学 位 論 文 の 要 旨

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学　位　論　文　名	Vitamin K_2 and Cotylenin A Synergistically Induce Monocytic Differentiation and Growth Arrest Along With the Suppression of c-*myc* Expression and Induction of Cyclin G2 Expression in Human Leukemia HL-60 Cells
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論 文 内 容 の 要 旨

INTRODUCTION

Acute myeloid leukemia (AML) is the most common type of leukemia in adults, occurs in approximately one third of newly diagnosed patients, and remains one of the most difficult hematological malignancies to treat. The use of differentiation therapy with all-*trans* retinoic acid (ATRA) for acute promyelocytic leukemia (APL) has revolutionized therapy for this disease by converting it from fatal to curable. However, ATRA is not effective other AMLs and many APL patients treated with ATRA fail to respond or invariably relapse. Therefore, the development of new drugs or effective combination therapy is urgently needed. We searched for substances capable of inducing cell differentiation and strongly suppressing the expression of *c-myc*, as well as inducing the expression of cyclin G2, which had a positive effect on the promotion and maintenance of cell cycle arrest, in HL-60 cells. In the present study, we found that vitamin K_2 (VK2) and cotylenin A (CN-A) (a fucicoccan-diterpene glycoside) synergistically induced monocytic differentiation and growth arrest along with the suppression of c-*myc* expression and induction of cyclin G2 expression in HL-60 cells.

MATERIALS AND METHODS

For assay of cell growth, HL-60 cells were plated in multidishes at a density of 2.5×10^4 cells/ml and incubated with or without the test compounds. Cell numbers were counted with a model Z1 Coulter Counter. Differentiation of HL-60 cells was evaluated by 1) NBT reducing

activity which is one of the typical myelo/monocytic differentiation markers of human leukemia cells, 2) induction of non-specific esterase which is a specific monocytic differentiation marker, and 3) induction of morphological changes of HL-60 cells. Following the treatment of HL-60 cells with or without VK2 plus CN-A, the cells were washed with phosphate-buffered saline (PBS) and fixed gently in 100% ethanol at 4°C for 30 min. Cells were suspended in propidium iodide (PI)-RNase solution for 30 min at room temperature. Then the cell cycle analysis was performed by flow cytometry (BD FACSCalibur). Gene expression was determined by reverse transcription-quantitative polymerase chain reaction (qPCR). qPCR using the SYBER Green method was carried out on a Thermal Cycler Dice Real Time PCR instrument (Takara Bio).

RESULTS AND DISCUSSION

VK2 and their analogs have been shown to inhibit the survival of various cancer cell lines and leukemia cells. Furthermore, previous studies reported that VK2 exhibited some differentiation-inducing activity in AML cell lines *in vitro*. We examined the effect of VK2 alone on the induction of differentiation of HL-60 cells in our culture condition. Only VK2 alone induced the intermediate stage of differentiation in HL-60 cells. However, as VK2 is a naturally-occurring, safe, and clinically-utilized agent, we searched for substances that could enhance the differentiation-inducing activity of VK2.

CN-A and VK2 synergistically induced the reduction of NBT. We then determined whether the induction of differentiation induced with VK2 plus CN-A was a granulocytic or monocytic lineage. HL-60 cells were cultured without or with CN-A, VK2, or VK2 plus CN-A for 5 days. Cells treated with CN-A plus VK2 synergistically became positive for non-specific esterase, whereas those treated CN-A or VK2 alone became weakly positive. The combined treatment of VK2 and CN-A also induced the marked morphological differentiation of HL-60 cells, whereas VK2 or CN-A alone induced the intermediate stage of differentiation. These results indicated that the treatment of HL-60 cells with VK2 and CN-A effectively induced monocytic differentiation.

The growth of HL-60 cells was moderately inhibited by VK2 or CN-A alone, but was still observed until at least 6 days; however, no significant changes were observed in the cell number after 4 days of the treatment with the combination of both VK2 and CN-A. We also examined the long-term effects of the combined treatment of VK2 and CN-A on the proliferation of HL-60 cells. Cell growth was greatly inhibited by the combined treatment of VK2 and CN-A, and the cell number was almost the same as that at day 5, whereas the cell number of VK2- or CN-A-treated cells still increased (100-fold between days 5 and 20). Cell cycle analysis showed that this combined treatment induced growth arrest at the G_1 phase.

Since the induction of differentiation and growth arrest in HL-60 cells was associated with the suppression of c-*myc* gene expression, we investigated whether the combined treatment of CN-A and VK2 synergistically inhibited c-*myc* gene expression in HL-60 cells. VK2 markedly enhanced the downregulation of c-*myc* gene expression induced by differentiation inducers, whereas VK2 alone at the doses used weakly suppressed gene expression. The combined treatment of VK2 and CN-A exhibited the most potent suppressive effects on c-*myc* gene expression among the inducers or their combinations tested. This combined treatment reduced the expression of c-*myc* to approximately one fortieth that of control levels, and synergistically induced differentiation and growth arrest. Although VK2 also effectively enhanced the suppressive effects of c-*myc* expression induced by 1α, 25-dihydroxyvitamin D_3 (VD3), VK2 plus VD3 reduced the expression of c-*myc* less than that of VK2 plus CN-A. Furthermore, no derivative of active VD3 has so far been used clinically as an anticancer agent because of the side effect of hypercalcemia. These results suggest that the combination of VK2 and CN-A has therapeutic value in the treatment of AML. Furthermore, since we previously found that CN-A was also capable of stimulating the functional and morphological differentiation of ATRA-resistant APL cell line cells, the combined treatment of VK2 plus CN-A may be useful for differentiation therapy in retinoid-resistant leukemia.

We also determined whether the differentiation of HL-60 cells induced with VK2 and CN-A was accompanied by the induction of cyclin G2 expression which had a positive role for cyclin G2 in the promotion or maintenance of cell cycle arrest. Cyclin G2 gene expression was markedly induced (>5-fold) in VK2 plus CN-A-treated HL-60 cells. On the other hand, the expression of cyclin G2 was approximately 2-fold higher in CN-A-treated HL-60 cells than in control cells, and VK2-treated cells showed only a marginal increase. We did not observe the marked induction (>2-fold) of the expression of p21/CIP1 in VK2-, CN-A-, or VK2 plus CN-A-treated HL-60 cells.

CONCLUSION

We have searched for substances that are capable of inducing cell differentiation and the expression of cyclin G2, and that also can strongly suppress the expression of *c-myc* in HL-60 cells. In the present study, we found that the treatment with VK2 plus CN-A induced functional and morphological differentiation as well as growth arrest in HL-60 AML cells. Furthermore, this treatment almost completely suppressed the expression of *c-myc* and markedly induced the expression of cyclin G2. Therefore, these results suggest that the combination of VK2 and CN-A has therapeutic value in the treatment of acute myeloid leukemia.