Vitamin K promotes mineralization, osteoblast-to-osteocyte transition, and an anticalcatabolic phenotype by γ-carboxylation-dependent and -independent mechanisms

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Atkins GJ, Welldon KJ, Wijenayaka AR, Bonewald LF, Findlay DM. Vitamin K promotes mineralization, osteoblast-to-osteocyte transition, and an anticalcatabolic phenotype by γ-carboxylation-dependent and -independent mechanisms. Am J Physiol Cell Physiol 297: C1358–C1367, 2009. First published August 12, 2009; doi:10.1152/ajpcell.00216.2009.—The vitamin K family members phylloquinone (vitamin K1) and the menaquinones (vitamin K2) are under study for their roles in bone metabolism and as potential therapeutic agents for skeletal diseases. We have investigated the effects of two naturally occurring homologs, phytomadione (vitamin K1) and menatetrenone (vitamin K2), and those of the synthetic vitamin K, menadione (vitamin K3), on human primary osteoblasts. All homologs promoted in vitro mineralization by these cells. Vitamin K1-induced mineralization was highly sensitive to warfarin, whereas that induced by vitamins K2 and K3 was less sensitive, implying that γ-carboxylation and other mechanisms, possibly genomic actions through activation of the steroid xenobiotic receptor, are involved in the effect. The positive effect on mineralization was associated with decreased matrix synthesis, evidenced by a decrease from control in expression of type I collagen mRNA, implying a maturational effect. Incubation in the presence of vitamin K2 or K3 in a three-dimensional type I collagen gel culture system resulted in increased numbers of cells with elongated cytoplasmic processes resembling osteocytes. This effect was not warfarin sensitive. Addition of calcine to vitamin K-treated cells revealed vitamin K-dependent deposition of mineral associated with cell processes. These effects are consistent with vitamin K promoting the osteoblast-to-osteocyte transition in humans. To test whether vitamin K may also act on mature osteocytes, we tested the effects of vitamin K on MLO-Y4 cells. Vitamin K reduced receptor activator of NF-κB ligand expression relative to osteoprotegerin by MLO-Y4 cells, an effect also seen in human cultures. Together, our findings suggest that vitamin K promotes the osteoblast-to-osteocyte transition, at the same time decreasing the osteoclastogenic potential of these cells. These may be mechanisms by which vitamin K optimizes bone formation and integrity in vivo and may help explain the net positive effect of vitamin K on bone formation.

phytomadione; vitamin K1; menatetrenone; vitamin K2; menadione; human primary osteoblast; osteocyte; MLO-Y4

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postmenopausal, nonosteoporotic women by maintaining bone mineral content of the femoral neck (33). Additionally, increased effectiveness of bisphosphonate has been reported in combination with vitamin K2 (26). The Yamaguchi Osteoporosis Prevention Study reported that vitamin K reduced vertebral fractures by 56% compared with placebo (24). Despite these positive associations between vitamin K and bone health, intake of phylloquinone (vitamin K1) in British children (and, presumably, adults as well) has decreased significantly since 1950, with a change from diets high in vegetables to diets with greater amounts of fats and oils (44).

In vitro experiments have demonstrated effects of vitamin K2 on osteoblast function. Vitamin K2, but not K1, inhibited receptor activator of NF-κB (RANK) ligand (RANKL) mRNA expression, together with inhibition of 1,25-dihydroxyvitamin D3-induced osteoclastogenesis from bone marrow cells (51). Menaquinone-7 was anabolic in rat bone organ cultures, and treatment of MC3T3-E1 mouse osteoblast-like cells increased alkaline phosphatase (AP) and OCN expression (55). In a more recent report, vitamin K2 induced RANKL, RANK, and osteoprotegerin (OPG) expression in MC3T3-E1 cells, with little effect on cell proliferation (32). There are few reports of the action of vitamin K in human osteoblasts. Vitamins K1 and K2 were reported to increase the number of colony-forming units-fibroblast expressing AP generated from human bone marrow cells (34). 1,25-Dihydroxyvitamin D3-induced mineralization of these cells was enhanced by vitamins K1 and K2. In contrast, vitamins K1 and K2 inhibited 1,25-dihydroxyvitamin D3-induced osteoclast formation in human bone marrow cultures, concomitant with decreased expression of RANKL mRNA and enhanced expression of OPG mRNA (34).

We hypothesized that vitamin K might exert its bone anabolic actions, at least in part, through direct actions on cells of the osteoblast lineage. Therefore, we investigated the effects of phytodiol (vitamin K1), menatetrenone (vitamin K2), also known as menaquinone-4, and the synthetic vitamin K analog menadione (vitamin K3) on adult human primary osteoblasts derived from trabecular bone and also the mouse osteocyte cell line MLO-Y4 (31). Vitamin K had suppressive effects on cell proliferation (32). There are few reports of the action of vitamin K in human osteoblasts. Vitamins K1 and K2 were reported to increase the number of colony-forming units-fibroblast expressing AP generated from human bone marrow cells (34). 1,25-Dihydroxyvitamin D3-induced mineralization of these cells was enhanced by vitamins K1 and K2. In contrast, vitamins K1 and K2 inhibited 1,25-dihydroxyvitamin D3-induced osteoclast formation in human bone marrow cultures, concomitant with decreased expression of RANKL mRNA and enhanced expression of OPG mRNA (34).

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**MATERIALS AND METHODS**

**Reagents.** Phytodiol (vitamin K1) and menatetrenone (vitamin K2) were kind gifts from Eisai (Tokyo, Japan). Menadione (vitamin K3), warfarin, and rifampicin were purchased from Sigma Chemical (St. Louis, MO).

**Cell culture.** Human primary osteoblast-like cells, which we previously termed NHBC, were obtained from a number of different individuals by growth from trabecular bone samples obtained at joint replacement surgery. Informed consent was obtained from the patients, and the study was approved by the Human Ethics Committee of the Royal Adelaide Hospital. The samples were processed for culture as described previously (5). Briefly, cells were cultured in α-MEM containing 10% FCS, l-glutamine (2 mM), HEPES (1 mM), and ascorbate 2-phosphate (100 μM) at 37°C and 5% CO2 in a humidified incubator. All experiments were performed with cells passaged a maximum of three times; the cells were enzymically removed from dishes using collagenase, dispase, and trypsin and plated onto tissue culture plastic flasks or wells. MLO-Y4 cells were passaged on type I collagen-coated plates, as previously described (31).

**Flow cytometric analysis.** Surface AP expression was analyzed by flow cytometry, as previously described (3). Briefly, NHBC were harvested and resuspended in blocking buffer [HBSS, 1% normal human AB blood type serum, 1% BSA (Sigma, St. Louis, MO), and 5% FCS] and then incubated on ice with saturating concentrations of...
MAB B-478 (anti-human bone/liver/kidney AP, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) or isotype-matched negative control MAB (3). Cells were washed three times by centrifugation in HBSS-5% FCS and incubated with goat anti-mouse IgG-FITC (1:50 dilution; Southern Biotechnology Associates, Birmingham, AL) for 45 min on ice. After they were incubated and further washed, cells were resuspended, fixed in PBS containing 1% (wt/vol) paraformaldehyde, and analyzed by flow cytometry (3).

**Mineralization in vitro.** A modification of methods reported previously (2, 16) was used to determine the ability of NHBC to form a mineralized matrix. Cells were incubated in triplicate in wells of a 96-well plate (8 × 10^3 cells/well) in α-MEM containing 10% FCS, dexamethasone (10^-8 M), KH2PO4 (1.8 mM), and HEPES (10 mM), in the presence or absence of various concentrations of vitamin K homolog or an equivalent vehicle (ethanol) dilution. Media were replaced at 4-day intervals, and incubation was continued as indicated for up to 6 wk before measurement of cell layer-associated calcium levels, as previously described (16). In separate experiments, NHBC cultures were established in eight-well chamber slides (Lab-Tek, Nalge Nunc International, Rochester, NY), and cells were grown for 6 wk under mineralizing conditions in the presence or absence of each vitamin K homolog. Supernatants were removed, and the cell layers were fixed in 4% paraformaldehyde for 10 min. The mineral layer was partially decalcified by incubation for 24 h in 2 mM EDTA solution. Cells were then stained with phalloidin-tetramethylrhodamine isothiocyanate (TRITC, Sigma) for 1 h at room temperature and then with 1 mg/ml of 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, Roche Diagnostics, Castle Hill, NSW, Australia) in PBS for 5 min at 37°C. Slides were mounted in Prolong-Gold-DAPI antifade reagent (Molecular Probes, Sunnyvale, CA) and then examined on a dual-laser confocal microscope (MRC-1000UV Confocal Scanning Microscope System, Bio-Rad Laboratories, Hercules, CA). In some experiments, cells were cultured in the added presence of rifampicin and/or warfarin. Calcium levels in the cell monolayers were determined from replicate cultures at the indicated time points using a colorimetric assay for calcium, as previously described (16).

**3-D collagen gel cultures.** NHBC or MLO-Y4 cells were plated into eight-well Permanox chamber slides (Lab-Tek) in a type I collagen gel (Cell Matrix, Nitta, Japan) in mineralization medium, as we previously described (6). Cells were cultured for up to 28 days; then they were fixed in 4% paraformaldehyde in PBS for 10 min, stained with phalloidin-TRITC as described above, and examined by confocal microscopy (6). In some experiments, gels were exposed overnight to calcium (5 μg/ml) before fixation and phalloidin staining to measure mineralization in situ.

**Energy dispersive spectroscopy.** NHBC were cultured in chamber slides (Nunc Nalgene) under mineralizing conditions with the addition of various doses of vitamins K2 and K1 for 42 days, as described above. Cells were fixed in 4% paraformaldehyde in PBS for 10 min, and the slides were rinsed three times in deionized water and then dehydrated in graded concentrations of ethanol. Slides were coated with carbon and analyzed by energy dispersive spectroscopy using eDXi System version 2.2 software (EDAX, Mahwah, NJ) on a scanning electron microscope (model XL20, Philips). Three separate readings for each sample were collected to determine the mean calcium-to-phosphorus ratio in each mineralized surface.

**Cell proliferation.** Cell proliferation was assessed using the carboxyfluorescein succinimidyl ester (CFSE) technique, as we described previously with modifications. Cells were labeled with CFSE and plated in standard 96-well tissue culture plates (54) or, alternatively, into collagen gels. After a 2-h seeding period, medium was replaced with medium containing vitamin K. Cells were cultured for 7 days and then removed from the plates or the gel with use of collagenase. Cells were then fixed and analyzed by flow cytometry and assessed for proliferation using ModFit software, as we described previously (54).

**Gene expression.** For gene expression experiments, cells (NHBC or MLO-Y4) were cultured in collagen gels seeded into 12-well tissue culture plates. At the time points indicated, gels were digested for 1 h

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**Fig. 2.** Effect of naturally occurring vitamin K homologs on osteoblast mineralization. NHBC from 4 individual donors (NHBC1, NHBC2, NHBC3, and NHBC4) were cultured under mineralizing conditions for up to 42 days untreated (UT) or in the presence of vitamin K1 or K2, each at 1 × 10^-8 M. Cell monolayers were assayed for adsorption of Ca^{2+}. Values are means ± SE of quadruplicate wells. *P < 0.05 vs. UT.
with 1 ml of collagenase and then pelleted by centrifugation at 200 g for 5 min at 4°C. Total RNA was extracted from the pellet using the TRizol method (Life Technologies, Gaithersburg, MD) (4). cDNA (Supercript III, Promega, Madison, WI) was prepared as previously described (4). Gene expression was analyzed by quantitative RT-PCR using the SYBR Green incorporation technique, as we described previously (4). Relative gene expression between samples was calculated using the comparative cycle threshold method ($\Delta\Delta CT$), with GAPDH used as a housekeeping gene. Oligonucleotide primers for quantitative RT-PCR were designed in-house to flank intron/exon boundaries and were purchased from Geneworks (Thebarton, SA, Australia). Sequences of PCR primers used to amplify human RANKL and human or mouse GAPDH (2), human type I collagen-1, OCN, E11, OPG, and mouse RANKL, have been published previously (6). To amplify mouse OPG, we used the forward primer 5'-agctggaaccccagagcgaa-3' and the reverse primer 5'-gcaggaggcaaatgtgctg-3' (product size 122 bp, annealing temperature 60°C), and for mouse E11, we used the forward primer 5'-aaacgcagacaacagataaagaaagat-3' and the reverse primer 5'-gttctgtttagctctttagggcga-3' (product size 158 bp, annealing temperature 60°C).

Statistical analysis. Differences between parametric data sets were tested using two-tailed (unpaired) Student’s t-tests or one-way ANOVA followed by Tukey’s post hoc test. *P < 0.05 was considered significant.

RESULTS

Effects of vitamin K on cell growth and differentiation. The effects of vitamin K on human primary osteoblast (NHBC) proliferation were assessed in experiments using the CFSE method, as we described previously. Both natural homologs vitamins K1 and K2 had mildly suppressive effects on cell proliferation (data not shown), implying a maturational effect. This possibility was explored further by determining the effects of the vitamin K homologs on cell phenotype. Changes in cell surface expression of the osteoblast phenotypic marker AP were tracked. In NHBC cultured for up to 14 days in the presence of vitamin K2 at 10^{-6} and 10^{-5} M and, to a lesser extent, in the presence of vitamin K1 at the highest concentration (10^{-5} M), we observed a decrease in cell surface AP expression (Fig. 1), consistent with the induction of a mature, postosteoblast phenotype (17, 18). We next assessed the effect of vitamin K on osteoblast differentiation. Deposition of a mineralized matrix provides an in vitro surrogate of bone formation. Time course studies were performed: NHBC were cultured under mineralizing conditions in the presence or absence of vitamins K1 and K2, and in vitro mineralization was assessed by energy dispersive spectroscopy (EDS). Ca/P, calcium-to-phosphorus ratio.

Table 1. EDS analysis of mineralized NHBC cultures

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ca/P</th>
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<tbody>
<tr>
<td>α-MEM</td>
<td>0.77±0.02</td>
</tr>
<tr>
<td>Control</td>
<td>1.66±0.07</td>
</tr>
<tr>
<td>K2 1×10^{-6} M</td>
<td>1.72±0.12</td>
</tr>
<tr>
<td>K2 5×10^{-6} M</td>
<td>1.63±0.08</td>
</tr>
<tr>
<td>K2 1×10^{-5} M</td>
<td>1.78±0.03</td>
</tr>
<tr>
<td>K1 1×10^{-6} M</td>
<td>1.58±0.01</td>
</tr>
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Values are means ± SD of 3 readings. Normal human bone-derived cells (NHBC) were cultured for 6 wk in the absence of phosphate (α-MEM), under control mineralization conditions, or in the additional presence of vitamin K2 or K1. Samples were prepared for and analyzed by energy dispersive spectroscopy (EDS). Ca/P, calcium-to-phosphorus ratio.

Fig. 3. Dependence of mineralization effect of vitamin K on γ-carboxylation. NHBC were cultured under mineralizing conditions for up to 42 days either untreated (UT) or in the presence of vitamin K1 (A), K2 (B), or K3 (C) at 1, 5, or 10 μM and in the presence or absence of warfarin at subtoxic concentrations (20 or 50 μM). D: cells were incubated with rifampicin (Rif) ± warfarin over the same concentration range. Cell monolayers were assayed for Ca^{2+} adsorption. Note differences in magnitude of Ca^{2+} adsorption (y-axes) between the various analogs. Values are means ± SE of quadruplicate wells. *P < 0.05 vs. UT.
was measured by assessment of calcium apposition. Cell matrix-associated calcium was increased in the presence of either analog at most time points, with vitamin K2, in particular, having a consistent effect as early as day 14, in all donors’ cells tested (Fig. 2). Interestingly, especially in the case of vitamin K2, a positive effect on mineralization was seen regardless of the intrinsic ability of each donor’s cells to mineralize (e.g., compare relative levels of calcium in “untreated” for donors 3 and 4 in Fig. 2). Energy dispersive spectroscopy analysis confirmed that the mineral deposited was a bone-like hydroxyapatite, with calcium-to-phosphorus molar ratios in the resulting mineral similar to that in control mineralized cultures (Table 1) and that reported for bone (1.63) (9).

To explore the involvement of the γ-carboxylation function of vitamins K1 and K2, as well as the synthetic homolog menadione (vitamin K3), cells were mineralized in the presence or absence of warfarin at two subtoxic concentrations (5 and 20 μM). Figure 3 shows that in vitro mineralization induced by vitamin K1 was dependent on γ-carboxylation activity. Vitamins K2 and K3, however, were only partially sensitive to warfarin, implying γ-carboxylation-independent mechanisms of vitamin K-induced mineralization, possibly genomic actions through the SXR. Interestingly, a classical activator of the SXR pathway, rifampicin, also had a mildly stimulating effect on mineralization in these assays. The positive effect of rifampicin was completely abrogated by warfarin (Fig. 3), suggesting that rifampicin, which is known to bear structural homology to vitamin K, may also participate in γ-carboxylation reactions.

**Effect on cell morphology.** The effect of vitamin K on in vitro mineralization, in the absence of profound effects on cell proliferation, implied that the effect may be on the post-proliferative stages of osteoblast differentiation, namely, the osteoblast-to-osteocyte transition. Therefore, we established 3-D cultures of NHBC in type I collagen gels in medium containing the necessary elements for differentiation, as we described previously (6) and with the addition of the most potent vitamin K homologs, vitamins K2 and K3. After 28 days of culture, gels were stained with phalloidin to enable visualization of cell bodies and cellular processes by confocal microscopy. Cells grown under control mineralizing conditions developed a stellate morphology (Fig. 4A), as we previously described (6). The effect on osteocyte-like morphology was notably increased in cultures of NHBC grown in the added presence of vitamin K2 or K3, with a greater incidence of cells with longer processes, <1 μm wide and up to 200 μm long (Fig. 4, B and C). To quantitatively this effect, we defined an “osteocyte-like morphology,” based on the properties of...
MLO-Y4 cells grown under identical culture conditions, as cells that had a stellate morphology with more than five dendritic processes >25 μm long. By these criteria, vitamin K3 resulted in significantly more osteocyte-like cells than were seen in cultures of NHBC without added vitamin K, and, furthermore, this effect was not sensitive to warfarin (Fig. 4D). To determine whether the increased number of osteocyte-like cells truly represented an effect on the osteoblast-to-osteocyte transition, we utilized a calcine-labeling technique for calcium phosphate crystal deposition to measured mineralization by these cells. Overnight exposure to calcine of 21-day cultures of NHBC treated with vitamin K2 (Fig. 4E) resulted in the appearance of spherical mineralized structures adjacent to cell bodies and also tracked alongside cell processes. This effect was more evident if cultures had been exposed to calcine for 7 days (Fig. 4F). Similar results were obtained with vitamin K3 (data not shown). These observations are consistent with the proposed mechanism by which preosteocytes mineralize the surrounding osteoid (7). Notably, this effect was vitamin K dependent, inasmuch as very little calcine deposition was observed in control untreated cultures (not shown).

We also performed CFSE labeling experiments to determine whether the inhibitory effect of vitamin K on cell growth in standard two-dimensional (2-D) cultures was recapitulated in the 3-D environment. As shown in Fig. 5, vitamin K2 mildly and vitamin K3 profoundly inhibited cell proliferation in 3-D cultures, as demonstrated by the increased proportion of undivided (parent) cells and the decreased percentage of cells that had undergone multiple cell divisions.

**Gene expression in 3-D cultures of NHBC and MLO-Y4 cells.** We demonstrated previously that NHBC undergo change in gene expression in 3-D cultures consistent with increased osteoblastic differentiation (6). To examine the added influence of vitamin K, we assayed the expression of a number of known osteoblast differentiation markers, with NHBC in 3-D cultures. Vitamins K2 and K3 caused a decrease in type I collagen-α1 mRNA expression, compared with control cultures, which was evident in early (3 and 7 days) and late (3 and 4 wk) cultures (Fig. 6A). Expression of OCN mRNA was profoundly diminished, compared with control, in late cultures of NHBC (Fig. 6B). Given that collagen and OCN expression were reduced in late osteoblast differentiation, these vitamin K effects are consistent with a maturational effect. Similar changes were observed in equivalently treated 2-D cultures (data not shown). Expression of the osteocyte marker E11 was increased in the presence of vitamin K3, but not vitamin K2, throughout the 28-day period (Fig. 6C). However, we saw no consistent effect of vitamin K on other osteocyte markers, such as sclerostin (SOST) or dentin matrix acidic phosphoprotein (DMP1; data not shown). Consistent with effects seen in bone marrow cells (34), vitamin K3 gave rise to a consistent increase in OPG mRNA expression (Fig. 6D). Vitamins K2 and K3 decreased RANKL mRNA expression, particularly at later time points (Fig. 6E). The overall RANKL-to-OPG mRNA ratio was decreased in these cultures in response to vitamin K, suggesting that the osteoclastogenic potential of the differentiated cells in these cultures was reduced. Warfarin had little discernible effect on vitamin K-induced gene expression (data not shown), supportive of a direct genomic action of vitamin K. Although this is also supportive of signaling via SXR, we found no expression of the classical reporter gene for SXR, CYP3A, in the presence or absence of any vitamin K homolog (data not shown).

Our results suggest that vitamin K was promoting the osteoblast-to-osteocyte transition, although it was also possible that vitamin K was also having direct effects on mature osteocytes in these cultures. To test the effects on osteocytes, we utilized the recognized osteocyte cell line model MLO-Y4. Vitamin K had no discernible effect on the morphology of MLO-Y4 cells grown for up to 21 days in collagen gels other than that due to 3-D culture (data not shown). However, vitamin K had a distinct effect at the level of expression of certain genes, with vitamins K2 and K3 profoundly downregulating RANKL mRNA expression and, thereby, the RANKL-to-OPG mRNA ratio (Fig. 7, A and B). Vitamin K3 also upregulated the already high levels of E11 mRNA in early cultures of MLO-Y4 cells, qualitatively consistent with the effects seen in human osteoblast cultures. These results suggest that vitamin K influences the behavior of mature osteocytes and promotes an anticyclic phenotype in these cells.

**DISCUSSION**

In the present study, we investigated the effects of vitamins K1, K2, and K3 on the differentiation and function of human primary osteoblasts (NHBC), since the evidence so far favors osteoblast lineage cells as targets of vitamin K, rather than a direct effect on osteoclasts (29, 32, 34, 51, 53, 55). We showed previously that NHBC acquire osteocyte-like properties and an osteoclastogenic phenotype in response to the anabolic
compound strontium ranelate (5). We also demonstrated that these cells differentiate into osteocyte-like cells in 3-D type I collagen gels and are capable of adopting a catabolic phenotype in response to polyethylene particles (6). Our present results are consistent with an effect of vitamin K to enhance the osteoblast-to-osteocyte transition. Vitamin K inhibited cell proliferation in standard 2-D cultures and decreased the expression of the osteoblast marker cell surface AP. Importantly, all vitamin K homologs increased the ability of NHBC cultures to form a bone-like mineralized matrix, a key functional hallmark of the mature osteoblast/preosteocyte phenotype (7). Our results suggest that the various vitamin K homologs may differ in their mode or extent of action. For example, vitamin K1 least potently induced mineralization and had negligible effects on gene expression (not shown). The activity of vitamin K1 was effectively abolished by the \( \gamma \)-carboxylation inhibitor warfarin, suggesting that vitamin K1 acts mainly through this pathway. Vitamins K2 and K3 had more potent effects on mineralization: at low doses they were only marginally inhibited by warfarin, and at high doses they were unaffected by warfarin. Some of the differences in effect between the homologs may relate to the intrinsic ability of cells to convert phylloquinones and menadiones to vitamin K2 (48), although this was not examined in the present study. Despite the difference in potency between the various homologs, our results indicate that the positive effect of mineralization is at least in part due to the ability of vitamin K to \( \gamma \)-carboxylate certain proteins, which in bone could include OCN (41), MGP (42), periostin (14), or some as yet unidentified Gla protein. Strikingly, when cultured in a type I collagen gel, the 3-D model, vitamin K analogs, particularly, vitamins K2 and K3, promoted the formation of cells with a mature, osteocyte-like morphology. Consistent with this, we observed in 3-D cultures deposition of calcein-binding mineral along cell processes in response to vitamin K (K2 and K3), providing strong evidence that vitamin K promotes the osteoblast-to-osteocyte transition. Importantly, mineralization in lamellar bone occurs at the late osteoblast/preosteocyte stage and is part of the process that transforms preosteocytes into mature osteocytes, as reviewed recently (7). An effect of this type in vivo may be facilitation of correct bone mineralization by vitamin K. Consistent with this, a recent study found that vitamin K2 corrected a mineralization defect caused by magnesium deficiency (1). A positive effect of vitamin K on mineral quality may also contribute to the protective effect of vitamin K against fracture (13). Morphological transformation in 3-D cultures was accompanied by decreased expression of osteoblast markers, including type I collagen-\( \alpha_1 \) and OCN, and vitamin K3 in particular increased the expression of the osteocyte marker E11 (57). E11 expression localizes to osteocyte processes and is thought to
new bone formation in response to increased loading of bone initiate bone repair in response to microcracks (12), as well as.

As reviewed recently (45), appropriate osteocyte density and optimal osteocyte density in newly formed or remodeled bone. "pool" imply that vitamin K sufficiency may also contribute to osteocyte-like phenotype from a given osteoblast precursor cultures increased the numbers of cells with a stellate, mature culture. Together, our results showing that vitamin K in 3-D cultures may undergo further differentiation with extended This implies that the cells that differentiate over 28 days in 3-D cultures treated with vitamins K2 and K3, respectively (37, 49). Vitamin K2 has also been proven effective in preventing bone loss in rat immobilization/disuse models (28, 29). In both of these examples of pathological bone loss, there is evidence for a central role for osteocyte apoptosis, and it is therefore plausible that the action of vitamin K was at least partly at the level of the osteocyte.

Our results also imply a direct functional effect on existing osteocytes, with respect to downregulation of RANKL relative to OPG mRNA expression. This finding is consistent with previous results obtained in more immature stromal populations present in mouse (51) and human (34) bone marrow cells. The principal effect on RANKL expression here differs from our observations in response to strontium ranelate, where the antiosteoclastogenic response in similar cells appeared to be driven principally by an increase in OPG production (5). The extent to which osteocytes contribute to osteoclastogenesis and to the activity of resorbing osteoclasts is understudied. Evidence suggests that osteocytes express RANKL in vivo (23, 46, 47). Also, MLO-Y4 cells are known to be capable of supporting osteoclast formation without the addition of recombinant RANKL (58) and in response to mechanical damage of a 3-D network of these cells (35). In addition, the osteoclastogenic potential of MLO-Y4 cells has been shown to decrease in response to mechanical loading (56).

Fig. 7. Effect of vitamins K2 and K3 on gene expression in 3-D cultures of MLO-Y4 cells over a 21-day culture period. Expression of individual genes was normalized to GAPDH mRNA levels. Total RNA was pooled from 3 identically treated wells of a 12-well plate. Values are means ± SD of triplicate reactions. a,bSignificant difference between untreated (control) cultures and cultures treated with vitamins K2 and K3, respectively (P < 0.05). Similar results were obtained from 2 independent experiments.

play a role in cell process extension (57) and may be mechanistically involved in the observed vitamin K effect on morphology. Interestingly, the expression of other osteocyte markers, such as DMP1 (30, 52) and SOST (40), was not reproducibly regulated by vitamin K in the 3-D model, although we have observed upregulation of these markers in longer-term (e.g., 42 day) 2-D cultures by vitamin K2 (data not shown). This implies that the cells that differentiate over 28 days in 3-D cultures may undergo further differentiation with extended culture. Together, our results showing that vitamin K in 3-D cultures increased the numbers of cells with a stellate, mature osteocyte-like phenotype from a given osteoblast precursor “pool” imply that vitamin K sufficiency may also contribute to optimal osteocyte density in newly formed or remodeled bone. As reviewed recently (45), appropriate osteocyte density and viability are essential for bone health. Osteocytes appear to initiate bone repair in response to microcracks (12), as well as new bone formation in response to increased loading of bone.
administered, the duration of administration, and the quality of the studies, including the sizes of the cohorts, the attrition rate, and other confounding variables (13). Another difficulty may relate to the expectation, until recently, that those agents that offer therapeutic advantage in osteoporosis will increase BMD. However, the observation that vitamin K supplements can change favorably circulating markers of bone turnover without changing BMD, as has been described for adolescents with cystic fibrosis (38), is reminiscent of calcitonin and other agents that have been reported to decrease fracture risk with little change in BMD (10). Since it is clear that the correct density and viability of osteocytes are essential for bone homeostasis and optimal bone strength, a role for vitamin K for these cells should be further explored.

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REFERENCES


Vitamin K promotes osteoblast-osteocyte transition


