Apoptosis induction of vitamin K2 in lung carcinoma cell lines: The possibility of vitamin K2 therapy for lung cancer

TSUYOSHI YOSHIDA, KEISUKI MIYAZAWA, IKUMA KASUGA, TOMOHISA YOKOYAMA, KAZUSHIGE MINEMURA, KENJU USTUMI, MASAHIRO AOSHIMA and KAZUO OHYASHI

First Department of Internal Medicine, Tokyo Medical University, Tokyo, Japan

Received March 14, 2003; Accepted May 2, 2003

Abstract. Vitamin K2 (menaquinone-4: VK2) has been reported to show apoptosis and differentiation-inducing effects on leukemia cells. Furthermore, the clinical benefits of using VK2 have been demonstrated for the treatment of the patients with acute leukemia and myelodysplastic syndromes. In the present study, we examined the in vitro effects of VK2 on lung carcinoma cell lines LU-139 and LU-130 for small cell carcinomas, PC-14 and CCL-185 for adenocarcinomas, LC-AI and LC-1/sq for squamous cell carcinomas, and IA-LM for large cell carcinoma, respectively. Treatment with VK2 for 48 to 96 h resulted in cell growth suppression in a dose-dependent manner in all cell lines tested. IC50 (50% inhibitory concentration) for VK2 ranged from 7.5 to 75 μM, and there was no relation between the efficacy of growth suppression by VK2 and tissue type of lung carcinoma cell lines. Morphologic features of the cells treated with VK2 were typical for apoptosis along with caspase-3 activation and becoming positive for APO2.7 monoclonal antibody, an antibody which specifically detects the cell undergoing apoptosis. In addition to the leukemia cell line, LU-139 cells accumulated into G0/G1 phase during 72 h exposure to VK2. Combined treatment of cisplatin plus VK2 resulted in enhanced cytocidal effect compared to the cells treated with either cisplatin or VK2 alone. Since VK2 is a safe medicine without prominent adverse effects including bone marrow suppression, our data strongly suggest the therapeutic possibility of using VK2 for the treatment of patients with lung carcinoma.

Introduction

Lung cancer has been ranked as the leading cause of death in recent years (1). More than 60% of patients with lung cancer have been diagnosed at the advanced stage when surgical resection cannot be carried out (2). Chemotherapy has been performed in these cases, however, it usually results in an unsatisfactory outcome without an apparent survival benefit (2-5). Since the prognosis of lung cancer patients at the inoperable stage is very poor, establishment of a novel strategy for treating these patients is an urgent issue.

Generically speaking, the term vitamin K (VK) is used for compounds that include phytonadione (VK1), the menaquinone series (VK2), and mendione (VK3). It is already known that physiologically, the natural K vitamins, VK1 and VK2 serve as cofactors in the γ-carboxylation of certain glutamates at the N-terminal of prothrombin and other VK-dependent coagulation factors (6). The VK congeners have in common a chemical structure that consists of a naphthoquinone nucleus that is capable of redox cycling. VK1 has a long phytol side chain, while VK2 has an unsaturated side chain made up of 4-13 isoprene units (polyprenylalcohols). VK3, in contrast, is a synthetic vitamin K congener that does not have the side chain. Greater detail on the structures of VK1, VK2, and VK3 can be found in the literature (6,7). Further research is starting to show that these congeners possess some growth-inhibitory effects on hepatoma cell lines such as Hep3B and Hep40, although the mechanisms of the growth inhibition are not fully elucidated (8-10). Furthermore, geranylgeraniol, a polyprenylalcohol which makes up the side chain of VK2, has already been reported to potently induce apoptosis in tumor cell lines (11). We have already reported that VK2 analogs such as menaquinone (MK)-3, MK-4, and MK-5 induce apoptosis in primary cultured leukemia cells in vitro (7), although VK1 does not. This apoptosis induction was even stronger than that induced by equivalent concentrations of polyprenylalcohols (7). The apoptosis-inducing effect of VK2 was more pronounced in leukemic blasts than in mature non-blastic cells (12). This suggests that VK2 is specific for leukemia cells.

In the present study, we demonstrate that VK2 potently induces apoptosis in lung carcinoma cell lines as well as in leukemia cells. In addition, combination of VK2 plus cisplatin (CDDP) further enhances the growth inhibition of a small cell lung carcinoma cell line as compared with the cells treated with VK2 or CDDP alone. Since VK2 is well known in Japan as a safe medicine for osteoporosis without prominent adverse effects including bone marrow suppression (13), our data suggest the clinical benefit of using VK2 for treating patients with lung carcinomas.

Materials and methods

Cell lines. Small cell lung carcinoma cell lines (LU-139, LU-130), lung squamous cell lines (LC-AI, LC-1/sq), lung...
adenocarcinoma cell lines (PC-14, CCL185), and a large cell line (IA-LM) were all obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in continuous culture in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% FCS (Hyclone, Logan, UT), 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (100 μg/ml). Exponentially growing cells were used for further experiments.

Reagents and antibodies. Menaquinon-4 was supplied by Eisai Co. (Tokyo, Japan). Cisplatin (CDDP) was purchased from Nippon Kayaku Co. (Tokyo, Japan). PC5-conjugated APO2.7 monoclonal antibody (mAb: clone 2.7), which was raised against the 7A6 antigen specifically expressed by cells undergoing apoptosis, was purchased from Immunotech (Marseille, France) (12,13).

Assessment of viable cell count. Viable cell numbers were analyzed by flow cytometry using EPICS XL II (Beckman-Coulter Japan, Tokyo, Japan). Since the cell lines LC-AI, LC-1/sq, PC-14, CCL-185 and IA-LM grow adherent on the bottoms of the plastic culture dishes, the cells treated with/without VK2 and/or CDDP were incubated with trisepine-EDTA solution (Gibco, Grand Island, NY) for 5 to 10 min at room temperature. The cells harvested were washed once with culture medium and passed through a 23 G needle 3 times using a plastic syringe to obtain the complete cell suspensions for the following flow cytometry. In LU-139 and LU-130 cell lines, which form the cell clots in suspension culture, the cells were spun down and suspended with trisepine-EDTA solution for 5 min at room temperature, passed through a 23 G needle 3 times, and re-suspended in PBS for further analysis. This procedure for obtaining the complete cell suspension was critical for all experiments using a flow cytometer.

The cells were stained with a solution containing 1% (v/v) propidium iodide (PI) (Sigma Chemical Co., St. Louis, MO) for 30 min at 4°C. First, the gating area of a cytogram for detecting the viable cells for each cell line was established based on the PI staining-negative area (indicating viable cells) and the forward- and side-scatter intensities. Then the cells were pipetted gently to obtain uniform cell suspension, and were introduced to a flow cytometer. The number of cells in the gating area for viable cells was analyzed by flow cytometry using a flow cytometer. The number relative to the cells treated with a control medium revealed to be well-correlated with the results obtained from a Cell Counting Kit (Dojin East, Tokyo, Japan) with absorption measurements at 450 nm (7).

Assessment of apoptosis. Flow cytometry using PC5-conjugated APO2.7 mAb (clone 2.7; Immunotech) was used to assess the cells undergoing apoptosis. We have reported that the percentage of APO2.7-positive cells detected was consistent with the results of the TUNEL method and DNA ladder formation by 2% agarose gel electrophoresis in a limiting dilution system that used both apoptotic and non-apoptotic cells (12). In order to observe the morphologic changes, Shandon Cytospin 2 (Shandon Inc., Pittsburgh, PA) was used for the cell suspension, and the prepared cells were stained with May-Giemsa.

 Assays for caspase-3 activity. Caspase-3 activity was assessed by flow cytometry using a substrate reagent kit containing PhiPhiLux-G6D2, a rhodamine-containing specific substrate with the amino acid sequence GDEVDGDI (OncorImmunin, College Park, MD). After treating with or without VK2, the cells were washed with PBS and incubated with a substrate reagent at 37°C for 60 min. Fluorescence of the profluorescent substrate cleaved by activated caspase-3 was analyzed by flow cytometry at FL2 channel with excitation at 488 nm (15).

Cell cycle analysis. Cells were fixed and stained with a solution containing 1% PI, 100 μg/ml digoxin, 0.01% NaN₃, 200 mg/ml RNAase (Sigma), and 2.5% FCS for 10 min at room temperature. Cells were analyzed by flow cytometry with a cell cycle analysis program, MultiCycle AV (Phoenix Flow Systems Inc, San Diego, CA) (16).

Statistics. Data are given as the mean ± SD. Comparisons between two groups were assessed with Student's t-test.

Results

VK2 induces growth suppression of lung carcinoma cell lines. We first examined the growth inhibitory effect of VK2 (menaquinone-4) in six lung carcinoma cell lines. After 96-h exposure to VK2 at various concentrations (0.1-500 μM), the cells cultured were harvested and variable cell number was assessed by flow cytometry as described in Materials and methods. As shown in Fig. 1, VK2 suppressed the cell growth of all lung carcinoma cell lines in a dose-dependent manner as well as HL-60 cells. Significant growth suppression was observed over 5 to 10 μM of VK2, and IC₅₀ was within 7.5-75 μM in the cell lines tested. Regarding the relationship of the efficacy of VK2 and tissue type of lung carcinomas, however, there was no apparent difference in sensitivity toward VK2 among tissue types such as small cells, squamous cells, adenocarcinoma, and large cell carcinoma.

Apoptosis induction of lung carcinoma cell lines in response to VK2. Since VK2 has apoptosis-inducing activity in leukemia cells and hepatoma cell lines (7-10), we next examined whether these growth inhibitions in these lung carcinoma cell lines were mediated through apoptosis induction. Cytospin preparation of each cell line after 72-h exposure to 10 μM of VK2 showed typical morphologic changes of the cells undergoing apoptosis such as apoptotic body and fragmented nucleus (Fig. 2). However, these apoptotic changes were observed in only some lung carcinoma cells.

As shown in Fig. 3, exposure to VK2 significantly increased the positive stained cells for APO2.7 mAb, a mAb that specifically detects the cells undergoing apoptosis (14). In addition, caspase-3 was significantly increased along with apoptosis induction in LU-139 cells. These effects were all consistent with our previous observations in leukemia cells (7,12,15,16).

G0/G1 arrest of lung carcinoma cells in response to VK2. VK2 treatment induces G0/G1 arrest of leukemia cells along with increased expression with p27KIP1 (16). We therefore
Figure 1. Growth inhibition of lung carcinoma cell lines in the presence of various concentrations of VK2. Lung cell lines LU-139, LU-130 (small cell), LC-AI, LC-1/sq (squamous cell carcinoma), PC-14, CCL-185 (lung adenocarcinoma), and IA-LM (lung large cell carcinoma) were cultured in the presence of 0.1-500μM of VK2 for 96h. Thereafter, a relative variable cell number was assessed as described in Materials and methods. All experiments were performed in triplicate. This is one set of representative results from 3 independent experiments.

Figure 2. Morphologic changes after 96-h exposure to VK2 in lung carcinoma cell lines. Lung carcinoma cell lines, LU-139, LC-AI, and PC-14, were cultured in the presence or absence of VK2 (10μM for LU-139 and LC-AI cell lines and 50μM for PC-14) for 96h. Cytospin preparations were made and stained with May-Giemsa. Original magnification x1,000. This is one set of representative results from 3 independent experiments.

performed the cell cycle analysis in LU-139 cells after 72-h exposure to VK2. As well as HL-60 cells, treatment of LU-139 cells with VK2 significantly increased the population of the cells in G0/G1 phase (Fig. 4). However, accumulation of LU-139 cells in G0/G1 phase was less than that in HL-60 cells in response to VK2. This was probably because of the longer doubling time for LU-139 cells than that for HL-60 cells (96h for LU-139 vs. 28h for HL-60).
YOSHIDA et al: VITAMIN K2 INDUCES APOPTOSIS IN LUNG CARCINOMA CELL LINES

Figure 3. Percentage of APO2.7 positive cells and activation of caspase-3 in response to VK2 in lung carcinoma cell lines. After 96-h treatment with VK2, LU-139 and LC-AI cells were processed to flow cytometry for measuring APO2.7 positive cells and caspase-3 activities as described in Materials and methods. This is one set of representative results from 3 independent experiments.

Figure 4. Cell cycle analysis after exposure to VK2. LU-139 and a leukemia cell line, HL-60 cells, were treated with 10 μM of VK2 for 72 h. Then cell cycle analysis was performed by flow cytometry as described in Materials and methods. This is one set of representative results from 3 independent experiments. Numbers represent the percentages of cells in G0/G1, S, and G2/M phases, respectively.

Combined effects of VK2 plus CDDP in LU-139 cells. Since VK2 induces apoptosis along with G0/G1 arrest in lung carcinoma cell lines, we next examined the combined effects of VK2 and CDDP in LU-139 cells. LU-139 cells were initially exposed to 5 or 10 μM of CDDP for 2 h, and then the cells were further treated with either 5 or 10 μM of VK2 for 96 h. As shown in Fig. 5, without pre-exposure to CDDP, 5 μM of VK2 treatment did not show any effects as compared to the control cells treated with control medium. However, it was noteworthy that once the cells were exposed to CDDP, 5 μM of VK2 treatment showed significant growth inhibition as compared with the cells treated with CDDP alone. In contrast, when the cells were treated with CDDP alone, 10 μM but not 5 μM of CDDP showed growth inhibition of LU-139 cells, whereas combined treatment of the cells with VK2 (5 μM)
plus CDDP (5 μM) resulted in a significant reduction of viable cell numbers. There was no difference in cell growth inhibition among treatment with CDDP (10 μM) alone, with CDDP (10 μM) plus VK2 (5 and 10 μM). These data suggest that combining sub-optimal doses of VK2 and CDDP enhance the growth inhibitory effects in LU-139 cells.

Discussion

The apoptosis-inducing effects of VK2 in leukemia cells were initially reported by us and have been studied using leukemia cell lines and primary cultured leukemia cells (7,12,15,16). In solid tumor cell lines, pretreatment with trypsin-EDTA solution under optimal conditions for preparing the single tumor cell suspensions has made it possible to analyze the series of VK2 effects using a flow cytometer as in the case with leukemia cells. By this procedure, we have demonstrated that VK2 induces growth inhibitory effect in all lung carcinoma cell lines tested. Morphologic features, positive staining for AP02.7 mAb and also caspase-3 activation all indicate the apoptosis-inducing activity of VK2 in lung cancer cells. Since VK2 is now widely used for the treatment of patients with osteoporosis in Japan, the safety of a long-term oral administration of VK2 45 mg/day has been proved (13). In addition, VK2 did not show any prominent adverse events including bone marrow suppression, unlike some other anti-cancer agents (13,17-19). These lines of evidence suggest the possibility of using VK2 for the treatment of patients with lung cancer.

Although VK2 shows apoptosis induction of lung carcinoma cell lines, a higher concentration of VK2 was required to exert the cell growth inhibition than that for HL-60 cells (Fig. 1). For a conventional therapeutic dose of 15 mg of VK2 taken orally for osteoporosis (13), Cmax has been reported to reach about 1 μM, which is a little lower than IC50 for the various lung carcinoma cell lines shown in this study (20). This may raise the question of whether these in vitro effects of VK2 shown in this study can also be reproduced in vivo. However, the pilot clinical trial at our institute revealed that oral administration of 15 mg of VK2 3 times a day to patients with lung carcinomas appear to show a survival benefit (Yoshida et al, unpublished data). In addition, by combined treatment with the sub-optimal dose of CDDP and VK2, the cell growth suppression of LU-139 became more prominent compared to the effects of either VK2 or CDDP alone (Fig. 5). This suggests that combining with other anti-cancer reagents can reduce the dosage of VK2 for exerting the therapeutic effect to a practical level. In addition, apoptosis induction in response to VK2 is reported to be mediated through depolarization of mitochondria membrane potential along with cytochrome-C release from mitochondria to the cytoplasm, which resulted in activation of the caspase cascade (16). Enforced overexpression of BCL-2 protein in HL-60 cells by gene transfection resulted in resistance against apoptosis induction (16). These data suggest that the mitochondria is one of the target cellular organ for apoptosis induction by VK2. As well as leukemia cells, it is likely that apoptosis induction in response to VK2 in lung carcinoma cells appears to be mediated via the same intracellular pathways. However, the signaling pathways towards mitochondria are still not clear (8,22). It is also not clear whether these biological effects by VK2 require binding of the vitamin to the cognate nuclear receptor as in the case of vitamin D3 (23-25). Elucidation of the precise molecular-based mechanism for apoptosis induction by VK2 will be important for designing the optimal therapeutic usage of VK2 for lung cancer. However, our data shown here appear to provide promise for VK2 therapy for patients with lung cancer.

References


