Vitamins K2, K3 and K5 exert antitumor effects on established colorectal cancer in mice by inducing apoptotic death of tumor cells

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Abstract. Although a number of studies have shown that vitamin K possesses antitumor activities on various neoplastic cell lines, there are few reports demonstrating *in vivo* antitumor effects of vitamin K, and the antitumor effect on colorectal cancer (CRC) remains to be examined. Therefore, antitumor effects of vitamin K on CRC were examined both *in vitro* and *in vivo*. Vitamins K2, K3 and K5 suppressed the proliferation of colon 26 cells in a dose-dependent manner, while vitamin K1 did not. On flow cytometry, induction of apoptosis by vitamins K2, K3 and K5 was suggested by population in sub-G1 phase of the cell cycle. Hoechst 33342 staining and a two-color flow cytometric assay using fluorescein isothiocyanate-conjugated annexin V and propidium iodide confirmed that vitamins K2, K3 and K5 induced apoptotic death of colon 26 cells. Enzymatic activity of caspase-3 in colon 26 cells was significantly up-regulated by vitamins K2, K3 and K5. The pan-caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone, substantially prevented vitamin K-mediated apoptosis. *In vivo* study using syngeneic mice with subcutaneously established colon 26 tumors demonstrated that intravenous administration of vitamins K2, K3 and K5 significantly suppressed the tumor growth. The number of apoptotic tumor cells was significantly larger in the vitamin K-treated groups than in the control group. These results suggest that vitamins K2, K3 and K5 exerted effective anti-tumor effects on CRC *in vitro* and *in vivo* by inducing caspase-dependent apoptotic death of tumor cells, suggesting that these K vitamins may be promising agents for the treatment of patients with CRC.

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Abbreviations: 5-FU, 5-fluorouracil; HCC, hepatocellular carcinoma; PBS, phosphate-buffered saline; MTT, tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PI, propidium iodide; FITC, fluorescein isothiocyanate; pNA, p-nitroanilide; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; ANOVA, analysis of variance; DCP, des-γ-carboxy prothrombin; Cdk, cyclin-dependent kinase

Key words: vitamin K, colorectal cancer, antitumor effect, apoptosis, caspase

Introduction

Worldwide, colorectal cancer (CRC) ranks third in cancer incidence after lung and breast, and is a major cause of cancer mortality (1). In 2000, there were 943,000 new cases diagnosed and 510,000 deaths worldwide, corresponding figures for Europe were 363,000 cases and 199,000 deaths (2). Although surgery alone is the standard approach in localized malignancy, approximately 50% of early-stage patients present disease relapse (3). Furthermore, approximately 30% of CRC is diagnosed when it is already at an advanced stage. There are also a considerable number of patients who cannot undergo surgery even at an early stage of the disease due to severe complications, such as chronic heart failure, chronic renal failure and chronic obstructive pulmonary diseases. Fluoropyrimidine-based chemotherapy is now considered the standard treatment for patients with inoperable CRC, but approximately 90% of patients do not respond to chemotherapy based on 5-fluorouracil (5-FU)/folinic acid (4). Thus, the development of new treatment modalities is necessary to improve the overall survival rate of patients with CRC.

Vitamin K is an essential vitamin that was discovered as a fat-soluble anti-hemorrhagic agent. Vitamin K is also known as an obligatory cofactor in the post-translational γ-carboxylation of glutamic acid residues (5-8). Vitamin K-dependent proteins include prothrombin (factor II) and coagulation factors VII, IX and X, and proteins C, S and Z. Vitamin K is a family of structurally similar fat-soluble 2-methyl-1,4-naphtoquinones, including phylloquinone (vitamin K1), menaquinone (vitamin K2), menadione (vitamin K3) and vitamin K5. All members of the vitamin K family possess an identical naphthoquinone skeleton with various side chains that distinguish them. Vitamin K1 is found in many higher plants as well as algae, with the

highest concentrations found in green leafy vegetables. Vitamin K2 also occurs naturally, but is not produced by plants. It is produced by a vast array of bacteria in the intestine. Vitamin K3 is not considered a natural vitamin K, but rather a synthetic analogue that acts as a provitamin. It possesses a much simpler structure, with no aliphatic chain prosthetic group at position 3. Vitamin K5 was previously examined as a fungistatic agent (9), and then used as an insulin mimicker (10,11).

It has been shown that vitamins K1, K2 and K3 could inhibit the growth of various rodent- and human-derived neoplastic cell lines *in vitro*, such as oral epidermal cancer, nasopharyngeal cancer, breast cancer, leukemia and hepatocellular carcinoma (HCC) cell lines (12-31). In marked contrast to numerous reports demonstrating *in vitro* antitumor effects of vitamin K, there are few reports demonstrating *in vivo* antitumor effects of vitamin K. Furthermore, it has not been examined so far whether vitamin K induces antitumor effects on CRC. In the present study, we evaluated antitumor effects of vitamins K1, K2, K3 and K5 on CRC both *in vitro* and *in vivo*. Furthermore, we also examined the mechanisms of antitumor actions of vitamin K on CRC.

Materials and methods

Cell culture. The N-nitroso-N-methylurethan-induced, undifferentiated and highly metastatic murine CRC cell line, colon 26 (32), which was originally established from a BALB/c mouse, was generously provided by the Chugai Pharmaceutical Co. (Tokyo, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (Sigma; St. Louis, MO, USA) supplemented with 10% (vol/vol) heat-inactivated (56˚C for 30 min) fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2 in air.

Vitamin K preparation. Vitamin K2 was purchased from Sigma, and vitamins K1, K3 and K5 were from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were also purchased from Wako Pure Chemical Industries, unless otherwise mentioned. Vitamins K1, K2, K3 and K5 were completely dissolved in 99.5% ethanol at a concentration of 10 mM, and then diluted to appropriate concentrations with phosphate-buffered saline (PBS) and 99.5% ethanol for *in vitro* experiments. For *in vivo* experiments, vitamins K2, K3 and K5 were completely dissolved in 99.5% ethanol at a concentration of 100 mM and then diluted to appropriate concentrations with PBS.

Animals. Eight-week-old male BALB/c mice, weighing approximately 20 g, were purchased from Japan SLC (Hamamatsu, Japan) and kept under a specific pathogen-free condition at 24±2˚C and in a 12-h day/night light cycle. Animal experiments were performed under approved protocols and in accordance with the institutional recommendations for the proper care and use of laboratory animals.

In vitro antitumor effects of K vitamins on CRC. Colon 26 cells were seeded in the absence or presence of various concentrations (0.05 μM-100 μM) of vitamin K at a density of $1x10^3/cm^2$ in 48-well tissue culture plates for 5 days. The final concentration of ethanol in media containing various concentrations of vitamin K was adjusted to 1%. Therefore, as controls, colon 26 cells were seeded in the presence of 1% ethanol without vitamin K. The cells were quantified by using the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) conversion assay, as described previously (33). Briefly, after the culture of colon 26 cells for 5 days with and without vitamin K in 48-well tissue culture plates, MTT was added to each well to a final concentration of 0.5 mg/ml. After a 4-h incubation at 37˚C with MTT, the unreacted MTT and medium were carefully removed and 300 μl of dimethyl sulfoxide was added to solubilize the MTT formazan. After gentle agitation for 10 min at room temperature, the optical density of each well was measured with a microplate reader (Microplate Reader MTP-120; Corona, Hitachinaka, Japan) equipped with a 540-nm filter for the measurement and a 630-nm filter for the reference. The spectrophotometer was calibrated at 0 absorbance using wells that had only contained the medium and MTT. Four separate experiments were performed.

Cell cycle synchronization and flow cytometric analysis. To synchronize cells at G0 phase of the cell cycle, colon 26 cells were incubated in a serum-free medium for 24 h. The cells were then trypsinized, washed twice with PBS and resuspended in the complete medium. Cells were immediately seeded at a density of $1x10^6$ cells per 100-mm dish, and vitamins K2, K3 and K5 were added at a final concentration of 40 μM, 2 μM and 2 μM, respectively. As controls, cells were cultured in the presence of 1% ethanol without vitamin K. After a 24-h cultivation, cells were harvested and washed twice with PBS. The cells were resuspended in 0.1% Triton X-100 and then stained in the dark with a propidium iodide (PI) staining solution containing 25 μg/ml PI and 250 μg/ml RNase A. Cell cycle progression was analyzed by flow cytometry using a FACSCalibur and CellQuest software (Becton-Dickinson Immunocytometry System; San Jose, CA, USA). Four separate experiments were performed.

Microscopic analysis for apoptotic cells. To microscopically examine the presence of apoptotic cells, chromatin condensation was detected by nucleus staining with Hoechst 33342 as described previously (34). Briefly, colon 26 cells cultured with or without 40 μM vitamin K2, 2 μM vitamin K3 or 2 μM vitamin K5 for 24 h were washed with ice-cold PBS and fixed with 4% formaldehyde in PBS. Cells were then stained with Hoechst 33342 (5 μg/ml) for 5 min at 4˚C. Nuclei were visualized using a Radiance 2100 confocal laser scanning microscope (Bio-Rad; Hercules, CA, USA).

Flow cytometric analysis for apoptotic and necrotic cells. To examine the presence of apoptosis and necrosis induced by vitamin K, $1x10^6$ colon 26 cells were cultured with 40 μM vitamin K2, 2 μM vitamin K3 or 2 μM vitamin K5 in a 100-mm dish for 24 h. As a control, colon 26 cells were cultured in the presence of 1% ethanol without vitamin K. Approximately $1x10^5$ cells were then stained for 15 min at room temperature in the dark with fluorescein isothiocyanate (FITC)-conjugated annexin V (1 μg/ml) and PI (0.5 μg/ml) in a Ca^{2+}-enriched binding buffer (Annexin V-FITC apoptosis

detection kit; BD Biosciences, San Diego, CA, USA), and analyzed by a two-color flow cytometric assay. Annexin V and PI emissions were detected in the FL1 and FL2 channels of a FACSCalibur flow cytometer, using emission filters of 525 and 575 nm, respectively. The annexin V^-/PI^- population were regarded as normal healthy cells, while annexin V^+/PI^- and annexin V^+/PI^+ were taken as a measure of apoptosis and necrosis, respectively.

Caspase-3 activity assay. To ascertain whether vitamin K induces apoptosis through caspase activation, enzymatic activity of caspase-3 was measured by using a colorimetric assay kit (BioVision, Mountain View, CA, USA) according to the manufacturer's instructions. The assay is based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate that recognizes optimal tetrapeptide sequence of individual activation sites. Briefly, $2x10^6$ colon 26 cells were incubated with 40 μM vitamin K2, 2 μM vitamin K3 or 2 μM vitamin K5 for 24 h before harvesting. As a control, cells were cultured in the presence of 1% ethanol without vitamin K. The cells were lysed in a cell lysis buffer for 10 min on ice and centrifuged at 10,000 x g for 1 min at 4˚C. Cell lysates (200 μg protein/100 μl reaction buffer) were incubated with 5 μl of 4 mM caspase-3-specific substrate, DEVD-pNA (200 μM final concentration), for 2 h at 37˚C. The pNA light emission was quantified by using a microtiter plate reader at 405 nm. Four separate experiments were performed.

Effect of the general caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk). To ascertain whether the suppression of caspase activities prevents vitamin K-mediated apoptotic death of CRC cells, colon 26 cells were pretreated with the pancaspase inhibitor, z-VAD-fmk (35), purchased from MP Biomedicals (Aurora, Ohio, USA). In brief, $1x10^6$ cells were pretreated for 4 h at 37˚C with 50 μM z-VAD-fmk prior to addition of vitamin K. After a 24-h cultivation with 40 μM vitamin K2, 2 μM vitamin K3 or 2 μM vitamin K5, the cells were stained with annexin V and PI, and analyzed by flow cytometry as described above.

In vivo antitumor effects of vitamin K on CRC. Colon 26 cells were suspended in PBS at a concentration of $1x10^7$ cells/ml, and 100 μl inoculum volumes were injected subcutaneously into the flank regions of syngeneic BALB/c mice. When the tumor became palpable, 100 μl of PBS containing 2 mM vitamin K2, K3 or K5 was injected into mice from the tail vein at a frequency of 5 days a week. Animals in the control group were given PBS containing only 2% ethanol without vitamin K, because vitamin K-containing PBS contained 2% ethanol. Each group consisted of 8 animals. After the initiation of the intravenous administration of vitamin K, the tumor growth was monitored by the same investigator, and the tumor diameters were measured every day using a graduated caliper. Two perpendicular tumor measurements, the largest and smallest diameters, were followed by calculation using the formula: tumor volume (mm^3) = the largest diameter (mm) x the smallest diameter $(mm)^2$ x 0.52. All animals were sacrificed on day 12 after treatment, because one of the animals in the control group died on day 12.

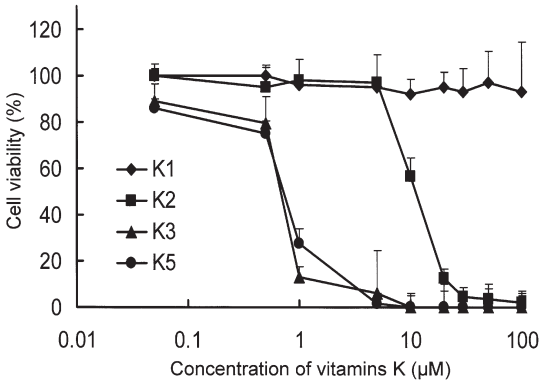

Figure 1. *In vivo* antitumor effects of vitamin K on CRC. Colon 26 murine CRC cells were cultured with various concentrations of vitamin K1, K2, K3 or K5 for 5 days. The final concentration of ethanol in a medium containing vitamin K was adjusted to 1%. Therefore, as controls, colon 26 cells were also cultured in the presence of 1% ethanol without vitamin K. The viability of cells treated with vitamin K was determined by an MTT assay and compared with that of control cells cultured in the medium containing only 1% ethanol. Four separate experiments were performed and results are expressed as means ± SD. Vitamin K1 did not show any cytotoxicity on colon 26 cells, while vitamins K2, K3 and K5 showed significant cytotoxicity on colon 26 cells in a dose-dependent manner. The *in vitro* antitumor effect of vitamin K3 and K5 on colon 26 cells was significantly stronger than that of vitamin K2.

Detection of apoptotic cells in vivo. When tumor-bearing mice treated with vitamin K were sacrificed in the above-mentioned experiment, subcutaneous tumors were excised from the animals, fixed in 10% buffered formalin, and then embedded in paraffin. To examine the presence of apoptotic cells in the tumor, apoptosis-related DNA fragmentation was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). Briefly, sections were cut from the paraffin blocks and deparaffinized, and protein was digested with 20 μg/ml proteinase K (Sigma) in 10 mM Tris (pH 7.4) for 15 min at room temperature. TUNEL analysis was performed by using a commercially available *in situ* apoptosis detection kit (ApopTag S7100; Chemicon, Temecula, CA, USA), according to the manufacturer's instructions.

Statistics. Results are expressed as means ± SD. All analyses were performed using the computer-assisted StatView program (SAS Institute, Gray, NC, USA). Standard descriptive statistics, Student's t test and Welch's t test, were used according to the distribution of experimental values. Tumor volume was statistically analyzed using analysis of variance (ANOVA) for multiple comparisons. Paired analysis between 2 groups was performed using the Scheffe test, where ANOVA indicated significance. A p value of <0.05 was considered to indicate a significant difference between groups.

Results

In vitro antitumor effects of vitamin K on CRC. In vitro antitumor effects of vitamin K on CRC were examined by exposing colon 26 murine CRC cells to various concentrations of vitamin K for 5 days. The viability of cells treated with vitamin K was determined by an MTT assay and compared with that of cells cultured in the medium containing only 1%

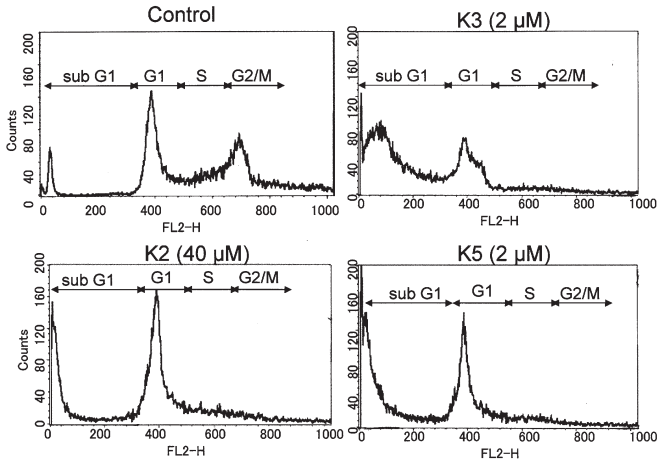

Figure 2. Flow cytometric analysis of cell cycle distribution of CRC cells treated with vitamin K. After synchronizing colon 26 cells at G0 phase of the cell cycle, the cells were cultured in the presence of 40 μM vitamin K2, 2 μM vitamin K3 or 2 μM vitamin K5. As controls, cells were also cultured in the presence of 1% ethanol without vitamin K. After a 24-h cultivation, cell cycle progression was analyzed by flow cytometry. Four separate experiments were performed. Representative results are shown. The ratios of cells in sub-G1 phase were markedly increased and the progression of the cell cycle was strongly inhibited by treatment with vitamin K2, K3 or K5.

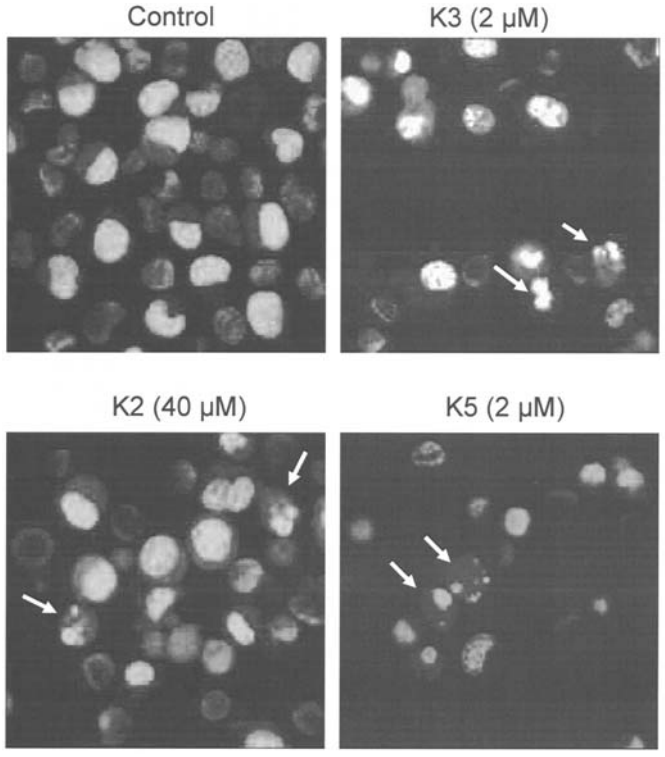

Figure 3. Morphological detection of apoptosis of CRC cells treated with vitamin K. Colon 26 cells were cultured in the presence of 40 μM vitamin K2, 2 μM vitamin K3 or 2 μM vitamin K5 for 24 h, and stained with the Hoechst 33342 dye to visualize nuclear morphology. As controls, the cells were also cultured in the presence of 1% ethanol without vitamin K. The cells treated with vitamin K2, K3 or K5 showed morphological changes characteristic of apoptosis, including shrinkage of cell bodies and condensation of nuclei, while few condensed nuclei were found in control cells.

ethanol. As shown in Fig. 1, colon 26 cells were susceptible to vitamins K2, K3 and K5 in a dose-dependent manner, while vitamin K1 did not show significant cytotoxicity on the cells. The values of IC_{50}, defined as the dose required for 50% cytotoxicity, of vitamins K2, K3 and K5 were approximately 20, 0.8 and 0.9 μM, respectively. The *in vitro* antitumor effects of vitamins K3 and K5 on CRC cells were significantly stronger than that of vitamin K2, and the cytotoxicity of vitamins K3 and K5 on CRC cells was approximately 20-fold stronger than that of vitamin K2. Concentrations of vitamins K2, K3 and K5 that had reduced the viability of colon 26 cells to 20% were approximately 40, 2 and 2 μM, respectively. These concentrations were applied to the whole of the subsequent *in vitro* experiments. Because vitamin K1 did not show any significant *in vitro* antitumor effects on CRC cells, it was not used in the subsequent *in vitro* or *in vivo* experiments.

Flow cytometric analysis of cell cycle distribution of CRC cells treated with vitamin K. The relative DNA content of colon 26 cells treated with vitamin K was analyzed by using a flow cytometric method to investigate the effect of vitamin K on the cell cycle. As shown in Fig. 2, the ratios of cells in sub-G1 phase were markedly increased and the progression of the cell cycle was strongly inhibited by treatment with vitamin K2, K3 or K5. The mean ratios of cells in sub-G1, G1, S and G2/M phases of the cell cycle in 4 separate experiments were 4.2%, 28.5%, 17.2%, and 23.7%, respectively, when the cells were cultured in the medium containing only 1% ethanol. In contrast, those of cells treated with 40 μM vitamin K2 were 23.1%, 42.4%, 14.7% and 5.9%, respectively. Those of cells treated with 2 μM vitamin K3 were 53.4%, 25.1%, 6.9% and 3.2%, respectively. Those of cells treated with 2 μM vitamin K5 were 44.6%, 30.1%, 10.4% and 4.4%, respectively. The ratios of cells in sub-G1 phase were markedly higher in vitamin K groups compared with the control group. These results suggest that vitamins K2, K3 and K5 induced apoptosis of CRC cells.

Morphological detection of apoptosis of CRC cells treated with vitamin K. To morphologically examine whether vitamins K2, K3 and K5 induce apoptosis of CRC cells *in vitro*, colon 26 cells were cultured with or without vitamin K. After a 24-h incubation in the presence of 40 μM vitamin K2, 2 μM vitamin K3 or 2 μM vitamin K5, cells were stained with the Hoechst 33342 dye to visualize nuclear morphology. As shown in Fig. 3, cells treated with vitamin K2, K3 or K5 showed morphological changes characteristic of apoptosis, including shrinkage of cell bodies and condensation of nuclei, while few condensed nuclei were found in cells cultured in the medium containing only 1% ethanol without vitamin K.

Flow cytometric analysis of apoptosis of CRC cells treated with vitamin K by annexin V and PI staining. To quantify the mode of cell death (apoptosis or necrosis) induced by vitamin K, colon 26 cells were treated with or without 40 μM vitamin K2, 2 μM vitamin K3 or 2 μM vitamin K5 for 24 h. Cells were then stained with annexin V and PI, and analyzed by flow cytometry. Annexin V^+/PI^- cells were taken as a measure of apoptosis. As shown in Fig. 4, vitamin K was shown to induce apoptosis of CRC cells. The ratios of apoptotic cells were 2.8%, 17.1% and 7.4% when the cells were treated with vitamins K2, K3 and K5, respectively, while it was only 0.4% when the

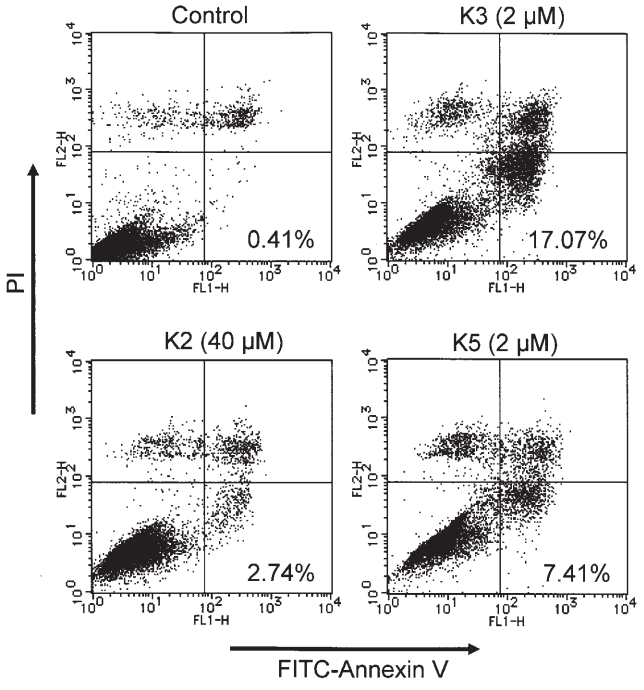

Figure 4. Quantitative determination of apoptotic CRC cells treated with vitamin K by annexin V and PI staining. Colon 26 cells were treated with 40 μM vitamin K2, 2 μM vitamin K3 or 2 μM vitamin K5 for 24 h, stained with annexin V and PI, and then analyzed by flow cytometry. As controls, the cells were cultured in the presence of 1% ethanol without vitamin K. Annexin V^+/PI^- cells were taken as a measure of apoptosis. The ratios of apoptotic cells were 2.74%, 17.07% and 7.41% when cells were treated with vitamins K2, K3 and K5, respectively, while it was only 0.4% when the cells were cultured in the medium containing 1% ethanol without vitamin K.

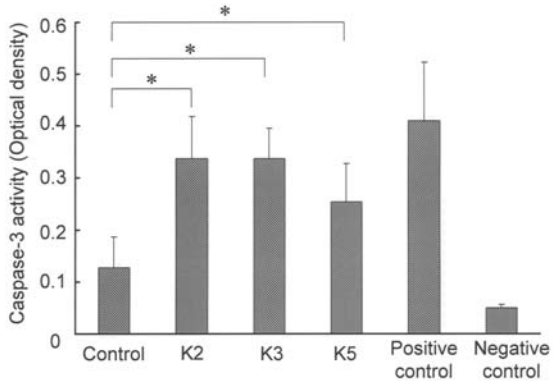

Figure 5. Activation of enzymatic activity of caspase-3 in CRC cells induced by vitamin K. Colon 26 cells were treated with 40 μM vitamin K2, 2 μM vitamin K3 or 2 μM vitamin K5 for 24 h. As controls, the cells were also cultured in the presence of 1% ethanol without vitamin K. Enzymatic activity of caspase-3 in cell lysates was evaluated based on spectrophotometric detection of the chromophore pNA. Enzymatic activity of caspase-3 was significantly increased in colon 26 cells by treatment with 40 μM vitamin K2, 2 μM vitamin K3 and 2 μM vitamin K5, compared with that in control cells. Each bar represents the mean ± SD of 4 separate experiments. $^*0.01<p<0.05$.

cells were cultured in the medium containing only 1% ethanol without vitamin K.

Effects of vitamin K on enzymatic activity of caspase-3 in CRC cells. Caspase-3 activation is considered essential for apoptotic

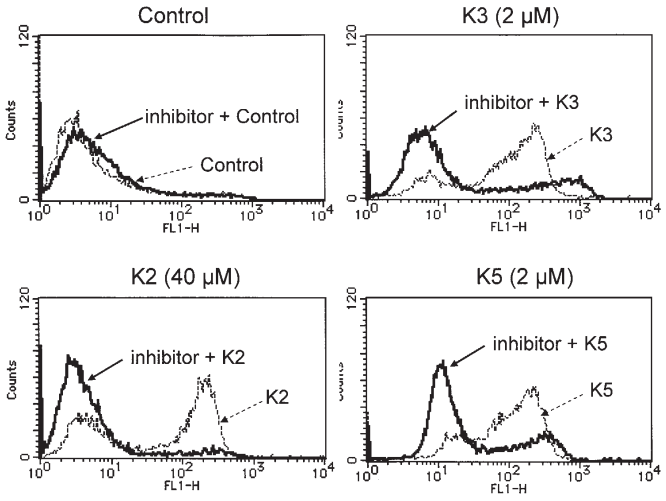

Figure 6. Effects of a pancaspase inhibitor on vitamin K-induced apoptosis of CRC cells. Colon 26 cells were treated with 50 mM z-VAD-fmk, a pancaspase inhibitor, for 4 h, followed by a 24-h cultivation in the presence or absence of 40 μM vitamin K2, 2 μM vitamin K3 or 2 μM vitamin K5. The cells were then stained with annexin V and PI, and analyzed by flow cytometry. As controls, the cells were also cultured in the presence of 1% ethanol without vitamin K. Pretreatment with z-VAD-fmk substantially blocked apoptotic cell death induced by vitamins K2, K3 and K5.

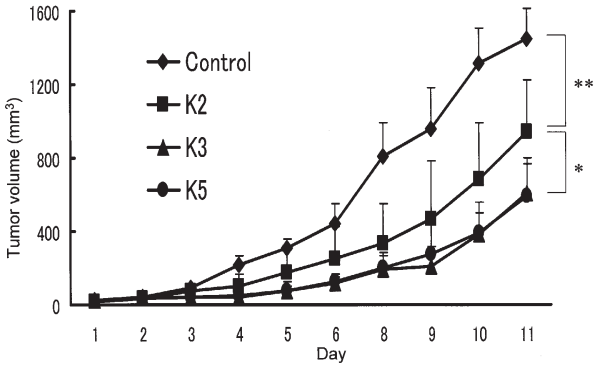

Figure 7. *In vivo* antitumor effects of vitamin K on established CRC in mice. Colon 26 cells were implanted subcutaneously into the flank regions of syngeneic BALB/c mice. When tumors became palpable, 100 μl of PBS containing 2 mM vitamin K2, K3 or K5 was injected into mice from the tail vein at a frequency of 5 days a week. Animals in the control group were given PBS containing only 2% ethanol without vitamin K, because vitamin K-containing PBS contained 2% ethanol. Animals in the control group developed rapidly growing subcutaneous CRC. In contrast, animals in the vitamin K groups exhibited significantly retarded tumor development compared with animals in the control group. The suppressive effects of vitamins K3 and K5 on established CRC were significantly stronger than that of vitamin K2. Each data point represents the mean ± SD of 8 animals. $^*0.01<p<0.05$, $^{**}p<0.001$.

cell death. Therefore, we examined whether enzymatic activity of caspase-3 was induced in CRC cells by the treatment with vitamin K by using a colorimetric assay. As shown in Fig. 5, enzymatic activity of caspase-3 was significantly increased in colon 26 cells by the treatment with 40 μM vitamin K2, 2 μM vitamin K3 and 2 μM vitamin K5, compared with that in the cells cultured in the medium containing only 1% ethanol.

Effects of a pancaspase inhibitor on vitamin K-induced apoptosis of CRC cells. To ascertain whether apoptosis of CRC

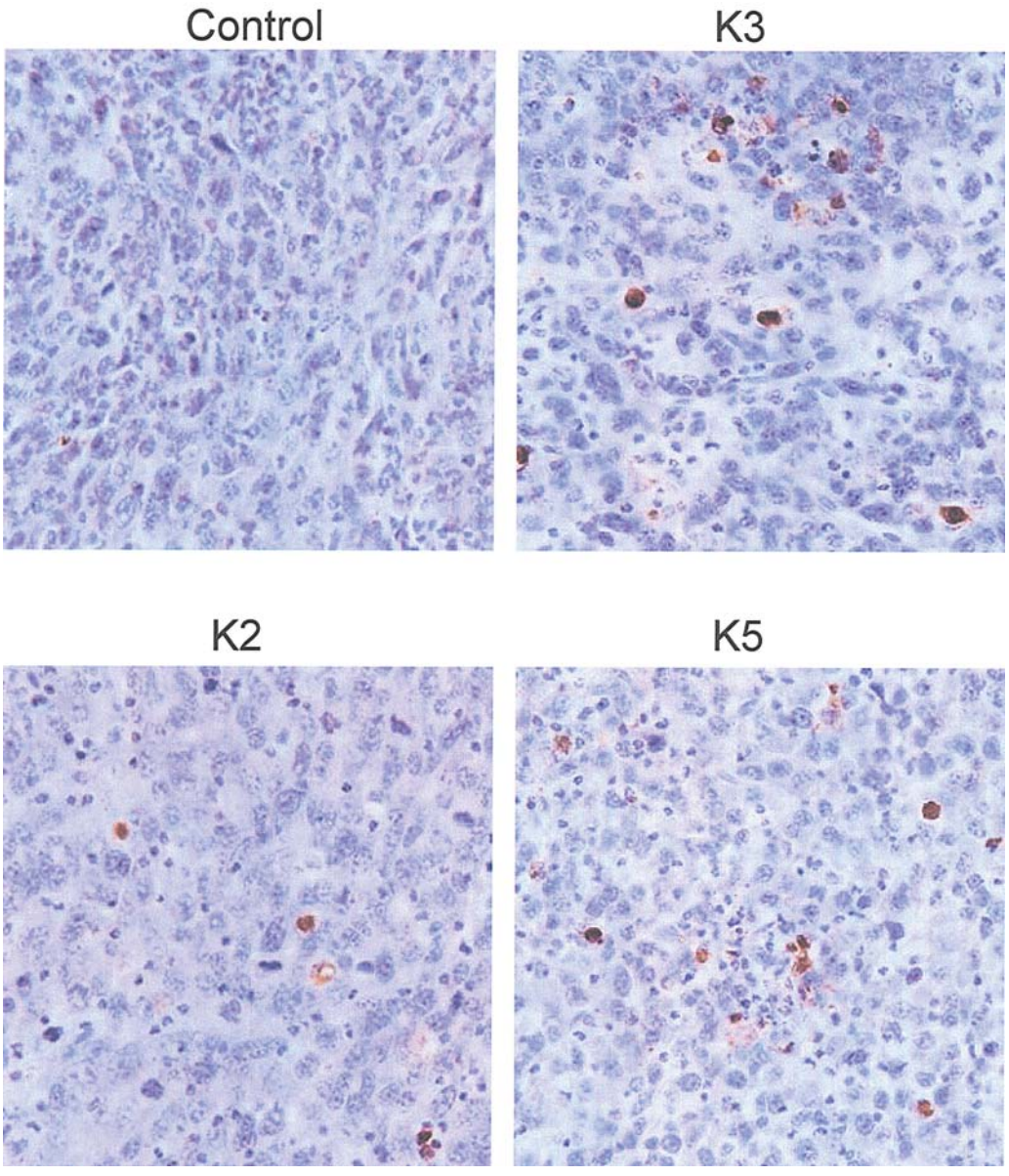

Figure 8. Detection of apoptotic CRC cells induced by vitamin K *in vivo*. When the animals with CRC were sacrificed on day 12 after treatment, subcutaneous CRC was removed and subjected to TUNEL staining. There were a few TUNEL-positive apoptotic cells in the tumors of animals treated with PBS containing only 2% ethanol. In contrast, there were an apparently larger number of TUNEL-positive cells in the tumors of animals that received intravenous administration of vitamin K2, K3 or K5.

cells induced by vitamin K is caspase-dependent, colon 26 cells were treated with 50 mM z-VAD-fmk, a pancaspase inhibitor, for 4 h, followed by a 24-h cultivation in the presence or absence of 40 μM vitamin K2, 2 μM vitamin K3 or 2 μM vitamin K5. The cells were then stained with annexin V and PI, and analyzed by flow cytometry as described above. As shown in Fig. 6, z-VAD-fmk pretreatment substantially blocked apoptotic cell death induced by vitamins K2, K3 and K5, indicating that apoptosis of CRC cells induced by vitamins K2, K3 and K5 was caspase-dependent.

Antitumor effects of vitamin K on established CRC in mice. Syngeneic BALB/c mice were implanted subcutaneously with colon 26 cells. When the animals developed palpable tumors, they were treated with intravenous administration of 100 μl of 100 μM vitamin K2, K3 or K5 at a frequency of 5 days a week. Animals in the control group received intravenous administration of PBS containing only 2% ethanol without vitamin K. As shown in Fig. 7, animals in the control group developed rapidly growing subcutaneous CRC. In contrast, animals in the vitamin K groups exhibited significantly retarded tumor development compared with animals in the control group. The suppressive effects of vitamins K3 and K5 on established CRC were significantly stronger than that of vitamin K2. Furthermore, animals in the vitamin K groups did not show any apparent changes, while those in the control group showed disheveled fur and body weight and one of the animals died on day 12 after treatment.

Induction of apoptosis of CRC cells in vivo by vitamin K. Because *in vitro* results demonstrated that vitamin K induced apoptotic death of CRC cells, it was examined whether anti-

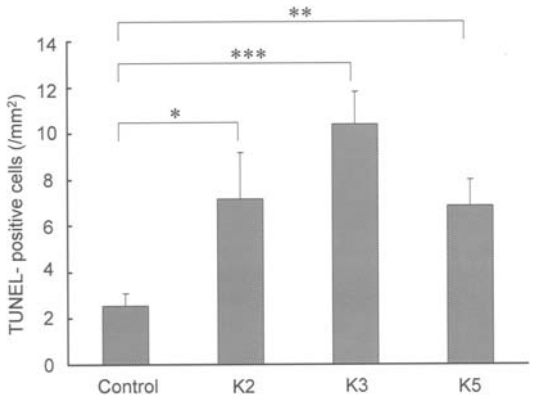

Figure 9. Quantitative determination of apoptotic CRC cells induced by vitamin K *in vivo*. In the above-mentioned experiments, the number of TUNEL-positive CRC cells in each tumor was quantitatively determined under a microscope. The number of apoptotic tumor cells was significantly larger in the vitamin K groups than in the control group. There were no significant differences in the number of apoptotic cells among vitamin K groups. Each data point represents the mean ± SD of 8 animals. $^{*}0.01<p<0.05$, $^{**}0.001<p<0.01$, $^{***}p<0.001$.

tumor effects of vitamin K on established CRC were mediated by induction of apoptosis. When the animals were sacrificed on day 12 after treatment in the above-mentioned experiments, subcutaneous CRC was removed and subjected to the TUNEL staining. As shown in Fig. 8, there were a few TUNEL-positive apoptotic cells in the tumors of animals treated with PBS containing only 2% ethanol. In contrast, there were an apparently larger number of TUNEL-positive cells in the tumors of animals that received intravenous administration of vitamin K2, K3 or K5. The number of TUNEL-positive cells determined under a microscope is shown in Fig. 9. The number of apoptotic tumor cells was significantly larger in the vitamin K groups than in the control group. There were no significant differences in the number of apoptotic cells among vitamin K groups.

Discussion

CRC is one of the most common visceral malignancies worldwide. Despite improvements in the management of patients with CRC, there has been little change in survival rates over the past 50 years (36). Therefore, a novel treatment modality is highly desired for patients with CRC. In the present study, we demonstrated that vitamin K inhibited the growth of CRC cells *in vitro* and induced the antitumor effect on established CRC in mice. It has been shown that vitamin K exhibited an antitumor effect on various rodent- and human-derived neoplastic cell lines. However, although there is only one report demonstrating that vitamin K3, but not vitamins K2 and K1, caused DNA damage in HT-29 human CRC cells (37), antitumor effects of vitamin K on CRC remain to be examined. Furthermore, there are few reports demonstrating *in vivo* antitumor effects of vitamin K. To the best of our knowledge, the present study is the first report demonstrating the antitumor effect of vitamin K on CRC both *in vitro* and *in vivo*.

Among various types of malignancy, antitumor activity of vitamin K on HCC has intensively been investigated, because lack of vitamin K2 induces elevation of serum levels of des-γ-carboxy prothrombin (DCP), a serum protein that increases at a notably high level in patients with HCC, and DCP was shown to have a mitogenic effect on human HCC cell lines (38). Nishiguchi and colleagues have reported that vitamin K2 had a preventive effect on the development of HCC in women with viral liver cirrhosis (39). Mizuta *et al* have reported that menatetrenone, a vitamin K2 analog, had a suppressive effect on recurrence of HCC and a beneficial effect on survival after curative treatment of HCC (40). We also recently demonstrated that vitamins K2, K3 and K5, but not vitamin K1, were able to induce antitumor effects on PLC/PRF/5 human HCC cells both *in vitro* and *in vivo* (41-43). When PLC/PRF/5 cells were cultured in the presence of 90 μM vitamin K1, no significant inhibition of cell growth was observed. In contrast, when the cells were cultured in the presence of 90 μM vitamin K2, the cell growth was significantly inhibited. Furthermore, when the cells were cultured with 30 μM vitamin K3 or K5, the cell growth was almost completely suppressed. In the present study, we demonstrated that vitamin K1 did not suppress the *in vitro* proliferation of colon 26 murine CRC cells at a concentration of 100 μM (the highest tested). In contrast, vitamins K2, K3 and K5 suppressed the proliferation of colon 26 cells in a dose-dependent manner, with the values of IC_{50} being 20, 0.8 and 0.9 μM, respectively. Consistent with our previous results using human HCC cells (41-43), vitamins K3 and K5 had significantly stronger *in vitro* antitumor effects on murine CRC cells than vitamin K2. It has been shown that IC_{50} values of vitamins K1, K2 and K3 for a panel of human neoplastic cell lines were in the range of 6000-9000, 800-2000 and 18-45 μM, respectively (26). Although the antitumor effect of vitamin K5 had not been examined, we demonstrated that vitamin K5 exhibited an antitumor effect on human HCC and murine CRC cells as potent as vitamin K3. Taken collectively, vitamins K3 and K5 are considered most promising anticancer agents among the family of vitamin K. However, when we examined the antitumor effects of vitamins K2, K3 and K5 *in vivo*, the growth of pre-established HCC of animals fed with drinking water containing 400 μM vitamin K2, K3 or K5 was markedly retarded, and there were no significant differences in tumor growth in animals treated with vitamin K2, K3 and K5 (43). In the present study, we intravenously administered 100 μl of PBS containing 2 mM vitamin K2, K3 or K5 into mice with established CRC. Although the *in vivo* antitumor effect on CRC of vitamins K3 and K5 was significantly stronger than that of vitamin K2, the differences were not as apparent as in the *in vitro* antitumor effect. Vitamin K2 has been used clinically for the treatment of osteoporosis, and its clinical safety is already established. Because safety is one of the most important issues for clinical application, vitamin K2 is ready to be evaluated as a new anticancer agent for the treatment of CRC.

Although the mechanisms of antitumor effects of vitamin K have been investigated intensively, they remain unclear, including the mechanism of *in vivo* antitumor effects of vitamin K. The original leading hypothesis focused on oxidative stress via redox-cycling of the quinine to produce reactive oxygen species such as the hydroxyl radical, superoxide radical and hydrogen peroxide (17). More sophisticated theories that do not fit into the oxidative model were recently proposed to explain puzzling aspects of the antitumor effects of K vitamins.

Especially, the induction of apoptosis and cell cycle inhibition are considered to be important mechanisms of antitumor actions of vitamin K.17 We previously demonstrated that vitamins K2, K3 and K5 suppressed the *in vitro* growth of PLC/PRF/5 human HCC cells by inducing G1 arrest of the cell cycle (41,42). Furthermore, we demonstrated in the subsequent *in vivo* experiments using athymic nude mice implanted subcutaneously with PLC/PRF/5 human HCC cells that vitamins K2, K3 and K5 suppressed the expression of the cell cycle-driving molecule, cyclin-dependent kinase 4 (Cdk4) and enhanced the expression of the cell cycle-suppressing molecules, Cdk inhibitor p16INK4a and retinoblastoma in HCC (43), indicating that vitamins K2, K3 and K5 induced G1 arrest of HCC cells *in vivo* as well as *in vitro*. In those reports, we suggested that vitamin K exerted antitumor actions by regulating the expression of G1 phase-related cell cycle molecules. It has been shown in the present study that the ratios of colon 26 murine CRC cells in sub-G1 phase of the cell cycle were markedly increased by treatment with vitamins K2, K3 and K5, suggesting that these K vitamins induced apoptotic death of CRC cells. Induction of apoptosis of CRC cells by vitamins K2, K3 and K5 was confirmed by Hoechst 33342 staining and by a two-color flow cytometric assay using annexin V and PI. The typical apoptotic features of nuclear fragmentation and condensation were observed by Hoechst 33342 staining, and annexin V-positive/PI-negative cells, namely apoptotic cells, were observed when the cells were treated with vitamins K2, K3 and K5. In the subsequent *in vivo* study, we examined the antitumor effect of vitamin K on CRC by using colon 26 murine CRC cells and immunocompetent syngeneic BALB/c mice instead of human CRC cells and immunodeficiency nude mice, because this model allows the involvement of immunological antitumor actions *in vivo*. It has been shown in the *in vivo* experiment that the number of TUNEL-positive tumor cells in established CRC was markedly increased by the treatment with vitamins K2, K3 and K5, indicating that these K vitamins induced apoptotic death of CRC cells *in vivo*. We are now examining whether the immunological mechanisms play a role in the vitamin K-induced apoptosis of CRC cells *in vivo*.

The caspases are cysteine proteases that play key roles in the execution phase of apoptosis. Among the family of caspases, caspase-3 is considered to play a crucial role in the apoptotic cell death process (44,45). We showed in the present study that vitamins K2, K3 and K5 enhanced the enzymatic activity of caspase 3. We also demonstrated that vitamin K-mediated apoptosis of colon 26 cells was almost completely inhibited by the treatment with the broad-spectrum caspase inhibitor zVAD-fmk. Taken collectively, these results indicate that vitamins K2, K3 and K5 caused apoptotic death of CRC cells *in vitro* in a caspase-dependent manner.

Although there are numerous studies that reported *in vitro* antitumor effects of K vitamins and vitamin K analogs (17), there are few studies that reported *in vivo* antitumor effects of K vitamins. We previously demonstrated that vitamins K2, K3 and K5 significantly suppressed the development of pre-established HCC in an animal model. In the present study, we examined the antitumor effect and mechanism of vitamin K on established CRC by using an animal model. *In vivo* development of established CRC in mice was apparently suppressed by treatment with vitamin K2, K3 or K5. When mice were treated with vitamins K2, K3 and K5, the number of apoptotic CRC cells was significantly larger compared with the control animals. These results suggest that vitamins K2, K3 and K5 were able to induce *in vivo* antitumor effects on CRC by inducing apoptotic death of cancer cells.

There are several studies demonstrating a synergistic effect of vitamin K when combined with other conventional agents. It has been shown that the combination therapy of vitamin K2 with a vitamin D3 derivate synergistically enhanced the differentiation of leukemia cells *in vitro* (46). The combination of vitamin K3 with vitamin C was shown to induce synergistic antitumor activity on ovarian cancer cells *in vitro* (47). *In vitro* synergistic action between vitamin K3, and doxorubicin, 5-FU and vinblastine was shown in nasopharyngeal cancer cells (48). *In vivo* synergistic effects of vitamin K3 and methotrexate have also been shown in tumor-bearing animals (49). We also showed that the combination of vitamin K2 and the angiotensin-converting enzyme inhibitor, perindopril, attenuated the liver enzyme-altered preneoplastic lesions in rats via angiogenesis suppression (50,51). These results indicate that vitamin K may play an adjuvant role in the treatment of cancer. Therefore, we are now examining whether the combination of vitamin K with 5-FU, a key chemotherapeutic agent for the treatment of CRC, can induce more potent antitumor effects on CRC than 5-FU monotherapy.

In conclusion, we demonstrated in the present study that vitamins K2, K3 and K5 exerted antitumor effects on CRC both *in vitro* and *in vivo* by inducing caspase-dependent apoptotic death of tumor cells. Although clinical studies are needed to evaluate the antitumor effects of vitamin K on CRC, vitamins K2, K3 and K5 may be useful agents for the treatment of patients with CRC, and may open up new avenues for the treatment of CRC.

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