP+ CFU-F colonies in bone marrow cells were not different from age-matched sedentary control. Thus it is suggested that the mechanisms of bone maintenance by reduced exercise may be associated with increased osteoblastic activity. Further studies are needed to determine the accurate mechanisms responsible for the responsiveness of skeleton to exercise and reduced exercise.

In this study, we showed that an exercise-induced high BMD could be enhanced subsequently with a reduced exercise schedule in growing male mice. Further studies are necessary to define whether the high BMD and bone formation would be maintained in other ages and to examine the effect of reduced exercise in longer periods.

In conclusion, moderate running exercise increases bone mass of the femur in growing mice as a result of increased bone formation that may be due to both osteoblast number and activity. The exercise-induced high bone formation was maintained, and bone mass was further increased by subsequent exercise even at a lower frequency in young adult period.

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REFERENCES


