別紙様式第４

学　位　論　文　要　旨

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[論文題名]

The HDAC inhibitor, SAHA, combined with cisplatin synergistically induces apoptosis in alpha-fetoprotein-producing hepatoid adenocarcinoma cells

HDAC阻害剤SAHAとシスプラチンはα-フェトプロテイン産生肝様腺癌のアポトーシスを相乗的に誘導する。

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[要　旨]

Introduction

Hepatoid adenocarcinoma (HAC) is a rare, aggressive cancer that is characterized by hepatic differentiation and alpha-fetoprotein (AFP) production. Clinically, HAC shows aggressive progression and a strong tendency to metastasize to lymph nodes and liver; and AFP production promotes neovascularization, high proliferative activity, and inhibition of apoptosis, which contribute to the poor prognosis. Currently, treatment for HAC is surgical resection followed by adjuvant chemotherapy; however, the efficacy is poor. Therefore, development of a novel treatment strategy for HAC is essential.

Both genetic and epigenetic changes, including DNA methylation and histone modification, play essential roles in initiation and progression of HAC. HDAC inhibitors (HDACi) are emerging as a new class of anticancer drugs. The HDACi, suberoylanilide hydroxamic acid (SAHA), was recently used to treat patients with relapsed cutaneous T-cell lymphoma, and has synergistic effects when combined with chemotherapeutic drugs, such as cisplatin. Epigenetic changes such as altered histone acetylation occur in AFP-producing cancers, which suggests that HAC could be treated with SAHA. However, the effects of SAHA in HAC are largely unknown.

Materials and Methods

VAT-39 cells, a newly established HAC cell line was used in this study. The cells were then treated with cisplatin (2 or 5 μM) or SAHA (1 or 2 μM) alone or with a combination of cisplatin and SAHA for 48 h. Cell viability and apoptosis were examined by MTT assay, flow cytometry and TUNEL assay. The expression of phosphorylated H3S10, cleaved caspase-3, and H3K9ac, H3K14ac, H3K18ac, H3K27ac, Bax, Bcl-2, AFP were examined by immunohistochemistry and western blotting, respectively. AFP concentration in culture media was measured with Architect AFP.

Results

Cell viability was examined by MTT assay to evaluate the antiproliferative effects of cisplatin and SAHA. Both drugs significantly decreased VAT-39 cell viability in a dose-dependent manner. Importantly, cisplatin in combination with SAHA decreased cell viability more efficiently than either treatment alone. Combinations of 2 μM cisplatin and 1 μM SAHA and 5 μM cisplatin and 2 μM SAHA decreased cell viability by 21.0±6.5% and 43.9±4.0%, respectively. Phosphorylated H3S10, a marker of cell mitosis, was also significantly decreased following combined treatment with cisplatin and SAHA compared to either treatment alone. These results indicate that cisplatin and SAHA have a synergistic effect in inhibiting proliferation of VAT-39 cells.

The effects of cisplatin and SAHA on acetylation of histone H3 in VAT-39 cells were evaluated by western blotting. SAHA increased acetylation of H3K9, H3K14, H3K18, and H3K27 dose-dependently, but cisplatin had no such effects. These results show that a low concentration of SAHA (1-2 μM) was sufficient to induce histone H3 hyperacetylation. Based on these results, the combination dose of 5 μM cisplatin and 2 μM SAHA was used for further experiments.

To analyze cell death, flow cytometry was performed to detect apoptotic and necrotic cells. Compared to control cells, the number of apoptotic cells was 2.2 times higher in cisplatin-treated cells, and 3.3 times higher in cells treated with cisplatin and SAHA in combination. There were no differences in the number of necrotic cells in all groups. Immunohistochemistry showed significantly increased cleaved caspase-3 expression in cisplatin and SAHA-treated cells, with a 12 times increase in cleaved caspase-3-positive cells compared to that in control cells. Western blotting confirmed these findings, including an increased cleaved caspase-3 level in cisplatin and SAHA-treated cells. Apoptosis was confirmed in a TUNEL assay. The number of TUNEL-positive cells was increased by cisplatin or SAHA alone compared to controls, but there was a marked increase in the number of TUNEL-positive cells in combination treatment with cisplatin and SAHA. These findings suggest that cisplatin and SAHA synergistically induce apoptosis in VAT-39 cells.

To investigate the mechanism of the induced apoptotic pathway, Bax and Bcl-2 expression was examined in cisplatin and SAHA-treated VAT-39 cells. A significant decrease in Bcl-2 expression was found in cells treated with the combination of cisplatin and SAHA, whereas Bax expression was unchanged in all groups. Densitometry showed that the Bax/Bcl-2 ratio was increased by 3 times in cells treated with cisplatin and SAHA in combination compared to control cells.

AFP production is the main characteristic of HAC cells. In control cells, strong expression of AFP was observed in the perinuclear area, which indicates AFP production. Decreased AFP expression was seen in cells treated with cisplatin alone or SAHA alone, and most VAT-39 cells lost AFP production with combined treatment with cisplatin and SAHA. The amount of secreted AFP was measured in supernatants of culture media. Since the decrease in AFP

production could be due to the decrease in cell number after cisplatin and SAHA treatment, western blotting analysis was performed to determine the relative amounts of AFP production in the groups. Collectively, these results indicate that VAT-39 cells have partial loss of AFP production after combined treatment with cisplatin and SAHA.

Discussion

In this study, we found a synergistic anticancer effect of apoptosis induction in combined treatment with cisplatin and SAHA in HAC cells. AFP production, a major characteristic of HAC, was markedly decreased after the combination treatment. These results indicate that cisplatin in combination with SAHA may be effective for treatment of HAC, an aggressive cancer with a poor prognosis.

SAHA is a pan-HDAC inhibitor that may open the chromatin structure and increase accessibility of cisplatin to DNA in cancer cells. Hyper- and hypoacetylation of histones are generally correlated with gene transcription that regulates many cellular processes. Acetylation at histone H3K9, H3K14, H3K18 and H3K27 is specifically associated with transcriptional activation of genes involved in apoptosis. Our western blotting results revealed that SAHA induces these hyperacetylation events, and this may support induction of apoptosis by cisplatin. Synergistic effects were observed at low doses of both cisplatin (5 μM) and SAHA (2 μM). Cisplatin is effective at a high dose, but patients experience side effects that include drug resistance, allergic reactions, and functional alteration of the gastrointestinal and urinary tracts. Therefore, our findings may be helpful for development of efficient anticancer therapy with minimal side effects.

Apoptotic cells, but not necrotic cells, significantly increased after combined treatment with cisplatin and SAHA. Bax/Bcl-2 ratio is an important indicator of apoptosis. In our findings, this ratio increased 3 times in VAT-39 cells treated with cisplatin and SAHA compared to control cells, suggesting that this treatment affects the mitochondrial apoptosis pathway in VAT-39 cells.

Expression and production of AFP in VAT-39 cells were significantly decreased after combined treatment with cisplatin and SAHA. AFP is a well-known marker of cancer with hepatoid differentiation, neovascularization and high proliferative activity. A high metastasis rate, aggressive phenotype, and poor prognosis are also related to AFP production in cancer cells. Thus, addition of SAHA to this chemotherapy may be effective.

The current study has the limitation of use of a single HAC cell line, but promising anticancer effects were obtained by combined treatment with cisplatin and SAHA. These drugs act synergistically to induce apoptosis of HAC cells, and the characteristic AFP production of these cells was markedly decreased after the combination treatment.

備考　論文要旨は、和文にあっては 2，000 字程度、英文にあっては 1，200 語程度