Vitamin K stimulates osteoblastogenesis and inhibits osteoclastogenesis in human bone marrow cell culture

Y Koshihara, K Hoshi, R Okawara, H Ishibashi¹
and S Yamamoto¹

Bone Research Group, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakaecho, Itabashi-ku, Tokyo, 173-0015, Japan

¹Department of Orthopedics, Tokyo Metropolitan Geriatric Hospital, Itabashi-ku, Tokyo, Japan

(Requests for offprints should be addressed to Y Koshihara; Email: ykoshi@tmig.or.jp)

Abstract

Accumulating evidence indicates that menaquinone-4 (MK-4), a vitamin K₂ with four isoprene units, inhibits osteoclastogenesis in murine bone marrow culture, but the reason for this inhibition is not yet clear, especially in human bone marrow culture. To clarify the inhibitory mechanism, we investigated the differentiation of colony-forming-unit fibroblasts (CFU-Fs) and osteoclasts in human bone marrow culture, to learn whether the enhancement of the differentiation of CFU-Fs from progenitor cells might relate to inhibition of osteoclast formation. Human bone marrow cells were grown in α -minimal essential medium with horse serum in the presence of MK-4 until adherent cells formed colonies (CFU-Fs). Colonies that stained positive for alkaline phosphatase activity (CFU-F/ALP⁺) were considered to have osteogenic potential. MK-4 stimulated the number of CFU-F/ALP⁺ colonies in the presence or absence of dexamethasone. The stimulation was also seen in vitamin K₁ treatment. These cells had

the ability to mineralize in the presence of α -glycerophosphate.

In contrast, both MK-4 and vitamin K₁ inhibited 1,25 dihydroxyvitamin D₃-induced osteoclast formation and increased stromal cell formation in human bone marrow culture. These stromal cells expressed ALP and Cbfa1. Moreover, both types of vitamin K treatment decreased the expression of receptor activator of nuclear factor κ B ligand/osteoclast differentiation factor (RANKL/ODF) and enhanced the expression of osteoprotegerin/osteoclast inhibitory factor (OPG/OCIF) in the stromal cells. The effective concentrations were 1.0 μ M and 10 μ M for the expression of RANKL/ODF and OPG/OCIF respectively.

Vitamin K might stimulate osteoblastogenesis in bone marrow cells, regulating osteoclastogenesis through the expression of RANKL/ODF more than through that of OPG/OCIF.

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Introduction

There is evidence that vitamin K has some beneficial effects on osteoporosis (Hodges *et al.* 1991, 1993, Feskanish *et al.* 1999). Menatetrenone (menaquinone-4, MK-4), a vitamin K₂ with four isoprene units at the 3 position of the quinone structure, has a significant therapeutic effect on involutional osteoporosis (Orimo *et al.* 1992, Iwamoto *et al.* 1999). The mechanism has been elucidated by *in vitro* studies showing that MK-4 inhibits osteoclastogenesis in murine bone marrow culture (Akiyama *et al.* 1994). Vitamin K₂ has been shown to inhibit bone resorption by affecting osteoclasts directly in tests using purified mature osteoclasts from bone of newborn rabbits. The fact that vitamin K₂ promotes apoptosis supported the finding of inhibition (Kameda *et al.* 1996). However, vitamin K₂ binding protein or localization of vitamin K₂ in the osteoclasts has not been

clarified, although vitamin K₂/K₁ binding protein has been demonstrated in human osteoblasts (Hoshi *et al.* 1999).

We previously demonstrated the effect of MK-4 on osteoblasts. MK-4 increased 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃)-induced mineralization by human osteoblasts *in vitro* (Koshihara *et al.* 1996), and MK-4 enhanced the accumulation of γ -carboxyglutamic acid-containing osteocalcin and osteocalcin synthesis in cultured human osteoblasts (Koshihara & Hoshi 1997). In fact, MK-4 was metabolized via the vitamin K cycle in cultured human osteoblasts as well as in the liver, to work as the cofactor for carboxylase (Miyake *et al.* 2001). In addition, 1,25(OH)₂D₃ promotes the metabolism of MK-4 in osteoblasts.

These findings show that MK-4 both inhibits osteoclastic bone resorption and stimulates osteoblastic bone formation in different assay systems of different species.

However, it has not been clarified whether MK-4 also has an inhibitory effect on osteoclastogenesis in human bone marrow culture. There is considerable evidence that bone marrow cells from the mouse and human respond differently in several circumstances, especially in their response to glucocorticoid (Greenberger *et al.* 1979, Croisille *et al.* 1994).

We hypothesized that the inhibition of osteoclastogenesis by MK-4 in human bone marrow followed the promotion of osteoblastogenesis, because osteoblasts originate from mesenchymal stem cells, which reside in bone marrow together with hematopoietic stem cells. These two stem cell types cooperate through direct cell-to-cell interaction and release of cytokines and growth factors. In this study, we investigated the effect of vitamin K₂ on osteoclastogenesis and osteoblastogenesis in human bone marrow culture by adding MK-4 to the culture. There are epidemiological data suggesting that vitamin K₁ also has a beneficial effect on bone metabolism (Feskanish *et al.* 1999). For example, women of any age with a low dietary intake of vitamin K₁ are at increased risk of fractures, and there is an association between vitamin K₁ intake and hip fracture among postmenopausal women. Given that, we also compared the potency of vitamin K₁ with that of MK-4 on osteoclastogenesis and osteoblastogenesis *in vitro*.

Materials and Methods

Formation of osteoclast-like multinucleated cells in human bone marrow culture

Multinucleated cells (MNCs) were formed by the method of Thavarajah *et al.* (1991) with modifications as follows. Bone marrow cells were obtained from the proximal region of the femur from elderly patients (aged 67–91 years) who gave their informed consent to be studied before undergoing joint replacement surgery because of hip fracture. Most of these patients suffered from type II osteoporosis, but none of them suffered from systemic disease likely to influence bone metabolism, or localized disease such as osteoarthritis and rheumatoid arthritis. Bone marrow fluid (about 2–6 ml) was diluted with 5–6 volumes of α -minimum essential medium (α -MEM) (Irvine Scientific, Santa Ana, CA, USA) containing 20% heat-inactivated horse serum (Gibco Brl, NY, USA), and filtered with a cell strainer (70 μ m, Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) to remove bone chips and blood clots. The filtered fluid was layered over an equal volume of Histopaque 1077 and centrifuged at 400 g for 30 min at 20 °C. The mononuclear-cell-rich fraction at the interface of the discontinuous gradient was transferred to new plastic tubes. The cells were washed three times by centrifugation with 5 volumes of α -MEM containing 5% horse serum. The precipitated cells ($2.7 \sim 5 \times 10^5$) were suspended in 0.4 ml α -MEM containing 20% horse serum and 10 nM 1,25(OH)₂D₃, and

seeded in an eight-well Lab-Teck chamber (0.8 cm²/well, Nalgen Nunk International, Rochester, IL, USA). A 1000-times greater concentration of 10 nM 1,25(OH)₂D₃ and 0.5, 1.0, 2.5 and 10 μ M MK-4 or vitamin K₁ was dissolved in ethanol and dimethyl sulfoxide respectively and added to the culture. The final concentration of the vehicle was 0.1% that of the culture medium. 1,25(OH)₂D₃ and vitamin Ks (MK-4 and vitamin K₁) were kindly supplied by Teijin (Tokyo, Japan) and Eisai Co. (Tokyo, Japan) respectively. Their vehicles were added to control cells. After 5 days, the conditioned medium containing floating cells was removed gently, and 0.6 ml fresh medium containing vitamins was added to the culture. Primary cultures were subsequently maintained by renewing half of the culture medium twice a week until MNCs were observed. The cultures were maintained for up to 16 days in a humidified atmosphere of 95% air with 5% CO₂.

In order to prepare MNC-rich cultures, stromal cells and other mononuclear cells that had adhered to the MNCs were removed on day 16 by treatment with 0.002% pronase (protease, Sigma Chemical Co.) and 0.02% EDTA for 5 min (Tezuka *et al.* 1992). Resulting adherent cells were cultured overnight for recovery from the treatment. Tartrate-resistant acid phosphatase (TRAP) activity in the putative osteoclast-like MNCs was detected by staining with an acid phosphatase detection kit (Sigma Chemical Co.), according to the instructions supplied by the company. TRAP-positive MNCs with more than three nuclei were counted as osteoclasts. The value is represented by the average number of TRAP-positive MNCs per well.

Detection of osteoclast apoptosis and cytotoxicity

The method used for detection of osteoclast apoptosis was that described previously by Kameda *et al.* (1996), with slight modifications. Isolated osteoclasts were seeded on the cover glass (15 mm diameter and 0.12–0.17 mm thick), and treated with or without 10 μ M MK-4 for 24 h. Cells were incubated with 3 mM Hoechst 33258 to visualize the localization of DNA for 2 h before fixation with 10% neutral-buffered formalin. Cells were examined under a fluorescence microscope (Axiophot, Carl Zeiss, Jena Gudvan, Vogel, Germany) for determination of osteoclasts with chromatin condensation, nuclear fragmentation, or both. Determinations were made on two fields on each cover glass of three replicate samples.

For assay of cytotoxicity during the formation of osteoclasts from isolated bone marrow monocytes, the monocytes were cultured with or without MK-4 in α -MEM containing 10 nM 1,25(OH)₂D₃ and 20% horse serum. When culture media were changed at day 13, the conditioned (4 days) media were kept for measurement of the activity of lactate dehydrogenase (LDH) released from damaged cells. The LDH reaction mixture (Wako Pure

Table 1 Oligonucleotide primers used in PCR

Target cDNA	Primer sequence	Product size (bp)	Annealing temperature (°C)	Cycles
ALP	S: 5'-ATCGCCTACCAGCTCATGCAT-3' AS: 5'-GACATGCAGTACGAGCTGAAC-3'	291	56	35
Cathepsin K	S: 5'-ATATGTGCAGAAGAACCGGG-3' AS: 5'-AGAGCAGGATGGATTTGGCT-3'	458	60	25
RANK	S: 5'-TTTGCAGATCGCTCCTCCAT-3' AS: 5'-AGGCATCAGAGAAGTAGCCT-3'	407	60	33
OPG/OCIF	S: 5'-AACCCAGAGCGAATAC-3' AS: 5'-AAGAATGCCTCCTCACAC-3'	219	60	28~32
RANKL/ODF	S: 5'-AGAGAAAGCGATGGTGGATG-3' AS: 5'-AGTAAGGAGGGGTGGAGAC-3'	471	63	40
Cbfa-1	S: 5'-CCGCCACGACAAACCGACCAT-3' AS: 5'-GCTCTGTGATAGGTAGCTAC-3'	365	54	32
γ -Carboxylase	S: 5'-TTGCAGATTTCCCTGGACTG-3' AS: 5'-GATACAAGTCATCAGGAAGC-3'	509	63	32
GAPDH	S: 5'-ACCACAGTCCATGCCATCAC-3' AS: 5'-TCCACCACCCTGTTGCTGTA-3'	452	60	25

S, sense strand; AS, antisense strand.

Chemicals, Osaka, Japan) was added to an equal volume of the conditioned medium. After the mixture had stood for 45 min at room temperature, its absorbance at 490 nm was measured using a microplate reader (Emax; Molecular Devices, Sunnyvale, CA, USA). The reference wavelength was 600 nm. The absorbance was compared between untreated control and vitamin K₂-treated samples, with four replicates.

Determination of osteoprotegerin/osteoclast inhibitory factor concentrations by enzyme-linked immunosorbent assay

Five days and 16 days after the start of bone marrow culturing, the conditioned media, from which floating cells had been removed by centrifugation, were used to determine osteoprotegerin/osteoclast inhibitory factor (OPG/OCIF) concentrations by enzyme-linked immunosorbent assay (ELISA). OPG ELISA kits were purchased from Cosmo Bio Co. (Tokyo, Japan). The concentration range of the assay supplied was 31.25~500 pg/ml. Monoclonal antibody against a mixture (1:1) of the monomeric and homodimeric forms of recombinant human OPG was used. The coefficient of variation was less than 10% when the same samples were measured five times at the same time. Details have been reported by Yano *et al.* (1999).

Separation of human stromal cells from osteoclasts

To investigate the effect of vitamin K on the expression of osteoclastogenesis-related genes, we first separated human stromal cells and osteoclasts on day 16 after starting the

Figure 1 Dose-response effect of MK-4 and vitamin K₁ on osteoclast formation in human bone marrow culture. Mononuclear cells ($2.7 \sim 5.0 \times 10^5$ cells/well in a Lab-Tek chamber) from human bone marrow were cultured with various concentrations of MK-4 and vitamin K₁ in the presence of 10 nM 1,25(OH)₂D₃ for 16 days. Each assay comprised four wells. TRAP-positive MNCs with more than three nuclei on a well were counted as osteoclasts. Three experiments were performed using bone marrow cells from three different patients. The values were expressed as (mean $\pm$ S.E.) % of control (non-addition) in each experiment and expressed as a graph. The control values were 3337, 2362 and 948 TRAP-positive MNCs/well for experiments 1, 2 and 3 respectively. MK-4 and vitamin K₁ inhibited osteoclast formation. *** $P < 0.001$, significant difference from non-addition.

Figure 2 Effect of MK-4 on long-term culture of human bone marrow. Mononuclear cells from human bone marrow were cultured in the presence of 10 nM $1,25(\text{OH})_2\text{D}_3$ with or without 10 μM MK-4 for 16 days as described above. Stromal cells were separated from the culture by a brief treatment with pronase/EDTA, re-seeded and cultured overnight. Undetached cells were also cultured overnight. The phase-contrast micrographs show that MK-4 inhibited osteoclast formation and enhanced stromal cell formation. Scale bar represents 100 μm .

bone marrow culture in the presence of MK-4. The bone marrow culture containing osteoclasts, stromal cells and some mononuclear cells was treated with 0.002% pronase and 0.02% EDTA for 5 min. Detached cells, which were mostly stromal cells, were collected by centrifugation. The

Figure 3 Production of OPG in long-term culture of human bone marrow. OPG concentration in the conditioned medium of the bone marrow culture depicted in Fig. 2 was determined by ELISA on days 5 and 16. High concentrations (10 μM) of MK-4 increased OPG secretion. The data are from four replicate experiments per patient. Similar results were obtained in another set of experiments. * $P < 0.05$, significant difference from non-addition.

resulting cell pellet was suspended in α -MEM containing 20% horse serum, re-seeded in the dish and cultured in the presence or absence of 0.5–10 μM MK-4 overnight. Adherent cells, which were mostly osteoclasts, were also cultured in the presence or absence of vitamins. Two days later, the second treatment with pronase/EDTA was carried out to prepare highly pure stromal cell and osteoclast fractions. Detached cells were pooled with the first set of stromal cells. The next day, total RNA was isolated by the acid guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski & Sacchi 1987). Reverse transcriptase-coupled polymerase chain reaction (RT-PCR) for the expression of alkaline phosphatase (ALP) and cathepsin K was performed to determine the purity in a stromal-cell-rich and an osteoclast-rich fraction.

Formation of ALP-positive colony-forming-unit fibroblast colonies

Colony-forming-unit fibroblast (CFU-F) colonies were formed by the method of D'Ippolito *et al.* (1999), with modifications. Briefly, mononuclear cells ($0.4 \sim 1.6 \times 10^5$ /well) isolated from human bone marrow were plated into 12-well dishes (3.8 cm^2 , Costar, Cambridge, MA, USA) in 1.0 ml α -MEM containing 20% horse serum. The next day, 0.5–10 μM MK-4 or vitamin K_1 was added in the

Figure 4 Separation of osteoclast- and stromal-cell-rich fractions. After long-term culture of mononuclear cells from bone marrow, osteoclasts and stromal cells were separated by pronase/EDTA treatment as described in Materials and Methods. Expression of mRNAs for ALP and cathepsin K of osteoclast- and stromal-cell-rich fractions were investigated by RT-PCR, using total RNA extracted from these fractions. Similar results were obtained in two other experiments. The levels of ALP and cathepsin K mRNA were compared with that of GAPDH mRNA by densitometry. The ratios of ALP and cathepsin K mRNA to GAPDH mRNA from three experiments are presented as mean $\pm$ S.E. under the gel photograph. ** $P < 0.01$, *** $P < 0.001$, significant differences between osteoclast- and stromal-cell-rich fractions on each treatment.

presence or absence of 1 nM dexamethasone (Sigma). After 1 week, half the growth medium in the culture was removed and replaced with fresh medium. Beginning on that day, the medium was changed twice a week. Cells were incubated for 24 days in a 100% humidified atmosphere of 95% air–5% CO₂ at 37 °C. For studies involving the production of mineralized matrix, cells were grown for another 3 weeks in the same medium supplemented with 2 mM α -glycerophosphate in the presence or absence of 1 nM 1,25(OH)₂D₃. α -Glycerophosphate is a stimulator of osteoblast mineralization (Koshihara *et al.* 1987).

To assay for ALP activity, cells were washed with Ca²⁺- and Mg²⁺-free phosphate-buffered saline (pH 7.4) and fixed with 10% neutral-buffered formalin for 10 min. ALP activity was assayed as described previously (Liu *et al.* 1987). The fixed cells were washed thoroughly with water and reacted with ALP substrate (Vector Blue; Vector Laboratories, Burlingame, CA, USA). After assay for ALP activity on day 21, CFU-F colonies with 50 or more cells (the conventional values for defining a colony; Freidenstein 1976) were scored visually as positive (blue stain, CFU-F/ALP⁺) using an Olympus BH-2 dissecting microscope (Olympus, Tokyo, Japan).

RT-PCR

Total RNA was isolated from cultured bone marrow cells and osteoclast- and a stromal-cell-rich fractions by the acid guanidinium thiocyanate–phenol–chloroform extraction method. The RT reaction was performed using an RNA LA PCR kit (Ver.1.1, Takara Biochemicals, Osaka, Japan). First, 1 μg total RNA from cells was hybridized to oligo dT-adaptor primer, and the RT reaction was carried out using AMV reverse transcriptase XL (Life Sciences, Boston, MA, USA) for 1 h at 42 °C. PCR of ALP, cathepsin K, receptor-activator of nuclear factor κ B (RANK), OPG and RANK ligand (RANKL) cDNA was performed using HotStarTaq (Qiagen Inc., Valencia, CA, USA). The synthetic forward primer and the reverse primer of each cDNA were designed as described in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as control cDNA samples. Most of the primers have been reported previously (cathepsin K and RANK: Higuchi *et al.* 1999; ALP and GAPDH: Togari *et al.* 1997; OPG/OCIF: Vidal *et al.* 1998; γ -carboxylase: Miyake *et al.* 2001). Human mRNA-specific primers for Cbfa1 and RANKL (synthesized by

Figure 5 Effect of MK-4 on expression of RANK in an osteoclast-rich fraction (a), and expression of RANKL (b) and OPG (c) in a stromal-cell-rich fraction. Human bone marrow mononuclear cells were cultured with various concentration of MK-4 for 16 days. Total RNA extracted from osteoclast- and stromal-cell-rich fractions was subsequently used in the mRNA RT-PCR. Expression of GAPDH mRNA did not differ much between samples. Similar results were obtained in two other experiments. The ratios of levels of mRNA for RANK/GAPDH, RANKL/GAPDH and OPG/GAPDH in each band in three experiments are presented as mean ± s.e. under the gel photograph. MK-4 treatment inhibited the expression of RANKL mRNA and stimulated that of OPG mRNA, but did not affect expression of RANK mRNA. **P*<0.05, ***P*<0.01, ****P*<0.001, significant differences from non-addition.

Life Technologies) were designated on the basis of published sequences (Genebank). The PCR profile followed was: denaturation at 95 °C for 15 min followed by each cycle of denaturation at 94 °C for 30 s, reannealing at the temperatures indicated in Table 1 for 30 s, and extension at 72 °C for 1 min. PCR products were separated by electrophoresis on a 2.0% agarose gel.

Results

Effect of MK-4 on long-term culture of human bone marrow

Human mononuclear cells isolated from bone marrow were cultured long term in the presence of 10 nM

1,25(OH)₂D₃ to form TRAP-positive MNCs. These TRAP-positive MNCs possessed osteoclast characteristics such as bone resorption activity, expression of calcitonin receptor (Nishikawa *et al.* 1999) and cathepsin K, and actin ring formation (Koshihara *et al.* 1999). 1,25(OH)₂D₃-induced osteoclast formation, shown by the presence of TRAP-positive MNCs, was inhibited by MK-4, a vitamin K₂ with four isoprene units. Osteoclast formation was also inhibited by vitamin K₁ in a dose-dependent manner from 0.5 to 10 μM (Fig. 1). Their potency was similar. Accordingly, further studies were performed using MK-4. A decrease in osteoclast formation by MK-4 treatment in bone marrow culture was not dependent on an increase in cell death: cell viability as determined by LDH activity in media conditioned for 4 days (from day 9 to day 13) was 0.289 ± 0.040 and 0.318 ± 0.036 optical density units at 490 nm for untreated control cells and 10 μM for MK-4-treated cells (*P*=0.5705, not significant). Moreover, apoptotic frequency as determined by Hoechst 33258 staining was 0.85 ± 0.39% (*n*=6) and 3.96 ± 1.1% (*n*=6) for untreated control cells and 10 μM MK-4-treated cells (*P*=0.023).

In contrast, stromal cells differentiated from mesenchymal progenitor cells were increased by the MK-4 treatment (Fig. 2). As osteoclasts adhere more tightly to the dish than do stromal cells, osteoclasts can be separated from stromal cells by short-term treatment with pronase/EDTA solution. Detached stromal cells re-seeded to dishes showed clearly different numbers of cells between 10 μM MK-4-treated dishes (683.3 ± 19.5 cells/cm²) and untreated dishes (258 ± 4.6 cells/cm²; *n*=3 replicate dishes from the same patient). This was statistically significant (*P*<0.001).

Stromal cells from mesenchymal progenitor cells, which differentiate into preosteoblasts and osteoblasts, regulate osteoclast formation positively by expressing RANKL on the cell membrane and negatively by producing OPG, a decoy receptor of RANKL, by interacting with RANK expressed on the osteoclast cell membrane (Suda *et al.* 1999). At day 16, just before separation of stromal cells from osteoclasts, production of OPG was significantly greater in a 10 μM sample of MK-4-treated bone marrow cells than in similar samples given other treatments (Fig. 3), but the stimulatory effect of MK-4 was not seen at day 5. Because the OPG concentration was measured in the bone marrow culture containing osteoclasts and stromal cells, these results implied either enhancement of OPG secretion by individual stromal cells or an increase in the number of stromal cells, or both.

To investigate whether vitamin K inhibits interaction between RANKL and RANK, we examined the effect of MK-4 on the expression of mRNAs for RANK, or for RANKL and OPG, in osteoclasts and stromal cells, respectively. First we tried to separate osteoclast- and stromal-cell-rich fractions in bone marrow culture at day 16 by brief treatment with pronase/EDTA, as described

Figure 6 Dose-dependent increase in CFU-F/ALP⁺ formation in response to vitamin Ks in bone marrow culture. Human mononuclear cells ($0.4 \sim 1.6 \times 10^5$ /well) from bone marrow (three experiments using bone marrow cells from three different patients) were cultured with various concentrations of MK-4 (a) and vitamin K₁ (b). CFU-F/ALP⁺ colonies in each assay ($n=3$ wells) were counted. The values are expressed as % of control (non-addition) in each experiment; means $\pm$ S.E. for three experiments are presented. The control values were 18.6, 21.3 and 11 colonies/well with MK-4-treatment, and 12.3, 22 and 8 colonies/well with vitamin K₁ treatment for experiments 1, 2 and 3 respectively. MK-4 and vitamin K₁ stimulated the formation of CFU-F/ALP⁺. ** $P<0.01$, *** $P<0.001$, significant differences from non-addition.

in Materials and Methods. We confirmed the purity of expression of ALP and cathepsin K mRNAs for stromal cells and osteoclasts respectively. Osteoclast-rich fractions clearly expressed cathepsin K in every fraction and scarcely expressed ALP. In contrast, stromal-cell-rich fractions clearly expressed ALP, but not cathepsin K (Fig. 4). These results indicated that osteoclasts and stromal cells had been clearly separated. We used RT-PCR to investigate the expression of RANK mRNA in the osteoclast fractions and the expression of RANKL and OPG mRNA in stromal-cell fractions. In these fractions, MK-4 did not affect the expression of RANK in osteoclasts (Fig. 5a). There was no difference in expression among MK-4 treatments in any of the PCR conditions. However, RT-PCR revealed that expression of RANKL in stromal cells was inhibited by MK-4 treatment in a dose-dependent manner (Fig. 5b). Conversely, 10 µM MK-4 enhanced expression of OPG in particular (Fig. 5c). When the expression was represented quantitatively as the ratio of RANKL and OPG to GAPDH of each band in arbitrary units of intensity, expression of RANKL was decreased approximately 80% by 10 µM MK-4, but expression of OPG was increased 200% by 10 µM MK-4.

These results implied that MK-4 regulates not only differentiation of progenitor cells into stromal cells, but also gene expression in stromal cells.

Increase in CFU-F/ALP⁺ formation in response to vitamin K treatment

To investigate whether vitamin K affects osteoblastogenesis, we studied the effects of MK-4 and vitamin K₁ on

ALP-positive CFU-F (CFU-F/ALP⁺) colony formation in bone marrow culture. CFU-F/ALP⁺ colony formation was increased threefold by 1 nM dexamethasone, a stimulator of proliferation and differentiation of osteoprogenitor cells (Bellows *et al.* 1990). The formation of CFU-F/ALP⁺ was enhanced by the MK-4 treatment, in either the presence or absence of dexamethasone. It was possible that the vitamin Ks might have promoted CFU-F/ALP⁺ colony formation independently of dexamethasone. Therefore we investigated the dose-response effects of MK-4 and vitamin K₁ on CFU-F/ALP⁺ colony formation in human bone marrow culture in the absence of dexamethasone. MK-4 enhanced CFU-F/ALP⁺ colony formation in a dose-dependent manner (from 1.0 to 10 µM) (Fig. 6a). Vitamin K₁ also enhanced CFU-F/ALP⁺ colony formation (Fig. 6b), with a potency almost the same as that of MK-4. RT-PCR analysis demonstrated that these cells expressed not only ALP mRNA, but also Cbfa-1 mRNA, which is an essential transcription factor for osteoblast differentiation and bone formation (Komori *et al.* 1997) (data not shown). These results suggest that MK-4 has the ability to induce the differentiation of osteoprogenitor cells to osteoblasts. To confirm whether CFU-F/ALP⁺ cells would indeed differentiate into osteoblasts, we further cultured CFU-F/ALP⁺ cells in the presence of 2 mM α -glycerophosphate to investigate their ability to mineralize. Von Kossa staining revealed that the presence of MK-4 or vitamin K₁ alone did not result in notable mineralization by CFU-F/ALP⁺ cells, but these vitamin Ks enhanced 1,25(OH)₂D₃-induced mineralization (Fig. 7).

Figure 7 Mineralization by CFU-F/ALP⁺ cells. CFU-F/ALP⁺ cells were cultured in medium containing 2 mM α -glycerophosphate for 3 weeks. The cells were cultured in the presence of 1 nM 1,25(OH)₂D₃, 10 μ M MK-4, or both. After fixation, cells were stained with Von Kossa solution. (a) Non-addition; (b) addition of 1,25(OH)₂D₃; (c) addition of 1,25(OH)₂D₃ plus MK-4; (d) addition of 1,25(OH)₂D₃ plus vitamin K₁. Similar results were obtained in at least two different experiments. Scale bar represents 100 μ m.

Discussion

In clinical practice, MK-4 (vitamin K₂) treatment in cases of osteoporosis has been shown to be successful in inhibiting the occurrence of new bone fractures and increased γ -glutamyl carboxylation of osteocalcin (Shiraki *et al.* 2000). There were no significant changes in markers of bone resorption, such as urinary excretion of pyridium. Moreover, *in vitro* studies have shown that MK-4 promotes 1,25(OH)₂D₃-induced mineralization by human osteoblasts (Koshihara *et al.* 1996, Koshihara & Hoshi 1997). These reports suggested a predominant effect of MK-4 on bone formation via osteoblasts. Our findings further support this: both types of vitamin K, vitamin K₁ and vitamin K₂, promoted osteoblastogenesis, with formation of CFU-F/ALP⁺ colonies from mesenchymal progenitor cells in human bone marrow culture (Fig. 6). Although CFU-Fs are a heterogeneous population of stem and progenitor cells (Owen & Friedenstein 1988), colonies with ALP activity are considered to have osteogenic potential (Owen *et al.* 1987) and thus are considered to be osteoprogenitors.

Conversely, osteoclast formation in human bone marrow culture was inhibited by treatment with both types of vitamin K, in the form of MK-4 and vitamin K₁. These inhibitory effects have also been reported in unfractionated bone cells from murine femurs and tibia in culture (Notoya *et al.* 1995). The effective concentration of MK-4 was more than 0.5 μ M, which was one-sixth lower than that in murine bone marrow culture as reported previously (Akiyama *et al.* 1994). This finding implies that human bone marrow cells are more sensitive to MK-4 than are the mouse cells.

MK-4 and vitamin K₁ both have a naphthoquinone skeleton, but they have different side chains, of geranyl-geranyl and phytol respectively. Accordingly, our data suggested that the effectiveness of the two types of vitamin K might originate in the naphthoquinone skeleton, not the side chains, as MK-4 and vitamin K₁ had similar potency.

A decrease in osteoclast formation by treatment of bone marrow cultures with MK-4 was not dependent on an increase in cell death, as 10 μ M MK-4 did not significantly increase LDH activity in the conditioned medium. Moreover, only 3.96% apoptosis was observed

for osteoclasts treated with 10 μ M MK-4, which was considerably different from the 6.0% previously reported for rabbit osteoclasts (Kameda *et al.* 1996). In contrast, MK-4 exhibited 80% inhibition of osteoclast formation in human bone marrow culture. Accordingly, these results suggested that MK-4 did indeed inhibit osteoclast formation in human bone marrow culture, and that the inhibition was not due to a decrease in cell viability and an increase in apoptosis.

As intake of vitamin K₁ decreases fracture frequency (Feskanish *et al.* 1999), MK-4 and vitamin K₁ both probably improve bone metabolism. However, conversion of dietary vitamin K₁ to tissue MK-4 in rats has been reported (Davidson *et al.* 1998). The conversion is not dependent on gut bacteria in a metabolic pathway. It is not yet known whether the conversion occurs in human beings.

The molecular mechanism underlying the ability of stromal cells/osteoblasts to support osteoclast formation was recently clarified by the identification of RANKL, a transmembrane protein that has proved to be an essential molecule for osteoclastogenesis (Lacey *et al.* 1998, Yasuda *et al.* 1998, Kong *et al.* 1999). The effect of RANKL is blocked by its soluble decoy receptor OPG (Simonet *et al.* 1997, Tsuda *et al.* 1997), which is produced by stromal cells/osteoblasts. Osteoclastogenesis is assumed to be controlled by the balance between RANKL and OPG *in vivo*; therefore, it would be important to evaluate the expression and generation of RANKL and OPG mRNAs simultaneously. To induce RANKL generation and osteoclastogenesis, we utilized 1,25(OH)₂D₃, which has often been used in models of osteoclast formation. MK-4 inhibited 1,25(OH)₂D₃-induced RANKL mRNA expression in stromal cells in a dose-dependent manner (1.0–10 μ M) (Fig. 5b), but expression and secretion of OPG mRNA in stromal cells were enhanced by the treatment with a concentration of MK-4 as high as 10 μ M (Fig. 5c). As expression of RANK mRNA, which is the real receptor of RANKL present on the membrane, was not affected by the MK-4 treatment, the inhibition of osteoclastogenesis by MK-4 might be due to a decrease in generation of RANKL in stromal cells, rather than an increase in production of OPG. We were able to demonstrate the involvement of RANKL in osteoclast differentiation in the human bone marrow culture used here, because osteoclast formation was inhibited about 50% by the addition of OPG 100 ng/ml (data not shown) – a concentration greater than that reported by others for murine bone marrow culture (IC₅₀ = 4–6 ng/ml) (Tsuda *et al.* 1997). In general, a relatively high concentration of OPG (25–100 ng/ml) was required to elicit the inhibitory effect on osteoclast formation in human cell cultures (Matsuzaki *et al.* 1998, Atkins *et al.* 2001).

Recently, Takeuchi *et al.* (2000) also reported that, in murine bone marrow culture, MK-4 inhibited the expression of RANKL mRNA and the formation of

osteoclast-like cells, but they did not investigate the expression and secretion of OPG. As their investigations were in bone marrow cells, not isolated stromal cells, their findings may reflect a decrease in the number of stromal cells.

In this study, we suggested that vitamin Ks (MK-4 and vitamin K₁) promoted the differentiation of bone marrow mesenchymal progenitor cells into osteoprogenitor cells, CFU-F/ALP⁺ cells, and inhibited osteoclast formation in human bone marrow culture. It is possible that these effects are interrelated. The mechanism by which vitamin Ks induces CFU-F/ALP⁺ formation in bone marrow culture should be investigated further.

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