主論文の要旨

Anti-tumor effects of suberoylanilide hydroxamic acid on Epstein-Barr virus-associated T cell and natural killer cell lymphoma

（EB ウイルス関連 T/NK リンパ腫における
スベロイラニリド・ハイドロザミック酸の抗腫瘍効果）

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Introduction

More than 90% of the world population is infected by the Epstein–Barr virus (EBV), which is an oncogenic γ-herpesvirus. Not only B cells but also T cells and natural killer (NK) cells can be infected by EBV. For the treatment and prophylaxis of B cell lymphoma and lymphoproliferative disorders, rituximab, a humanized monoclonal antibody (Ab) against CD20, targets B cell-specific surface antigens and has been used with marked success. However, novel approaches to molecular targeted therapy are required to effectively treat T and NK cell malignancies. Histone deacetylase (HDAC) inhibitors induce acetylation of histones, thus affecting transcription, and selectively induce tumor-suppressive genes. Suberoylanilide hydroxamic acid (SAHA) is an FDA approved HDAC inhibitor, and its efficacy has been confirmed by clinical trials for malignant diseases such as non-Hodgkin lymphoma, acute myeloid leukemia, breast cancer, and cutaneous T cell lymphoma. In the present study, we evaluate the antitumor effects of SAHA on EBV-positive and -negative T and NK cell lines and analyze induction of apoptosis and the expression of EBV-encoded genes and proteins. We also evaluate the effect of SAHA on an *in vivo* xenograft model of NOG (NOD/ Shi-scid/IL-2Rγ^{null}) mouse, which is completely immunodeficient and lacks T, B, NK, and dendritic cells, as well as macrophages.

Methods

EBV-positive and -negative T and NK cell lines were treated with various concentrations of SAHA. The cell number and viability were quantified by trypan blue exclusion. Apoptosis was measured by flowcytometry. Acetyl-histone H3, poly (ADP-ribose) polymerase (PARP), latent membrane protein (LMP) 1, and EBV nuclear antigen (EBNA) 1 were identified by immunoblotting. Viral mRNA expression was quantified by one-step multiplex real-time RT-PCR. *In vivo* effects were confirmed using the mouse xenograft model. EBV-encoded small RNA (EBER) was detected by *in situ* hybridization (ISH).

Results

Effect of SAHA on the viability of T and NK cell lines

EBV-positive and -negative T and NK cell lines were cultured with various concentrations of SAHA. SAHA increased acetylated histone H3 levels, confirming that SAHA worked as an HDAC inhibitor (Fig. 1A). SAHA reduced the viability of all treated cell lines in a dose-dependent manner (Fig. 1B). Next, the same six cell lines were treated with 5 μM SAHA and assessed at different time points. The viability of all six cell lines was reduced by treatment with SAHA for 96 h (Fig. 1C). The effects of SAHA did not differ between EBV-positive and EBV-negative cell lines.

Effects of SAHA on apoptosis of T and natural killer cell lines

To determine whether apoptosis was induced by SAHA in the tested cell lines, early apoptotic cells were quantified by annexin V and 7-AAD staining. SAHA increased early apoptotic cells in the Jurkat, KAI3, and KHYG1 cell lines (Fig. 2A). In other cell lines, the proportions of early apoptotic cells were not increased. Next, the cleavage of PARP was analyzed by immunoblotting. With the exception of the SNT16 cell line, SAHA induced the cleavage of PARP in the five cell lines (Fig. 2B).

Effects of SAHA on EBV-encoded genes and proteins of EBV-positive T and natural killer cell lines

The expression of eight EBV-related genes, including lytic genes (BZLF1 and gp350／220) and latent genes (EBNA1, EBNA2, LMP1, LMP2, EBER1 and Bam HI-A rightward transcripts [BART]) were analyzed using real-time RT-PCR. In the SNT13, KAI3 and SNK6 cell lines, the expression of BZLF1, which is an immediate-early gene in the lytic infection cycle, was increased by SAHA (Fig. 3A). However, the expression of the late lytic gene gp350／220 was increased only in the SAHA-treated SNT13 cell line. Of the EBV latent genes tested, the expression of EBNA1, LMP1 and BART was decreased in most of the cell lines, whereas that of LMP2 was increased by SAHA (Fig. 3A). Next, the EBNA1 and LMP1 protein levels were determined by immunoblotting. SAHA decreased the EBNA1 protein level in all cell lines, and that of LMP1 in the SNT16, KAI3 and SNK6 cell lines (Fig. 3B).

***In vivo* effects of SAHA using the mouse xenograft model**

After confirmation of the *in vitro* effect of SAHA, we extended our work to an *in vivo* xenograft model. Initially, we inoculated six T and NK cell lines into immunodeficient NOG mice via various routes. Of the EBV-positive T or NK cell lines used, only the SNK6 cell line was engrafted after subcutaneous or intravenous inoculation. Because evaluation of the former was easier, the subcutaneous model was used in subsequent experiments. We subcutaneously inoculated 1x 10^6 SNK6 cells into NOG mice. All of the mice developed tumors at the site of inoculation. Four days after the inoculation, mice were treated with SAHA daily up to day 28. The treated mice normally tolerated SAHA without showing any obvious toxicity. During this period, no significant difference in the body weights of SAHA-treated and control mice was noted. Until the end of the experiment, the size of tumors in SAHA treated mice increased gradually, but the tumor volume was significantly less than the control group (Fig. 4A). Additionally, SAHA-treated mice showed a significantly lower plasma EBV-DNA level (Fig. 4B). Furthermore, SAHA showed significant inhibitory effects on most EBV-encoded genes in tumor tissues (Fig. 4C). Finally, we collected samples from organs at 30 days after inoculation and performed EBER ISH. EBER-positive cells were detected in the organs of control mice, but not SAHA-treated mice (Fig. 4D).

Discussion

HDAC inhibitors affect tumor cell growth and survival through the induction of cell death by their characteristics of apoptosis. HDAC inhibitors can also reduce the expression of proangiogenic factors, resulting in the suppression of angiogenesis. In the present study, SAHA markedly suppressed the proliferation of T and NK lymphoma cell lines, irrespective of the presence of EBV. In several T and NK cell lines, SAHA-induced apoptosis was confirmed by the increase in annexin V-positive cells and cleavage of PARP. The mechanism of killing appeared to differ among the cell lines. SAHA has been reported to induce EBV lytic infection in EBV-positive gastric and nasopharyngeal carcinoma cells. For the treatment of EBV-associated malignant diseases, induction of lytic infection is advantageous because it causes lysis of EBV-infected tumor cells. Furthermore, lytic infection should produce viral proteins with antigenicity that could induce host cellular responses. BZLF1 is an immediate-early gene and a hallmark to switch from latent gene to lytic infection. In the present study, SAHA increased the expression of BZLF1 in most EBV-positive T and NK cell lines, although the late lytic gene gp350／220 was increased in only one cell line. SAHA also decreased the expression of the LMP1 gene and protein in some EBV-positive T and NK cell lines. LMP1 is a major oncoprotein that is responsible for the immortalization of primary human B lymphocytes and activation of the NF-κB, PI3K, and JNK pathways. Expression of LMP1 induces several pleiotropic effects, including the upregulation of adhesion molecules, anti-apoptotic proteins and cytokines. SAHA also decreased the expression of EBNA1 in all of the cell lines. EBNA1 is essential for the maintenance of the viral episome, as well as for the initiation of latent viral replication. EBNA1 also plays an important role in inhibiting apoptosis. Downregulation of EBNA1 may also be associated with the suppressive effect on the proliferation of EBV-positive T and NK cell lines. We applied the murine xenograft model to further evaluate the efficacy of SAHA. Using this model, we have shown that SAHA prevented not only tumor growth but also metastasis of EBV-positive NK cell lymphoma.

Conclusion

SAHA suppressed the proliferation of T and NK cell lines, although no significant difference was observed between EBV-positive and EBV-negative cell lines. SAHA induced apoptosis in T and NK cell lines. Furthermore, SAHA inhibited tumor progression and metastasis in a murine xenograft model. Thus, SAHA had a marked suppressive effect against EBV-associated T and NK cell lymphomas, which were mediated by the induction of apoptosis and could represent an alternative treatment.