Inhibitory effects of vitamin K3 on DNA polymerase and angiogenesis

KIMINORI MATSUBARA[1,2], TOMOKO KAYASHIMA[1], MASAHARU MORI[3], HIROMI YOSHIDA[2,4] and YOSHIYUKI MIZUSHINA[2,4]

[1]Department of Human Life Sciences Education, Graduate School of Education, Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8524; [2]Cooperative Research Center of Life Sciences, Kobe-Gakuin University, Nishi-ku, Kobe, Hyogo 651-2180; [3]Department of Nursing, Faculty of Health and Welfare Science, Okayama Prefectural University, 111 Kuboki, Soja, Okayama 719-1197; [4]Department of Nutritional Science, Kobe-Gakuin University, Kobe, Hyogo 651-2180, Japan

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Abstract. Vitamins play essential roles in cellular reactions and maintain human health. Recent studies have revealed that some vitamins including D3, B6 and K2 and their derivatives have an anti-cancer effect. As a mechanism, their inhibitory effect on cancer-related angiogenesis has been demonstrated. Vitamin K2 (menaquinones) has an anti-cancer effect in particular for hepatic cancer and inhibits angiogenesis. In the current study, we demonstrated that sole vitamin K3 (menadione) selectively inhibits the *in vitro* activity of eukaryotic DNA polymerase γ, which is a mitochondrial DNA polymerase, and suppresses angiogenesis in a rat aortic ring model. The anti-angiogenic effect of vitamin K3 has been shown in angiogenesis models using human umbilical vein endothelial cells (HUVECs) with regard to HUVEC growth, tube formation on reconstituted basement membrane and chemotaxis. These results suggest that vitamin K3 may be a potential anti-cancer agent like vitamin K2.

Introduction

The importance of vitamins for human health is well established. Our research interest has focused on the relationship between vitamins and vascular function, in particularly, angiogenesis (1). The various functions of vitamin B6 on endothelial cells, DNA topoisomerase and embryonic stem cell differentiation (2,3) led us to investigate whether other vitamins have anti-angiogenic activity. It has been demonstrated so far that some vitamins, including D3, E and K2, and their derivatives, have anti-angiogenic activity (4-7). We have found that vitamin B6 not only inhibits angiogenesis but also DNA topoisomerase (2), and that anti-angiogenic agents strongly inhibit DNA polymerase (8,9).

DNA polymerases are indispensable for maintaining the integrity of the genome, both through faithful replication of DNA and by repairing damage to DNA. Among the 16 highly specialized mammalian polymerases, 15 are involved in maintaining nuclear genetic information (10,11). Replication and maintenance of the mitochondrial genome relies on a unique polymerase, DNA polymerase γ (11). DNA polymerase γ is responsible for all aspects of mtDNA synthesis, including all replication, recombination of the mitochondrial genome, and repair of mtDNA damage.

Angiogenesis and DNA polymerase are critical targets for anti-cancer drug development, and an agent inhibiting both activities would be a potent anti-cancer agent or a good model for anti-cancer drug design. Thus, we screened low-molecular weight compounds, which have both anti-angiogenic and DNA polymerase inhibitory activities. Notably, vitamin K3, a synthetic vitamin K, showed strong anti-angiogenic activity and inhibited DNA polymerase γ activity. In this study, we demonstrated the effects of vitamin K3 on angiogenesis and DNA polymerase activity.

Materials and methods

Materials. Vitamin K1 (phylloquinone), K2 (menaquinones) and K3 (menadione) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). The chemical structures of vitamin K compounds are shown in Fig. 1. Nucleotides and chemically synthesized DNA template-primers such as poly(dA), poly(rA), and oligo(dT)$_{12-18}$ and [^{3}H]-dTTP (deoxythymidine 5'-triphosphate, 43 Ci/mmol) were purchased from GE Healthcare Bio-Sciences (Little Chalfont, Buckinghamshire, UK). Human recombinant vascular endothelial growth factor (VEGF) was obtained from R&D Systems (MN, USA). Other reagents were of special grade as commercially available.

Correspondence to: Dr K. Matsubara, Department of Human Life Sciences Education, Graduate School of Education, Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8524, Japan
E-mail: kmatsuba@hiroshima-u.ac.jp

Abbreviations: HUVEC, human umbilical vein endothelial cell; VEGF, vascular endothelial growth factor

Key words: angiogenesis, aortic ring, endothelial cells, vitamin K3

Enzymes. DNA polymerase α was purified from calf thymus by immunoaffinity column chromatography as described by Tamai *et al* (12). Recombinant rat DNA polymerase ß was purified from *E. coli* JMpß5 as described by Date *et al* (13). The human DNA polymerase γ catalytic gene was cloned into pFastBac. Histidine-tagged enzyme was expressed using the Bac-to-Bac HT Baculovirus Expression System according to the supplier's instructions (Life Technologies, MD, USA) and purified using ProBoundresin (Invitrogen Japan, Tokyo, Japan) (14). Human DNA polymerases δ and ε were purified by the nuclear fractionation of human peripheral blood cancer cells (Molt-4) using the second subunit of polymerases δ and ε-conjugated affinity column chromatography, respectively (15). Recombinant human DNA polymerases η and ι tagged with His6 at their C-terminal, were expressed in SF9 insect cells using the baculovirus expression system, and were purified as described previously (16,17). A truncated form of DNA polymerase κ (i.e., hDINB1DC) with 6xHis-tags attached at the C-terminal, was overproduced using the Bac-to-Bac Baculovirus Expression System kit (Gibco BRL) and purified as described previously (18). Recombinant human His-DNA polymerase λ was overexpressed and purified according to a method described previously (19). Fish DNA polymerases α and δ were purified from the testis of cherry salmon (*Oncorhynchus masou*) (20). Fruit fly DNA polymerases α, δ and ε were purified from the early embryos of *Drosophila melanogaster* as described previously (21,22). DNA polymerase α from a higher plant, cauliflower inflorescence, was purified according to the methods outlined by Sakaguchi *et al* (23). Recombinant rice (*Oryza sativa* L. cv. Nipponbare) His-DNA polymerase λ was overexpressed and purified according to a method described previously (24). Human immunodeficiency virus type-1 (HIV-1) reverse transcriptase (recombinant) and the Klenow fragment of DNA polymerase I from E. coli were purchased from Worthington Biochemical Corp. (Freehold, NJ, USA). Taq DNA polymerase, T4 DNA polymerase, T7 RNA polymerase and T4 polynucleotide kinase were purchased from Takara (Kyoto, Japan). Calf thymus terminal deoxynucleotidyl transferase and bovine pancreas deoxyribonuclease I were obtained from Stratagene Cloning Systems (La Jolla, CA, USA).

DNA polymerase assays. The reaction mixtures for DNA polymerases α and ß, plant polymerases and prokaryotic DNA polymerases have been described previously (25,26). Those for DNA polymerase γ, and polymerases δ and ε were as described by Umeda *et al* (14) and Ogawa *et al* (27), respectively. The reaction mixtures for DNA polymerases η, ι and κ were the same as that for polymerase α, and the reaction mixture for DNA polymerase λ was the same as that for polymerase α. For DNA polymerases (i.e., DNA-dependent DNA polymerases), poly(dA)/oligo(dT)$_{12-18}$ (A/T = 2/1) and dTTP were used as the DNA template-primer and nucleotide (i.e., deoxyribonucleotide 5'-triphosphates, dNTP) substrate, respectively. For HIV-1 reverse transcriptase (i.e., RNA-dependent DNA polymerase), poly(rA)/oligo(dT)$_{12-18}$ (A/T = 2/1) and dTTP were used as the template-primer and nucleotide substrate, respectively. For terminal deoxynucleotidyl transcriptase, oligo(dT)$_{12-18}$ (3'-OH) and dTTP were used as the DNA template-primer and nucleotide substrate, respectively.

Vitamin K compounds were dissolved in distilled dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 sec. Aliquots of 4 μl of sonicated samples were mixed with 16 μl of each enzyme (final amount 0.05 units) in 50 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol, 50% glycerol and 0.1 mM EDTA, and kept at 0˚C for 10 min. These inhibitor-enzyme mixtures (8 μl) were added to 16 μl of each of the enzyme standard reaction mixtures, and incubation was carried out at 37˚C for 60 min, except for Taq DNA polymerase which was incubated at 74˚C for 60 min. Activity without the inhibitor was considered 100%, and the remaining activity at each concentration of the inhibitor was determined relative to this value. One unit of polymerase activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of dNTP (i.e., dTTP) into synthetic DNA template-primers in 60 min at 37˚C under the normal reaction conditions for each enzyme (25,26).

Other enzyme assays. The DNA primase activity of DNA polymerase α, the activities of T7 RNA polymerase, T4 polynucleotide kinase and bovine deoxyribonuclease I were measured in standard assays according to the manufacturer's specifications as described by Tamiya-Koizumi *et al* (28), Nakayama and Saneyoshi (29), Soltis and Uhlenbeck (30), and Lu and Sakaguchi (31), respectively.

Ex vivo angiogenesis assay. Male Wistar rats (6 weeks old, Charles River Laboratories, Kanagawa, Japan) were housed in metal cages in a room with controlled temperature (24±1˚C) and a 12-h light/dark cycle (lights on, 08:00-20:00). They had free access to diets and deionized water. The rats were maintained according to the 'Guide for the Care and Use of Laboratory Animals' established by Hiroshima University. The *ex vivo* angiogenesis assay was performed according to slightly modified methods as described before (32,33). Briefly, a male Wistar rat (body weight ~200 g) was sacrificed by bleeding from the right femoral artery under anesthesia with diethyl ether. A thoracic aorta was removed and washed with RPMI-1640 medium (Gibco, New York, USA) to avoid contamination with blood. It was then turned inside out, and cut into short segments of about 1-1.5 mm. Collagen gel (gel matrix solution) was made with 8 volumes of porcine tendon collagen solution (3 mg/ml) (Cellmatrix Ia, Nitta Gelatin Co., Osaka, Japan), 1 volume of 10X Eagle's MEM (Gibco), 1 volume of reconstitution buffer (0.08 M NaOH and 200 mM HEPES). These solutions were mixed gently at 4˚C. Each aortic segment was placed in the center of a well on a 6-well culture plate and covered with 0.5 ml of gel matrix solution reconstituted as described. The solution was allowed to gel at 37˚C for 20 min. Culture medium [RPMI-1640 medium containing 1% of ITS+ (Becton Dickinson Labware, MA, USA)] with various concentration of vitamin K or vehicle (DMSO) was prepared as described above. The collagen gel was then overlaid with 2 ml of culture medium. Incubation was carried out for 10 days in a fully humidified system of 5% CO_2 in air at 37˚C. The medium was changed on day 7 of the culture. An estimation of the length of the capillary was performed under phase-contrast microscopy by measuring

the distance from the cut end of the aortic segment to the approximate mean point of the capillary. Microscopic fields were photographed with a digital camera (Olympus DSE330-A System). The length of the capillary was measured using Adobe Photoshop software. Each reported value represents the average of culture samples.

Endothelial cells. Human umbilical vein endothelial cells (HUVECs) were purchased from Kurabo Industries (Osaka, Japan). Cells were grown in the medium, HuMedia EG-2 (Kurabo Industries, Osaka, Japan), which was modified MCDB 131 medium containing 2% fetal bovine serum (FBS), 10 ng/ml recombinant human epidermal growth factor (EGF), 1 μg/ml hydrocortisone, 50 μg/ml gentamicin, 50 ng/ml amphotericin B, 5 ng/ml recombinant human basic fibroblast growth factor (bFGF) and 10 μg/ml heparin, at 37˚C in humidified 5% CO_2. Subcultures were obtained by treating the HUVEC cultures with 0.025% trypsin-0.01% EDTA solution. HUVECs at passage three to seven were used in this experiment.

HUVEC tube formation assay. Tube formation assay was performed using BD Matrigel™ (Becton Dickinson and Company, Tokyo, Japan). Briefly, solid gels were prepared according to the manufacturer's instructions on a 96-well tissue culture plate. HUVECs ($1x10^5$ cells/ml) in HuMedia EG-2 medium containing vitamin K_3 or vehicle (DMSO) were seeded 100 μl/well onto the surface of the solid gel, BD Matrigel. The cells were incubated for 12 h at 37˚C in a CO_2 incubator. Tube formation was observed under an inverted light microscope at 40x magnification. Microscopic fields were photographed with a digital camera (Olympus DSE330-A System). The total length of tube structures in each photograph was measured using Adobe Photoshop software. Each reported value represents the average of three samples.

HUVEC proliferation assay. For HUVEC proliferation assay, HUVEC were dispersed with trypsin and suspended in HuMedia EG-2 medium. A cell suspension (15,000 cells/ml) was plated onto 96-well culture plates (100 μl/well), and incubated at 37˚C in a humidified 5% CO_2 for 24 h. The medium was replaced with fresh HuMedia EG-2 containing vitamin K_3. After 72 h, 10 μl of WST-1 reagent was added into each well of a 96-well plate and incubated for 4 h at 37˚C. The absorbance at 450 nm was measured using a microplate spectrophotometer

HUVEC chemotaxis assay. This was carried out by a modified Boyden chamber assay (34). The microporous membranes (8 μm) of 24-well cell culture inserts (BD Biosciences, MA, USA) were coated with 0.1% gelatin. HUVECs were detached with cell dissociation buffer (Invitrogen Corp., Carlsbad, CA, USA), collected by centrifugation, re-suspended in Medium 199 (Invitrogen) with 0.1% bovine serum albumin (BSA), and seeded in triplicate in the chamber ($1.0x10^5$ cells/400 μl). The well was filled with 400 μl of Medium 199 containing 0.1% BSA and 10 ng/ml of VEGF with or without vitamin K_3. The assembled chamber was incubated for 6 h at 37˚C in humidified 5% CO_2. Non-migrated cells on the upper surface of the membrane were removed by scrubbing with a cotton swab. The cells on the lower surface of the membrane were fixed with methanol, and stained with Diff-Quik™ stain. Migrated cells were counted in five fields of each membrane under a microscope at 200x magnification, and the average number in a field was calculated. The experiments were performed in triplicate.

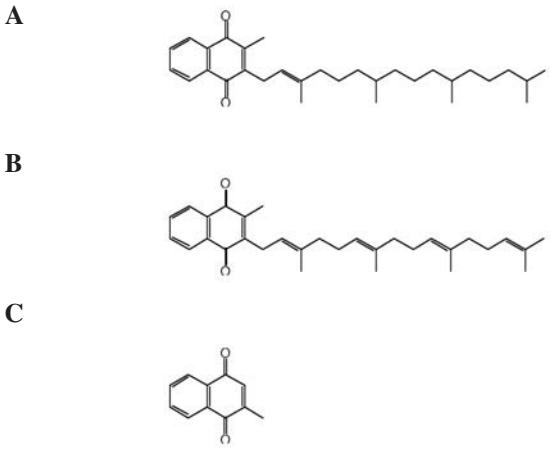

Figure 1. Chemical structures of vitamin K compounds. (A) Vitamin K_1, (B) vitamin K_2 and (C) vitamin K_3.

Statistical analysis. Values are presented as means ± SEM. Data were analyzed by one-way analysis of variance (ANOVA). Differences of $p<0.05$ were considered significant.

Results

Effects of vitamin K compounds on DNA polymerase activities. First, the inhibition of the activities of mammalian DNA polymerases by vitamin K compounds was investigated (Fig. 2). In the three vitamin Ks (i.e., K_1, K_2 and K_3) tested, 100 μM of vitamin K_3 indicated DNA polymerase γ inhibitory activity, but the same concentration of vitamins K_1 and K_2 did not influence the activity of any DNA polymerases tested including polymerase γ. Vitamin K_3 selectively and dose-dependently inhibited DNA polymerase γ activity, with 50% inhibition observed at doses of 6.0 μM. DNA polymerase γ is the sole polymerase in animal mitochondria. Biochemical and genetic evidence document a key role for polymerase γ in mitochondrial DNA replication, whereas DNA repair and recombination were thought to be limited or absent in animal mitochondria (35). This compound had not influenced the activities of other mammalian polymerases such as α, ß, δ, ε, η, ι, κ and λ at all. When activated DNA (i.e., DNA digested by bovine deoxyribonuclease I) was used as the template-primer, the mode of inhibition by these compounds did not change (data not shown). These results suggested that longer isoprenoid chains of the benzquinone ring in vitamin K (Fig. 1) weaken the inhibitory effect on DNA polymerase γ.

All vitamin K compounds had no inhibitory effect on fish (i.e., cherry salmon) DNA polymerases α and δ, insect (i.e., fruit fly) polymerases α, δ and ε, plant (i.e., cauliflower and rice) DNA polymerases α and λ, or prokaryotic DNA polymerases, such as the Klenow fragment of *E. coli* polymerase I, Taq DNA polymerase and T4 DNA polymerase (Table I).

Table I. IC_{50} values of vitamin K compounds for the activities of various DNA polymerases and other DNA metabolic enzymes.

Enzyme	IC_{50} values (μM)		
	K1	K2	K3
Mammalian DNA polymerases			
Calf DNA polymerase α	>200	>200	>200
Rat DNA polymerase ß	>200	>200	>200
Human DNA polymerase γ	>200	>200	6.0±0.3
Human DNA polymerase δ	>200	>200	>200
Human DNA polymerase ε	>200	>200	>200
Human DNA polymerase η	>200	>200	>200
Human DNA polymerase ι	>200	>200	>200
Human DNA polymerase κ	>200	>200	>200
Human DNA polymerase λ	>200	>200	>200
Fish DNA polymerases			
Cherry salmon DNA polymerase α	>200	>200	>200
Cherry salmon DNA polymerase δ	>200	>200	>200
Insect DNA polymerases			
Fruit fly DNA polymerase α	>200	>200	>200
Fruit fly DNA polymerase δ	>200	>200	>200
Fruit fly DNA polymerase ε	>200	>200	>200
Plant DNA polymerases			
Cauliflower DNA polymerase α	>200	>200	>200
Rice DNA polymerase λ	>200	>200	>200
Prokayotic DNA polymerases			
E. coli DNA polymerase I (Klenow fragment)	>200	>200	>200
Taq DNA polymerase	>200	>200	>200
T4 DNA polymerase	>200	>200	>200
Other DNA metabolic enzymes			
Calf Primase of DNA polymerase α	>200	>200	>200
Calf terminal deoxynucleotidyl transferase	>200	>200	>200
HIV-1 Reverse transcriptase	>200	>200	>200
T7 RNA polymerase	>200	>200	>200
T4 Polynucleotide kinase	>200	>200	>200
Bovine deoxyribonuclease I	>200	>200	>200

These compounds were incubated with each enzyme (0.05 units). Enzymatic activity was measured as described in Materials and methods. Enzyme activity in the absence of the compound was taken as 100%. Data are expressed as the mean ± SEM; n=3.

Inhibitory effect of vitamin K compounds on the activities of other DNA metabolic enzymes. Vitamin K compounds did not inhibit the activities of other DNA-metabolic enzymes, such as calf primase of DNA polymerase α, calf terminal deoxynucleotidyl transferase, HIV-1 reverse transcriptase, T7 RNA polymerase, T4 polynucleotide kinase and bovine deoxyribonuclease I (Table I). These results may suggest that vitamin K3 selectively inhibits the activity of mitochondrial DNA polymerase γ in the polymerases and DNA metabolic enzymes tested.

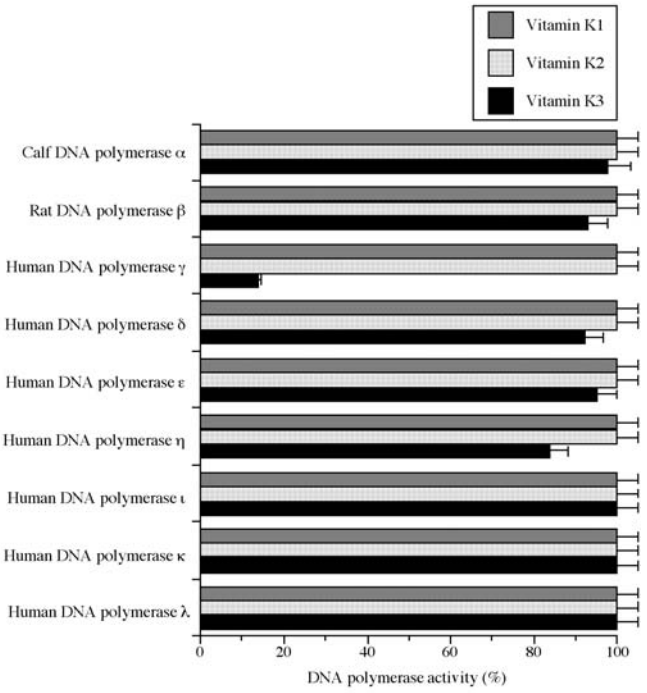

Figure 2. Effect of vitamin K compounds on the activities of mammalian DNA polymerases. Each vitamin K compound (100 μM) was incubated with each DNA polymerase (0.05 units). The DNA polymerase activities were measured as described in the text. Enzymatic activity in the absence of the compound was taken as 100%. Data are expressed as the mean ± SEM for four independent experiments.

We would, however, like to emphasize here that vitamin K3 at first intercalates into the DNA molecule as the substrate (i.e., DNA template-primer), and subsequently inhibits both activities indirectly through the induction of a conformational change in the DNA. This compound effected no thermal transition of melting temperature (data not shown), thus, no vitamin K3 bound to the double stranded DNA, suggesting that this must inhibit enzyme activities by interacting with DNA polymerase γ directly. We then investigated whether an excessive amount of poly(rC) or bovine serum albumin (BSA) prevented the inhibitory effect of vitamin K3 to determine whether the effect resulted from their non-specific adhesion to DNA polymerase γ, or selective binding to specific sites. Poly(rC) and BSA had little or no influence on the effect of vitamin K3, suggesting that the binding to DNA polymerase γ occurs selectively.

Effects of vitamin K compounds on ex vivo angiogenesis. An anti-angiogenic effect of vitamin K2 has been reported by Yoshiji *et al* (7). Thus, we examined the effect of vitamin K compounds in a rat aortic ring assay. In this angiogenesis assay model, vitamin K3 also showed strong anti-angiogenic activity (Fig. 3A). However, vitamin K1 had no inhibitory effect on angiogenesis (data not shown). Vitamin K3 significantly inhibited angiogenesis in this model at more than 25 μM (Fig. 3B)

Effect of vitamin K3 on HUVEC functions. The effect of vitamin K3 was examined in an *in vitro* angiogenesis model

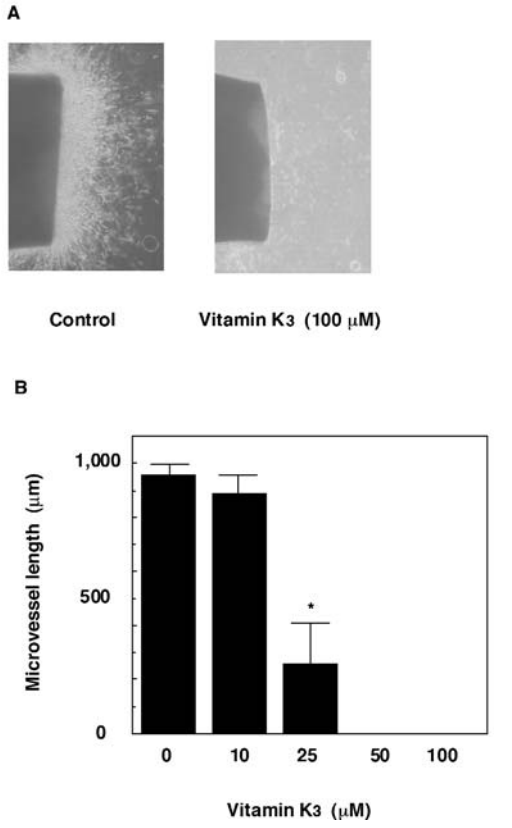

Figure 3. Effect of vitamin K3 on *ex vivo* angiogenesis using a rat aortic ring. (A) Representative result of the inhibitory effect of vitamin K3 (100 μM). (B) Microvessel length was measured on day 7 of culture. Values are means ± SEM (n=6-12). *Significantly different from control ($p<0.01$).

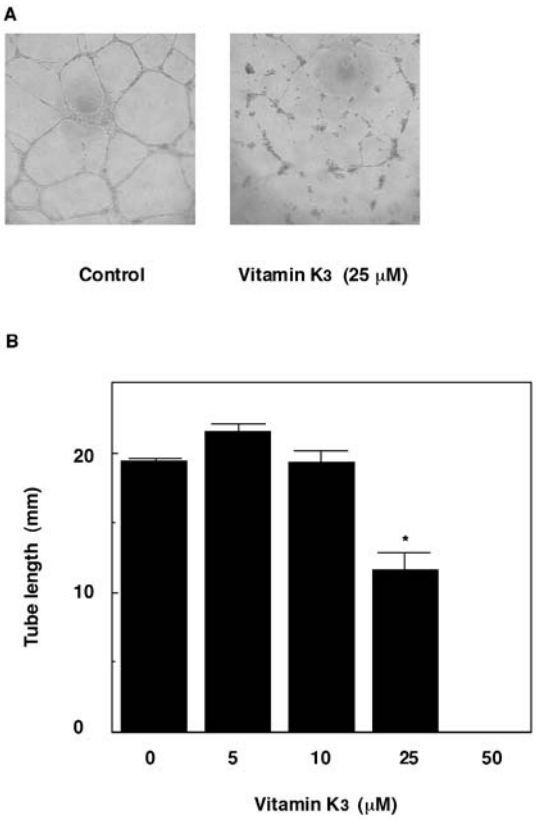

Figure 4. Effect of vitamin K3 on HUVEC tube formation on reconstituted basement membrane. (A) Cells were plated on reconstituted gel and observed after 12 h. (B) Capillary length was measured, and values are means ± SEM (n=3). *Significantly different from control ($p<0.01$).

using HUVECs. HUVECs inoculated on reconstituted basement membrane (Matrigel) migrated, then attached to each other, and finally formed tube structures (Fig. 4A). Vitamin K3 suppressed HUVEC tube formation significantly at 25 μM and completely inhibited it at 50 μM (Fig. 4A and B). This result was consistent with that in an aortic ring assay.

As many angiogenesis inhibitors suppress endothelial cell proliferation, we examined the effect of vitamin K3 on HUVEC proliferation. HUVECs were treated for 72 h with various concentrations of vitamin K3. Significant differences ($p<0.05$) were found between control and vitamin K3-treated HUVECs (5-25 μM) (Fig. 5).

Finally, the effect of vitamin K3 on HUVEC migration stimulated with VEGF was examined on gelatin-coated Boyden chambers. VEGF strongly stimulated HUVEC migration as shown in Fig. 5. HUVEC migration stimulated with VEGF was moderately inhibited by vitamin K3 (Fig. 6).

Discussion

Vitamin K3 (menadione) is 2-methyl-1,4-naphthoquinone and vitamins K1 (phylloquinone) and K2 (menaquinones) are polyisoprenoid-substituted naphthoquinones. Vitamin K3 is not found naturally, but is converted to vitamin K2 *in vivo*. The functions of these vitamins in cellular reactions are as well known as those of other vitamins. Vitamin K is a co-factor of γ-glutamylcarboxylase, which converts glutamic acid residues of blood coagulation factors and bone matrix proteins (36,37). Recent studies show that vitamin K2 functions as an anti-cancer agent, in particular, for hepatic cancer (7,38-41). However, the anti-cancer mechanism of vitamin K2 on hepatic cancer has not yet been elucidated.

In our study, we demonstrated that vitamin K3 selectively inhibits DNA polymerase γ and suppresses angiogenesis in some models. Although an anti-angiogenic effect of vitamin K2 has been demonstrated (7), an inhibitory effect of vitamin K3 on DNA polymerase would be a novel finding for vitamin K compounds. In addition, vitamin K3 selectively inhibits DNA polymerase γ, which is a mitochondrial DNA polymerase. We previously reported that coenzyme Q, which is an isoprenoid quinine in the mitochondrial respiratory chain, selectively inhibited both DNA polymerase γ and DNA topoisomerase II and suppressed human cancer cell growth (42). Similarly, vitamin K3 could be useful for cancer treatment or for the design of a new anti-cancer drug.

As a new function of vitamin K3, we found that it inhibited angiogenesis. Angiogenesis is a process forming new blood vessels, and the growth of many solid tumors depends on it (43). Thus, an anti-angiogenic activity of vitamin K3 inhibiting DNA polymerase γ may contribute to its function as an anti-cancer agent. In addition, anti-angiogenic activity of vitamins including D3, B6, K2 and vitamin E derivative has been reported so far (1,4,7,41,44) and has attracted attention with regard to cancer prevention and treatment, as vitamins are relatively safe agents without side effects compared to anti-cancer drugs. Although vitamin K3 is a

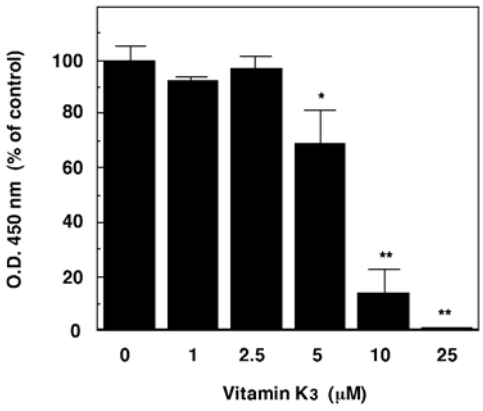

Figure 5. Effect of vitamin K3 on HUVEC proliferation. Values are means ± SEM (n=3). Significantly different from control: *p<0.05, **p<0.01.

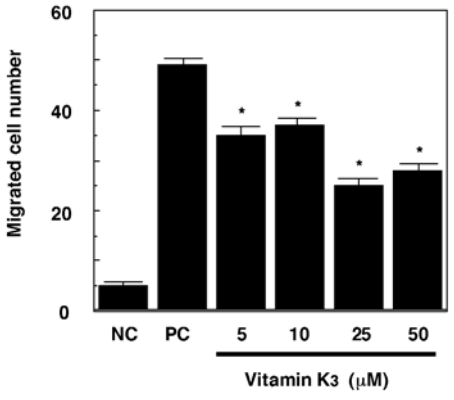

Figure 6. Effect of vitamin K3 on HUVEC chemotaxis. HUVECs migrated after a 6-h incubation to the lower surface of the filter were counted in five 200x filter fields. Means of a field of three filters ± SEM (n=3) are shown. NC, medium without VEGF and vitamin K3 (negative control); PC, VEGF containing medium without vitamin K3 (positive control). *Significantly different from control (p<0.01).

synthetic vitamin, it is converted to vitamin K2, which has anti-angiogenic activity but no effect on DNA polymerase γ and is used in clinical treatment. Therefore, vitamin K3 could be used for such a purpose.

We have noted that most of these vitamins and derivative affect DNA polymerases and angiogenesis (1,2,4-7,44-46), which led us to consider the usefulness of vitamins inhibiting both DNA polymerases and angiogenesis to prevent and treat cancer. Proper consumption of these vitamins could reduce the risk of cancer.

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