

Menatetrenone Prevents Osteoblast Dysfunction in Unilateral Sciatic Neurectomized Rats

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Received February 14, 2002 Accepted July 5, 2002

ABSTRACT—Menatetrenone (MK-4) inhibits bone resorption and enhances osteoblast-induced mineralization. In this study, we examined whether MK-4 administration had beneficial effects on osteoblast dysfunction and trabecular microstructure as well as on bone volume loss in a rat model of osteopenia. Male Sprague-Dawley rats were neurectomized and administered MK-4 as a daily supplement. On Day 21 after neurectomy, significant bone loss was observed in the positive control rats. MK-4 prevented the decrease in bone mineral density of the distal metaphysis of the femur. The osteoclast surface per bone surface (Oc.S/BS) and the number of osteoclasts per bone perimeter (N.Oc/B.Pm) were reduced and the mineral apposition rate (MAR) decreased in the immobilized rats on Day 42, suggesting suppression of bone turnover. In contrast, administration with a low dose of menatetrenone led to an increase of MAR and bone formation rate (BFR), while Oc.S/BS and N.Oc/B.Pm remained at normal levels. These data suggested that MK-4 reduced the loss of trabecular bone, prevented osteoblast dysfunction to a certain extent, and contributed to preservation of the trabecular microstructure in this rat model of osteopenia induced by sciatic neurectomy.

Keywords: Menatetrenone, Immobilization, Bone mineral density, Osteoblast function

Many studies have suggested that vitamin K participates in bone metabolism. In previous reports, circulating levels of vitamin K in osteoporotic patients with fracture were significantly lower than those in control subjects matched for age (1–4). Vitamin K is essential for the γ -carboxylation of osteocalcin. Undercarboxylation of osteocalcin is related to a higher risk of fractures in osteoporotic patients (5, 6) and vitamin K-depletion induces an increase in the urinary excretion of calcium and hydroxyproline in rats (7). Vitamin K is known to promote the post-translational modification of vitamin K-dependent proteins such as osteocalcin. Osteocalcin is a protein that contains gamma-carboxyglutamic acid and it is synthesized only by osteo-

blasts. Plasma osteocalcin is used clinically as a biochemical marker of bone formation.

Two types of vitamin K occur in nature. Vitamin K₂ (menaquinone) is a series of vitamers with multi-isoprene units at the 3-position. Menatetrenone (MK-4) is a vitamin K₂ homologue with unique characteristics; MK-4 inhibits bone resorption (8–11) and enhances osteoblast-induced mineralization (12) in vitro. The effects of MK-4 on bone metabolism were demonstrated in a model of postmenopausal osteoporosis (13–15). It is generally accepted that bone resorption is increased in ovariectomized rats. Various studies have suggested that MK-4 prevents to some extent the bone loss induced by ovariectomy by inhibiting bone resorption (13–15). However, it has not been clarified as yet whether MK-4 influences the function of osteoblasts in vivo.

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Bone volume loss due to long-term bed rest has long been recognized as one of the major health problems affecting elderly patients with fracture. Unloading of bones by immobilization leads to systemic or local bone loss known clinically as disuse osteoporosis. Immobilization-related bone loss in rats subjected either to tenotomy or sciatic neurectomy is a rapid process. Previous reports revealed that the mineral apposition and bone formation rates decreased while the number of osteoclasts increased, resulting in an imbalance between bone formation and bone resorption that led to a rapid loss of bone (16–18).

The purpose of this study was to evaluate the effect of MK-4 on osteoblasts *in vivo*. We measured bone mineral density (BMD) and bone histomorphometric parameters to assess the effects of MK-4.

MATERIALS AND METHODS

Experimental protocol

Seventy-eight male Sprague-Dawley rats (Nippon Bio Supply Center Co., Ltd., Tokyo) weighing approximately 440 g were acclimated to local vivarium conditions (12-h light/dark cycle at $23 \pm 1^\circ\text{C}$) for 7 days. During the experimental period, the rats were allowed free access to water and standard powder feed (#92095; Harlan Teklad, Madison, WI, USA), which contained 0.7% (w/w) calcium and 0.6% (w/w) phosphorus. They were checked daily and their body weight and food intake were measured three times per week to monitor their general health. Six animals were sacrificed at the beginning of the experiment as basal controls. The rest of the rats were divided into four groups (18 animals each) and were subjected to unilateral sciatic neurectomy or sham operation. Sciatic neurectomy was performed on the right hindlimb. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium, a dorsolateral incision was made on the right hip through which the sciatic nerve was exposed, and a 0.5-cm segment was excised. The muscle and skin were sutured to its case. The sham-operated control rats underwent an operation similar to that of the unilateral sciatic neurectomy group, except that the nerve was mobilized but not transected. The right hindlimb immobilized rats (IM) were administered either the vehicle (IM + control), 10 mg/kg MK-4 (IM + LK) or 30 mg/kg MK-4 (IM + HK). MK-4 (menatetrenone; Eisai Co. Ltd., Tokyo) was given as a dietary supplement (17.5 mg/100 g or 52.5 mg/100 g of the diet, respectively). The actual intake of menatetrenone was 10 or 30 mg/kg body weight/day, respectively, as calculated by the average diet intake and menatetrenone content in the diet. The dosages of MK-4 were selected based on the results of earlier studies (13, 15). Sham-operated and immobilized rats were sacrificed on Days 7, 21 and 42.

The animals used in this study were treated in compli-

ance with the guiding principles for the care and use of the animals established by the American Journal of Physiology. Protocols of the experiments were approved by the institutional animal care committee.

Bone size

The length (mm) and middiaphyseal width (mm) of the tibia were measured with a micrometer.

Serum biochemistry

Blood samples were obtained from the abdominal aorta at sacrifice. Serum Ca, Pi, and alkaline phosphatase activity were measured with an autoanalyzer (Hitachi736; Hitachi Co., Ltd., Hitachi-City). Serum PTH levels were measured with an immunoradiometric assay kit for rat PTH (Nichols Institute, San Juan Capistrano, CA, USA). Serum levels of $1,25(\text{OH})_2\text{D}_3$ were measured by radioreceptor assay using vitamin D receptors derived from calf thymocytes. Serum MK-4 concentration was measured by high-performance liquid chromatography according to the method of Langenberg and Tjaden (19) on the samples of Day 42.

Bone mineral densitometry (BMD)

At sacrifice, femora and tibiae were separated from adherent muscles and connective tissues other than the periosteum. BMD of the right femora was measured by dual energy X-ray absorptiometry (DXA) using QDR-1500 (Hologic, Waltham, MA, USA) with ultra high-resolution mode (rat mode, version 4.59 software). The coefficients of variation of BMD were less than 1% in the rat mode using the step phantom for small animals. The scan image of each femur was divided into four portions of equal length. BMD values were obtained for the distal one-fourth of the femur including the epimetaphyseal region (20).

Bone histomorphometry

On Day 42, the right proximal tibia was removed from each rat, fixed in 70% ethanol and embedded in glycol-methacrylate (Wako Pure Chemical Industries, Ltd., Osaka) without decalcification. Then serial sections ($3 \mu\text{m}$ in thickness) were cut longitudinally using a microtome (Model 2050; Reichert Jung, Buffalo, NY, USA), and sections were further stained with the Villanueva Goldner stain to discriminate between mineralized and unmineralized bone and to identify cellular components. All rats were subcutaneously injected with calcein (Wako Pure Chemical Industries, Ltd.) (8 mg/kg body weight) 7 and 3 days prior to sacrifice for labeling. Five-micron sections with no-staining were used to clearly visualize calcein labels under fluorescent light microscopy.

Histomorphometric analysis of secondary spongiosa of the proximal metaphysis between 1.2- and 3.6-mm distal to the growth plate-epiphyseal junction was performed using

semiautomated systems (Osteoplan II; Carl Zeiss, Thornwood, NY, USA), and measurements were made at $\times 200$ magnification. Total tissue area, cancellous bone area and perimeter were used to calculate percentage cancellous bone area (BV/TV, %), trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, /mm), and trabecular separation (Tb.Sp, μm). Dynamic parameters were as follows: Single- and double-labeled and total bone surface in the secondary spongiosa were traced at $200\times$, and then single-labeled (sLS/BS, %) and double-labeled (dLS/BS, %) surfaces were calculated as a percentage of the total bone surface. Labeling width was calculated as the average distance between the double-labels, and mineral apposition rate (MAR, $\mu\text{m}/\text{day}$) was calculated by dividing the labeling width by the number of days between the two calcein administrations. Bone formation rate per bone surface (BFR/BS, mm^3/cm^2 per year) was the product of (sLS/2+dLS) \times MAR/BS. Parameters for bone resorption were as follows: In sections subjected to TRAP staining, TRAP-positive cells that formed resorption lacunae on the surface of the trabeculae and more nucleus were identified as osteoclasts. Trabecular osteoclast surface (Oc.S/BS, %) and number of osteoclasts (N.Oc/B.Pm, per 100 mm) were determined. The nomenclature, symbols and units used in

this study are those recommended by the American Society for Bone Mineral Research (ASBMR) Nomenclature Committee (21).

Statistical analyses

All data were expressed as the mean \pm S.D., and the statistical analyses were performed by one-way analysis of variance (ANOVA). A *P* value less than 0.05 was considered statistically significant.

RESULTS

General conditions and bone size

All the rats remained healthy during the experimental period. No difference in final body weight, food intake, body weight gain, behavior, or appearance was observed among the groups. The middiaphyseal width in the sham group and the groups administered MK-4 was significantly greater than that in the IM + control group (Table 1).

Serum biochemistry

There was no difference in serum levels of Ca, Pi or PTH among these four main groups; i.e., sham-operated, IM + control, IM + LK and IM + HK at each determination

Table 1. Effects of sciatic neurectomy and MK-4 on body weight and bone size at the end of the experimental period

	Body weight (g)	Denervated right tibia	
		Length (mm)	Middiaphyseal width (mm)
Day 0			
Basal controls	448.2 \pm 27.0	38.0 \pm 0.4	3.7 \pm 0.1
Day 7			
Sham	459.3 \pm 23.9	38.5 \pm 0.4	3.8 \pm 0.2*
IM + control	420.0 \pm 17.3	38.0 \pm 0.9	3.5 \pm 0.1
IM + LK	416.0 \pm 41.3	38.1 \pm 0.3	3.7 \pm 0.2
IM + HK	452.7 \pm 16.4	39.0 \pm 0.7	3.7 \pm 0.1
Day 21			
Sham	503.3 \pm 15.3	40.1 \pm 0.8	3.9 \pm 0.3
IM + control	466.7 \pm 15.3	40.4 \pm 0.7	3.7 \pm 0.1
IM + LK	460.7 \pm 24.2	39.2 \pm 0.2	3.6 \pm 0.1
IM + HK	482.7 \pm 12.7	39.7 \pm 0.6	3.6 \pm 0.2
Day 42			
Sham	559.3 \pm 12.0	42.3 \pm 0.3	4.2 \pm 0.1
IM + control	492.7 \pm 47.0	42.1 \pm 0.8	3.8 \pm 0.1
IM + LK	496.7 \pm 25.2	42.6 \pm 0.9	3.9 \pm 0.1
IM + HK	544.7 \pm 23.4	42.8 \pm 1.0	4.1 \pm 0.1*#

Vitamin K₂ (menatetrenone) was given as a dietary supplement. IM, the right hindlimb immobilized rats; IM + control, IM rats were administered with vehicle; IM + LK, IM rats were administered 10 mg/kg MK-4; IM + HK, IM rats were administered 30 mg/kg MK-4. Each value represents the mean \pm S.D. of 6 animals. **P*<0.05, compared with IM + control; #*P*<0.05, compared with IM + LK.

point (data not shown). The serum concentration of MK-4 on Day 42 of the administration was 2.32 ± 1.74 ng/ml in the sham group, 3.35 ± 2.36 ng/ml in the IM + control group, 440.14 ± 141.09 ng/ml in the IM + LK group, and 2666.67 ± 828.00 ng/ml in the IM + HK group.

BMD as assessed by DXA

On Day 21 after the operation, BMD of the femoral distal metaphysis was significantly decreased in control rats compared with that of sham-operated rats. On Day 42, the BMD in femoral distal metaphysis was nearly kept at in the same level as that sham-operated rats in the IM + HK group (Fig. 1).

Bone histomorphometry

The trabecular bone volume decreased after neurectomy (Table 2). The volume of trabecular bone and trabecular thickness decreased significantly, whereas trabecular free space increased in control rats as compared with that of sham-operated rats. Histomorphometric parameters indicated that bone loss was suppressed in the IK + HK group.

Results of the bone turnover parameters are summarized in Table 3. MAR and BFR, parameters of osteoblast function, were significantly lower in IM + control rats. Administration of 10 mg MK-4 prevented such a decrease in these parameters. N.Oc and Oc.S, bone resorption parameters, were significantly decreased in the IM + control group.

Decrease of these parameters was prevented and maintained at normal levels by administration of 10 mg/kg MK-4.

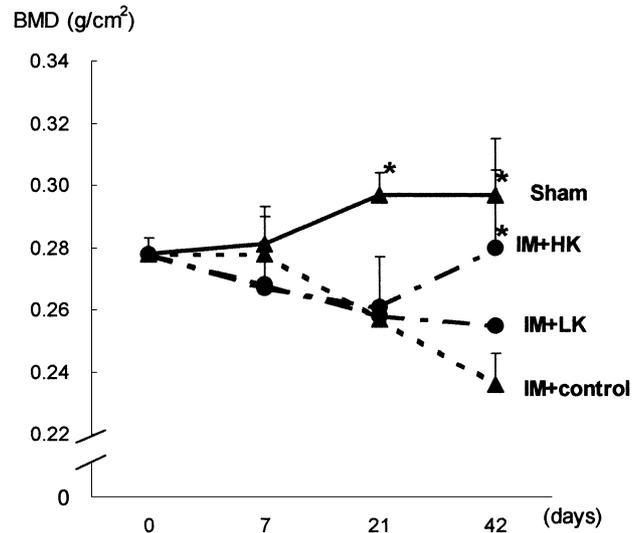


Fig. 1. Prevention of BMD decline by MK-4 administration. Data are each the mean value \pm S.D. of 6 animals. * $P < 0.05$, compared with IM + control group.

Table 2. Protection by MK-4 against disuse osteopenia: histomorphometric parameters of the right tibial proximal metaphysis

	BV/TV (%)	Tb.N (/mm)	Tb.Th (μ m)	Tb.Sp (μ m)
Sham	$17.53 \pm 4.12^*$	$3.01 \pm 0.70^*$	58.28 ± 0.10	$283.64 \pm 79.97^*$
IM + control	9.06 ± 1.17	1.83 ± 0.05	49.15 ± 5.13	496.96 ± 18.78
IM + LK	14.77 ± 5.07	$2.33 \pm 0.22^*$	62.68 ± 15.95	$368.53 \pm 55.91^*$
IM + HK	$33.63 \pm 5.04^{*#}$	$4.52 \pm 4.12^*$	$74.25 \pm 58.88^*$	$147.70 \pm 21.62^{*#}$

BV/TV, Bone volume per tissue volume; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation. These parameters are used to evaluate the trabecular structure of the cancellous bone area. * $P < 0.05$, compared with IM + control group; # $P < 0.05$, compared with IM + LK group.

Table 3. Effects of MK-4 on bone turnover: histomorphometric analysis of the right tibial proximal metaphysis

	MAR (μ m/day)	BFR/BS (mm^3/cm^2 per year)	N.Oc/B.Pm (/100 mm)	Oc.S/BS (%)
Sham	$2.38 \pm 0.57^*$	$23.84 \pm 5.34^*$	$222.68 \pm 30.29^*$	$7.04 \pm 1.50^*$
IM + control	1.59 ± 0.12	11.61 ± 3.96	143.86 ± 24.53	4.98 ± 0.89
IM + LK	$2.72 \pm 0.38^*$	$21.6 \pm 4.62^*$	$198.57 \pm 50.65^*$	$7.17 \pm 0.89^*$
IM + HK	$2.35 \pm 0.01^*$	15.38 ± 2.32	$45.12 \pm 10.26^{*#}$	$2.21 \pm 0.73^{*#}$

MAR, mineral apposition rate; BFR/BS, bone formation rate per bone surface; N.Oc/B.Pm, number of osteoclasts per bone perimeter; Oc.S/BS, osteoclast surface per bone surface. * $P < 0.05$, compared with IM + control group; # $P < 0.05$ compared with IM + LK group.

DISCUSSION

Sciatic neurectomized rats are used as an experimental model of immobilization-induced osteopenia. Immobilization-related bone loss is a rapid process. Within the first 10 days of immobilization, significant bone resorption is observed, as evidenced by the increase in the number of osteoclasts and the extent of resorption, but bone formation, estimated from the extent of formation area and mineral apposition rate, is decreased (17, 18). Disuse osteopenia is known to frequently develop in patients with long-term bed rest, paralysis after spinal cord injury, and plaster cast fixation. Sato et al. reported that bone mineral density decreased on the hemiplegic and contralateral sides in stroke patients (22), and treatment with MK-4 could increase the BMD in disuse osteopenia and vitamin K deficient hemiplegic bone by increasing the concentration of vitamin K. They also reported that serum calcium levels decreased through inhibition of bone resorption (23).

Recent studies indicate that MK-4 can act on bone metabolism (9–13). MK-4 inhibits bone resorption and enhances osteoblast-induced mineralization. However, the effect of MK-4 on bone metabolism has not been extensively examined by bone histomorphometry.

This study clearly demonstrated that in the tibiae of immobilized rat administered MK-4, bone mass increased and the structure of secondary spongiosa was maintained. Loss of trabeculae as the result of immobilization was obvious in the secondary spongiosa. In the group administered MK-4, the number of trabeculae in secondary spongiosa was maintained. In the IM + HK group, both the number of trabeculae and trabecular thickness increased, resulting a larger bone volume, above the levels observed in the sham-operated group. The bone microstructure was maintained by MK-4 administration and BMD was increased in the high-dose administration group.

MK-4 administration may have beneficial effects on bone turnover and trabecular microstructure as well as on bone volume loss in sciatic neurectomized rats. Bone histomorphometry analysis revealed that bone turnover was decreased on Day 42 after neurectomy. In the MK-4 administration groups, mineral apposition rate and bone formation rate were increased and the number of osteoclasts and osteoclast surface were kept at normal levels.

The effects of MK-4 may be partly assessed by gamma-carboxylation of osteocalcin. A recent report by Shiraki and associates suggest that 200 times higher serum concentration of vitamin K₂ (MK-4) lead to increase of serum Gla-osteocalcin (24). In our study, serum MK-4 concentration reached levels comparable with those of their report. Although we could not measure serum levels of Gla-osteocalcin because no assay system for rat Gla-osteocalcin is available at the moment, it is highly possible that mainte-

nance of bone change in neurectomized rats was the result of MK-4 administration.

On the other hand, Koshihara et al. reported the effects of vitamin K₂ on mineralization by human osteoblasts in vitro (12). They observed that vitamin K₂ at 2.5 μM enhanced in vitro mineralization in the presence of 1,25(OH)₂D₃. In our study, the serum concentration of MK-4 in the IM + LK group was in the order of 10⁻⁶ M. Our results were in agreement with the in vitro data obtained by Koshihara et al. MK-4 is thought to prevent bone loss through the maintenance of the functions of osteoblasts.

In conclusion, our findings suggest that MK-4 administration protects against the loss of trabecular bone volume and its structure in the osteoporotic rats induced by sciatic neurectomy. Treatment and prevention of bone abnormalities should be aimed not only at the restoration of bone volume, but also at that of bone structure and metabolism. MK-4 may be useful for preventing disuse osteopenia in humans.

Acknowledgments

This work was supported in part by a Special Grant for Metabolic bone disease, Japan (to MF). We are grateful to Mr. Naohisa Hanawa for technical assistance.

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