Vitamin K2 augments 5-fluorouracil-induced growth inhibition of human hepatocellular carcinoma cells by inhibiting NF-κB activation

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Abstract. Although 5-fluorouracil (5-FU) is one of the most commonly used chemotherapeutic agents in various cancers including hepatocellular carcinoma (HCC), chemoresistance has precluded single use of 5-FU in clinical settings. Since menatetrenone, an analogue of vitamin K2 (VK2), inhibits growth of cancer cells including HCC cells in vitro and in vivo, we examined VK2 modulation of HCC cell response to 5-FU. VK2 pretreatment dose-dependently enhanced growth-inhibition by 5-FU through a G1 cell cycle arrest. VK2 inhibited 5-FU-induced NF-κB activation and cyclin D1 expression. Therefore, combination of VK2 and 5-FU might represent a new therapeutic strategy for patients with HCC.

Introduction

Among chemotherapeutic agents against hepatocellular carcinoma (HCC), most commonly used 5-fluorouracil (5-FU) possesses significant cell-killing activity. Yet as with other anticancer agents, chemoresistance is a major clinical obstacle to successful 5-FU chemotherapy for HCC patients. Among various factors that confer chemoresistance, attention has focused on transcription factor nuclear factor (NF)-κB, which promotes tumor development and progression mainly by enhancing cell proliferation and interfering with apoptosis (1-3). Anticancer drugs have been reported to induce the nuclear translocation and DNA binding activity of NF-κB in cancer cells. This drug-induced NF-κB activity inhibits killing of cells by the chemotherapeutic agents (4,5). Furthermore, inhibition of NF-κB activity by transfecting dominant-negative IκBα has been shown to enhance the cytotoxicity of anticancer drugs in vitro and in vivo (2,4,6). These results implicate NF-κB in resistance to cancer treatment, suggesting importance of finding ways to reduce NF-κB activity induced by anticancer drugs.

Menatetrenone is an analogue of vitamin K2 (VK2), first identified as a critical factor in blood coagulation and a cofactor in bone metabolism. VK2 has been found to inhibit in vitro and in vivo growth of cancer cells including glioma, lung cancer, leukemia, and HCC cells (7-10). Cell cycle arrest and apoptosis are believed to be involved in this action of VK2, but precise molecular mechanisms underlying VK2-induced growth inhibition and killing in HCC cells remain to be elucidated. Recently VK2 also was found to inhibit hepatoma cell invasiveness and metastasis (11). More importantly, we recently demonstrated that administration of VK2 to HCC patients significantly decreased recurrence of HCC after curative local ablation therapy (12). Habu et al (13) reported that VK2 suppressed development of HCC from the cirrhotic liver in patients infected with hepatitis virus. These observations strongly suggest that VK2 has profound influence on HCC development and progression. We reported that VK2 inhibited HCC cell proliferation by down-regulating cyclin D1 through the inhibition of NF-κB activation (14). Although these results obtained with VK2 in experimental and clinical studies are promising, one question is whether VK2 could impede development of chemotherapy-induced drug resistance to preserve 5-FU cytotoxicity in HCC.

In the present study we examined the effects of combined 5-FU and VK2 treatment on growth of HCC cells. Our results indicated that VK2 pretreatment rendered tumor cells more susceptible to growth inhibition by 5-FU. Enhancement of growth-inhibitory effect was related to increased G1 cell cycle arrest involving suppression of NF-κB activation and cyclin D1 expression by VK2. Combining VK2 with conventional chemotherapy such as 5-FU treatment might improve outcome in HCC patients.

Materials and methods

Cells and reagents. The human HCC cell lines HepG2, Huh7, HLE, and Hep3B were obtained from the Japanese
Cancer Research Resources Bank (Osaka, Japan). Cells were cultured and maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (Invitrogen). 5-FU was purchased from Sigma (St. Louis, MO). Menatetrenone, a VK2 analogue, was obtained from Eisai (Tokyo, Japan). Dominant-negative IxB vector was purchased from Clontech Laboratories (Mountain View, CA).

Cell proliferation assay. Cell growth was determined by the WST-1 proliferation assay kit (Takara, Kyoto, Japan) as previously described (14). Cells were seeded in 24-well culture plates at a density of 1x10^5 cells/well, and incubated at 37°C for 24 h. After various treatments, the cells were incubated further for 48 h. Then the cells were incubated with the WST-1 reagents, and absorbance of formazan product was measured with a CS-9300 microtiter plate reader (Shimadzu, Tokyo, Japan).

FACS analysis. FACS analysis of propidium iodide (PI)-stained nuclei was performed as previously described (14,15). FACS analysis of propidium iodide (PI)-stained nuclei was performed as previously described (14,15). Briefly, cells were plated in 96-well plates (2x10^5 cells/well); treated with 5-FU, VK2, or both for 24 h; stained with Hoechst 33342, and then observed using fluorescence microscopy. One thousand cells were observed in five randomly chosen fields. Cells with condensed or fragmented nuclei were considered apoptotic.

Electrophoretic mobility shift assay (EMSA). NF-κB activity in nuclei isolated from hepatoma cells was determined by electrophoretic mobility shift assay (EMSA). The extraction of nuclear and cytoplasmic proteins and EMSA were performed as described previously (14,15). Briefly, 5 μg of nuclear protein was incubated for 30 min at room temperature with binding buffer (20 mM HEPES-NaOH, pH 7.9, 2 mM EDTA, 100 mM NaCl, 10% glycerol, 0.2% NP-40), poly (dl-dC), and 32P-labeled double-strand oligonucleotide containing the NF-κB binding motif (Promega Corp., Madison, WI, USA). The double-stranded oligomer used for EMSA had the sequence: 5′-AGTTGAGGGGACCTTCCCCAGGC-3′ (only the sense strand is shown). Reaction mixtures were loaded onto a 4% polyacrylamide gel and electrophoresed with a running buffer of 0.25X TBE. After the gel had dried, DNA-protein complexes were visualized by autoradiography. Intensity of electrophoretic bands was measured using a BAS 2000 image analyzer (Fujifilm, Tokyo, Japan).

Western blotting. Protein expression of p65, p18, p21, p27, cyclin D1, and β-actin were investigated by Western blotting. Cells (2x10^6) were collected and lysed with extraction buffer containing 50 mM Tris (pH 7.5), 130 mM NaCl, 0.1% SDS, 5 mM EDTA (pH 8.0), 1 mM PMSF, 10 μg/ml trypsin inhibitor, and 50 mM iodoacetamide. After 30 min at 4°C, cell debris was eliminated by centrifugation at 15,000 rpm for 20 min, and the supernatant was collected. Western blot analysis was performed using 5% skim milk containing 50 mM Tris (pH 8.0), and 0.5% Triton X-100 at 4°C for 10 min and centrifuged at 16,000 rpm for 20 min. The supernatant was treated with RNase A at 37°C for 1 h and proteinase K at 50°C for 30 min and then precipitated with isopropanol. The DNA was resuspended, and electrophoresed in a 2% agarose gel at 50 V for 3 h. DNA was visualized by ethidium bromide staining and photographed under ultraviolet light. Morphologic changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with Hoechst 33342 (Wako, Osaka, Japan). Briefly, cells were plated in 96-well plates (2x10^5 cells/well); treated with 5-FU, VK2, or both for 24 h; stained with Hoechst 33342, and then observed using fluorescence microscopy. One thousand cells were observed in five randomly chosen fields. Cells with condensed or fragmented nuclei were considered apoptotic.

RNA isolation and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from cultured hepatoma cells using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. RNA concentration was determined spectrophotometrically, and integrity of all samples was confirmed by visualizing 28S and 18S ribosomal RNA bands under ultraviolet light after gel electrophoresis. Semiquantitative RT-PCR for p21,
Luciferase reporter gene assay. Luciferase assays were performed using the method described by the manufacturer (Dual-Luciferase Reporter Assay System; Promega). Briefly, cells were seeded onto 24-well plates at 10^4 cells/well without antibiotics. After 24 h, cells were washed twice with Opti-MEM medium (Life Technologies, Rockville, MD), followed by the addition of 0.5 ml of Opti-MEM medium containing 0.4 μg of NF-κB-luciferase reporter plasmid, 0.1 μg of control luciferase plasmid, and 4 μl of Lipofectamine reagent (Invitrogen). After 4-h incubation, the medium was changed to a fresh medium, again free of antibiotics. Then cells were treated with 5-FU with or without VK2 for 12 h, washed twice with PBS, and carefully scraped into 1X passive lysis buffer. Cell extracts were immediately assayed for luciferase activity using a Berthold Luminometer (MLR-100 Micro Lumino Reader, Corona Electric, Ibaragi, Japan).

Plasmids and transfection. To study the role of NF-κB induced by 5-FU in hepatoma cell proliferation, hepatoma cells were transiently transfected with dominant-negative IκBα p65 subunit of NF-κB, or empty vector. The plasmid encoding dominant-negative IκBα was purchased from Clontech Laboratories. Plasmid encoding p65 NF-κB subunit was described previously (14). HCC cells were transfected using Lipofectamine reagents (Invitrogen) according to the manufacturer's instructions. For proliferation analysis, cells were treated with 5-FU for 6 h after transfection, and then further incubated for 48 h. Subsequently, cell growth was examined by WST-1 proliferation assay.

Statistical analysis. Differences were analyzed using Student's t-test, and a p-value <0.05 was considered indicative of significance. All experiments were performed in triplicate as a minimum. Data are shown as the mean ± standard deviation (SD).

Results

VK2 enhanced 5-FU-induced growth inhibition in HCC cells through cell cycle arrest at G1. To investigate whether VK2 altered susceptibility of cells to 5-FU, HCC cells were treated with various concentrations of VK2 prior to 5-FU (5 μg/ml). BrdU incorporation during DNA synthesis by these cells was then assayed. As shown in Fig. 1, DNA synthesis of HCC cells was inhibited by 5 μg/ml of 5-FU alone to 40-70% of synthesis in control. Preincubation with VK2 dose-dependently enhanced the growth-inhibitory effect of 5-FU. Similar findings were observed when cell survival was quantitated by the WST-1 proliferation assay (data not shown).
G1 cells, presumably apoptotic cells, did not differ between 5-FU alone and in combination with VK2. This result was supported in findings by DNA laddering and Hoechst 33342 staining (data not shown). Similar results were obtained in Huh7, HLE, and Hep3B cells.

VK2 decreases 5-FU-induced cyclin D1 expression and counteracts reduction of cyclin-dependent kinase (Cdk) inhibitor expression by 5-FU in HCC cells. The above FACS analyses indicated that VK2 increased 5-FU-induced growth inhibition through a G1 arrest in the cell cycle. We next examined by Western blot analysis whether VK2 affects the G1 cell cycle control protein cyclin D1 and the Cdk inhibitors p18, p21, and p27. As shown in Fig. 3A, inhibition of p21 and p27 and slight induction of cyclin D1 were observed after exposure to 5-FU alone, while p18 was not changed. When cells were treated with 5-FU plus VK2, significant reduction in cyclin D1 expression and induction in p21 and p27 expression were observed. Expression of p18 did not change in treated cells. Further, when mRNA expression for cyclin D1, p21, and p27 was examined by RT-PCR, and pattern of change was similar to that seen by Western blotting (Fig. 3B), indicating that expression of these cell cycle regulatory proteins was regulated at the mRNA level by combined treatment with 5-FU plus VK2.

Figure 2. VK2 enhances 5-FU-induced growth inhibition by inducing G1 cell cycle arrest in Hep3B cells. Cells were incubated in 5-FU (5 μg/ml) with or without various concentrations of VK2. After 48 h of incubation, cells were collected and stained with PI. DNA content was analyzed by FACS. Histograms represent total DNA content, percentages of cells in pre-G1 and G1 phases are shown as M1 and M2, respectively.

Figure 3. VK2 up-regulates 5-FU-induced p21 and p27 reduction and inhibits 5-FU-induced cyclin D1 promotion. (A) Cells were treated with 5-FU with or without various concentrations of VK2 for 48 h. Forty micrograms of extracted total cell lysate was used for Western blot analysis of p18, p21, p27, cyclin D1, or β-actin. (B) Cells were treated with 5-FU with or without various concentrations of VK2 for 24 h, and transcription of p21, p27, and cyclin D1 was determined by RT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used as a control. M, 100-base-pair DNA molecular marker.

VK2 inhibits 5-FU-induced NF-κB activation in HCC cells. Since NF-κB is essential to up-regulation of proliferation in various cell types (1-3) and cyclin D1 is known to be up-regulated by NF-κB, we investigated involvement of NF-κB activity in the VK2-mediated increase in susceptibility of HCC cells to growth inhibition by 5-FU by performing EMSA to assess DNA-binding activity. Western blotting to detect nuclear translocation, and a luciferase reporter gene assay to determine promotion of transcriptional activity, also were carried out. As shown in Fig. 4A, Hep3B cells constitutively showed NF-κB DNA-binding activity. Treatment with 5-FU alone increased this NF-κB binding
Figure 4. VK2 inhibits 5-FU-induced NF-κB activation in Hp3B cells. (A) After cells were incubated in 5-FU (5 μg/ml) with or without various concentrations of VK2 for 12 h, nuclear protein was extracted for analysis of NF-κB binding activity by electrophoretic mobility shift assay (EMSA). Quantitative analysis of NF-κB bands measured by BAS 2000 is presented in the lower panel, which is a representative result among 3 similar experiments. (B) VK2 inhibited 5-FU-induced p65 nuclear translocation. Cells were treated with 5-FU with or without various concentrations of VK2. The p65 protein expression in nuclear extracts was detected by Western blotting. Nuclear protein loading was standardized by Coomassie blue staining. (C) VK2 inhibited 5-FU-induced NF-κB transcriptional activity. After cells were transiently transfected with NF-κB-luciferase reporter plasmid or control luciferase plasmid for 4 h, cells were treated with 5-FU with or without various concentrations of VK2 for 12 h. The luciferase activity was measured by a Dual-Luciferase Reporter Assay System. Data are shown as the mean ± SD obtained from 3 independent experiments. *P<0.05, **P<0.01 compared with the cells treated with 5-FU alone.
To determine how VK2 enhances 5-FU-induced growth inhibition, we transiently transfected the HCC cells with an NF-κB probe (10-fold and 100-fold), but not an NF-κB mutant probe abolished the binding of NF-κB, demonstrating that binding of the NF-κB probe was specific. Furthermore, results of a supershift assay suggested that the 5-FU-activated complex consisted of p50 and p65 subunits. Densitometric analysis of the autoradiographic bands showed that combined treatment with 5-FU and 10⁻⁶, 10⁻⁵, 10⁻⁴ M of VK2 decreased 5-FU-induced NF-κB activation by 25, 38, 49%, respectively (Fig. 4A, lower panel).

To test the effect of 5-FU and VK2 on expression of nuclear p65 subunit, cells were exposed to 5-FU with or without various concentrations of VK2. As shown in Fig. 4B, 5-FU increased amounts of p65 in nuclei of Hep3B cells. Consistent with results observed by EMSA, VK2 reduced this effect upon nuclear p65 protein in a dose-dependent manner. Equivalent nuclear protein loading in each lane was verified by Coomassie blue staining.

To determine the effect of VK2 on 5-FU-induced NF-κB transcriptional activity, we transiently transfected the HCC cells with an NF-κB luciferase reporter gene construct, having treated some of them with VK2 for 2 h before 12-h treatment with 5-FU. With 5-FU treatment alone luciferase activity was about 1.8-times higher than in untreated controls. Preincubation of Hep3B cells with VK2 prior to 5-FU abolished this induction of luciferase expression by 5-FU (Fig. 4C).

**Inhibition of NF-κB by mutant IκBα transfection augments 5-FU-induced growth inhibition in HCC cells.** To determine whether activation of NF-κB by 5-FU is directly associated with 5-FU-induced growth inhibition in hepatoma cells, we examined the effect of mutant IκBα which is resistant to degradation and inhibits translocation of NF-κB to the nucleus, upon proliferation of cells exposed to 5-FU. After introduction of mutant IκBα into cells by transient transfection, we stimulated cells with 5-FU for 48 h, then examining the cell growth using a WST-1 proliferation assay. As shown in Fig. 5, transfection of mutant IκBα enhanced growth inhibition in 5-FU-treated Hep3B cells, while vector control did not augment 5-FU-induced growth inhibition in Hep3B cells. When HCC cells were transfected with the p65 subunit of NF-κB, 5 μg/ml of 5-FU failed to inhibit cell growth. These results suggested that NF-κB activation antagonized 5-FU- and VK2-induced growth inhibition in HCC cells.

**Discussion**

HCC is highly resistant to currently available chemotherapeutic regimens including those with 5-FU. In the present study, pretreatment of HCC cells with VK2 resulted in significant enhancement of 5-FU-mediated growth inhibition of cells. Furthermore, the results suggested a possible signal transduction mechanism underlying VK2 enhancement of growth inhibition. VK2 inhibited activation of NF-κB otherwise induced by 5-FU, and up-regulated Cdk inhibitors as well as down-regulating cyclin D1. The result was cell cycle arrest in G1. Thus, our findings delineate a possible mechanism for synergistic induction of growth inhibition by 5-FU given in combination with VK2.

While 5-FU is the first-line drug in chemotherapy of HCC, chemoresistance is the major obstacle to successful 5-FU chemotherapy. In an effort to improve the therapeutic index of 5-FU, several investigators have studied efficacy of 5-FU combined with other agents such as cisplatin, methotrexate, and doxorubicin in the treatment of patients with HCC, but use of multiple chemotherapeutic agents in these patients induced various degrees of toxicity (16-18). Although combination therapy using 5-FU and IFN-α has shown effectiveness in HCC, disagreement was reported by some investigators (19-21). High associated costs limit extensive use of IFN-α combination regimens in clinical treatment, particularly in developing countries. Our results indicated that combined treatment with VK2 may enhance sensitivity to 5-FU in HCC cells, without problems of toxicity that are typical of other combined regimen including 5-FU. Thus, VK2 might be uniquely beneficial in combination with 5-FU in chemotherapy for patients with HCC.

VK2 has been reported to show anticancer effects inducing apoptosis and cell cycle arrest in several tumor cell types such as leukemia cells, osteosarcoma cells, HCC cells, glioma cells, and lung cancer cells, also a number of striking anecdotal clinical reports supports use of VK2 as an anticancer agent (7-13, 22). However, therapies combining VK2 with established anti-tumor agents have not been investigated. Our present results showed augmentation of cell growth suppression induced by 5-FU in HCC cells by adding VK2.

To determine how VK2 enhances 5-FU-induced growth inhibition, we investigated the activity of NF-κB, a key mediator in promoting proliferation and inhibiting apoptosis. We found that exposure of cells to 5-FU alone activated NF-κB activity in a dose- and time-dependent manner (data not shown). Several chemotherapeutic agents such as daunorubicin, cisplatin, and paclitaxel have been reported to...
activate NF-κB in HCC cells, resulting in marked suppression of the cell-killling activity of the tested drug (23,24). We first demonstrated that 5-FU induced NF-κB activation in HCC cell lines, ruling out inhibition of NF-κB as a mechanism for 5-FU-induced growth inhibition. Indeed, NF-κB activation after chemotherapeutic treatments has been reported in various tumor cell lines, presumably representing a defense mechanism tending to counteract the cell death cascades inhibited by these cancer therapies (2,4,25). This NF-κB-based defense therefore represents a potentially important molecular target in enhancing sensitivity of certain cancer cells that otherwise show resistance to various drugs. Our results showed that a VK2 analogue, on the other hand, significantly inhibited NF-κB activity in 5-FU treated cells in a dose-dependent manner, including NF-κB nuclear translocation, DNA binding activity, and transcriptional activity.

This suggests that enhancement by VK2 of 5-FU anticancer effects is attributable to inhibition of the NF-κB activation ordinarily induced by chemotherapeutic agents. Furthermore, dominant-negative IκBα transfection in HCC cells enhanced 5-FU-induced growth inhibition, again implicating NF-κB activation in resistance to 5-FU-induced growth inhibition. Like this blockade-inducing transfection, the more attainable VK2-mediated inhibition of NF-κB activation by 5-FU enhanced growth inhibition by a conventional drug. VK2 has not shown any prominent side effects, and safety of long-term oral administration has been proven. In addition to NF-κB-related concerns, some advanced HCC tumors harbor p53 mutations that confer chemoresistance (26). Our results showed that VK2 enhanced 5-FU cytotoxicity in cell lines with a variety of forms of p53: wild-type (HepG2), mutant (HLE, Huh7), and null (Hep3B). These data further suggest that usefulness of VK2 as a potential sensitizer for drug-induced HCC cell growth suppression when included in combination therapy.

Constitutive activation of NF-κB which is characteristic of in HCC cells, exerts influence upon a signaling network, that includes expression of cell cycle regulatory genes, anti-apoptotic genes, and genes encoding cell surface receptors (27,28). Regulation of these genes by NF-κB prevents induction of apoptosis and promotes cell proliferation. Since our results showed VK2 enhanced 5-FU-induced cell growth inhibition through a G1 cell cycle arrest, we examined the expression of cell cycle regulatory proteins after treatment with 5-FU plus VK2. We found that the combination of 5-FU and VK2 induced expression of the Cdk inhibitors p21 and p27 and inhibited expression of cyclin D1. All of these changes block the cell cycle at the G1 checkpoint in contrast to the effect of 5-FU alone. These data are consistent with other previous reports suggesting that induction of cell cycle regulatory proteins is an important part of the anticancer effect of VK2 (29). Cyclin D1 has been suggested to be transcriptionally regulated by NF-κB to induce tumor progression through the G1 phase of the cell cycle (30). Indeed, overexpression of cyclin D1, which has been found in human HCC, is sufficient to initiate hepatocarcinogenesis in transgenic mice (31,32). Therefore, as both NF-κB and cyclin D1 function as oncogenes in the liver, down-regulation of NF-κB activity and down-regulation of genes that NF-κB apparently regulates, such as cyclin D1 are likely to be productive molecular goals in HCC treatment. Our results suggested that VK2-mediated cyclin D1 reduction might result from VK2-mediated NF-κB inhibition, although additional studies are required to determine conclusively whether cyclin D1 is NF-κB dependent in our system.

In summary, we demonstrated that VK2 may augment growth inhibition induced by 5-FU in hepatoma cells through inhibition of the NF-κB signaling pathway, and that combining VK2 with conventional anticancer agents might improve results in the treatment of HCC patients.

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References


