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Vitamin K_2 and geranylgeraniol, its side chain component, inhibited osteoclast formation in a different manner

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Abstract

We comparatively examined the mechanism by which vitamin K_2 (Menatetrenone, MK4) and its side chain component, geranylgeraniol (GGO), inhibited osteoclast formation in the co-culture system of stromal cells with spleen cells. Both MK4 and GGO inhibited osteoclast formation induced by 1α,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$). MK4, but not GGO, inhibited cyclooxygenase-2 (COX-2) expression and prostaglandin E_2 (PGE_2) production in the co-culture system. To elucidate the precise mechanism of the inhibitory effect of GGO on osteoclast formation, the co-cultured cells were stimulated with PGE_2. GGO, but not MK4, inhibited osteoclast formation via suppression of the receptor activator of NF-κB ligand (RANKL) expression. Moreover, GGO abolished the disruption of osteoclastic actin rings induced by nitrogen-containing bisphosphonate (N-BP), whereas MK4 did not affect it at all. These data suggest that MK4 inhibited osteoclast formation independently of GGO, and that MK4, but not GGO, has no competitive action on the anti-osteoporotic effect of N-BP.

Keywords: Osteoporosis; Osteoclast formation; Vitamin K_2 (Menatetrenone, MK4); Geranylgeraniol; 1α,25-dihydroxyvitamin D_3; Prostaglandin E_2 (PGE_2); COX-2; RANKL; Alendronate; Risedronate

Osteoporosis characterized by loss of bone mass is a major public health problem, especially in elderly women. It is widely accepted that osteoporosis is a syndrome that may result from a combination of numerous risk factors such as estrogen loss and aging [1], and many anti-osteoporosis drugs have therefore been developed. Among them, estrogen, selective estrogen receptor modulators (SERM), calcitonin, calcium compounds, vitamin D, bisphosphonates (BPs), and vitamin K are widely used throughout the world [1,2]. BPs have been shown to prevent spine and non-vertebral fractures associated with osteoporosis, and the molecular targets of nitrogen-containing bisphosphonates (N-BPs) have recently been identified [3,4]. They inhibit cholesterol biosynthesis pathway enzymes, farnesyl diphosphate (FPP) synthetase, and geranylgeranyl diphosphate (GGPP) synthase. By inhibiting these enzymes in osteoclasts, N-BPs interfere with the geranylgeranylation of small GTPases such as Ras, Rho, and Rac. It is well known that small GTPases are important signaling proteins regulating a variety of cell processes important for osteoclast formation, including cell morphology. Hence, N-BPs indirectly prevent the protein prenylation of small GTP-binding proteins, disrupt the cytoskeleton, abolish the ruffled border, and induce apoptosis in osteoclasts [5–7] and preosteoclasts [8] in vivo and in vitro.

Vitamin K_1 (2-methyl-3-phenyl-1,4-naphthoquinone) and K_2 (MK4) are also used as anti-osteoporosis drugs. Vitamin K is essential for the γ-carboxylation of osteocalcin, that is, it converts glutamic acid residue (Glu) into γ-carboxyglutamic acid residue (Gla) in osteocalcin molecules [9]. γ-Carboxylation of osteocalcin is important for bone metabolism, because it is impossible for non-carboxylated osteocalcin to bind to hydroxyapatite [10,11]. Therefore, the serum level of osteocalcin is regarded as a biochemical marker of bone turnover [12]. MK4 is a potent homologue of vitamin K_2 in preventing bone loss induced by ovariectomy and prednisolone

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treatment in rats [13,14]. MK4 affects metabolism by stimulating bone formation and inhibiting bone resorption [15,16]. We have already reported that MK4 inhibited osteoclastogenesis in the mouse bone marrow culture system [17] and co-culture system [18]. In these reports, we found that GGO, a side-chain component of MK4 at the 3-position of naphthoquinone, inhibited osteoclast formation to the same degree as MK4 [18]. Collectively these results suggest that the GGO chain of MK4 plays an important role in the inhibitory effect of MK4 on bone resorption. These findings lead us to speculate that treating osteoporosis with MK4 causes a decrease in the potency of N-BPs.

In this paper, we comparatively examined the mechanism by which MK4 and GGO inhibited osteoclast formation, in order to clarify the involvement of GGO in the anti-osteoporotic effects of MK4.

Materials and methods

Materials

α-Minimum essential medium (α-MEM) and fetal bovine serum were obtained from Gibco (Life Technologies, Grand Island, NY), and 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) was obtained from Teijin (Tokyo, Japan). Menatetrenone (all-*trans* form), geranylgeraniol, and Alendronate were kindly provided by Eisai (Tokyo, Japan). Risedronate was kindly provided by Ajinomoto (Tokyo, Japan). Prostaglandin E_2 was kindly provided by Ono Pharmaceutical (Osaka, Japan). The soluble receptor activator of the NF-κB ligand (sRANKL) was purchased from PeproTech EC (London, UK).

Methods

Osteoclast-like cell formation in the co-culture system of stromal cells with spleen cells. TMS-12 cells isolated from bone marrow support osteoclast-like cell formation with bone resorbing factors such as prostaglandin E_2, $1,25(OH)_2D_3$, parathyroid hormone, and interleukin-1 as described previously [19,20]. The spleens of 5- to 7-week-old male ddy mice (Saitama Experimental Animals, Saitama, Japan) were minced and a single cell suspension was prepared. The cell suspension was centrifuged at 1200 rpm, 20 °C for 15 min using a density gradient prepared with Ficoll (type 400, Sigma Chemical) and 60% Urografin (Nihon Schering K.K., Osaka, Japan). A layer of monocytes was collected and used for subsequent experiments. TMS-12 cells (1×10^4 cells/well in 24-well tissue culture plates, 0.5×10^4 cells/well in 48-well tissue culture plates) were cultured for 24 h. The purified spleen cells (5×10^5 cells/well) were then seeded onto the TMS-12 cells. Either $1,25(OH)_2D_3$ (10^{-8} M) or PGE_2 (10^{-8} M) was added to the culture from day 1. sRANKL (30 ng/ml) was added to the culture from day 3. The cultures were incubated for 7 days, with the medium changed twice a week (only 65% of the medium was changed each time).

Characterization of osteoclast-like cells formed in the co-culture system. Numerous osteoclast-like cells were formed in the co-culture system, and osteoclast-like cell formation was measured by tartrate-resistant acid phosphate (TRAP) staining. The cells were washed by phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} and fixed with ethanol:acetone (1:1) for 1 min. The well surface was dried and then stained for TRAP according to the method modified by Burstone [21]. The cells were incubated at 37 °C for 60 min in a TRAP-staining solution: 0.1 M sodium acetate buffer (pH 5.0) containing 0.1% (w/v) of naphthol AS-BI phosphate (Wako Pure Chemical Industries) and 0.6% (w/v) of fast red ITR salt (Sigma Chemical) in the presence of 1.5% (w/v) sodium tartrate. The staining solution was then removed and the wells were washed with water and dried. TRAP-positive cells containing three or more nuclei were counted as osteoclast-like MNC. As reported previously [22], the TRAP-positive MNC observed in this study possessed calcitonin receptors identified with an auto-radiographic technique using ^{125}I-labeled CT.

Actin ring formation in osteoclasts. Actin rings formed in osteoclast-like cells were detected by staining actin filaments with fluorescein isothiocyanate (FITC)-conjugated phalloidin. Osteoclast formation was induced by 10^{-8} M of $1,25(OH)_2D_3$ in the co-culture of spleen cells with TMS-12 cells. After a 6-day incubation, the co-cultured cells were treated with or without MK4 or GGO (5×10^{-6} M) in the presence or absence of Alendronate and Risedronate (5×10^{-5} M) for 24 h. At the end of incubation, cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 min and permeated with 0.1% Triton X-100 in PBS for 5 min. After washing with PBS, F-actin was stained with 0.3 μM FITC-conjugated phalloidin. The distribution of F-actin in the osteoclast was visualized and detected under a fluorescence microscope.

Measurement of immunoreactive PGE_2. The level of PGE_2 in the conditioned medium were measured by using an enzyme immunoassay kit (Amersham Biosciences, USA) according to the manufacturer's protocol.

Reverse-transcription polymerase chain reaction. Total RNA was extracted from the co-culture of spleen cells and TMS-12 treated with or without MK4 and GGO in the presence or absence of $1,25(OH)_2D_3$ using TRIZOL Reagent (Invitrogen, USA). Two micrograms of total RNA was reverse-transcribed using oligo(dT) as a primer (250 nM final concentration) in a final 20 μl reverse-transcription (RT) solution. Two microliters of RT solution was amplified by polymerase chain reaction (PCR) within the exponential phase of amplification. The specific primer pairs were mouse RANKL (GenBank Accession No. AB008426), 5′-CGCTCTGTTCCTGTACTTTCGAGCG-3′ (forward) and 5′-TCGTGCTCCCTCCTTTCAGGTT-3′ (reverse), mouse OPG (GenBank Accession No. U94331), 5′-GTGTGAGGAAGGGCGTT ACC-3′ (forward) and 5′-TTTTGGGAAAGTGGGATGTT-3′ (reverse), and mouse COX-1 (GenBank Accession No. NM008969), 5′-CTCACAGTGCGGTCCACC-3′ (forward) and 5′-CAAGCACC TGGTACTTAAG-3′ (reverse), mouse COX-2 (GenBank Accession No. NM011198), 5′-TCAGCCAGGCAGCAAATCCTTG-3′ (forward) and 5′-TAGTCTCTCCTATGAGTATGAGTC-3′ (reverse). PCR consisted of 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C (RANKL), 56 °C (OPG), and 55 °C (COX) for 30 s, and extension at 72 °C for 30 s. The PCR product (8 μl) was electrophorosed with 2% agarose gel, which contained 0.2% ethidium bromide, and specific DNA bands were examined under an ultraviolet transilluminater.

Results

Effect of MK4 and GGO on osteoclast formation induced by $1,25(OH)_2D_3$ in cloned TMS-12

In our previous papers, MK4 suppressed osteoclast formation as well as GGO, a side chain of MK4. We investigated whether the inhibitory effect of MK4 on osteoclast formation is dependent on GGO, using a co-culture system of spleen cells and cloned TMS-12 stromal cells. We isolated two different clones, named TMS-12A and -B, from TMS-12 cells. In TMS-12A and the spleen cell co-culture system, either MK4 or GGO significantly inhibited the osteoclast formation induced

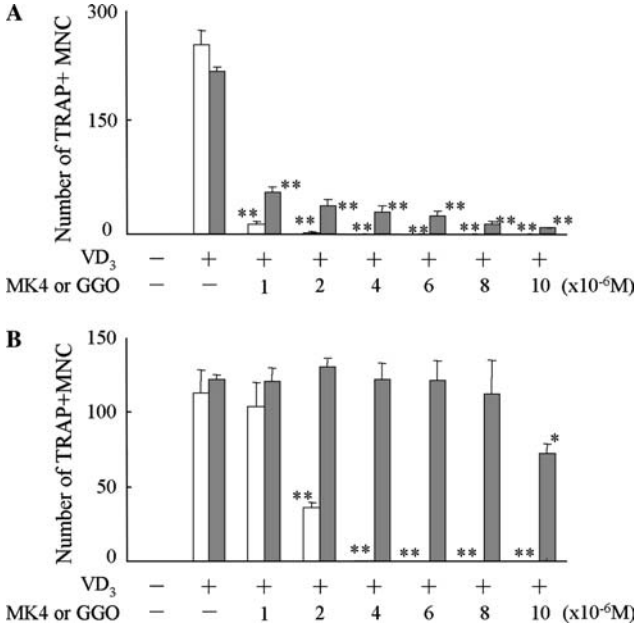

Fig. 1. (A,B) Effect of MK4 (open column) or GGO (filled column) on TRAP-positive MNC formation induced by $1,25(OH)_2D_3$. TRAP-positive MNC formation was induced by $1,25(OH)_2D_3$ (10^{-8} M) in the co-culture system of spleen cells with cloned TMS-12 stromal cells (A, TMS-12A and B, TMS-12B). (C) Effect of MK4 (open column) or GGO (filled column) on TRAP-positive MNC formation induced by PGE_2. TRAP-positive MNC formation was induced by PGE_2 (10^{-8} M) in the co-culture system of spleen cells with cloned TMS-12A. The culture continued for 7 days with MK4 (open column) or GGO (filled column) at the indicated concentration, and the medium was replaced twice a week. Data are expressed as the number of TRAP-positive MNC in each dish and represent means ± SEM ($n = 4$). $^*p < 0.05$, $^{**}p < 0.01$ vs. VD_3 alone.

by $1,25(OH)_2D_3$ (Fig. 1A). This result supported the hypothesis that the inhibitory effect of MK4 on osteoclast formation is dependent on GGO, as previously reported [18]. Surprisingly, in TMS-12B and the spleen cell co-culture system, only GGO did not inhibit $1,25(OH)_2D_3$-induced osteoclast formation (Fig. 1B). These data strongly suggested that the inhibitory mechanism of MK4 on osteoclast formation differed from that of GGO.

Effects of MK4 and GGO on the PGE_2 production of cells stimulated by $1,25(OH)_2D_3$

PGE_2 is a potent substance inducing osteoclast formation and is induced by $1,25(OH)_2D_3$. To reveal the different mechanisms by which MK4 and GGO inhibit $1,25(OH)_2D_3$-induced osteoclast formation, we measured the PGE_2 production of the co-cultured cells of TMS-12A with spleen cells in the presence or absence of MK4 or GGO. $1,25(OH)_2D_3$ increased the PGE_2 production of the co-cultured cells ~8-fold. MK4 inhibited $1,25(OH)_2D_3$-induced PGE_2 production at the basal level. In contrast, GGO did not affect $1,25(OH)_2D_3$-induced PGE_2 production at all (Fig. 2A). These findings suggest that MK4, but not GGO, inhibits osteoclast formation via the suppression of PGE_2 production induced by $1,25(OH)_2D_3$.

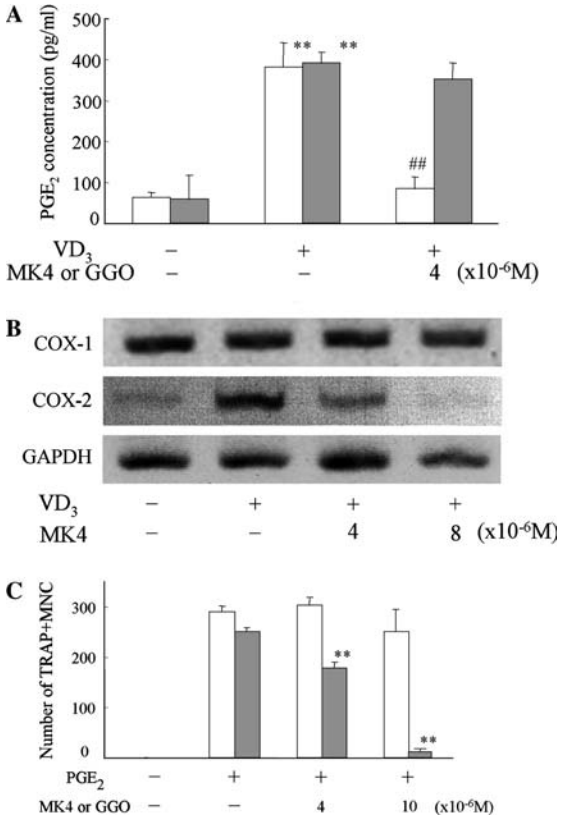

Fig. 2. (A) Effect of MK4 and GGO on PGE_2 production induced by $1,25(OH)_2D_3$ in the co-culture system. Mouse spleen cells were co-cultured with TMS-12A in the presence or absence of 10^{-8} M $1,25(OH)_2D_3$. The coculture was treated with 4×10^{-6} M MK4 (open column) or 4×10^{-6} M GGO (filled column) for 5 days. The media were collected for the last two days. Data are expressed as the immunoreactive PGE_2 concentration of the conditioned medium and represent means ± SEM ($n = 3$). $^{**}p < 0.01$ vs. without VD_3, $^{\#\#}p < 0.01$ vs. VD_3 alone. (B) Effect of MK4 and GGO on COX mRNA expression induced by $1,25(OH)_2D_3$ in the co-culture system. After TMS-12A cells were cultured for 24 h, spleen cells were co-cultured with or without MK4 in the presence or absence of 10^{-8} M $1,25(OH)_2D_3$ for the following 24 h. The mRNA levels of COX-1, COX-2, and GAPDH were estimated by RT-PCR. The results are representative of three independent experiments. (C) Effect of MK4 (open column) or GGO (filled column) on TRAP-positive MNC formation induced by PGE_2. TRAP-positive MNC formation was induced by PGE_2 (10^{-8} M) in the co-culture system of spleen cells with cloned TMS-12A. The culture continued for 7 days with MK4 (open column) or GGO (filled column) at the indicated concentration, and the medium was replaced twice a week. Data are expressed as the number of TRAP-positive MNC in each dish and represent means ± SEM ($n = 4$). $^{**}p < 0.01$ vs. PGE_2 alone.

Effect of MK4 on COX-1 and COX-2 expressions induced by $1,25(OH)_2D_3$

To investigate the mechanism by which MK4 inhibited PGE_2 production, we examined the effect of MK4

on the expression of COX-1 mRNA and COX-2 mRNA in the co-culture system using a RT-PCR technique. Both COX-1 and COX-2 mRNA levels were measured in the co-culture system 24 h after treatment with $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ induced COX-2 mRNA expression, however, it did not affect COX-1 mRNA expression. MK4 decreased the expression of COX-2 mRNA induced by $1,25(OH)_2D_3$ in a dose-dependent manner (Fig. 2B). On the other hand, GGO failed to decrease the expression of COX-2 mRNA induced by $1,25(OH)_2D_3$ (data not shown). These results suggest that the decrease in PGE_2 production by MK4 is due to the suppression of the COX-2 mRNA level in co-cultured cells.

Effect of MK4 and GGO on osteoclast formation induced by PGE_2

To confirm the involvement of PGE_2 in the inhibition of osteoclast formation by MK4, we examined the effect of MK4 or GGO on the osteoclast formation induced by PGE_2 in the co-culture system. Even the maximal dose of MK4 (10^{-5} M) did not inhibit PGE_2-induced osteoclast formation. In contrast, PGE_2-induced osteoclast formation was significantly inhibited by GGO (Fig. 2C). These results suggested that the inhibitory effect of MK4 on osteoclast formation induced by $1,25(OH)_2D_3$ was responsible for the decreased production of PGE_2. On the other hand, GGO inhibited an event downstream from the PGE_2 signal during osteoclast formation in this co-culture system of TMS-12A with spleen cells.

Effect of MK4 and GGO on RANKL expression and sRANKL-induced osteoclast formation

To clarify the inhibitory mechanism of GGO on osteoclast formation induced by $1,25(OH)_2D_3$, we investigated the effect of MK4 or GGO on the expression of RANKL. Using a RT-PCR technique, the expression of RANKL mRNA was examined in the co-culture system of spleen cells with TMS-12A or B for 48 h. In the co-culture system of spleen cells with TMS12-A, GGO, but not MK4, suppressed the mRNA expression of RANKL induced by $1,25(OH)_2D_3$ (Fig. 3A). However, GGO as well as MK4 failed to suppress the mRNA expression of RANKL induced by $1,25(OH)_2D_3$ in the co-culture system of spleen cells and TMS-12B. OPG mRNA was not affected by $1,25(OH)_2D_3$, and neither MK4 nor GGO affected the mRNA expression of OPG (Fig. 3A). To test whether the inhibitory effect of GGO on $1,25(OH)_2D_3$-induced osteoclast formation is a result of decreased RANKL expression, GGO alone or in combination with soluble RANKL (sRANKL) was added to the co-cultured spleen cells with TMS-12A. sRANKL restored the inhibitory effect of GGO on

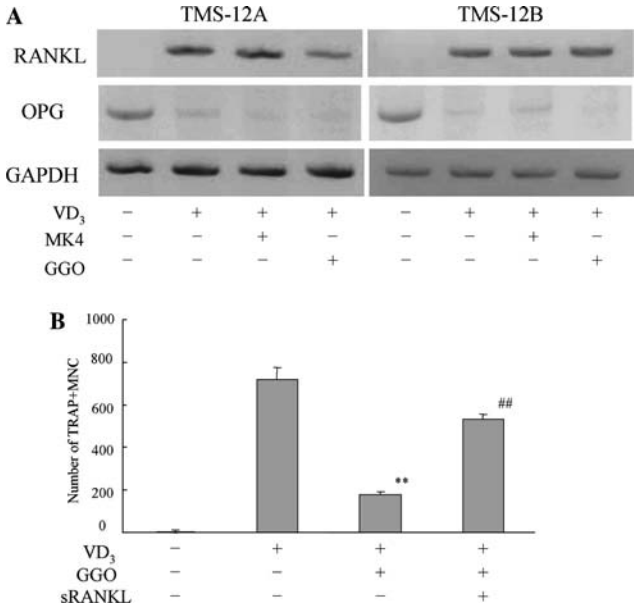

Fig. 3. (A) Effect of MK4 and GGO on the expression of RANKL and OPG mRNA induced by $1,25(OH)_2D_3$ in the co-culture system. The co-culture of TMS-12 cells with spleen cells was treated with or without MK4 and GGO at a concentration of 5×10^{-6} M in the presence or absence of $1,25(OH)_2D_3$ (10^{-8} M) for 48 h. The mRNA levels of RANKL, OPG, and GAPDH were estimated by RT-PCR. The results are representative of three independent experiments. (B) Effect of sRANKL on TRAP-positive MNC formation inhibited by GGO. The co-culture of TMS-12A with spleen cells was treated with GGO (5×10^{-6} M), in the presence of $1,25(OH)_2D_3$ (10^{-8} M) for 3 days. The cells were then successively treated with GGO (5×10^{-6} M) and $1,25(OH)_2D_3$ in the presence or absence of sRANKL (30 ng/ml) for 4 days. The medium was replaced three times a week. Data are expressed as the number of TRAP-positive MNC in each dish and represent means ± SEM ($n = 4$). $^{**}p < 0.01$ vs. VD3 alone, $^{\#\#}p < 0.01$ vs. VD_3 + GGO.

osteoclast formation induced by $1,25(OH)_2D_3$ (Fig. 3B). These findings suggested that the effect of GGO on osteoclast formation was closely related to the expression of RANKL.

Effect of MK4 and GGO on osteoclast apoptosis induced by N-containing bisphosphonate

It has been reported that GGO can rescue the N-BP-induced apoptosis of osteoclasts [23]. Therefore, we next examined whether or not MK4 or GGO prevents the disruption of actin rings in osteoclasts induced by N-BP. Osteoclast formation was induced by 10^{-8} M of $1,25(OH)_2D_3$ in the co-culture system of spleen cells with TMS-12A. After a 6-day incubation, these cells were treated with MK4 or GGO (5×10^{-6} M, respectively) in the presence or absence of N-BPs (alendronate or residronate; 5×10^{-5} M, respectively) for 24 h. MK4 did not abolish residronate- and alendronate-induced actin disruption, but GGO abolished actin disruption as expected (Figs. 4A and B). These results suggest that an

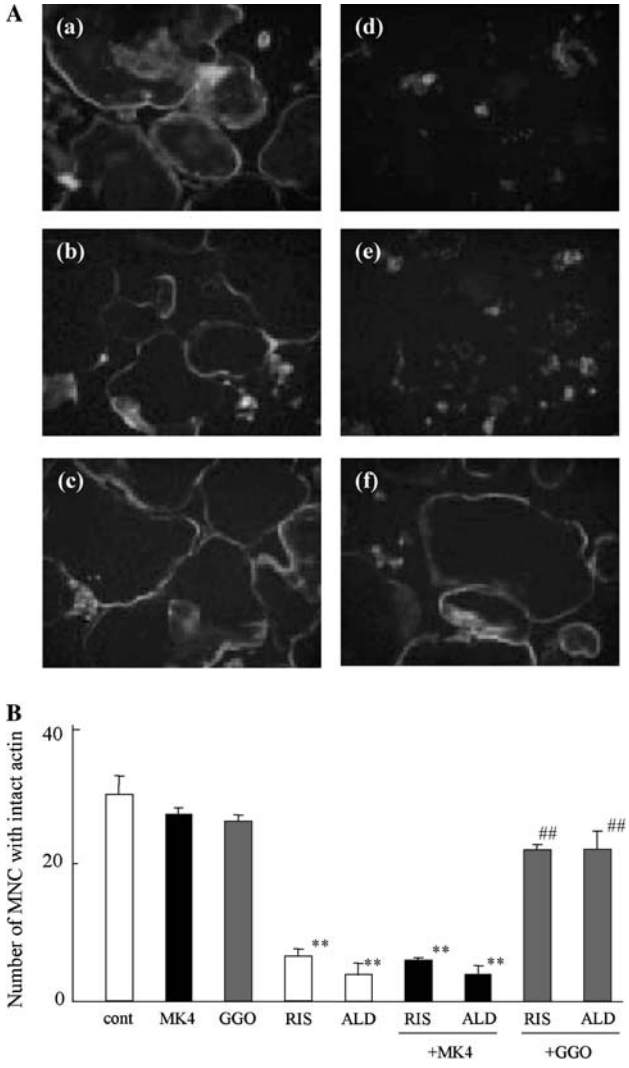

Fig. 4. (A) Effect of MK4 and GGO on actin disruption in osteoclasts induced by N-BP. TRAP-positive MNC formation was induced by 10^{-8} M of $1,25(OH)_2D_3$ in the co-culture of spleen cells with TMS-12 cells. After a 6-day culture, the cells were treated with or without MK4 (5×10^{-6} M) and GGO (5×10^{-6} M), in the presence or absence of risedronate (RIS, 5×10^{-5} M) or alendronate (ALD, 5×10^{-5} M) for 24 h. F-actin distribution was observed by staining with FITC-conjugated phalloidin. (a) control, (b) MK4, (c) GGO, (d) risedronate, (e) risedronate + MK4, and (f) risedronate + GGO. (B) Effect of MK4 and GGO on the number of live osteoclasts. Experimental conditions were identical to those described above. Intact F-actin-distributed MNC and actin-disrupted MNC were counted up to 35 cells in each dish ($n = 4$). Data are expressed as the number of intact MNC and represent means ± SEM. $^{**}p < 0.01$ vs. cont, $^{\#\#}p < 0.01$ vs. N-BP alone.

additive effect of MK4 and N-BP on osteoporosis is predicted.

Discussion

Osteoporosis occurs most commonly in menopausal women as a result of lowered estrogen levels [1]. Of various types of fractures, bone fractures of the vertebra and femoral neck were associated with pronounced morbidity, resulting in high mortality. Thus, the prevention and treatment of osteoporosis should be investigated. Combination therapy using an inhibitor of bone resorption and a stimulator of bone formation is thought to be useful for the treatment of osteoporosis. It has been reported that VK_2-induced bone formation through the effect of γ-carboxylation [9] and inhibited bone resorption through the suppression of osteoclast formation. With regard to osteoclast formation, we have already reported that the inhibitory effect of VK_2 (MK4) on bone resorption may be related to its side chain, geranylgeraniol. However, in a recent study, Tabb et al. [24] demonstrated that the regulation of bone homeostasis by VK_2 is mediated by the steroid and xenobiotic receptor, SXR. This report led us to the hypothesis that the mechanism for the inhibition of osteoclast formation by MK4 differs from that by GGO. To examine this possibility, we cloned mouse stromal cells, which are able to support osteoclast formation in co-culture with spleen cells. As a result, we were able to clone two types of stromal cells; in one, both MK4 and GGO suppressed osteoclast formation induced by $1,25(OH)_2D_3$ (TMS-12A), and in the other, it was suppressed by MK4, but not GGO (TMS-12B). As shown in Fig. 1, MK4 inhibited $1,25(OH)_2D_3$-induced osteoclast formation, but MK4 failed to suppress PGE_2-induced osteoclast formation. These results suggest that MK4 suppresses osteoclast formation via the inhibition of PGE_2 production. PGE_2 has long been considered to play a crucial role in bone physiology [25], and it has recently been reported that PGE_2 bound to the EP2/EP4 subtype in stromal cells, resulted in the induction of osteoclast formation [26] via stimulation of the RANKL expression. Moreover, it has been reported that PGE_2 can enhance RANKL-induced osteoclast formation [27], indicating that PGE_2 produced by $1,25(OH)_2D_3$ will enhance osteoclast formation. The marked decrease in the $1,25(OH)_2D_3$-mediated PGE_2 production and no change of RANKL mRNA expression by MK4 indicate the possibility that MK4 abolishes only the synergistically effect of PGE_2 on osteoclast formation induced by $1,25(OH)_2D_3$. PGE_2 synthesis is mediated by COX-1 and COX-2. COX-1 is constitutively expressed in cells and COX-2 is an inducible enzyme in response to various cytokines. According to recent experiments using COX-2 knocked out mice, COX-2 is required for osteoclast formation [28]. As shown in Fig. 2, MK4, but not GGO, inhibited PGE_2 production induced by $1,25(OH)_2D_3$. The inhibition of PGE_2 production by MK4 is dependent on the inhibition of COX-2 expression (Fig. 2B). We concluded that the inhibitory effect of MK4 on osteoclast formation was a result of the suppression of COX-2 induced by $1,25(OH)_2D_3$.

GGO as well as MK4 inhibited osteoclast formation induced by $1,25(OH)_2D_3$ in the co-culture system of

spleen cells and TMS-12A (Fig. 1A), but GGO suppressed neither the PGE_2 production induced by $1,25(OH)_2D_3$ (Fig. 2A) nor the expression of COX-2 (data not shown). On the other hand, GGO inhibited the expression of RANKL induced by $1,25(OH)_2D_3$ in the co-culture system of spleen cells with TMS-12A (Fig. 3A). Moreover, GGO inhibited PGE_2-induced osteoclast formation, but it could not inhibit sRANKL-induced osteoclast formation (Figs. 2C and 3B). These data suggest that MK4 and GGO, a side chain component, inhibited osteoclast formation in a different manner. This hypothesis was supported by data in which MK4, but not GGO, suppressed osteoclast formation in other stromal cells (TMS-12B) co-cultured with spleen cells (Fig. 1B). It has not been clarified why GGO inhibited RANKL expression induced by $1,25(OH)_2D_3$. However, it has been reported that RAS-transformed keratinocytes were resistant to the growth inhibitory action of $1,25(OH)_2D_3$ [29]. Considering this report, it is likely that geranylgeranylated RAS by GGO results in the inhibition of RANKL expression mediated by vitamin D signaling.

N-BP induces osteoclast apoptosis via the inhibition of small GTP-binding proteins [30]. We examined whether GGO prevented N-BP-induced osteoclast apoptosis. As expected, N-BP dramatically induced actin ring disruption in osteoclasts. Moreover, GGO significantly restored the actin ring disruption induced by N-BP (Fig. 4). The different mechanisms for suppressing osteoclast formation between MK4 and GGO lead us to propose that MK4 cannot prevent N-BP-induced osteoclast apoptosis. As shown in Fig. 4, MK4 did not affect N-BP-induced osteoclast apoptosis.

In conclusion, we found that the anti-bone-resorptive effects (apoptosis and the inhibition of osteoclast formation) of MK4 differ from those of GGO. We observed no competitive action of MK4 on the anti-osteoporotic effect of N-BP. Therefore, our data suggest that combination therapy with BP and MK4 will be useful in the future.

References

[1] G.A. Rodan, T.J. Martin, Therapeutic approaches to bone diseases, Science 289 (2000) 1508–1514.

[2] P.D. Delmas, Treatment of postmenopausal osteoporosis, Lancet 359 (2002) 2018–2026.

[3] J.E. Fisher, M.J. Rogers, J.M. Halasy, S.P. Luckman, D.E. Hughes, P.J. Masarachia, G. Wesolowski, R.G.G. Russell, G.A. Rodan, A.A. Reszka, Alendronate mechanism of action: geranylgeraniol, an intermediate in the mevalonate pathway, prevents inhibition of osteoclast formation, bone resorption, and kinase activation in vitro, Proc. Natl. Acad. Sci. USA 96 (1999) 133–138.

[4] M.J. Roger, J.C. Frith, S.P. Luckman, F.P. Coxon, H.L. Benford, J. Mönkkönen, S. Auriola, K.M. Chilton, R.G.G. Russel, Molecular mechanisms of action of bisphosphonate, Bone 24 (Suppl. 5) (1999) 73S–79S.

[5] F.P. Coxon, M.H. Helfrich, R.V. Hof, S. Sebti, S.H. Ralston, A. Hamilton, M.J. Rogers, Protein geranylgeranylation is required for osteoclast formation, and survival: inhibition by bisphosphonates and GGTI-298, J. Bone Miner. Res. 15 (2000) 1467–1476.

[6] M. Ito, N. Amizuka, T. Nakajima, H. Ozawa, Ultrastructural and cytochemical studies on cell death of osteoclasts induced by bisphosphonate treatment, Bone 25 (1999) 447–452.

[7] D.E. Hughes, K.R. Wright, H.L. Uy, A. Sasaki, T. Yoneda, G.D. Roodman, G.R. Mundy, B.F. Boyce, Bisphosphonates promote apoptosis in murine osteoclasts in vitro and in vivo, J. Bone Miner. Res. 10 (1955) 1478–1487.

[8] E.R. Van Beek, C.W.G.M. Löwik, S.E. Papapoulos, Bisphosphonate suppress bone resorption by a direct effect on early osteoclast precursors without affecting the osteoclastogenic capacity of osteogenic cells: the role of protein geranylgeranylation in the action of nitrogen-containing bisphosphonate on osteoclast precursors, Bone 30 (2002) 64–70.

[9] R.K. Menon, D.S. Gill, M. Thomas, P.B.A. Kernoff, P. Dandona, Impaired carboxylation of osteocalcin in warfarin-treated patients, J. Clin. Endocr. Metabol. 64 (1987) 59–61.

[10] P.V. Hauschka, S.A. Carr, Calcium-dependent α-helical structure in osteocalcin, Biochemistry 21 (1982) 2538–2547.

[11] P.A. Price, M.K. Williamson, J.W. Lothringer, Origin of the vitamin K-dependent bone protein found in plasma and its clearance by kidney and bone, J. Biol. Chem 256 (1981) 12760–12766.

[12] P.A. Price, J.G. Parthemore, L.J. Deftos, S.K. Nishimoto, New biochemical marker for bone metabolism, J. Clin. Invest. 66 (1980) 878–883.

[13] Y. Akiyama, K. Hara, I. Ohkawa, T. Tajima, Effect of Menatetrenone on bone loss induced by Ovarietomy in rats, Jpn. J. Pharmacol. 62 (1993) 145–153.

[14] K. Hara, Y. Akiyama, T. Tajima, M. Shiraki, Effect of Menatetrenone on prednisolone-induced bone loss in rats, Bone 14 (1993) 813–818.

[15] Y. Koshihara, K. Hoshi, Vitamin K_2 enhances osteocalcin accumulation in the extracellular matrix of human osteoblasts in vitro, J. Bone Miner. Res. 12 (1997) 431–438.

[16] Y. Takeuchi, M. Suzawa, S. Fukomoto, T. Fujita, Vitamin K_2 inhibits adipogenesis, osteoclastogenesis, and ODF/RANK ligand expression in murine bone marrow cell culture, Bone 27 (2000) 769–776.

[17] Y. Akiyama, K. Hara, S. Murota, I. Morita, Effect of vitamin K_2 (Menatetrenone) on osteoclast-like cell formation in mouse bone marrow cultures, Eur. J. Pharmacol. 263 (1994) 181–185.

[18] K. Hara, Y. Akiyama, T. Nakamura, S. Murota, I. Morita, The inhibitory effect of vitamin K_2 (Menatetrenone) on bone resorption may be related to its side chain, Bone 16 (1995) 179–184.

[19] I. Morita, K. Sakaguchi, T. Kurachi, S. Murota, Ipriflavone inhibits murine osteoclast formation in vitro, Calcif. Tissue Int. 51 (Suppl. 1) (1992) S7–S10.

[20] K. Sakaguchi, I. Morita, S. Murota, Relationship between the ability to support differentiation of osteoclast-like cells and adipogenesis in murine stromal cells derived from bone marrow, Prostaglandins, Leukot. Essent. Fatty Acids 62 (2000) 319–327.

[21] M.S. Burstone, Histochemical demonstration of acid phosphatases with naphtol AS-phosphates, J. Natl. Cancer Inst. 21 (1958) 523–539.

[22] N. Takahashi, T. Akatsu, T. Sasaki, G.C. Nicholson, J.M. Moseley, T.J. Martin, T. Suda, Induction of calcitonin receptors by 1α,25-dihydroxyvitamin D_3 in osteoclast-like multinucleated cells formed from mouse bone marrow cells, Endocrinology 123 (1988) 1504–1510.

[23] A.A. Reszka, J.M. Halay-Nagy, P.J. Masarachia, G.A. Rodan, Bisphosphonates act directly on the osteoclast to induce caspase

cleavage of mst1 kinase during apoptosis. A link between inhibition of the mevalonate pathway and regulation of an apoptosis-promoting kinase, J. Biol. Chem 274 (1999) 34967–34973.

[24] M.M. Tabb, A. Sun, C. Zhou, F. Gruuuuuun, J. Errandi, K. Romero, H. Pham, S. Inoue, S. Mallick, M. Lin, B.M. Forman, B. Blumberg, Vitamin K_2 regulation of bone homeostasis is mediated by the steroid and xenobiotic receptor, SXR J. Biol. Chem. 278 (2003) 43919–43927.

[25] H. Kawaguchi, C.C. Pilbeam, J.R. Harrison, L.G. Raisz, The role of prostaglandins in the regulation of bone metabolism, Clin. Orthop. 313 (1995) 36–46.

[26] T. Suzawa, C. Miyaura, M. Inada, T. Maruyama, Y. Sugimito, F. Ushikubi, A. Ichikawa, S. Narumiya, T. Suda, The role of prostaglandin E receptor subtypes (EP1, EP2, EP3, and EP4) in bone resorption: an analysis using specific agonists for the respective EPs, Endocrinology 141 (2000) 1554–1559.

[27] M.R. Wani, K. Fuller, N.S. Kim, Y. Choi, T. Chambers, Prostaglandin E_2 cooperates with TRANCE in osteoclast induction from hemopoietic precursors: synergistic activation of differentiation, cell spreading, and fusion, Endocrinology 140 (1999) 1927–1935.

[28] Y. Okada, J.A. Lorenzo, A.L. Freeman, M. Tomita, S.G. Morham, L.G. Raisz, C.C. Pilbeam, Prostaglandin G/H synthase-2 is required for maximal formation of osteoclast-like cells in culture, J. Clin. Invest. 105 (2000) 823–832.

[29] C. Solomon, R. Kremer, J.H. White, J.S. Rhim, Vitamin D resistance in RAS-transformed keratinocytes: mechanism and reversal strategies, Radiat. Res. 155 (1 Pt 2) (2000) 156–162.

[30] S.P. Luckman, D.E. Hughes, F.P. Coxon, R.G.G. Russell, M.J. Rogers, Nitrogen-containing bisphosphonates inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras, J. Bone Miner. Res. 13 (1998) 581–589.