Note

Mechanism in Inhibitory Effects of Vitamin K₂ on Osteoclastic Bone Resorption: In Vivo Study in Osteopetrotic (op/op) Mice

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Summary Osteoclast deficiency in op/op mice was cured by a single injection of 5μg recombinant human macrophage colony-stimulating factor (M-CSF). On d 5, the osteoclast number reached a maximum value. By d 15, the osteoclast number had decreased to about 70% of the maximum level. Moreover, by d 20, the osteoclast number had decreased to about 30% of its maximum level. On d 5, the osteoclast number of vitamin K₂ 12 h previously had decreased to about 30% of the M-CSF-only injected mice. Moreover, on d 5, the osteoclast number of the mice receiving a single injection of vitamin K₂ 24 h previously had decreased to about 15% that of mice injected only with M-CSF. These results indicate that vitamin K₂ inhibits in vivo osteoclast formation. On d 20, the osteoclast number of the mice injected with a single dose of vitamin K₂ 12 or 24 h previously had decreased to 0% compared with those receiving only M-CSF. The present results suggest that the vitamin K₂ “causes cell death” to mature osteoclasts and inhibits in vivo osteoclast formation.

Key Words inhibit osteoclast formation, op/op mouse, osteoclast cell death, vitamin K₂, M-CSF

Vitamin K exists in two forms, vitamin K₁ (VK₁) and vitamin K₂ (VK₂). The major dietary form of VK is VK₁ of plant origin. VK₂ is produced by bacteria in the bowel, and its proteins are suggested to have an important function in bone metabolism. The precise role of VK in the skeletal system remains unclear. In patients with osteoporotic fracture, VK in serum has been reported to be reduced

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Moreover, the tissue distribution of VK is selective, and its highest level is observed in bone tissue. Recently, the effects of VK on bone resorption and formation have been reported. In contrast to VK₁, VK₂ inhibits bone resorption and osteoclast-like multinucleate cell formation.

Osteopetrosis is a congenital disease, closely linked to the bone metabolism, observed in several animal species and in humans. In the osteopetrotic (op/op) mutant mouse, both the number and size of osteoclasts are much smaller than in normal mice. Recent studies have revealed that the deficiency of osteoclasts, monocytes, and macrophages in op/op mice essentially results from a defect in the production of functional macrophage colony-stimulating factor (M-CSF). Furthermore, it has been reported that an injection of purified recombinant human rhM-CSF (M-CSF) elicits osteoclast differentiation and cures the bone sclerosis condition in mutant mice.

In this study, VK₂ was subcutaneously injected into op/op mice once a day at 0, 1, 1.5, 2, 2.5, 4, 4.5, 5, 5.5, 14, 14.5, 19, 19.5, 24, and 24.5 d after a single injection of 5 µg M-CSF. The injected op/op mice were used in each experimental stage.

In the present study, we assessed, in vivo, the effects of VK₂ on the inhibition of osteoclast differentiation, and determined whether the induction of osteoclast cell death is involved in this mechanism.

Experimental

Animals. B6C3Fe-a/a-op/+ male and female mice, as breeding pairs, were imported from Jackson Laboratory (Bar Harbor, ME, USA). Newborn op/op male mice were kept in metal cages (22 × 32 × 11 cm) with autoclaved wood chips for bedding in an animal room (temperature, 24 ± 2°C; relative humidity, 50 ± 5%). The study was approved by the Hiroshima University Animal Use Committee, and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Hiroshima University. Homozygous recession of op/op mice was identified by failure in tooth eruption and a characteristic domed skull at 10 d old. The op/op mice were fed a granulated diet, while normal mice were fed a solid diet (CE2: Clea Japan, Tokyo, Japan). In each of the experimental and control groups, three op/op mice were sacrificed for histological examination at each of 60, 62, 63, 65, 66, 75, 80, and 85 d old.

Injection of drugs into op/op mice. Macrophage colony-stimulating factor, 5 µg (rhM-CSF; Austral Biologicals Co., CA, USA), was injected into 60-d-old op/op mice, according to the method designed by Kodama et al. Two-methyl-3-all-trans-tetra-prenyl-1,4-naphthoquinone 3 mg/kg (VK₂; Sigma Chemical Co., St. Louis, MO, USA) was subcutaneously single-injected into op/op mice at 0, 1, 1.5, 2, 2.5, 4, 4.5, 5, 5.5, 14, 14.5, 19, 19.5, 24 and 24.5 d after the single injection of 5 µg M-CSF, according to the method designed by Akiyama et al. The VK₂ was dissolved in 95% corn oil. The 5 µg M-CSF was injected into the op/op mice. The VK₂ 3 mg/kg-injected op/op mice were sacrificed 12 h after injection (2, 3, 5, 6, 15, 20 and 25 d after single M-CSF injection). The VK₂...
3 mg/kg-injected op/op mice were sacrificed 24 h after injection (2, 3, 5, 6, 15, 20 and 25 d after single M-CSF injection). One male mouse from each group was anesthetized via intraperitoneal injection of sodium pentobarbital (0.06 mg/g body weight).

Light microscopic observations. The femora were removed from the mice, fixed with 4% formaldehyde for 12 h at 4°C, decalcified in 5% ethylenediamine tetraacetic acid (EDTA) (pH 7.4) for 1 wk, embedded in paraffin, and cut into longitudinal sections of 7μm in thickness. The sections were stained with hematoxylin and eosin (HE) and tartrate-resistant acid phosphatase (TRAP), which is generally acknowledged as a cytochemical marker for osteoclasts, and finally counter-stained with hematoxylin. The number of positively stained cells in sections of the median portion of whole femora was enumerated for the three groups. Kruskal-Wallis testing was carried out, followed by Dunn Procedure tests to detect pairwise differences among three groups.

Results and discussion

In this study, we assessed, in vivo, the effects of VK₂ on the inhibition of osteoclast differentiation and determined whether the induction of osteoclast cell death is involved in the mechanism. Figure 3 shows the time course of the appearance of osteoclasts after a single injection of 5μg M-CSF. A few osteoclasts appeared at 2 d after the single M-CSF injection (Figs. 1-A2 and 3). Five days after a single injection of rhM-CSF, a large number of osteoclasts were observed on the trabeculae bone surface in the femora (Figs. 1-A3 and 3). The number of osteoclasts in the op/op mice approached a maximum 5 d after a single M-CSF injection and then gradually decreased up to 25 d after injection, although the numbers were substantially different for all ages (Fig. 3). These observations are consistent with the findings of Kodama et al (11). On d 5, after the single M-CSF injection, the osteoclast number reached its maximum value. On d 5, the osteoclast number of the mice injected with VK₂ 12 h previously had decreased to about 30% that of the mice receiving only M-CSF (Figs. 1-B3 and 3). Moreover, on d 5, the osteoclast number of the mice injected with VK₂ 24 h previously had decreased to about 15% compared with those receiving only M-CSF (Figs. 1-C3 and 3). These results indicate that VK₂ inhibits osteoclast formation in vivo.

On d 15, the osteoclast number in the mice that were injected once with VK₂ 12 h before had decreased to about 25% compared with the mice receiving only M-CSF (Figs. 2-B4 and 3). On d 15, the osteoclast number of the mice injected with VK₂ 24 h previously had decreased to about 5% of that compared with the mice receiving only M-CSF (Figs. 2-C4 and 3). Moreover, on d 25, the osteoclast number of the mice injected with a single dose of VK₂ 12 or 24 h previously had decreased to 0% compared with the mice receiving only M-CSF (Figs. 2-B6, C6 and 3). The present results suggest that VK₂ “causes cell death” to mature osteoclasts and inhibits osteoclast formation in vivo. If osteoclasts induced by a single M-CSF injection to op/op mice disappeared after VK₂ administration, we would be able
Fig. 1. Resorption of bone trabeculae in the femora of op/op mice after a single injection of M-CSF. M-CSF (5 μg) was injected into 60-d-old op/op mice. Uninjected (A), 12 h before single VK₂ (3 mg/kg) injection (B) and 24 h before single VK₂ injection (C) mice were sacrificed at 60 (1), 62 (2) and 65 d old (3). Longitudinal sections of the femora were stained for TRAP activity (arrowheads) and counterstained with hematoxylin. Each micrograph represents a group of femora from three mice. (× 230)
Fig. 2. Resorption of bone trabeculae in the femora of op/op mice after a single injection of M-CSF. M-CSF (5 μg) was injected into 60-d-old op/op mice. Uninjected VK₂ (3 mg/kg) (A), 12 h before single VK₂ injection (B) and 24 h before single VK₂ injection (C) mice were sacrificed at 66 (4), 80 (5) and 85 d old (6). Longitudinal sections of the femora were stained for TRAP activity (arrowheads) and counterstained with hematoxylin. Each micrograph represents a group of femora from three mice. (×230)
M-CSF (5 μg) was injected into 60-d-old op/op mice. Uninjected VK₂ (3 mg/kg) (○), 12 h before single VK₂ injection (▲) and 24 h before single VK₂ injection (□) mice were sacrificed on various days after treatment. Longitudinal sections of the femora, 7 μm-thick, were stained for TRAP activity and counterstained with hematoxylin. TRAP-positive cells containing two or more nuclei were counted as osteoclasts. Results represent the mean ± standard deviation (SD) of three sections. The result shows mean ± SD from 3 animals. *p < 0.05 and **p < 0.01.

To demonstrate that VK₂ “causes cell death” to osteoclasts in vivo. Kameda et al (13) reported that VK₂ inhibits osteoclastic bone resorption in vitro by targeting osteoclasts to undergo apoptosis and that VK₂-induced osteoclast apoptosis is active cell death and not due to toxic effects or cellular sectioning.

In conclusion, we suggest that VK₂ “causes cell death” to mature osteoclasts and that the drug inhibits osteoclast formation in vivo.

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REFERENCES


