Mode of Action of Vitamin K_2 in Osteoblastic Cells

骨芽細胞におけるビタミンK_2の作用機序

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Contents

Introduction

Vitamin K was discovered by H. Dam *et al.* in 1929 as a factor which has an important role in blood coagulation, and was named by taking the first letter of "Koagulation" (1). In 1939, P. Karrer *et al.* purified vitamin K_1 and R.W. McKee *et al.* separated vitamin K_2 from fish powder. In 1940, E.A. Doisy determined chemical structures of vitamin K_1 and K_2. Vitamin K forms a family of vitamin Ks, which are classified as K_1 through K_7 according to different side chains attached to the common structure of naphtho-kinone ring (2, 3) (Table 1). Most abundant vitamin Ks in nature are phylloquinone known as vitamin K_1 and menaquinone as vitamin K_2. Vitamin K_1 is produced by green plants and is contained at low concentration in milk and milk products. Vitamin K_2 is a name for a group of microbial-derived compounds consisting of 14 homologous chemicals with different length in their side chains (different in the number of prenyl groups), and vitamin K_2 is found in fermented foods and in intestines where intestinal bacteria produce vitamin K_2. In the experiments with rats (4), vitamin K_2 was found more effective than vitamin K_1 in recovery of elongated PT time which is caused by vitamin K defficiency. Although majority of vitamin K found in human liver is vitamin K_2 (5), it is not established which vitamin K, K_1 or K_2 , plays more significant role in prevention and therapy for vitamin K defficiency. Vitamin K_1 has mainly been used in Europe for clinical treatment and *in vitro* experiments, while Menaquinone-4, one of the vitamin K_2

derivatives with four isoprenoid units in the side chain, has been used in Japan.

During the first 40 years since the discovery of vitamin K, vitamin K was studied based on the knowledge that it has a function of stimulating *de novo* synthesis of blood coagulating factors (6, 7). In 1970s after the discovery of prothrombin precurcor, Stenflo *et al.* (1974) discovered a new amino acid, γ-carboxyglutamic acid (Gla), in prothrombin molecule (8). Since then, Gla-containg proteins have been screened for Gla as a marker, and four vitamin K-dependent coagulation factors have been discovered in serum (II, VII, IX, X) and other serum proteins have also been discovered such as protein C, protein S, protein M and protein Z. Since these discoveries, mode of action of vitamin K was considered to activate carboxylase present in microsomal fractions of cells and to function as a cofactor for the enzyme facilitating γ-carboxylation reaction at γ-carbon of glutamic acid (Glu) moiety near the amino terminus of precursor proteins (9, 10, 11) (Figure 1). γ-Carboxy glutamic acid (Gla) is known to have an affinity and to bind to calcium ion and hydroxyapatite (12), and all Gla-proteins are considered as Ca-binding proteins. It is interesting to note that Gla-proteins such as blood coagulation factors are produced in the liver, but Gla-proteins are also found in other organs such as bone (13), kidney and aorta (14, 15). Furthermore, γ-glutamic acid carboxylase, an enzyme involved in biosynthesis of Gla-proteins, is also found in many eukaryotic cells such as cells in spreen (16), pancreas (17), placenta (18), thymus, cartilage, tendon and uterus, and kidney cell culture (19), fibroblast cell culture and tumor cells (9, 20).

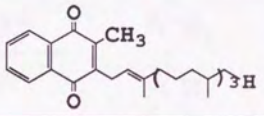
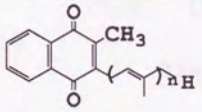
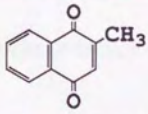
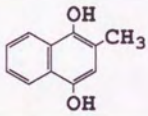
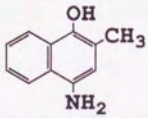
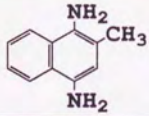
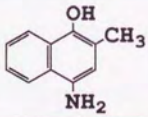
These findings suggest that the role of vitamin K would be considered not only from a viewpoint of blood coagulation factors, but the role of vitamin K could possibly be viewed as a biochemical factor involved in a wide variety of biological phenomena including those with Gla-proteins and calcium ion.

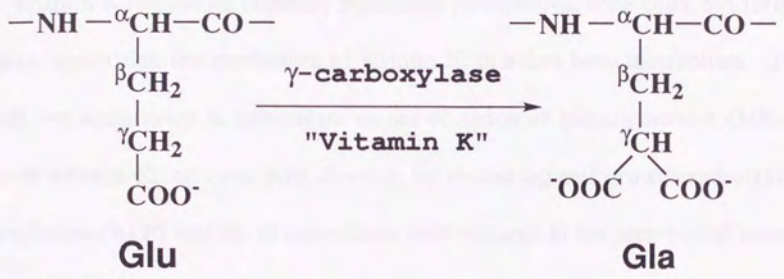
In 1975, Hauschka *et al.* reported on osteocalcin as a γ-carboxyglutamic acid (Gla) containing protein derived from bone (21), and in 1976, Price *et al.* extracted, purified and identified matrix-Gla protein (MGP) from bone (22). Since these Gla-proteins are found as principle bone proteins, and Gla-proteins are known to have an affinity to calcium ion and hydroxyapatite (12), these Gla-proteins in bone are considered to have an important role in bone metabolism. As mentioned above, vitamin K functions as a cofactor for γ-carboxylase which converts glutamic acid moiety in protein into γ-carboxyglutamic acid (Gla) in post-translational modification (Figure 1). Findings on relationship between vitamin K and bone have been accumulated. In 1960, administration of vitamin K resulted in earlier healing of bone fracture in rabbits (23), and in 1975, chondrodysplasia was found in baby born from a mother who had taken warfarin, a vitamin K antagonist, during her pregnancy (24). After Hauschka and Price made direct demonstration of an existence of Gla-proteins in bone as described above, Hart *et al.* (1985) made a clinical observation that osteoporosis patients, especially those patients with bone fracture, exhibited a low level of serum vitamin K_1 (25), and in 1991, Delmas *et al.* reported that serum concentration of non-carboxylated osteocalcin was found

higher with post-menopausal women as compared with that before menopause, and that this increase became more significant as women became older (26). Thus, from the above observations, it has been expected that vitamin K exerts some effects, direct and indirect, on bone metabolism. In 1992, Orimo *et al.* conducted clinical studies measuring changes in bone mass by double-blind methods and reported that vitamin K_2 (2-metyl-3-all-trans-tetraphenyl-1,4-naphtoquinone; menaquinone-4:MK-4) showed therapeutic effects in the treatment of involutional osteoporosis by increasing bone mass better than vitamin D_3 (27). Since then, studies have been directed toward an elucidation of mechanisms of actions by which vitamin K exerts its effects in bone metabolism. In bone resorption system, MK-4 was reported to maintain bone strength and bone mass in ovariectomized rat model under steroid administration (28,29), MK-4 was observed to suppress bone resorption in mouse calvaria culture (30), MK-4 suppressed a vitamin D_3-induced formation of multinuclear cells in organ culture (31, 32), and MK-4 suppressed the formation of resorption lacuna by osteoclast cells on dentine slice (31). These accumulated observations indicated that the action of MK-4 on the bone resorption system would involve suppression of osteoclast cell formation and that MK-4 would suppress this osteoclast formation not by directly intervening the differentiation steps from precursor cells to mature osteoclast cells (31), but partly by suppressing PGE_2 formation by osteoblast cells and interstitial cells (31,32). In bone formation system, MK-4 was observed to enhance mineralization in human osteoblast cells and the

mineralization and osteocalcin formation were greatly stimulated by the presence of both MK-4 and vitamin D_3 (33). There are, however, very few other cell biological studies on the action of MK-4 in osteoblast cells, and it is not clearly understood that the role of MK-4 can solely be explained by its known function as a cofactor for γ-carboxylase. This study was, therefore, designed to examine and elucidate the mode of action of MK-4 in *in vitro* osteoblast cell culture system.

Table 1. Chemical Structure of Vitamin K Family

Vitamin K	Chemical Name	Molecular Formular
K_1	Phylloquinone	
K_2	Menaquinone-n	
K_3	Menadione	
K_4	Menadiol	
K_5	4-amino-2-methyl-1-naphthol	
K_6	2-methyl-1,4-naphthalene-diamine	
K_7	4-amino-3-methyl-1-naphthol	



Glu Gla

Figure 1. Reaction of γ-carboxylase in the presence of vitamin K

Part 1: Effects of MK-4 on the proliferation and alkaline phosphatase activity of osteoblastic cells.

Introduction:

Vitamin K has shown clinically significant influence on bone cells, but little is known concerning the mechanism of vitamin K_2 to affect bone metabolism. This study was undertaken to investigate modes of action of menaquinone-4 (MK-4), one of vitamin K_2, on bone cells *in vitro*, by measuring cell growth and alkaline phosphatase (ALP) activity of osteoblastic cells cultured in the presence of various doses of MK-4.

Materials and Methods:

A murine osteoblastic cell line, MC3T3-E1, was maintained as previously described (34). A human osteosarcoma cell line, HOS-TE85 (35), were obtained from American Type Culture Collection. These cells were cultured in an a-modified minimum essential (α-MEM, GIBCO) supplemented with 10% (v/v) heat-inactivated fetal bovine calf serum (FCS) (Bioserum, Victoria, Australia) and 60 g/ml gentamycin sulfate at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Both cell lines were seeded in 6-well multiplates (Becton Dickinson Labware, Lincoln Park, NJ) with an initial concentration of 5 $x10^4$ cells in 2ml of α-MEM containing 10% FCS per well until a semi-confluence state was obtained. The culture medium was then replaced with 2ml of α-MEM containing 0.1% FCS and various concentrations (10^{-8}M to 10^{-5}M) of menaquinone-4 (MK-4, a

kind gift from Eizai Co., Gunma, Japan). In some experiments, 3-(α-acetonyl benzyl)-4-hydroxy-coumarin (warfarin, Sigma, St. Louis, MO) was added to the culture at the concentration of 10 μg/ml. After cells were incubated for another 3 days, the cells were washed twice with phosphate-buffered saline (PBS, pH7.4), and then treated with 0.25% trypsin- 0.02%-EDTA solution (Immunobiological Laboratories, Tokyo, Japan). The trypsin-treated cell suspension was passed several times through pipette trips in order to separate into individual cells. The number of cells were determined with use of hemo-cytometer. ALP activity was measured as previously reported (36). After washed twice with PBS, the cells were collected with a cell scraper and transferred to 1.5ml conical tubes. Cell pellets were obtained by centrifugation at 15,000 rpm at 4°C. Whole cell lysates were prepared with a solution containing 0.2% (v/v) Nonidet P-40 and 1mM $MgCl_2$. The lysate combined with a reaction mixture (1M diethanolamine, pH9.8, 1mM $MgCl_2$ and 10mM p-nitrophenyl phosphate) was incubated at 37° C for 30 min. NaOH (0.1N) was then added to the solution in order to quench the reaction. Absorbance at 405nm was read by an EIA reader 2550 (Bio-Rad Lab., Richmond, CA) and the ALP activity was then calculated. ALP activity was corrected for protein content which was determined with Bio-Rad Protein Assay Kit (Bio-Rad Lab.).

Statistical analysis:

Data were evaluated by Student's t-test after analysis of variance testing. A p

value of less than 0.05 was considered to be significant. All data presented in the text and figures were expressed as the mean±SEM.

Results:

As shown in Figure 2, MK-4 suppressed the growth of both osteoblastic cells in a dose-dependent manner. The maximal suppressive effect was obtained at 10^{-7}M of MK-4 for HOS TE85 cells where the growth was suppressed down to about 56% of the control (Figure 2-A), while the same effect for MC3T3-E1 cells was obtained at 10^{-6}M of MK-4 where the growth was suppressed down to about 84% of the control (Figure 2-B). The suppressive effect of MK-4 on the cell growth was not due to its toxic effects, which was confirmed when viability of cells was checked by Trypan Blue staining. On the other hand, specific activity of alkaline phosphatase (ALP) corrected for protein content showed a bell-shaped curve as a function of MK-4 concentration and the activity was increased in a dose-dependent manner as MK-4 concentrations were increased to a certain level (Figure 3). In the experiments with HOS TE85 cells, the maximal ALP activity in the lysates was observed at 10^{-6}M of MK-4 where the ALP activity was approximately 5 times higher than that of the control culture (Figure 3-A). The maximal ALP activity in MC3T3-E1 cells was observed at $3x10^{-7}$M of MK-4, which was about 1.5 times higher than the control (Figure 3-B). At higher concentration of MK-4, the increase in ALP activity was not observed in both cells, which was probably due to an excess amount MK-4 beyond

pharmacological concentration. Warfarin reversed the suppressive effects of MK-4 on the proliferation of both osteoblastic cells (Figure 4-A, B). The increase of ALP activity by MK-4 was, however, not affected by warfarin. Warfarin itself did not affect the growth and the ALP activity of these two osteoblastic cells.

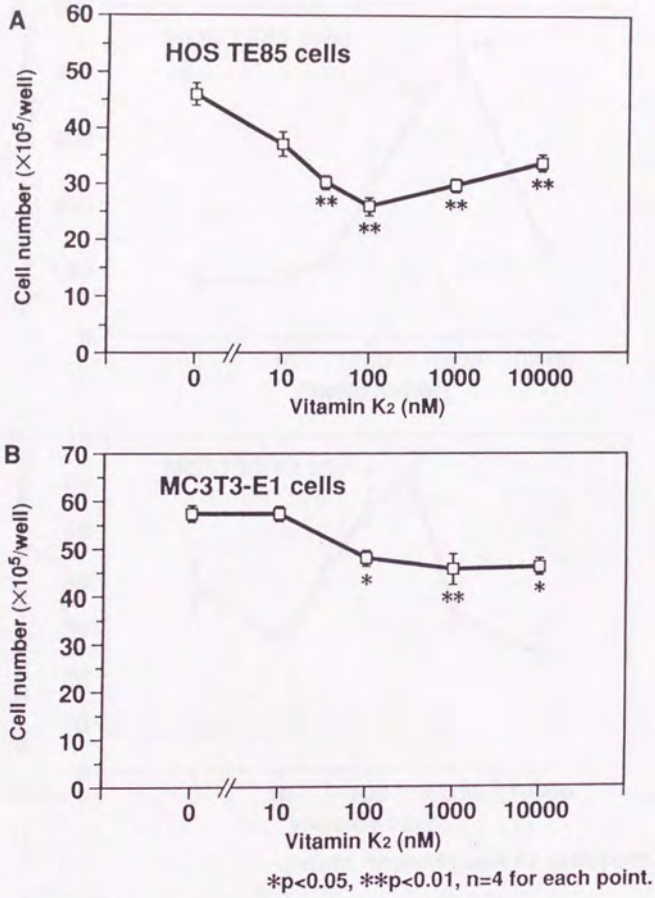


Figure 2. Effect of MK-4 on the growth of osteoblast cells.
A human osteosarcoma cell line, HOS TE85 (A), and a murine osteoblastic cell line, MC3T3-E1 (B), were cultured in the presence of various concentrations of MK-4. After 3 days culture, the number of cells were counted. Statsitical analysis was made by an analysis of variance. (*p<0.05, **p<0.01, n=4)

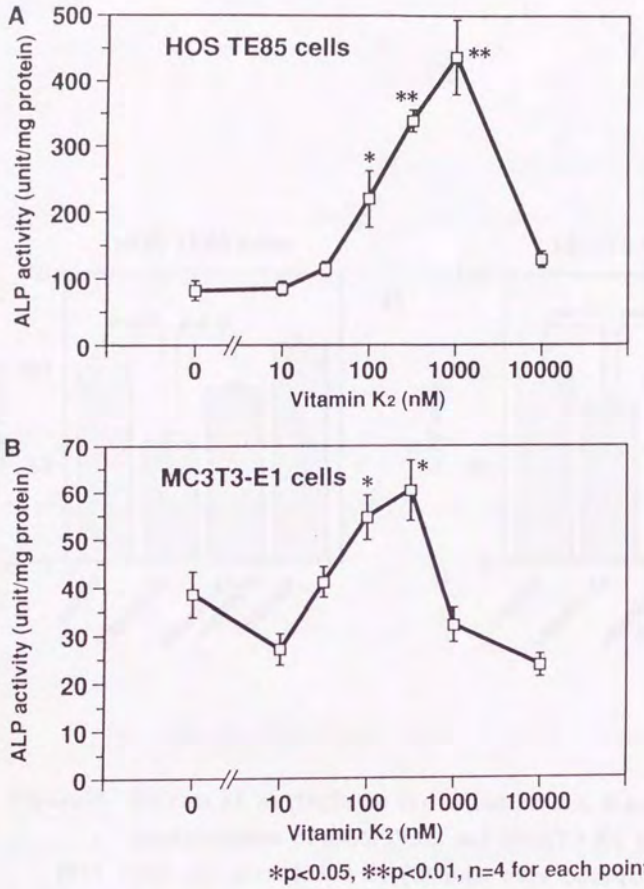


Figure 3. Effect of MK-4 on ALP activity of osteoblast cells.

HOS TE85 (A) and MC3T3-E1 (B) cells were cultured in the absence or presence of MK-4 for 3 days after semi-confluent state of growth. ALP activity of whole cell lysates was measured and expressed as units/mg protein. (*p<0.05, **p<0.01, n=4)

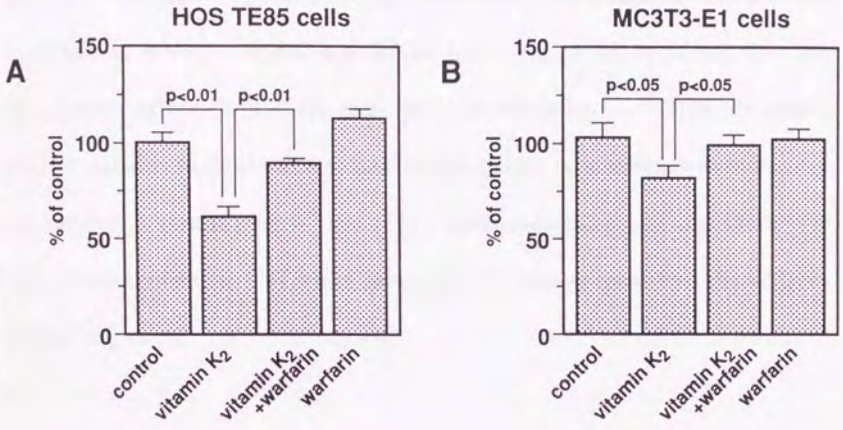


Figure 4. Effects of warfarin on the action of MK-4 on the proliferation of HOS TE85 and MC3T3-E1 cells.

HOS TE85 (A) and MC3T3-E1 (B) cells were cultured in the absence or presence of MK-4 (10^{-7}M). After 3 days of incubation, the number of cells were counted and expressed as mean ± of % control (n=4). Statistical analysis was performed by an analysis of variance.

Part 2: Effects of MK-4 on the cell cycle and on function associated with the cell cycle in osteoblastic cells

Introduction:

As shown in Part 1, MK-4 was demonstrated to modify the growth and function of osteoblastic cells. In order to elucidate mechanisms involved in this modification, it was examined how MK-4 will affect on cell cycle and how this cell cycle effect will influence this modification. G1/S boundary synchoronization method was employed and cell cycle progression was monitored by growth measurement and by immunohistological detection of bromodeoxyuridine (BrdU) which is specifically incorporated by cells at DNA synthesizing phase.

Materials and Methods:

Experiment 1: Measurement of Cell Growth and Cellular ALP Activity after Synchronization

HOS TE85 cells were seeded and grown to a semi-confluent level in six-well culture plates in α-MEM containing 10% FCS at 37°C under 5% CO_2 and 95% air. Optimum concentrations for thymidine and hydroxyurea were determined to give double cell numbers in one cell cycle without cellular toxicity. The results were shown in Figure 5, where the optimum concentration for thymidine was found as $5x10^{-4}$M and that for hydroxyurea was 10^{-4}M. These concentrations were regarded as optimum concentrations for thymidine and hydroxyurea which

were used in subsequent experiments. The cells were synchronized in the G1/S phase boundary by treating cells intermittently with $5x10^{-4}$M thymidine for 24 hours and then with 10^{-4}M hydroxyurea for 18 hours. The hydroxyurea treatment followed 10 hours after the thymidine treatment. After the removal of hydroxyurea, the synchronized cells of HOS TE85 were changed in α-MEM containing 0.1% FCS in the absence or presence of MK-4 (10^{-7}M). Cell numbers and ALP activities were determined at 0, 1.5, 3, 6, 9, 12, 15, 18, 21, 24, and 30 hours (Figure 6). The number of cells were determined with the use of a hemo-cytometer as described in Part 1 and ALP activity was measured by the same method as described in Part 1. For control group (in the absence of MK-4), BrdU incorporation into DNA was also measured by the methods described in the Experiment 2 below.

Experiment 2: Incorporation of Bromodeoxyuridine (BrdU) into DNA

HOS TE85 cells were mounted and grown on chambered glass slides (Lab-Tek Chamber Slide, Nunc, Napervill, IL) to a semi-confluent level. The cells were synchronized in the G1/S phase boundary by the methods mentioned in the Experiment 1 above. After the removal of hydroxyurea, BrdU was added to the medium of α-MEM plus 0.1% FCS in the presence of 10^{-7}M of MK-4. BrdU incorporation was determined at 0, 6, 12, 21, and 24 hours by the following methods. In order to investigate BrdU incorporation at various points after the removal of hydroxyurea, the cells were incubated with RPMI-1640 containing

10μM BrdU for 30 min at 37°C and washed three times with PBS. Cells were then fixed with 0.1M phosphate buffer (pH 7.4) containing 4% paraformaldehyde and 10% sucrose for 10 min at room temperature, and washed three times with PBS. Cells were treated with acetone-ethanol (1 : 1) for 1 min and washed three times with PBS. The cells were treated with 4N HCl for 20 min at 37°C, and then treated with 0.1M sodium tetraborate. After washing with PBS three times, the cells were treated with 0.03% hydrogen peroxide in 100% methanol for 30 min in order to inhibit endogenous peroxidase. After washing cells with PBS three times, cells were incubated with PBS containing 10% FCS for 30 min at room temperature in order to minimize the nonspecific binding of reagents in subsequent steps. The cells were incubated with the monoclonal anti-BrdU antibody overnight at 4°C (37). After washing cells with PBS three times at room temperature, incubation with the biotin-labelled second antibody (anti-mouse IgG) for one hour at room temperature. Cells were again washed three times with PBS. Horseradish peroxidase-conjugated streptavidin (Histofine Sab-Po kit, Nichirei, Tokyo, Japan) was added to the cells and the reaction mixture was incubated for one hour at room temperature. After washing the slide three times with PBS, the presence of the bound antibody was visualized by the additon of peroxidase substrate, 3,3'-diaminobenzidine (DAB kit, Vector Laboratories, Burlingame, CA) (38). BrdU incorporated cells were examined by the immuno-histological microscopy.

Experiment 3: Analysis by FACS (fluorescence-activated cell sorter) Methods

HOS TE85 cells were seeded and grown to a subconfluent level in plastic dish culture plates (10 cm in diameter; Falcon, Oxford, CA) in α-MEM containing 10% FCS at 37°C under 5% CO_2 and 95% air. The medium was then changed to α-MEM containing 0.1% FCS together with 10^{-6}M or 10^{-7}M of MK-4 and incubation was continued for 24 hours at 37°C. After washing cells with PBS, pulse labeling with BrdU was made by incubation of the cells in RPMI-1640 for 30 min at 37°C in the presence of 16.7μM BrdU. Cells were washed twice with PBS, and removed by trypsin-EDTA treatment. Cells were harvested by centrifugation at 1,000-1,500 rpm for 5 min, and the pellet was washed with PBS. The cells were fixed by the dropwise addition of 10ml of ice cold 70% EtOH under Vortex mixing. The resulted cell suspension was centrifuged at 1,000-1,500 rpm for 5 min, and cells were resuspended in 1 ml of PBS. The resuspended cells were transferred to a microtube and centrifuged at 1,500g for 30 sec at room temperature. 1 ml of 4N-HCl was added dropwise to the cells under mixing for DNA denaturation and the cell suspension was incubated at room temperature for 20 min. Cells were then harvested by centrifugation at 1,500g for 30 sec. 1 ml of 0.1M sodium tetraborate was added to the cells and the cells were collected by centrifugation at 1,500g for 30 sec. Cells were washed with 1 ml of 0.5% Tween 20/PBS, and again centrifuged at 1,500g for 30 sec. The supernatant was checked for pH 7.0 by the conventional pH paper, and then the supernatant was discarded. The cells were resuspended in 50 μl of PBS,

and 20μl of the FITC (fluorescein isothiocyanate)-conjugated mouse monoclonal anti-BrdU antibody (Becton Dickinson, Mountain View, CA) were added and the reaction mixture was incubated overnight at 4°C. After washing cells with PBS twice by centrifugation, 0.05 ml of propidium iodide solution (50μg/ml) were added as non-specific DNA binding fluorescent dye (39), and the preparation was incubated at 4°C for 15 min. Cell cycle analysis was made by a Becton-Dickinson FACS flow cytometer.

Statistical analysis:

Experimental valhes are given as mean±SEM. Statistical significance of the results was evaluated by Student's t-test. A p value smaller than 0.05 was considered to be a significant difference.

Results:

Experiment 1: HOS TE85 cells were synchronized to the G1/S boundary by intermittent treatment with $5x10^{-4}$M of thymidine and 10^{-4}M of hydroxyurea. In Figure 7, the number of cells and the percentage of BrdU-positive cells are plotted against time, where the cell numbers are shown by bar graph and the BrdU cells are by line graph. After S phase synchronized cells were allowed to move on the cell cycle by hydroxyurea removal, the number of cells became doubled in 24 hours, showing that one cell division has been occurred. Since cells actively synthesizing DNA will only incorporate BrdU, the percentage of

BrdU-positive cells expresses a population of cells at DNA synthesis phase. At very early stage of culture (around 1.5 hours) after S phase synchronized cells were allowed to start growing, a great majority of cells were found as BrdU-positive, indicating that S phase synchronization was successfully achieved. Around 9-12 hours after hydroxyurea removal, the population of BrdU-positive cells dropped down to about 10%, indicating that few cells were remained at S phase while most of the cells were assumed to enter into G2 phase. At around 18-21hours the cells were considered to enter into G1 phase again, because the cell number was approximately doubled after the removal of hydroxyurea. After 24 hours of culture, BrdU-positive cells were increased to about 40% of the cell population. The cell cycle stages were then determined based on the cell number determination and the percentage of BrdU-incorporated cells. The results from more than 10 synchronization experiments showed that the cells were in S phase from 0hr to 9hr, G2/M phase from 9hr to 15hr, G1 phase from 15 hr to 21hr, and that S phase of the next cell cycle restarted at around 21 hr to 24hr (Figure 8). These cells will move along the cell cycle into the next phase. With use of these synchronized cells, an effect of MK-4 on cell cycle was examined in the following experiment. S-phase synchronized HOS TE85 cells were grown in the presence or absence of MK-4 (10^{-7}M), and the number of cells were determined in the course of time. In Figure 9-A, a similar cell growth curve was obtained for control group as observed in Figure 7. There were little differences in cell numbers among all two groups at early G2 phase (around 12 hours). It was

observed, however, that at G2/M phase (around 15 hours) cell growth was suppressed among MK-4 groups, and this growth suppression was continued on around 36 hours. This growth suppression was not due to cellular toxic effect by vitamin K_2, which was confimed by dye exclusion method with Trypan Blue stain (0.4%) (GIBCO). While the cell number for control group became twice as much in 24 hours completing the cell cycle to go into another cycle, it took about 36 hours for MK-4 (10^{-7}M) to have their cell numbers doubled. Intracellular ALP activities were observed to decrease toward M phase and to increase from G1 phase for all groups, but the rate of this ALP activity increase after G1 phase was slowed down by the presence of MK-4 (Figure 9-B).

Experiment 2: Cells at S phase incorporated BrdU into nuclei, which was visualized by the immuno-staining method (Figure10). In control group, at 6 hours of culture most cells were stained indicating that cells were at S-phase, but at 12 hours few cells were stained indicating that cells were no longer at S phase. At 21 hours of culture, cells were beginning to be stained again showing that cells were again entering into S phase. In vitamin K group, however, even at 12 hours of culture, most cells remanined staining showing that cells were still at S phase. In other words, cells in control group were observed to go into the next S phase at a new cell cycle in about 20 hours, but cells grown in the presence of 10^{-7}M MK-4 were experienced elongated S phase and as a result, cells were delayed entering into the next S phase.

Experiment 3: The amounts of incorporated BrdU were plotted against the amounts of DNA which were associated with propidium iodide (Figure11), where G1 phase was represented by the area surrounded by the square #1, G2/M phase by the square #2, and S phase by the square #3. After 24 hour culture of the control cells, 46.68± 3.03% of cell population were at G1 phase, 8.43± 0.70% were at G2/M phase and 45.20±2.65% were at S phase. For cells grown in the presence of MK-4 (10^{-7}M or 10^{-6}M), however, 51.46± 3.54% of cell population were at G1 phase with 10^{-7}M MK-4 and 52.19± 2.05% were at G1 phase with 10^{-6}M MK-4, respectively. Similarly, 9.90±0.64% cells were at G2/M phase with 10^{-7}M MK-4 and 10.47±2.16% were at G2/M phase with 10^{-6}M MK-4, and 38.60 ±2.95% cells were at S phase with 10^{-7}M MK-4 and 37.48±0.55% were at S phase with 10^{-6}M MK-4. These above observations indicated that MK-4 delayed progression of the cell cycle resulting in less population of cells at S phase.

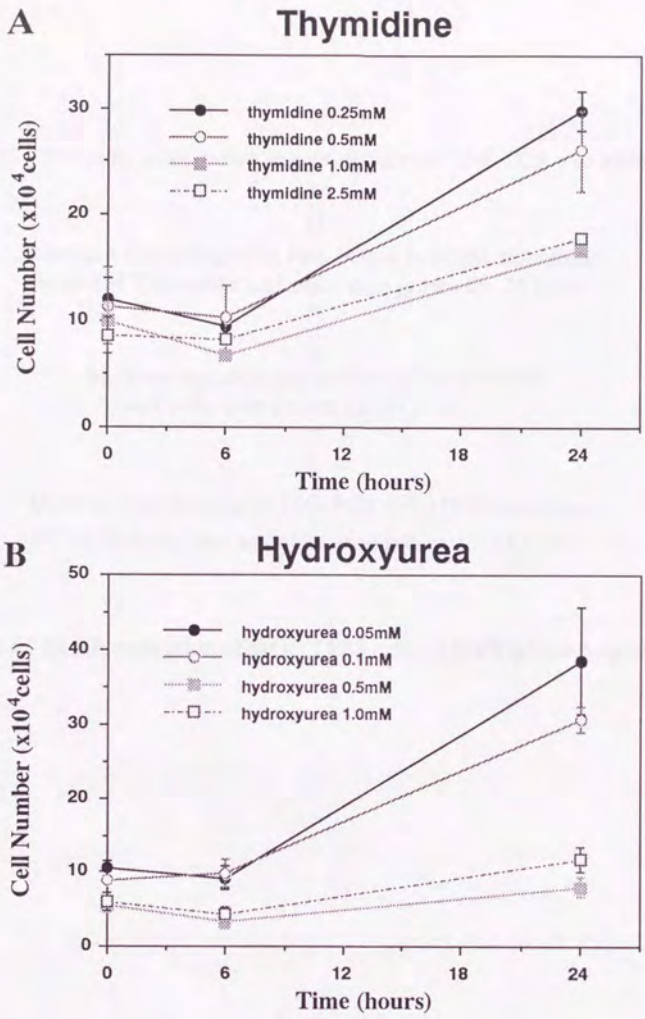


Figure 5. Determination of optimal concentrations for thymidine and hydroxyurea for synchronization of HOS TE85 cells.

HOS TE85 cells were grown in the presence of various concentrations of thymidine (A) or hydroxyurea (B) and the growth was monitored up to 24 hours. (mean ± S.E., n=4)

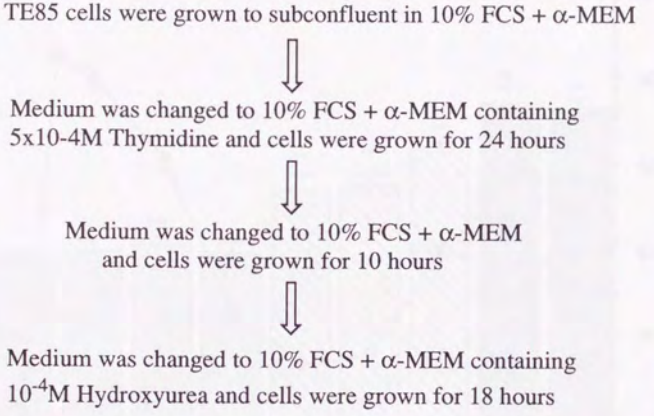


Figure 6. Synchronization of HOS TE85 cells at G1/S-phase boundary

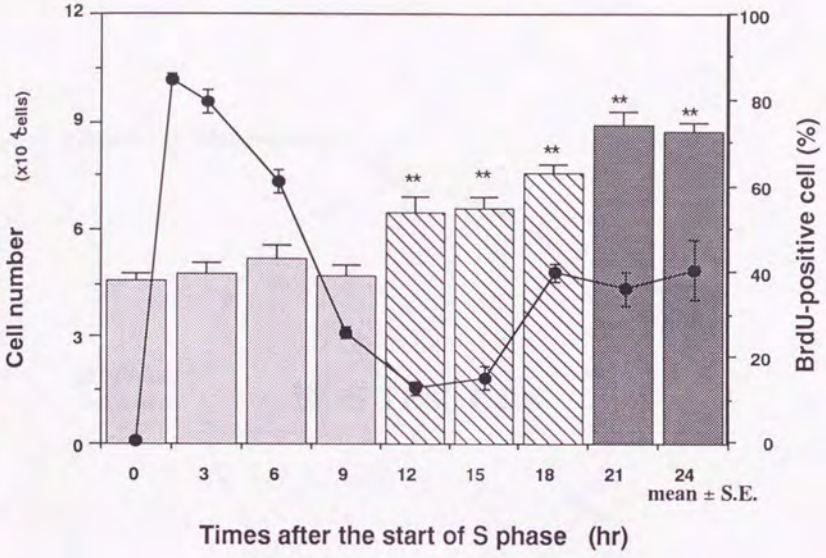


Figure 7. Changes in the total cell number and BrdU-positive cells after synchronization.

HOS TE85 cells were synchronized to the G1/S boundary by intermittent treatment with 5 x 10^{-4}M of thymidine and 10^{-4}M of hydroxyurea. The total cell number was shown by bar graph and the BrdU-positive cells were shown by line graph. (**p<0.01, n=4)

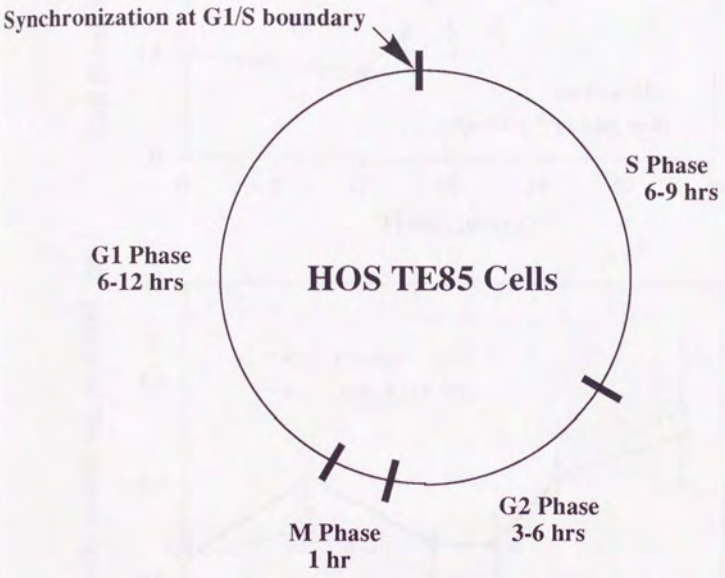


Figure 8. Cell cycle for HOS TE85 cells

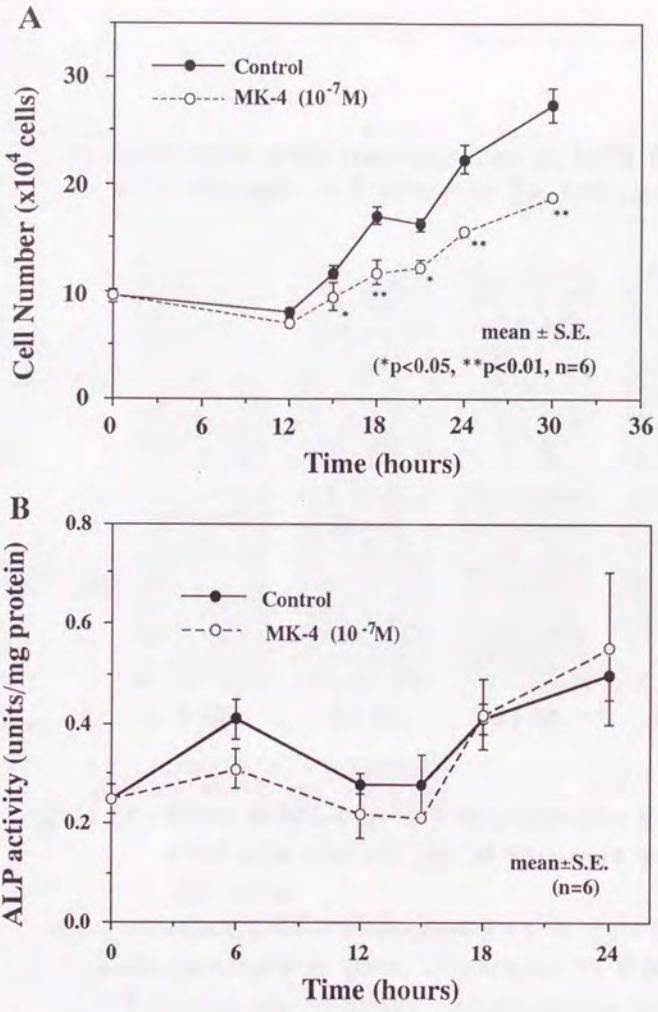


Figure 9. Effect of MK-4 on cell proliferation and ALP activity in synchronous HOS TE85 cells.

Synchronized HOS TE85 cells at G1/S phase boundary were incubated in the absence or presence of MK-4 (10^{-7}M), and the number of cells were determined (A). ALP activity was measured after synchronization in the absence or presence of MK-4 (10^{-7}M) which was expressed as units/mg protein (B). (*p<0.05, **p<0.01, n=6)

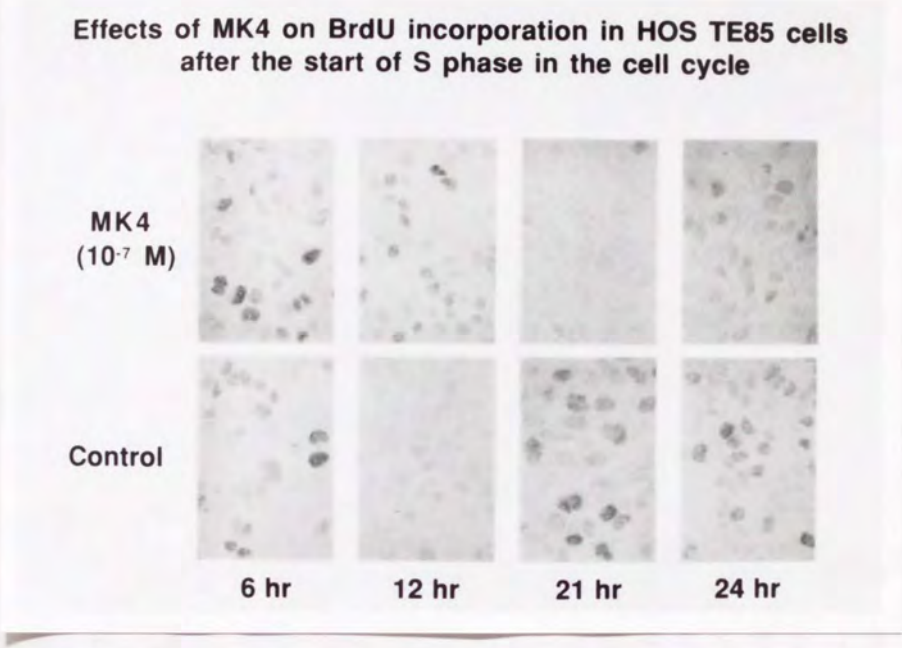
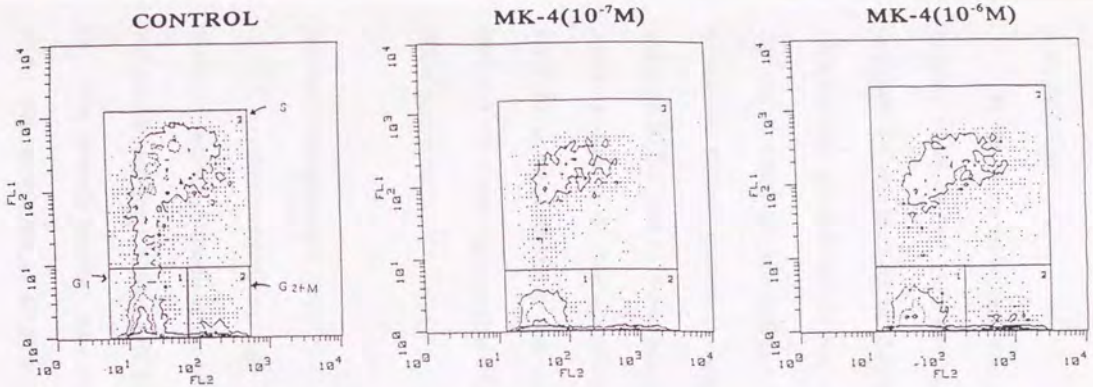


Figure 10. Effect of MK-4 on BrdU incorporation HOS TE85 cells after the start of S phase in the cell cycle.

Synchronized HOS TE85 cells were incubated with 10mM BrdU (bromodeoxyuridine) in the absence or presence of 10^{-7}M MK-4, and BrdU incorporation into DNA at various incubation time was determined by monoclonal anti-BrdU antibody plus biotin-avidin-peroxidase methods. Visualization of incorporated BrdU was made by microscope.



	CONTROL mean	CONTROL s.e.	MK-4(10^{-7}M) mean	MK-4(10^{-7}M) s.e.	MK-4(10^{-6}M) mean	MK-4(10^{-6}M) s.e.
S :	45.20	2.65	38.60**	2.95	37.48**	0.55
G2/M :	8.43	0.70	9.90	0.64	10.47	2.16
G1 :	46.68	3.03	51.46	3.54	52.19*	2.05

Figure 11. FACS analysis for MK-4 effect on the cell cycle of HOS TE85 cells.

HOS TE85 cells grown in the absence or presence of MK-4 (10^{-7}M and 10^{-6}M) were pulse-labelled with 16.7mM BrdU, and BrdU incorporation was determined by the addition of FITC-conjugated monoclonal anti-BrdU antibody. 0.05 ml of propidium iodide (50mg/ml) were used to monitor the total DNA contents. Cell cycle analysis was made by FACS (fluorescence-activated cell sorter) flow cytometer.

Part 3: Intracellular free calcium and MK-4

Introduction:

It has been observed that menaquinone-4 (MK-4) modifies growth and function of osteoblast cells and that MK-4 delays each phase of the cell cycle observed by the method of fluorescence activated cell sorter (FACS). With regard to cell growth in relation to intracellular free calcium ion, it is reported that an increase in intracellular free calcium ion concentration resulted in stimulation of cell growth in mouse fibroblast (Balb/c3T3 cells) (40), human leukemic HL60 cells (41) and human keratinocytes (42). In order to analyze the mode of actions of MK-4, this study examined MK-4 effect on changes in intracellular free calcium ion concentration ($[Ca^{2+}]_i$) and discussed possible involvement of the signal transduction system in osteoblast cells for expression of MK-4 activities.

Materials and Methods:

A murine osteoblastic cell line, MC3T3-E1, was grown in α-modified minimum essential medium (α-MEM, GIBCO) supplemented with 10% (v/v) heat-inactivated fetal bovine calf serum (FCS) at 37°C under 5% CO_2 and 95% air. The growth medium was changed to α-MEM with 0.1% FCS 24 hours before the measurement of $[Ca^{2+}]i$. Cells were harvested by trypsin-EDTA treatment and resuspended in HEPES solution. 2mM of acetoxy-methyl ester of Fura-2, Fura-2/AM (Dojin Co.) a fluorescent Ca^{2+} indicator, were added to this

cell suspension and loading was made for 60 min at 37°C under 5% CO_2 and 95% air (43). Cells were resuspended in Na-solution (130mM NaCl, 1.5mM $CaCl_2$, 5mM KCl, 1mM $MgCl_2$, 20mM HEPES) with bovine serum albimin (BSA) and incubated for 30 min at 37°C under 5% CO_2 and 95% air in order to convert unhydrolyzed Fura-2/AM to Fura-2. Cell concentration was then adjusted to give 1-3 $x10^6$ cells/ml in a solution containing Na-solution+BSA+glucose, and 300 μl of this cell preparation were used for the experiments. Measurement of intracellular free calcium ion concentration $[Ca^{2+}]i$ was made with use of FS-100 equipment (Kowa Co.) by monitoring the ratio of emissions at 510nm which resulted from alternating excitation wavelengths (340nm/380nm). The intracellular free calcium ion concentration $[Ca^{2+}]i$ was calculated by the equation (43),

$$[Ca^{2+}]i=Kd \times (Ratio-Rmin) /(Rmax-Ratio) \times sf2/sb2,$$

where $Kd=2.24 \times 10^{-7}M$, sf2=380max, sb2=380min, and Ratio was the values obtained above. The Maximum fluorescence (Rmax) was obtained by the addition of 20mM of digitonin and the minimum fluorescence (Rmin) was obtained by the addition of 10mM of EGTA.

The cell preparation was preincubated for 5 min in the FS-100 system to bring the reaction mixture to an equilibrium before the addition of MK-4. With use of various concentrations of MK-4 ($10^{-13}M$-$10^{-5}M$), effect of MK-4 on $[Ca^{2+}]i$ was examined. $10^{-10}M$ of 1α,25-$(OH)_2$ vitamin D_3 (1α,25-$(OH)_2D_3$) were used together with MK-4 to see an additive effect. Cells were preincubated with $10^{-5}M$

or 10^{-4}M of verapamil, Ca^{2+} voltage channel blocker, 30 min just before the addition of MK-4 in order to block Ca^{2+} influx from outside of the cells. 2mM of EGTA, a chelating agent for extracellular calcium, were also used.

Statistical analysis:

Data were evaluated by analysis of variance, ANOVA. A p value less than 0.05 was considered to be significant. All data presented in the text and figures were expressed as the mean±SEM.

Results:

Figure 12 showed the actual data diagram for time course values of $[Ca^{2+}]i$ after the additon of MK-4 (10^{-8}M). The $[Ca^{2+}]i$ value at the time of MK-4 addition was read as zero and the time course changes of $[Ca^{2+}]i$ were measured from the actual curve, where the time of MK-4 addition was set as zero time. The time course changes in $[Ca^{2+}]i$ values in the presence and absence (background) of MK-4, thus obtained, were plotted against time to give Figure 13. The net $[Ca^{2+}]i$ changes for which MK-4 was responsible were obtained by subtracting the background values from the values with MK-4. In MC3T3-E1 cells, after the addition of MK-4 the $[Ca^{2+}]i$ increased and reached to a plateau at around 120 seconds. In the following experiments, the $[Ca^{2+}]i$ values at 1/2 time to reach the maximum $[Ca^{2+}]i$ were used as experimental data.

The change in $[Ca^{2+}]i$ was observed as dose-dependent manner with

bell-shaped curve (Figure 14), where maximal $\Delta[Ca^{2+}]i$ was observed as 25.0± 2.23nM at the concentration of 10^{-8}M of MK-4. Since $1\alpha,25\text{-}(OH)_2D_3$ is known to increase the $[Ca^{2+}]i$ in MC3T3-E1 cells (44), $1\alpha,25\text{-}(OH)_2D_3$ was used as a positive control. As shown in Figure 15, an addition of $1\alpha,25\text{-}(OH)_2D_3$ resulted in a significant increase in $[Ca^{2+}]i$ where 27.5± 1.4nM was obtained for $1\alpha,25\text{-}(OH)_2D_3$ versus 5.0± 2.2nM for control. When 10^{-8}M of MK-4 were added together with 10^{-10}M of $1\alpha,25\text{-}(OH)_2D_3$, $\Delta[Ca^{2+}]i$ was observed as 40.0± 2.3nM (Figure 15), which was higher than an addition of MK-4 alone or $1\alpha,25\text{-}(OH)_2D_3$ alone.

In order to analyze a source of the increased Ca^{2+} by MK-4, EGTA was first added to remove extracellular Ca^{2+} and then MK-4 (10^{-8}M) was added. $[Ca^{2+}]i$ increase by Mk-4 was completely suppressed (Figure 16) suggesting that an increase in $[Ca^{2+}]i$ by MK-4 could possibly be derived from an extracellular environment. Verapamil, a calcium channel blocker, was then used in the next experiments to examine how verapamil would affect MK-4 induced increase of $[Ca^{2+}]i$. The $\Delta[Ca^{2+}]i$ increase by MK-4 was suppressed by preincubation of cells with 10^{-5}M or 10^{-4}M of verapamil by 53% and 70% respectively (Figure 17) indicating that a majority of $[Ca^{2+}]i$ increase by MK-4 was derived from an extracellular medium through a verapamil-sensitive calcium channel.

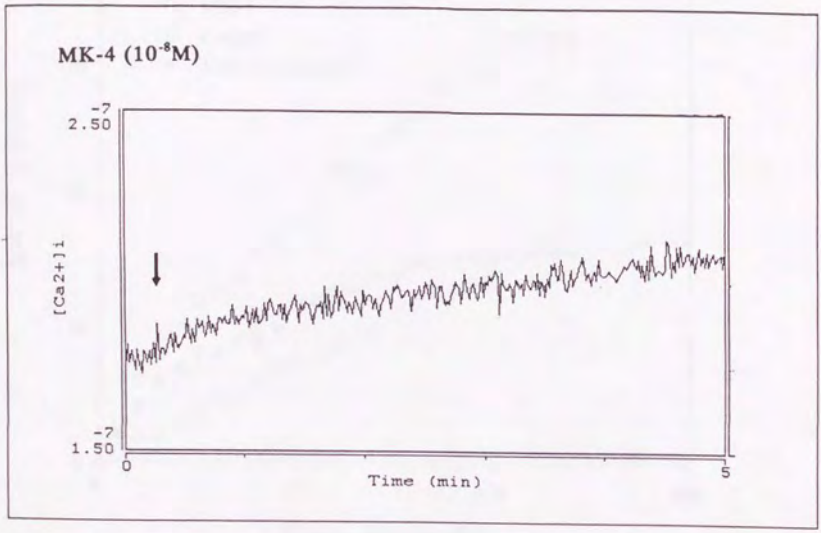


Figure 12. Time course changes in intracellular free Ca^{2+} concentration $[Ca^{2+}]i$ after the addition of MK-4 in MC3T3-E1 cells

MC3T3-E1 cells were treated with Fura-2/AM and incubated for 60 min at 37°C. After removal of unhydrolyzed Fura-2/AM, 10^{-8}M of MK-4 were added to the cell suspension and intracellular free calcium ion concentration $[Ca^{2+}]i$ was recorded by a fluorimeter.

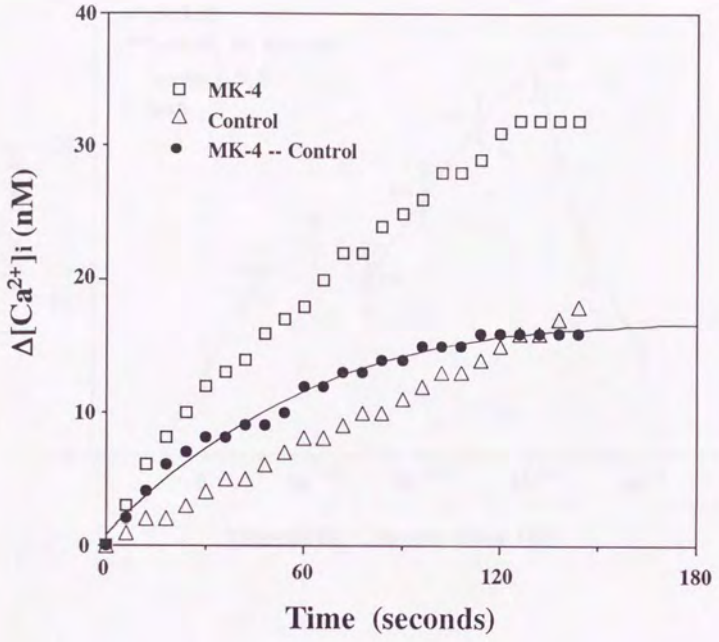


Figure 13. Effect of MK-4 on intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$ of MC3T3-E1 cells as a function of time.

After treatment with Fura-2/AM for 60min at 37°C and removal of unhydrolyzed Fura-2/AM, intracellular free calcium ion concentration $[Ca^{2+}]i$ was measured by a fluorimeter in the presence and absence of MK-4 (10^{-8}M). The net increase in $[Ca^{2+}]i$ was obtained by subtracting the background values from the MK-4 values.

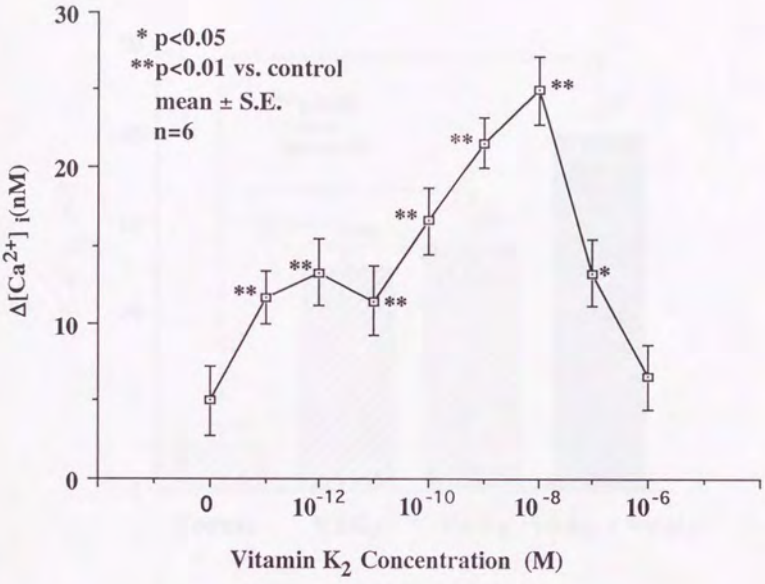


Figure 14. Effect of MK-4 om intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$ of MC3T3-E1 cells as a function of MK-4 concentration.

After treatment with Fura-2/AM for 60 min at 37°C, MC3T3-E1 cells were incubated with increasing amounts of MK-4 (10^{-13}M to 10^{-6}M), and changes in intracellular free Ca^{2+}concentration $\Delta[Ca^{2+}]_i$ was measured by a fluorimeter.

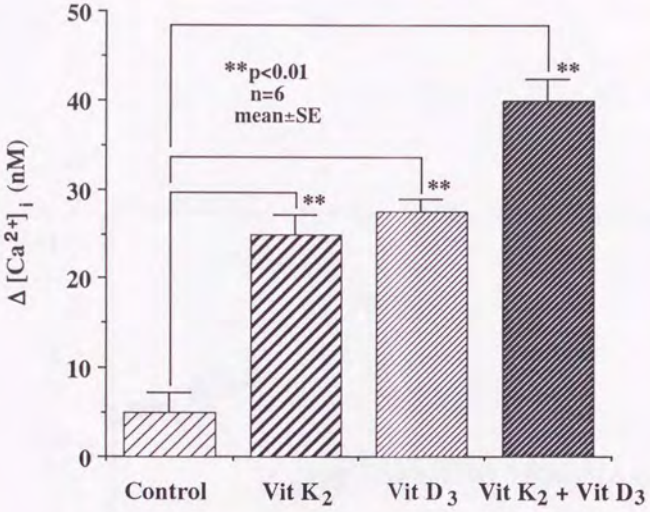


Figure 15. Effect of MK-4 on intracellular free Ca^{2+} concentration of MC3T3-E1 cells in the presence of 1α,25-(OH) 2 vitamin D3.

After treatment with Fura-2/AM for 60 min at 37°C, MC3T3-E1 cells were incubated with 10^{-10}M of 1α,25-(OH)2 vitamin D3 in the absence or presence of 10^{-8}M of MK-4. Changes in intracellular free Ca^{2+} concentration $\Delta[Ca^{2+}]_i$ was measured by a fluorimeter.

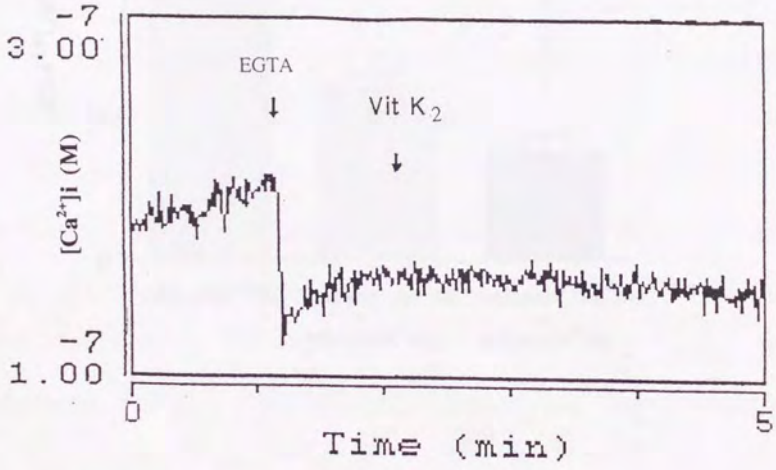


Figure 16. Effect of EGTA on increase in intracellular free Ca^{2+} concentration $[Ca^{2+}]i$ caused by MK-4 in MC3T3-E1 cells.

After treatment with Fura-2/AM for 60 min at 37°C, MC3T3-E1 cells were incubated with 2mM of EGTA and then 10^{-8}M of MK-4 were added. Changes in intracellular free Ca^{2+} concentration $\Delta[Ca^{2+}]i$ was measured by a fluorimeter.

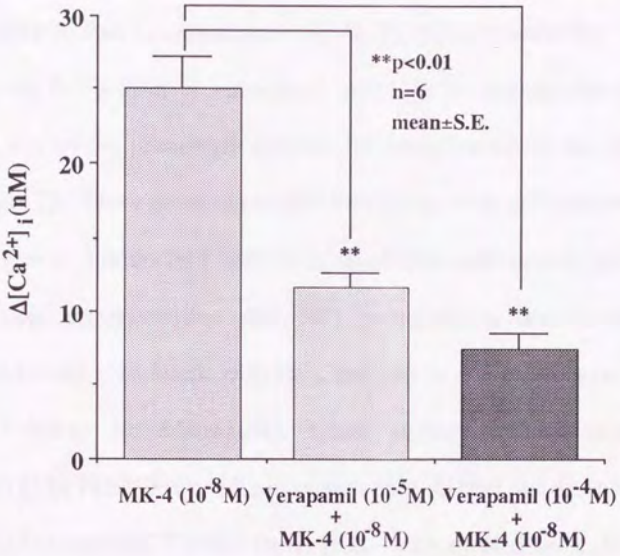


Figure 17. Effect of verapamil on increase in intracellular free Ca^{2+} concentration $\Delta[Ca^{2+}]_i$ caused by MK-4 in MC3T3-E1 cells.

After treatment with Fura-2/AM for 60 min at 37°C, MC3T3-E1 cells were incubated with 10^{-5}M or 10^{-4}M of verapamil for 30 min, and then 10^{-8}M of MK-4 were added. Changes in intracellular free Ca^{2+}concentration $\Delta[Ca^{2+}]i$ was measured by a fluorimeter.

Discussion

As shown in Part 1, menaquinone-4 (MK-4), one of vitamin K_2s, was observed to suppress the growth of osteoblastic cells and to increase the cellular ALP activity, one of the phenotypic markers for osteoblastic cells *in vitro* (Figure 2 and Figure 3). There are accumulated observations as to the relationship between the cell growth and the ALP activity in osteoblastic cells such as MC3T3-E1 (45, 46) human osteoblast-like cells (47), osteosarcoma cell lines (48, 49), marrow-derived osteoblastic cells (50), and also in non-osteoblastic cells such as human cultured fibroblasts (51), human urinary bladder carcinoma cells (JTC-32) (52), HeLaS3 cervical cancer cells (53), Caco-2 human colon carcinoma cell line (54) and MCF-7 breast cancer (55). With all these cells, the two factors, cell growth and ALP activity, are inversely related each other, namely when the cell growth was suppressed, ALP activity was increased. On the other hand, there are other reports which show that, while the cell growth was suppressed, ALP activity was not increased as observed with MC3T3-E1 (56), newborn rat periosteal cells (57), human HCT-116 colon tumor cell clones (58), and HBL-100 normal breast cell lines (55). The observations presented in this study is in good agreement with majority of the above reports. The concentration of MK-4 to obtain the maximal effect on the cell growth was almost the same as that to obtain the maximal effect on the cellular ALP activity, which was about 10^{-7}M to 10^{-6}M.

There is no report on the physiological level of MK-4 concentration in blood in healthy adults except that Usui *et al.* (1994) described it as 10^{-10}M in Japanese people (59). It turned out, hence, that 1000 times higher concentration of MK-4 than its physiological concentration were used in Part 1 studies. When MK-4 (4mg/kg) was orally administrated to OVX rat models once a day for 10 days, MK-4 concentration in bone tissue was found to reach somewhere around 10^{-6}M (Sano, personal communication). Those effective concentrations of MK-4 *in vitro* osteoblastic cells on growth and ALP activity were, therefore, within pharmacologically viable concentration range.

Although the function of MK-4 is not clearly elucidated yet, the data presented here suggest that the mechanism of the action of MK-4 in the treatment of involutional osteoporosis (27) would include an anabolic effect of MK-4 on osteoblastic cells rather than a mitogenic effect. The only known biochemical role of MK-4 in mammalian cells is as a co-factor for γ-glutamyltransferase (9, 10, 11). Based on this background, the effects of MK-4 on the osteoblastic cells observed in Part 1 study have to be assessed from the view point of the possible activation of γ-carboxylation reaction by exogenous MK-4. In order for vitamin K to serve as a cofactor for γ-carboxylase, 1,4-naphthoquinone ring in the vitamin K molecule has to be first reduced to give vitamin K-hydroquinone, which is then oxidized during its role as a cofactor for γ-carboxylation reaction resulting in vitamin K-2,3-epoxide. This 2,3-epoxide moiety of vitamin K is then reduced to the original 1,4-naphthoquinone ring for another cycle (Figure 18).

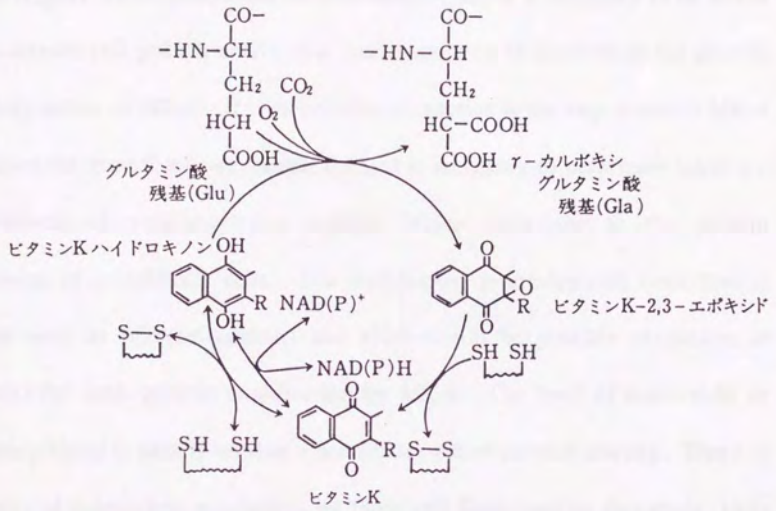


Figure 18. Metabolic Cycle of Vitamin K

Warfarin, widely used in clinics, is known as an inhibitor of vitamin K action by blocking the two reduction steps in the vitamin K metabolic cycle. The observation that warfarin reversed the suppressive effect of MK-4 on the cell growth (Figure 4), suggested that the activation of MK-4 is necessary in its action on osteoblastic cell growth and/or that γ-carboxylation is involved in the growth inhibitory action of MK-4. If γ-carboxylation reaction is the step at which MK-4 suppresses the growth of osteoblastic cells, it is necessary to determine what are the products of γ-carboxylation reaction which contribute to the growth suppression of osteoblastic cells. The well-known γ-carboxylated bone matrix proteins such as BGP(osteocalcin) and MGP would be possible candidates as mediators for such growth modification by MK-4. The level of osteocalcin in circulating blood is usually used as a marker for osteoblast cell activity. There is no report of osteocalcin production by those cell lines used in this study, HOS TE85 cells and MC3T3-E1 cells, and the currently available assay system for osteocalcin has some drawbacks in measuring all of carboxylated and non-carboxylated osteocalcin and osteocalcin degradation products. It was, therefore, not possible to measure osteocalcin production by these two cell lines.

The results of G1/S boundary synchronization experiments (Figure 6) and FACS analysis indicated that MK-4 would modify the cell cycle in such a way as MK-4 makes the initial S phase longer and also elongates the subsequent cell cycle phases resulting in an overall extention of the cell cycle (Figure 9-A, Figure 10 and Figure 11). Several proteins are known to be involved in the regulation of

cell cycle progression. One such protein, $p34^{cdc2}$ kinase, acts as a key catalytic subunit of mitotic promoting factor (MPF) (60) and also catalyzes phosphorylation of such multiple substrates *in vitro* and *in vivo* like H1 histone (61), retinoblastoma RB protein (62), SV40 large T antigen (63), RNA polymerase II (64) and cyclin B (65). The action of $p34^{cdc2}$ itself is controlled by phosphorylation at Thr-14, Tyr-15 and Thr-167 residues in the enzyme molecule. Dephosphorylation at the Tyr-15 residue of the enzyme by protein-tyrosine phosphatase (PTPase) can activate the MPF function (66). $p34^{cdc2}$ kinase is also implicated in an activation of several cellular processes such as chromosome condensation, mitotic spindle formation, and DNA replication (67). C-C. Juan *et al.* (68) reported that vitamin K_3 suppressed the growth of HepG2 cells by delaying the cell cycle progression. Possible mechanism for this observation was that a decrease in the activity of PTPase suppressed dephosphorylation of $p34^{cdc2}$ kinase resulting in deactivation of MPF, which caused a delay in cell cycle progression into M phase. Prolonged S phase by MK-4 was due to delayed progresson of the cell cycle from G2 phase into M phase, and it can be considered that dephosphorylation of $p34^{cdc2}$ kinase may have been dropped. Since G2 cyclin is considered to be invloved in the cell cycle progression from G2 into M phase, it is interesting to analyze the molecular mechanisms by which G2 cyclin forms a complex with $p34^{cdc2}$ kinase to function as MPF.

The role of ALP is not well known except that it would serve as a supplier of inorganic phosphate. ALP activity was monitored as an indicator for

differentiation because its activity is known to increase during the onset of differentiation (69). It was observed that the ALP activity was increased by MK-4 in osteoblast cells. The fact that this increase was not affected by warfarin, would suggest that MK-4 effect of increasing ALP activity could involve other functions than γ-carboxylation. One such possible function would be MK-4's involvement in gene expression at the level of transcription as seen from the observations that MK-4 increased both ALP activity and the amount of ALP mRNA in rat osteosarcoma cells (ROS 17/2.8) (70) and that the level of osteocalcin mRNA in human osteoblast cell culture was higher in the presence of both MK-4 and 1,25-$(OH)_2$ vitamin D_3 than 1,25-$(OH)_2$ vitamin D_3 alone (71). In order to explore a possible mechanism of cellular ALP activity increase by MK-4, cells were synchronized at S phase by the G1/S boundary method and ALP activity was analyzed during the cell cycle. In HOS TE85 cells in the presence or absence of MK-4, low ALP activity was found at M phase (mitosis) and high ALP activity was at G1 phase (pre-DNA synthesis). This observation was in agreement with Fedarko *et al.* (72) who reported that in human trabecular bone cells, alkaline phosphatase (ALP) activity dropped at M phase and returned during G1 phase. Two observations that MK-4 did not show significant increase in ALP activity at any phases of the cell cycle as compared with the control and that the ratio of G1 phase cell population was higher in the MK-4 added group as measured by FACS analysis, could possibly explain the ALP activity increase by MK-4 in the Part 1 study by considering that G1 phase cells associated with

higher ALP activity would increase its population proportion resulting in an overall increase in cellular ALP activity. Since MK-4 delayed the cell cycle and the number of cell division hence decreased, the total number of cells were reduced in the presence of MK-4. It is, however, not entirely possible to explain the MK-4 action to increase ALP activity in osteoblast cells solely by the results of the cell cycle analysis because FACS analysis was made on asynchronized cells at 24 hours after the addition of MK-4 whereas ALP activity was measured after 3 day incubation with MK-4, thus cell population was a likely mixture of various cell cycle phases.

With regard to the cell growth in relation to intracellular free calcium ion, it is reported that an increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) stimulated the growth of mouse fibroblast (Balb/c3T3 cells) and other cells (40, 41, 42). This increase in intracellular free Ca^{2+} is known to occur through the action of epidermal growth factor (EGF) or insulin-like growth factors (IGFs). It is comfirmed in Balb/c3T3 cells that an influx of free Ca^{2+} into the cell is, in fact, an intracellular signal for IGF's growth stimulation action. This conclusion was made from the observations in the experiments designed for investgating the relation between IGF action on cell growth and Ca^{2+} influx that the degree of growth stimulation action by IGF was dependent on the degree of intracellular Ca^{2+} increase and that stimulation of DNA synthesis and subsequent increase in the cell number were achieved even by an artificial increase in Ca^{2+} influx with use of BAYK8644 (40). Therefore, in order to further investigate the mode of

action of MK-4, the initial intracellular response to the addition of MK-4 in the movement of intracellular free Ca^{2+}, known as the second messenger in the intracellular signal transduction system, was examined. In the cell suspension of murine osteoblastic cell line (MC3T3-E1), MK-4 induced a rapid increase in the level of $[Ca^{2+}]i$ and maintained its elevated level (Figure 12). The degree of an increase in $[Ca^{2+}]i$ by MK-4 was observed as 25± 2.3 nM, which was the same extent as 1α,25-$(OH)_2D_3$ exhibited (27.5±1.4 nM) (Figure 15). 1α,25-$(OH)_2D_3$ is known to increase the level of $[Ca^{2+}]i$ in various cells (73, 74, 75, 76), which was also observed in osteoblastic cell system such as mouse osteoblasts (77), rat osteoblast-like cells (78, 79) and mouse osteoblast-like cells (44). There are two distinctly different patterns of $[Ca^{2+}]i$ increase by 1α,25-$(OH)_2D_3$. In one case where an immediate transient increase in $[Ca^{2+}]i$ was followed by rapid return to a basal level (44, 77, 78), while in another case where a rapid increase in $[Ca^{2+}]i$ was sustained for a prolonged period of time as observed with our studies as well as with colonal rat sarcoma cell line (ROS 17/2.8) stimulated by a low concentration (10^{-10}M) of 1α,25-$(OH)_2D_3$ (64) and with studies by Baran *et al.* with ROS 17/2.8 (77). In case of the sustained type of $[Ca^{2+}]i$ increase by 1α,25-$(OH)_2D_3$, this increase was explained, on one hand, to be due to an influx of extracellular Ca^{2+} (78), and on the other hand, to be due to Ca^{2+} release from intracellular Ca^{2+}pool (79). In case of the transient type of $[Ca^{2+}]i$ increase, both an influx of extracellular Ca^{2+} and release from intracellular Ca^{2+} pool such as endoplasmic reticulum are reported to be involved at the same time (77, 78).

Even with osteoblast cells, depending on 1α,25-$(OH)_2D_3$ concentrations and investigators, there are variations in patterns of $[Ca^{2+}]i$ increase and in the origin of $[Ca^{2+}]i$. This may be due to differences in $[Ca^{2+}]i$ measurement system, cell types and cell cycle stages.

Pre-treatment with verapamil, a voltage-operated Ca^{2+} channel blocker, resulted in significant suppression of MK-4-induced $[Ca^{2+}]i$ increase, and pre-treatment with EGTA, a chelating agent for extracellular Ca^{2+}, resulted in little increase in $[Ca^{2+}]i$ by MK-4 (Figure 16 and Figure 17). These observations suggested that MK-4 would open a verapamil-sensitive calcium channel and thus increase in the level of $[Ca^{2+}]i$. Verapamil did not, however, completely block the influx of extracellular Ca^{2+}, it is hence considered that a pathway insensitive to verapamil might also be involved. As we observed with MK-4, 24,25-$(OH)_2D_3$, a metabolite of 1α,25-$(OH)_2D_3$, induced in osteoblasts (ROS 17/2.8 cells) a rapid increase in $[Ca^{2+}]i$ which was then sustained for 5 min (77). Since this increase in $[Ca^{2+}]i$ in ROS 17/2.8 cells was due to release of Ca^{2+} from intracellular stores, which is caused by an activation of phospholipase C and subsequent hydrolysis of membrane-associated phosphatidylinositol-4,5-bisphosphate (PIP_2), it could also be considered that a part of an increase in $[Ca^{2+}]i$ by MK-4 may possibly invlove phospholipid metabolism.

Regardless of differences in kinds of cells, patterns of $[Ca^{2+}]i$ increases and origin of intracellular Ca^{2+} sources, it is commom in all observations that 1α,25-$(OH)_2D_3$ was observed to increase $[Ca^{2+}]i$. In the experimental system used

in this study, it was also confirmed that 1α,25-$(OH)_2D_3$ (10^{-10}M) indeed increased $[Ca^{2+}]i$. With respect to the relation between 1α,25-$(OH)_2D_3$ (10^{-11}M~10^{-9}M) and the cell growth in MC3T3-E1 cells and human bone cells, it has been reported that 1α,25-$(OH)_2D_3$ decreased thymidine uptake and thus suppressed the cell growth (80, 81). It is then considered that both MK-4 and 1α,25-$(OH)_2D_3$ will increase the level of intracellular Ca^{2+}, an intracellular second messenger, and will act on the cell growth rather suppressively. In the experiments on $[Ca^{2+}]i$ changes presented here, use of both MK-4 and 1α,25-$(OH)_2D_3$ (10^{-10}M) resulted in a larger increase in $[Ca^{2+}]i$ as compared to the cases where MK-4 or 1α,25-$(OH)_2D_3$ was used alone (Figure 15), showing that, as long as the movement of the intracellular Ca^{2+} was concerned, both MK-4 and 1α,25-$(OH)_2D_3$ would work in the same direction on the behavior of intracellular Ca^{2+} movement. There are observations suggesting a possibility of "cooperative action" by vitamin K and vitamin D_3. On the cellular aspects, it is reported that [1] MK-4 synergistically facilitated 1α,25-$(OH)_2D_3$-induced calcium deposition and osteocalcin formation in the cell layer of human oasteoblast cells (33), [2] the increase in the level of osteocalcin mRNA by 1α,25-$(OH)_2D_3$ in human osteoblast cells was more significant when 1α,25-$(OH)_2D_3$ was used together with MK-4 (71), and [3] an attachment of 1α,25-$(OH)_2D_3$ to DNA was influenced by vitamin K_1-catalyzed γ-carboxylation of vitamin D receptor (VDR) (82). For clinical observations, [1] significantly better clinical efficacy (bone density increase) by MK-4 was obtained with the group which had a higher level of blood

1α,25-$(OH)_2D_3$ (27), and [2] those elderly osteoporosis patients who were likely to cause bone fracture were found to have a low level of both vitamin K_1 and 25-$(OH)_2D_3$ (83). Thus, the above collective observations may suggest the cooperative action by vitamin K and vitamin D_3, but no insights on this cooperative action has not been analyzed. There remains a need for further investigation to know how these two vitamins will interact with each other in their respective metabolic pathways.

The rapid increase in $[Ca^{2+}]i$ by 1α,25-$(OH)_2D_3$ which was observed in various osteoblastic cell system (44, 77, 78, 79) is considered as a non-genomic action independent of nuclear receptor from observations that [1] this rapid increase occurs immediately after the addition of hormones, [2] this $[Ca^{2+}]i$ increase was observed with osteoblast-like cells (ROS 24/1) which lacks the vitamin D receptor and does not express osteoblastic phenotype, and [3] 1β,25-$(OH)_2D_3$ inhibited 1α,25-$(OH)_2D_3$ -induced increases in $[Ca^{2+}]i$, which does not bind nor displace 1α,25-$(OH_2)D_3$ from its nuclear receptor. The observations, that the inactive epimer, 1β,25-$(OH)_2D_3$, inhibited $[Ca^{2+}]i$ increase by 1α,25-$(OH)_2D_3$ and osteocalcin mRNA transcription at the same time (84), suggest a possibility that non-genomic action of vitamin D may modify its genomic action. Further observations, that vitamin D receptor (VDR), which is scattered in cytoplasm, will aggregate and move into the nucleus in a short time after the addition of vitamin D (85, 86), suggest a possibility that non-genomic action of vitamin D may occur via VDR. Since MK-4 rapidly increased $[Ca^{2+}]i$

immediately after the addition, MK-4 could exhibit its function by non-genomic action, whereas the observations that MK-4 increased transcription of alkaline phosphatase (ALP) mRNA as well as specific activity of ALP in ROS 17/2.8 cells (70), suggest that MK-4 may also be involved in gene regulation at the level of transcription. With regard to MK-4 involvement in gene regulation at the level of transcription, Koshihara *et al.* has reported that MK-4 by itself did not influence the level of osteocalcin mRNA and vitamin D receptor (VDR) mRNA in osteoblast culture system, but when MK-4 was used together with $1\alpha,25\text{-}(OH)_2D_3$, an increased level of osteocalcin mRNA was observed as compared to $1\alpha,25\text{-}(OH)_2D_3$ alone (71). It has been established that $1\alpha,25\text{-}(OH)_2D_3$ controls an expression of osteocalcin mRNA by up-regulating the vitamin D responsive element (VDRE) on the promotor region of osteocalcin gene (87), and phosphorylation of VDR by protein kinase C is essential for the vitamin D's transactivation function (88). An increased transcription level of osteocalcin mRNA in the presence of both MK-4 and vitamin D may suggest that MK-4 could modify vitamin D's genomic action.

Intracellular Ca^{2+} is one of many factors which control the cell growth, and the cell growth is materialized by the sum of actions of these factors resulting in the direction of growth promotion or growth suppression. IGF, one of those factors controling the cell growth, is formed and secreted by osteoblast cells, and IGFs are complexed to IGF binding proteins (IGFBPs), which may modulate IGF biological activities (80, 89, 90). At the same time IGF controls the cell growth

by modifying the intracellular $[Ca^{2+}]i$ through the IGF's non-voltage-operated Ca^{2+} channel. In this way, various factors are involved even in an expression of IGF activities on the cell growth, and thus it would not be possible to discuss the cell growth control by MK-4 only uniformly by the movement of intracellular Ca^{2+}. It can be at least noted that an increase in $[Ca^{2+}]i$ by MK-4 would be an early event in the cellular mechanisms leading to modulation of cell growth and ALP activity.

This study demonstrated that the mode of action of MK-4 on bone metabolism is not only attributed to its function as a cofactor for γ-carboxylase, but some other mechanisms are also possibly involved in exerting its effects on bone. Just like an example of retinoic acid or phorbol ester which promotes cell differentiation while suppressing the cell growth and in proliferating cell system, the clinically demonstrated efficacy of MK-4 in increasing bone mass may well be considered from the results of this study that MK-4 induces cell differentiation in osteoblast cells while suppressing the cell growth. The mechanisms of MK-4 effects observed in many basic and clinical studies are, however, not competely elucidated and there remains many unsoloved questions.

Conclusion

In *in vitro* osteoblast cell culture system, menaquinone-4 (MK-4) was observed to suppress the growth of murine osteoblastic cells (MC3T3-E1) and human osteosarcoma cells (HOS TE85), while MK-4 increased the cellular ALP activity which is a marker for cell differentiation. This is the first observation that MK-4 modifies growth and function of osteoblast cells. By employing G1/S-phase synchronization method and FACS analysis, it was observed that MK-4 elongated the initial S phase and also caused the subsequent phases in the cell cycle longer resulting in an overall extention of the cell cycle, suggesting that MK-4 exerts its effect on cellular function by modifying the cell cycle. With regard to the cell growth suppression by MK-4, warfarin counter-reversed the MK-4's suppressive effect on the cell growth, suggesting that MK-4 action of growth suppression would involve the function of MK-4 as a cofactor for γ-carboxylation. With regard to the intracellular ALP activity, MK-4 increased its activity. This can be explained by considering the increasing ratio of G1 phase population with higher ALP activity. It was not, however, entirely possible to explain the MK-4 action to increase ALP activity solely by the results of the cell cycle analysis and FACS analysis. The increase in ALP activity was not affected by warfarin and it was hence considered that MK-4 would exert its effect of increasing ALP activity by involving its other functions than γ-carboxylation.

The observations above may suggest that MK-4 would modulate the growth and function of the osteoblast cells by modifying the cell cycle. In order to further elucidate a mode of action of MK-4 on cellular function, a possibility of an involvement of intracellular signal transduction system was investigated. MK-4 induced a rapid increase in $[Ca^{2+}]i$ to the same extent as $1\alpha,25\text{-}(OH)_2D_3$ did. Experiments with verapamil indicated that MK-4 would induce Ca^{2+} influx from an extracellular environment through a verapamil-sensitive calcium channel thus resulting in an increased $[Ca^{2+}]i$. Just like MK-4, $1\alpha,25\text{-}(OH)_2D_3$ is also reported to suppress the cell growth of MC3T3-E1 cells and other cells. From the results of this study, therefore, both MK-4 and $1\alpha,25\text{-}(OH)_2D_3$ will exhibit their activities in the direction of increasing the intracellular Ca^{2+} and suppressing the cell growth. The observation that intracellular Ca^{2+} was significantly increased by the presence of both MK-4 and $1\alpha,25\text{-}(OH)_2D_3$ as compared to the increase in $[Ca^{2+}]i$ by MK-4 or $1\alpha,25\text{-}(OH)_2D_3$ alone, suggested that a possibility of cooperative action by the two vitamins. It is generally known that an increase in $[Ca^{2+}]i$ is related to the cell growth promotion, but since the cell growth is controlled by such growth factors like IGFs (insulin-like growth factor) and the cell growth is appeared as a sum of actions by various factors, it was not quite possible to discuss the cell growth suppression by MK-4 solely by the movement of intracellular Ca^{2+}. Just like an example of retinoic acid or phorbol ester which promotes cell differentiation while suppressing the cell growth in proliferating cell system, the clinical efficacy of MK-4 in increasing the bone mass can be

considered from the results of this study that MK-4 will induce cell differentiation in osteoblast cells while suppressing the cell growth.

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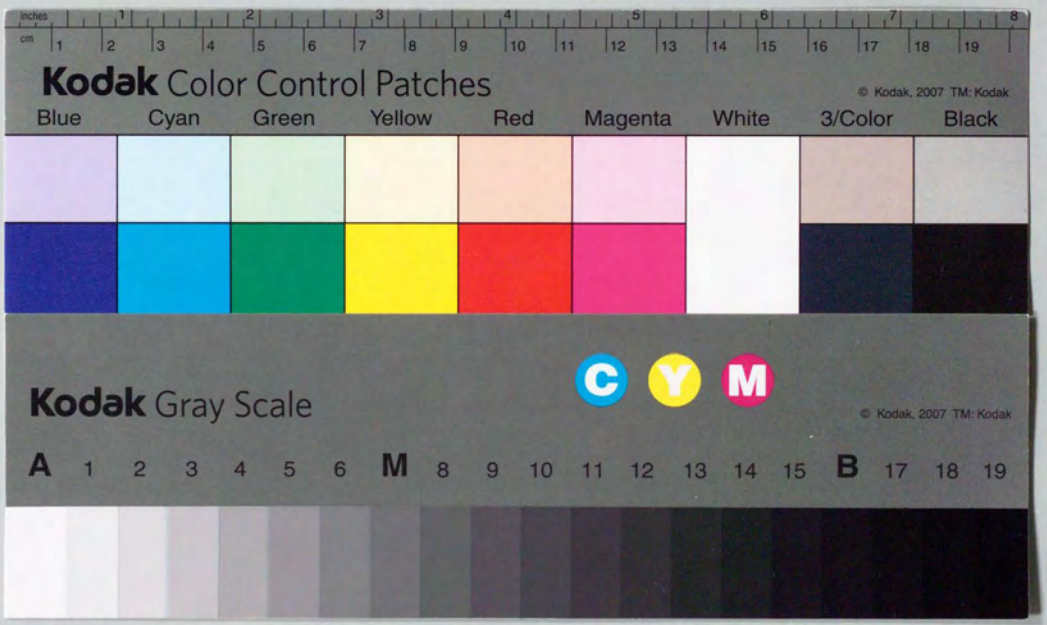
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