学 位 論 文 の 要 旨

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学　位　論　文　名	Cotylenin A and Tyrosine Kinase Inhibitors Synergistically Inhibit the Growth of Chronic Myeloid Leukemia Cells
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論 文 内 容 の 要 旨

INTRODUCTION

Chronic myeloid leukemia (CML), a clonal disease affecting hematopoietic stem cells, is driven by the BCR/ABL oncoprotein, a constitutively active tyrosine kinase. Patients with CML in chronic phase are treated with imatinib (IM) or other tyrosine kinase inhibitors (TKIs), which are highly effective at inducing remission and prolonging survival. However, TKIs do not completely eliminate leukemia stem cells (LSCs), even in patients who achieve deep molecular responses. Thus, the identification of drugs that can target these LSCs is of primary importance in order to achieve the eradication of CML. Transcriptomic and proteomic analyses have revealed that p53 and c-Myc play defining roles in CML stem cell survival, suggesting that the dual targeting of p53 and c-Myc may selectively eliminate LSCs in CML. Since downregulation of c-Myc and upregulation of p21 (a target gene of p53) are commonly observed during differentiation of acute myeloid leukemia (AML) cells, we hypothesized that differentiation-inducing agents may be useful in regulating c-Myc and p53 expression in CML cells.

In this study, we examined the effects of agents that induce the differentiation of AML cells on proliferation of CML cells in the presence of TKIs. Among these agents, cotylenin A (CN-A) was found to be the most effective at inhibiting clonogenic potential and proliferation of CML cells in long-term culture in the presence of TKIs. These results suggest that CN-A may be useful in CML therapy in combination with TKIs.

MATERIALS AND METHODS

Human CML cell lines (K562 and KU812) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 80 μg/ml gentamicin at 37 ˚C in a humidified atmosphere of 5% CO_2 in air. Suspensions of cells ($2x10^4$ cells/ml) in 1 ml of culture medium were incubated with or without the test compounds in multidishes. Morphological changes were examined in cell smears stained with May-Grünwald-Giemsa solution. The surface expression of CD38 was determined by monoclonal antibody labeling and flow cytometry. In the colony-forming assay, K562 or KU812 cells ($3x10^3$ per dish) were plated into 2 ml of a semi-solid medium with 0.8% methylcellulose and 20% fetal bovine serum in multiwell plates for 7-14 days. A solution of 0.1 ml of phosphate-buffered salin (PBS) containing various concentrations of drugs was added to the semi-solid medium. To determine the colony-forming ability of leukemia cells from CML patients, heparinized bone marrow aspirations were diluted with culture medium, overlaid on 15 ml of Ficoll-Paque Plus and centrifuged at 500 x *g* for 30 min. The mononuclear cells were washed twice and suspended in culture medium, plated into semi-solid culture medium with 20% serum at 10^5 cells/dish, and incubated at 37˚C in a humidified atmosphere of 5% CO_2 in air. For cumulative cell number assay, CML cells ($2x10^4$/ml) were cultured in medium with the test compounds. Thereafter, the cell density of the treated cells was kept at 1-$8x10^5$/ml to maintain the growth phase. The medium of treated cultures was replaced with fresh medium with or without the test compounds at least every 7 days. The cumulative cell number was calculated from the cell counts and the dilution used when feeding the culture. Equal amounts of protein (10 μg) were separated by SDS/PAGE (10% gels) prior to transfer to polyvinylidene fluoride membranes, and then blocked with Block Ace for 60 min at room temperature. The membranes were then immunoblotted with anti-p21, anti-cMyc and anti-β-actin antibodies. Horseradish peroxidase (HRP)-conjugated antibody was used as a secondary antibody. The bands were developed by treatment with the Immun-Star HRP Chemiluminescent kit for 5 min at room temperature. Total RNA was extracted from CML cells and was converted to first-strand cDNA primed with random hexamer in a reaction volume of 20 μl using an RNA PCR kit. The quantitative RT-PCR reaction was performed using a real-time PCR system.

The study protocol was approved by the Ethics Committee of Shimane University and written informed consent was obtained from all subjects.

RESULTS AND DISCUSSION

KU812 cells were treated with IM for 14 days, washed with PBS, resuspended in drug-free culture medium, and cultured for an additional 7 days. While continuous IM treatment effectively suppressed proliferation, depletion of IM from the medium allowed the cells to re-grow, indicating that IM did not completely block the ability of the cells to repopulate. We

then examined the effects of several agents that are known to induce differentiation of AML cells on the proliferation of CML cells. CN-A was the most potent at inhibiting cell proliferation for 14 days, suggesting that CN-A effectively suppressed the self-renewal ability of these cells. CN-A and IM cooperatively inhibited cell proliferation. Similar results were observed when the cells were treated with CN-A plus other TKIs such as dasatinib and nilotinib. These compounds also exerted combined effects on clonogenic activity of the cells. The colony-forming ability of K562 cells was markedly inhibited by treatment with CN-A alone at a concentration of 4 μg/ml, and IM cooperatively inhibited the colony-forming ability of the cells treated with CN-A. The effectiveness of combined treatment with CN-A and IM on primary CML cells was examined and compared with that of treatment with either IM or CN-A alone. CN-A significantly inhibited colony formation of leukemia cells from CML patients. Although IM alone did not significantly suppress colony formation, it significantly enhanced the inhibitory effects of CN-A on colony formation.

Since CN-A is a potent inducer of the differentiation of AML cells, we examined the effects of CN-A on differentiation-associated phenotypes of K562 and KU812 cells. CN-A induced morphological changes in these cells, whereas ATRA, AraC or IM alone did not. The enlargement of the cytoplasm, decreased cytoplasmic basophilicity and compact nuclei were observed in the CN-A-treated cells. Since CD38 is a marker of hematopoietic committed progenitor and more differentiated cells, we examined the expression of CD38 in the CN-A-treated cells. CN-A at concentrations as low as 0.5 μg/ml efficiently converted the KU812 cells from $CD38^-$ to $CD38^+$. IM did not affect CD38 expression even in the presence of CN-A.

A previous study indicated that the dual targeting of p53 and c-Myc selectively eliminated CML stem cells. Since p21 is a target gene of p53, we examined p21 expression as a marker of the p53 signal transduction pathway. CN-A effectively decreased c-Myc protein level and increased p21 protein level in KU812 cells, while ATRA did not affect c-Myc or p21 protein level. IM did not affect protein levels of p21 or c-Myc in KU812 cells, even in the presence of CN-A. Next, we examined the effect of CN-A on c-Myc mRNA expression in KU812 cells. The downregulation of c-Myc mRNA by CN-A was observed within 6 hours, suggesting that it is an early event in the action of CN-A. Neither IM nor ATRA affected mRNA expression.

CONCLUSION

CN-A effectively suppressed the self-renewal of CML cells and combination with CN-A and TKI resulted in significantly greater inhibition of cell growth than that with TKI or CN-A alone. These results suggest that CN-A may have potential to promote the elimination of stem cells in CML.