The mechanisms of vitamin K2-induced apoptosis of myeloma cells

Background and Objectives. Physiologically, vitamin K compounds act as co-factors for $\gamma$-carboxylation of selected glutamates at the N-terminus of prothrombin and other VK-dependent coagulation factors. These congeners have some growth inhibitory effects on human neoplastic cells. Furthermore, vitamin K2 (VK2) causes apoptosis of some leukemic cells. In search for a new candidate agent to use in the maintenance treatment of myeloma, we analyzed the growth inhibitory effects and apoptosis-inducing capacity of VK2 in human myeloma cells.

Design and Methods. We assayed the growth of myeloma, lymphoma and non-lymphoid cells cultured with various concentrations of VK2 with or without dexamethasone or allopurinol. Flow cytometry was used to detect apoptotic cells, activated caspase-3 and -9, the generation of superoxide by hydroethidine, and mitochondrial membrane potential ($\Delta$Ψm). In addition, the activation of apoptosis-inducing MAPK, p38 and JNK, release of cytochrome c from mitochondria, and change in the relative Bcl-XL/XI expression balance were analyzed by Western blotting.

Results. Myeloma cells and B-cell lymphoma cells were sensitive to VK2. The growth inhibition was caused by apoptosis and activation of caspase-3. The generation of superoxide, and inhibitory effects of the xanthine oxidase inhibitor allopurinol, were demonstrated in myeloma cells. The phosphorylation of MAPK was increased by VK2 in myeloma cells. In addition, the mitochondrial apoptotic pathway was activated.

Interpretation and Conclusions. VK2 could be a possible treatment for myeloma patients, particularly those who are not suitable candidates for intensive cytoreductive chemotherapy due to age and/or complications.

Key words: myeloma vitamin K2, apoptosis, ROS mitochondria.

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Multiple myeloma (MM) is a hematologic malignancy characterized by the clonal expansion of malignant plasma cells in the local bone marrow environment and by extensive destruction of bone. Recently, a reduction of myeloma cell numbers in the bone marrow, decrease of clonal malignant immunoglobulin, and improvement of clinical symptoms such as bone pain, anemia, hypercalcemia, and renal dysfunction, have been achieved by initial chemotherapy, for example, with melphalan and prednisolone, multi-drug chemotherapies, and high-dose chemotherapies supported by stem cell transplantation. However, over 80% of patients with MM die within 5 years. Several novel agents have shown therapeutic potential in MM, including angiogenesis inhibitors such as thalidomide, and a proteasome inhibitor, although the evaluation of these drugs may take several years. In addition, new drugs for remission induction and maintenance therapy are needed in order to prolong survival.

Vitamin K (VK) is a generic term for compounds such as phylloquinone (VK1), menaquinone (VK2) and naphthoquinone (VK3). Physiologically, the natural VK, VK1 and VK2, act as cofactors for the $\gamma$-carboxylation of selected glutamates at the N-terminus of prothrombin and other VK-dependent coagulation factors. It has been shown that these congeners have some growth inhibitory effects on human neoplastic cells such as lung cancer, glioblastoma and hepatocellular carcinoma cells. In addition, VK2 has been reported to induce in vitro apoptosis of leukemic cell lines derived from patients with myelodysplastic syndrome (MDS) and acute leukemia, as well as freshly isolated leukemic cells. In this article, we describe the effects of VK2 on growth inhibition and apoptosis of human myeloma cells (primary cells and cell lines), examining the role of the mitochondrial apoptotic pathways involving activation of apoptosis-inducing mitogen-activated protein kinase (MAPK) and p38.

Design and Methods

Cell lines and primary cells

Twelve human myeloma cell lines (HMCL), seven non-myelomatous lymphoid cell lines (NMLCL), and three non-lymphoid cell lines (NLCL) were used in this study. The HMCL were ten lines established in our laboratory (KMM-1, KMS-11, KMS-12PE, KMS-12BM, KMS-20, KMS-24, KMS-26, KMS-27, KMS-28PE, and KMS-34) and two widely used
The NMLCL were SU-DHL-4 (derived from transformed follicular lymphoma), Raji and Daudi (Burkitt's lymphoma), CEM, Molt4 and Jurkat (T-cell acute leukemia), and MT-1 (adult T-cell leukemia). The NLCL were HL60 (promyelocytic leukemia), U937 (histiocytic leukemia) and K562 (chronic myelogenous leukemia). All lines were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO2. Primary myeloma cells collected by positive selection using CD138 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) (purity; more than 95%) from the bone marrow of five myeloma patients were also used in this study. These patients had given informed consent to the collection and use of their bone marrow samples. The Institutional Ethics Committee of Kawasaki Medical School approved the project.

**The assay of cell growth**

The water-soluble tetrazolium salt (WST-1) assay was used to assess the growth of myeloma cell lines cultured with 0 (ethanol as vehicle for VK2), 3, 10, and 30 µM VK2 with or without 10−6 M dexamethasone or preincubation with 200 µM allopurinol for 48 or 72 hours. The growth of cells was assessed using the Premix WST-1 Cell Proliferation Assay System (Takara Biochem., Tokyo, Japan) according to the manufacturer's instructions and as reported previously. Primary myeloma cells were cultured with or without 10 µM VK2 for 48 hours with 10 nM recombinant human interleukin-6 (rhIL-6) to support basic growth and subjected to the WST-1 assay. In one case (case #5), primary myeloma cells were seeded onto culture dishes with or without covering the dishes with a feeder layer, which consisted of 30 Gy-irradiated fibroblasts or with 0, 10, or 30 µM VK2 for 72 hours. The effect of VK2 on their growth was assayed using the 3H-thymidine incorporation assay to ensure that the primary cells were proliferating and that their proliferation was enhanced by the feeder layer.

**The detection of apoptosis**

Apoptosis in human myeloma cell lines cultured with 0, 3, 10, or 30 µM VK2 for 72 h was detected using the TUNEL method (in situ cell death detection kit, Roche Applied Science, Indianapolis, IN, USA) and FACSCalibur flow cytometry (Becton Dickinson Immunocytometry Systems, Franklin Lakes, NJ, USA) as reported previously. In addition, active caspase 3 in KMS-34 cells cultured with 0, 10 or 30 µM VK2 for 72 hours was detected as another indicator of apoptosis by flow cytometry using a CaspGLOW™ Fluorescein Active Caspase-3 Staining Kit (Biovision, Research Products, Mountain View, CA, USA) according to the manufacturer's instructions.

**The generation of reactive oxygen species (ROS)**

The production of superoxides in the KMS-26, KMS-28PE, and KMS-54 cells cultured with 0, 10, or 30 µM VK2 for 48 hours with 5 µM hydroethidine (Molecular Probes Inc., Eugene, OR, USA) added for the last 30 minutes was measured based on the change in the fluorescence of hydroethidine after oxidation by the superoxides using FACSCalibur flow cytometry as reported previously. In addition, the effects of the xanthine oxidase inhibitor (allopurinol) were assayed based on growth characteristics and the change of hydroethidine in these cells.

**Western blotting**

Whole cell lysate (10 µg) from KMS-34 cells cultured with or without 30 µM of VK2 for 30 or 60 minutes was electrophoresed on a 12.5% acrylamide gel, and the resolved proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech., Little Chalfont, Buckinghamshire, UK) as reported previously. After subsequent blocking, the membrane was reacted with antibodies against GAPDH, α-tubulin, Bcl2, Bax, Bcl-X (which detects both Bcl-XL and Bcl-Xs), p38, phosphorylated p38 (p-p38), c-Jun N terminal protein kinase (JNK), p-JNK, and caspase-9 (which reacts with precursor and cleaved forms). All antibodies, including horseradish peroxidase (HRP)-conjugated antimouse, and anti-rabbit immunoglobulin (Ig) G secondary antibodies, were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), except the anti-GAPDH antibody, which was purchased from BD Bioscience Pharmingen (San Diego, CA, USA). The staining for control molecules such as tubulin and GAPDH was always performed by re-hybridization after stripping the primary antibodies. To examine the cytoplasmic and mitochondrial expression of cytochrome-c, a Cytochrome c Assay Kit™ (B-Bridge International Inc.) was employed to separate the cytoplasmic and mitochondrial fractions. After the measurement of protein concentrations, western blotting for both proteins was performed as described above. All blots were analyzed using a FAS-II UV-image analyzer (TOYOBO Co. Ltd., Tokyo, Japan), and the densities of the bands were quantified using Quantity One™ version 2.5 (PDI Inc., Huntington Station, NY, USA).

**Assay of the mitochondrial apoptotic pathway**

To analyze the status of the mitochondrial apoptotic pathway, assays for the mitochondrial membrane potential, activation of caspase-9, and release of cytochrome c were employed. KMS-34 and KMS-26 cells cultured with 0, 10, or 30 µM VK2 for 72 hours were stained with 40 nM 3,3'-dihexyloxacarbocyanine (DiOC6) in phosphate-buffered saline (PBS) for 20 minutes and analyzed by flow cytometry. The decrease in mitochondrial membrane potential (∆Ψm) was calculated as the mean fluorescent intensity (MFI) of VK2-treated samples minus that of untreated cells divided by the MFI of control samples (VK2-untreated) minus that of a cell only sample. Then, the activation of caspase-9 was assayed by western blotting as described above with a CaspGLOW™ Fluorescein Active Caspase-9 Staining Kit (Biovision, Research Products) according to the manufacturer's instructions. The method used to detect the cytochrome c release was as described above.

**Statistical analyses**

The growth rate of individual cell lines cultured with various concentrations of VK2 with or without combined treatment with dexamethasone or allopurinol, the comparison of the growth inhibitory effects of VK2 among
groups of cell lines (HMCL, NMLCL, and NLCL), the percentage of TUNEL-positive fractions, active caspase-3 and -9-positive fractions, and hydroethidine-positive fractions, and densitometric analyses of the ratios of p-p38/p38, pJNK/JNK, cytosolic/mitochondrial cytochrome c, and Bcl XL/Xs were analyzed using Fisher’s parametric least significant difference test.

Results

Growth inhibitory effects of VK2

As shown in Figure 1, most of the cell lines studied showed growth inhibition when cultured with 10 and 30 µM VK2, with several exceptions, such as K562 and HL60. Although most previous investigations using VK2 as a cytocidal agent against hematologic malignancies were targeted at leukemic cells derived from patients with MDS or acute leukemia, VK2 seemed to inhibit the growth of HMCL and NMLCL, but not of NLCL. Primary myeloma cells derived from four patients showed growth inhibition when cultured with 10 µM VK2 (Figure 2). Whether or not the growth inhibitory effects of VK2 on myeloma cells were the results of overcoming growth support from the bone marrow microenvironment was investigated in patient #5. In this case, growth inhibition was not observed in the culture without a feeder layer; however, VK2 inhibited myeloma cell growth remarkably when the cells were cultured on a feeder layer, even though the feeder layer enhanced the growth of these cells approximately three fold. The growth inhibitory effects of the combination of VK2 with dexamethasone on myeloma cells were also analyzed. As shown in Figure 3, the combination of these agents had an additive effect in both cell lines studied. The combination of VK2 with bisphosphonate (alendronate) or interferon-α was not observed to have any effect (data not shown).

The detection of apoptotic cells

Flow cytometric TUNEL analysis of KMS-28PE and KMS-34 cells cultured with 0, 3, 10 or 30 µM VK2 for 72 hours showed a dose-dependent increase of the percentage of TUNEL-positive cells (Figure 4A). In addition, the activated fraction of caspase 3 was increased in a dose-dependent manner in KMS-34 cells (Figure 4B) and KMS-28PE cells.

The generation of superoxide

As shown in Figure 5A, KMS-34 cells produced superoxide, as indicated by stronger fluorescence from...
hydroethidine, in a dose-dependent manner. Similar findings were obtained for KMS-28PE and KMS-26 cells (data not shown). Figure 5B illustrates that myeloma cells recovered from VK2 (10 nM)-induced growth inhibition when pretreated with 200 μM allopurinol (a xanthine oxidase inhibitor). In addition, the generation of superoxide was reduced when KMS-26 cells cultured with 30 μM VK2 were pretreated with 200 μM allopurinol (Figure 5C). These results indicate that the apoptosis of myeloma cells induced by VK2 was mediated by the production of reactive oxygen species.

The activation of apoptosis-inducing MAPK and the mitochondrial apoptotic pathway

To gain insight into the signaling involved in the observed generation of reactive oxygen species and induction of apoptosis, we examined whether the apoptosis-inducing MAPK, p38and/or JNK, and mitochondrial apoptotic pathway were activated. First, the status of p38 and JNK MAPK was analyzed. As shown in Figure 6A (actual images of the western blot) and 6B (densitometric analyses of the western blot of Figure 6A), the phosphorylation of p38 kinase was increased 30 to 60 minutes after the addition of 30 μM VK2 to the culture of KMS-34 cells, whereas no significant increase in the phosphorylation of JNK kinase was observed. Next, decreases in mitochondrial membrane potential (Δψm) and activation of caspase-9 were analyzed, since both indicate the activation of the mitochondrial apoptotic pathway. The MFI of KMS-34 cells and KMS-26 cells (data not shown) decreased when the cells were cultured with 0, 10 or 30 μM VK2 for 72 hours (Figure 7A). The relative Δψm of KMS-34 and KMS-26 cells decreased in a dose-dependent manner (Figure 7B). The overlaid histograms (Figure 7C) and percentage of activated caspase-9 fraction (Figure 7D) in KMS-34 cells cultured with 0, 10 or 30 μM VK2 for 72 hours indicate that caspase-9 was activated in a dose-dependent manner. The activation of caspase-9 was also detected by western blotting (data not shown). We then analyzed the release of cytochrome c from mitochondria to the cytoplasm, since the decrease of Δψm enables this release and the release initiates the activation of caspase-9. Figure 8A (actual gel images of mitochondrial and cytosolic cytochrome c in KMS-34 and KMS-26 cells cultured with 0 and 10 μM of VK2 for 72 hours) and Figure 8B (calculated relative localization of cytochrome c in these cells) show that cytochrome c was released into the cytoplasm when myeloma cells were cultured with VK2.

The change in the balance of Bcl-XL/XS expression

When the mitochondrial apoptotic pathway is activated, the balance between the pro- and anti-apoptotic molecules of the Bcl2 family tends to shift toward a pro-apoptotic balance. To evaluate this balance, Bcl2 and Bax expression was initially analyzed by western blotting. However, neither protein was altered in cultures with VK2, although both were expressed in the myeloma cell lines studied. Therefore, the Bcl-XL/XS balance was examined using not only western blotting (Figure 9A) but...
The expression was calculated as the intensity of the growth band divided by that of the Bcl-X. Various molecules were involved in this process.

In Figure 7A, DiOC6 (40 nM) was added to the KMS-34 cells cultured with 0, 10 or 30 mM VK2 for 72 hours, and then the cells were subjected to flow cytometry to detect the change of ∆ψm. This panel shows the overlaid histograms. B. KMS-34 and KMS-26 cells were used for the assay to detect the change of ∆ψm as described in the Design and Methods. The relative ∆ψm decreased in a dose-dependent manner in both cell lines. C. KMS-34 cells under the above-described conditions were used to detect active caspase-9 by flow cytometry. D. The increase in the active caspase-9 fraction in KMS-34 cells cultured with 0, 10 or 30 mM VK2 was dependent on the dose of VK2.

Also by densitometric analysis (Figure 9B), the relative Bcl-Xi/XS expression was calculated as the intensity of the Bcl-Xi band divided by that of the Bcl-XS band in the same culture with or without (control culture) 10 µM VK2 for 72 hours. As shown in Figure 9B, all the myeloma lines studied showed a decrease in relative Bcl-Xi/XS expression when cultured with 10 µM of VK2. These results indicate that Bcl-Xi/XS molecules were involved in the VK2-induced apoptosis of myeloma cells.

**Discussion**

Recent efforts to find new agents for therapeutic applications have led to the introduction of several drugs into the clinical field, such as thalidomide (as an angiogenesis inhibitor), bortezomib (as a proteasome inhibitor), and bisphosphonate (as an anti-cancerous agent), as well as to the improvement of bone lesions. However, the search for new drugs, not only for remission induction but also for maintenance therapy, should continue. We, therefore, investigated the growth inhibitory effects of VK2 in myeloma cells, since this agent has been reported to have anticancerous effects in various human neoplastic cells as well as human leukemic cells derived from patients with MDS and acute leukemia. VK2 may be a good candidate therapeutic agent for myeloma patients since it caused growth inhibition, induced apoptosis via the mitochondrial pathway, activated apoptosis-inducing caspase-9, and generated reactive oxygen species. The clinical advantages of this agent, such as the ability to administer it orally and its few side effects, enables its use in elderly patients. In addition, although the effective concentration used in this study (10 µM) is higher than the plasma concentration of VK2 administered as a medication, VK2 may accumulate in the space beneath bone-dissolving osteoclasts, where myeloma cells are frequently found, as reported for bisphosphonate. Various investigations have shown the clinical usefulness of VK2 in MDS patients, even though the in vitro growth inhibitory and apoptosis-inducing effects of VK2 on human leukemic cells derived from leukemia cell lines...
This has tended to shift toward α56 proteins. B. The relative Bcl-L

Figure 8. A. KMS-34 and KMS-26 cells were cultured with or without 10 μM of VK2 for 72 hours and mitochondrial and cytosolic proteins were extracted according to the instructions of the manufacturer of the Cytochrome c Assay Kit™ (B-Bridge International Inc.). Western blotting was then performed. B. The change of the relative localization of cytochrome c was calculated as described in the Design and Methods. Cytochrome c was released from mitochondria to cytoplasm in both cell lines.

and MDS cell lines or patients were achieved only at relatively high concentrations of VK2, similar to those used in this study. C–E

However, caution is required when using VK2 clinically. The exogenous administration of high doses of VK2 could cause adverse effects such as thromboembolic events. This is of particular clinical relevance, because it is now recognized that thalidomide leads to increased rates of thromboembolic events in MM patients, especially when used in combination with other anti-myeloma agents (e.g., dexamethasone or alkylators). This has prompted clinicians to employ various different approaches for prophylactic anti-coagulation (including coumadin) in MM patients, which would appear to be pharmacologically incompatible with the administration of high doses of VK2. In view of the cardinal role that thalidomide has assumed in the management of MM, and the general concern about thromboembolic events in the myeloma patient population due to other concomitant factors (e.g., advanced age), if VK2 were to be used clinically as one of the therapeutic agents, it would have to be started at a low dose and with meticulous care to avoid thrombotic events. Several reports have demonstrated that VK2 induces apoptosis of leukemic cells derived from patients with MDS or acute leukemia via the mitochondrial pathway. In our investigation, VK2 induced myeloma cell apoptosis through the mitochondrial pathway initiated by the apoptosis-inducing activation of p38 MAPK and the generation of reactive oxygen species. This notion is supported by the observed increase of phosphorylated p38, the decrease of Δψm, release of cytochrome c from mitochondria to the cytoplasm, activation of caspase-9, and increase of superoxide production and its inhibition by xanthine oxidase inhibitor. This series of alterations of the mitochondrial pathway caused the activation of caspase-3 and subsequently apoptosis, as detected using the TUNEL method. However, perturbation of the cell cycle in myeloma cells cultured with various concentrations of VK2 did not have any particular effect on check-points such as the G1/G0, G0/S, and G1/S transitions (data not shown). These observations suggest that VK2 may be effective against most myeloma cells, since sub-populations of myeloma cells categorized by chromosomal translocations such as t(11:14)(q13;q32) with the overexpression of cyclin D1, and t(4:14)(q16.3;q32) with the overexpression of fibroblast growth factor receptor 3, are known to have an advantage with respect to progression through the cell cycle. Although growth inhibitory effects of VK2 combined with other candidate agents for myeloma therapy, such as bisphosphonates and interferon-α7 were not demonstrated in this study (except for dexamethasone), the long-term (months to years) effects of these combinations should be studied, since the in vitro experiments reported here were short-term and involved high-dose combinations. Although meticulous care would be needed, it might be worth considering the clinical usage of VK2 for myeloma patients, particularly elderly individuals or those who have other complications, since these patients cannot complete intensive cytoreductive therapy and stem cell transplantation due to side effects and there are many more such patients than patients with other hematologic malignancies.

TO conceived the study; TT and YM performed all the pilot experiments and most of the analysis. The study was performed in TO’s group under the direction of TS. TT and YM performed the benchwork and statistics; NY, FH and HW discussed all the steps of the study. TT wrote the manuscript with contributions from the other authors. The authors reported no potential conflict of interest. The authors thank Ms. Haruko Sakaguchi, Tamayo Hatayama, Satomi Hatada, and Sakura Eda for their skillful technical assistance. This work was supported by JSPS KAKENHI grants (14570811 and 16390175), Subsidies for Ordinary Expenses of Private Schools, and a Kawasaki Medical School Project Grant (15–101A). Manuscript received April 28, 2005. Accepted February 10, 2006.
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