

Simvastatin Induces Anti-Tumor Effects on Systemic CAEBV

In Vitro and In Vivo

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Abstract

Background: Systemic chronic active Epstein-Barr virus infection (sCAEBV) is a rare and fatal lymphoid neoplasm characterized by sustained systemic inflammation and clonal proliferation of EBV-infected T or NK cells. Because the mechanisms responsible for the development of the disorder have not been elucidated to date, its optimal chemotherapy has not been established, and the prognosis of sCAEBV has remained very poor. We previously found and reported that simvastatin, an inhibitor of HMG-CoA reductase, inhibits prenylation of intracellular signal-mediating molecules in adult T-cell lymphoma/leukemia cells. On the basis of these findings, we hypothesized that simvastatin could also induce apoptosis in EBV-positive T or NK cells from sCAEBV.

Methods: We examined two clinical samples of EBV-positive T-cell lines derived from sCAEBV, SNT8 and SNT15. The samples were obtained from six patients with sCAEBV. The *in vitro* effects of simvastatin on the cells were examined by XTT and Annexin V assay, and the *in vivo* effects of simvastatin on sCAEBV were examined in xenograft models of sCAEBV generated from NOD/Shi-*scid*/IL-2R γ^{null} mice.

Results: Simvastatin suppressed proliferation and induced apoptosis in the cell lines in a dose-dependent manner. Simvastatin also suppressed proliferation and induced apoptosis of peripheral blood mononuclear cells derived from patients with sCAEBV with infected cell types of CD4 in 3 patients, CD8 in 2 patients, and CD56 in 1 patient. The presence of farnesyl pyrophosphate, one of the intermediates in the mevalonate pathway, restored the number of viable SNT8 and SNT15 cells treated with simvastatin. Furthermore, oral administration of simvastatin reduced EBV-DNA in the peripheral blood of sCAEBV xenograft models.

Conclusions: Simvastatin may be an attractive reagent for sCAEBV.

Key words: Systemic chronic active Epstein-Barr virus infection, proliferation, apoptosis, simvastatin, xenograft model

Introduction

Systemic chronic active Epstein-Barr virus in-

fection (sCAEBV) is a rare and fatal lymphoid neoplasm characterized by sustained systemic inflammation and clonal proliferation of EBV-infected T or

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NK cells¹⁾. sCAEBV is defined in the most recent World Health Organization (WHO) classification revised in 2017 (WHO2017) as an EBV-positive T- or NK-cell lymphoproliferative disease²⁾. Its mechanisms of development have not been elucidated to date, and consequently, optimal chemotherapy has not been established for it. The prognosis of sCAEBV remains very poor. Although hematopoietic stem cell transplantation (HSCT) is the only effective treatment strategy for now³⁾, not all patients with sCAEBV can receive transplantation because many cases are accompanied by organ damage that is too severe.

Simvastatin is an inhibitor of HMG-CoA reductase. In lymphoblastoid cell lines, it dissociates a viral protein called EBV latent membrane protein 1 (LMP1) from lipid rafts of the cell membrane and suppresses LMP1-mediating intracellular molecular signals⁴⁾. As LMP1 is indispensable for EBV-induced transformation, the effects of simvastatin on LMPs lead to apoptosis in EBV-positive neoplastic B-cell lines. Furthermore, we previously found that simvastatin induces apoptosis by prenylation of anti-apoptotic protein AKT in adult T-cell lymphoma/leukemia cells⁵⁾. On the basis of these findings, we hypothesized that simvastatin might also induce apoptosis in EBV-positive T or NK cells from sCAEBV. We investigated the effects of simvastatin on EBV-positive T- or NK-cell lines established from cell lines of patients with sCAEBV, primary cells of patients with sCAEBV and xenograft models of sCAEBV.

Materials and Methods

Cells and reagents

Lymphoblastoid cell line was cultured in RPMI containing 10% fetal calf serum (10% FCS-RPMI). EBV-infected T- or NK-cell (EBV-T/NK cells) lines SNT8 and SNT15 were cultured in Artemis Medium-2 manufactured by Nihon Techno Service Co., LTD. (Ushiku, Japan). SNT8 and SNT15 were established from EBV-positive lymphoproliferative diseases⁶⁾. Simvastatin was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). Simvastatin was activated by dissolving in ethanol and treating with 0.1 M NaOH at 50°C for 2 h. The pH was then adjusted to 7.0 with HCl. Farnesyl pyrophosphate (FPP) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

Diagnosis of sCAEBV

sCAEBV was diagnosed based on the criteria suggested by a research group of Measures Against

Intractable Diseases in the Ministry of Health, Labour and Welfare of Japan, which conform to the definition of CAEBV in WHO2017²⁾:

(1) elevated EBV-DNA load in peripheral blood (PB) ($>10^{2.5}$ copies/ μ g DNA);

(2) EBV infection of T or NK cells (see below) in the affected tissues or PB;

(3) systemic inflammatory symptoms (such as fever, lymphadenopathy, liver dysfunction, progressive skin lesions, vasculitis, and uveitis) persisting for more than three months; and

(4) exclusion of other possible diseases: primary infection of EBV (infectious mononucleosis), autoimmune disease, congenital immunodeficiency, HIV, and other immunodeficiencies or underlying diseases with potential immunosuppression.

Patients who fulfilled all four criteria were diagnosed as having sCAEBV. Patients who were pathologically diagnosed with extranodal NK/T-cell lymphoma, nasal type (ENKL), aggressive NK-cell leukemia (ANKL), or peripheral T-cell lymphoma (PTCL) simultaneously or prior to diagnosis with sCAEBV were excluded.

Detection of EBV-infected cells in patients with sCAEBV

The detection and isolation of infected cells were performed as described in our previous study⁷⁾. In brief, peripheral blood mononuclear cells (PB-MCs) from patients were isolated by density gradient centrifugation using Separate-L[®] (Muto Pure Chemical Co., Ltd., Tokyo, Japan) and sorted into CD19-, CD4-, CD8-, or CD56-positive fractions using antibody-conjugated magnetic beads (Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany). The EBV-DNA level in each fraction was measured by real-time RT-PCR using a TaqMan[®] system (Thermo Fisher Scientific Inc., Waltham, MA, USA). The fraction with the highest titer was determined to contain infected cells. The clonality of infected cells was examined by Southern blotting.

XTT assay

The XTT assay was performed with the sodium 3V-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)-benzene sulfonic acid hydrate (XTT) colorimetric method by using a Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's instructions. The kit was used to measure cellular metabolic activity as an indicator of viable cells.

Annexin assay

Annexin V apoptosis detection assay was performed with a TACS[®] Annexin V-FITC apoptosis detection kit (Bio-Techne Corporation, Minneapolis, MN, USA) according to the manufacturer's instruction.

Generation of xenograft model

The generation of an NOD/Shi-*scid*/IL-2R γ^{null} (NOG) mouse model was as described in our previous study⁸⁾. In brief, male NOG mice were purchased from Central Institute for Experimental Animals (Kawasaki, Japan) and maintained under pathogen-free conditions. The models were generated by injecting PBMCs from patients with sCAEBV into 6-week-old mice via the tail vein. The establishment of the models was confirmed by detecting EBV-DNA load in the PB of the mice.

In vivo effect of simvastatin on sCAEBV

Simvastatin and ddH₂O were administered orally to mice six days a week for two weeks. The EBV-DNA titer in their PB was measured by RQ-PCR as described in our previous study⁹⁾. Tribromoethanol anesthesia was administered intravenously to minimize suffering. After the experiment, mice were euthanized via CO₂ inhalation and subjected to analysis.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, USA). The significance was calculated by Student *t*-test.

Ethics statement

The study complied with the Declaration of Helsinki and was approved by the ethical committees of Tokyo Medical and Dental University (TMDU) (G2000-176) and St. Marianna University School of Medicine (4709). Written informed consent was obtained from each patient. The experiments with the NOG mice were performed in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science and the guidelines of Animal Research: Reporting of In Vivo Experiments¹⁰⁾. The experiments with the xenograft models were approved by the Institutional Animal Care and Use Committees of TMDU (A2018-293C5) and performed at TMDU.

Results

Simvastatin suppressed proliferation and induced apoptosis in EBV-positive T or NK cells

First, we investigated the effects of simvastatin on two cell lines, SNT8 and SNT15, established from sCAEBV. As shown in **Figure 1A**, simvastatin suppressed proliferation of the cell lines in a dose-dependent manner. We also investigated the effects of simvastatin on apoptosis of the cell lines. Simvastatin induced apoptosis in SNT8 and SNT15 in a dose-dependent manner (**Figure 1B, C**).

Next, we examined the effects on EBV-positive cells from patients with sCAEBV. The characteristics of these patients are shown in **Table 1**. sCAEBV was diagnosed according to the criteria mentioned in the Materials and Methods section. We investigated six patients (three males and three females aged 24 to 64 years old) with sCAEBV. Five were of T-cell type, and one was of NK-cell type: CD4 type, n=3; CD8 type, n=2; and CD56 type, n=1. sCAEBV patient-derived PBMCs were used because they contain EBV-positive T or NK cells. As in the cell lines, simvastatin suppressed the proliferation of patients' cells in a dose-dependent manner (**Figure 2A**). We performed apoptosis assay using the PBMCs of Cases 2, 3, and 5, of which the number of cells were enough to perform the assay. Simvastatin induced apoptosis in the cells in a dose-dependent manner (**Figure 2B**). These results indicated that simvastatin could suppress the proliferation of and induce apoptosis in EBV-T/NK cells of sCAEBV.

Simvastatin suppressed the survival of sCAEBV cell lines by inhibiting the mevalonate pathway

The mevalonate pathway is shown in **Figure 3A**. Statins reduce the isoprenylation of proteins such as PI3K, Akt and small guanosine triphosphatases (GTPases) by blocking the synthesis of farnesyl and geranyl isoprenoids¹¹⁾. These proteins mediate survival-promoting molecular signals, and their constitutive activation plays a role in the development of malignant neoplasms. The activated forms of the proteins are localized to the cell membrane. Prenylation of these proteins with farnesyl or geranylgeranyl groups is essential for their localization and for their biological functions¹²⁾. Therefore, we investigated whether sCAEBV cells treated with simvastatin would restore viability in the presence of FPP, one of the intermediates in the mevalonate pathway. As shown in **Figure 3B**, adding FPP restored the number of viable SNT8

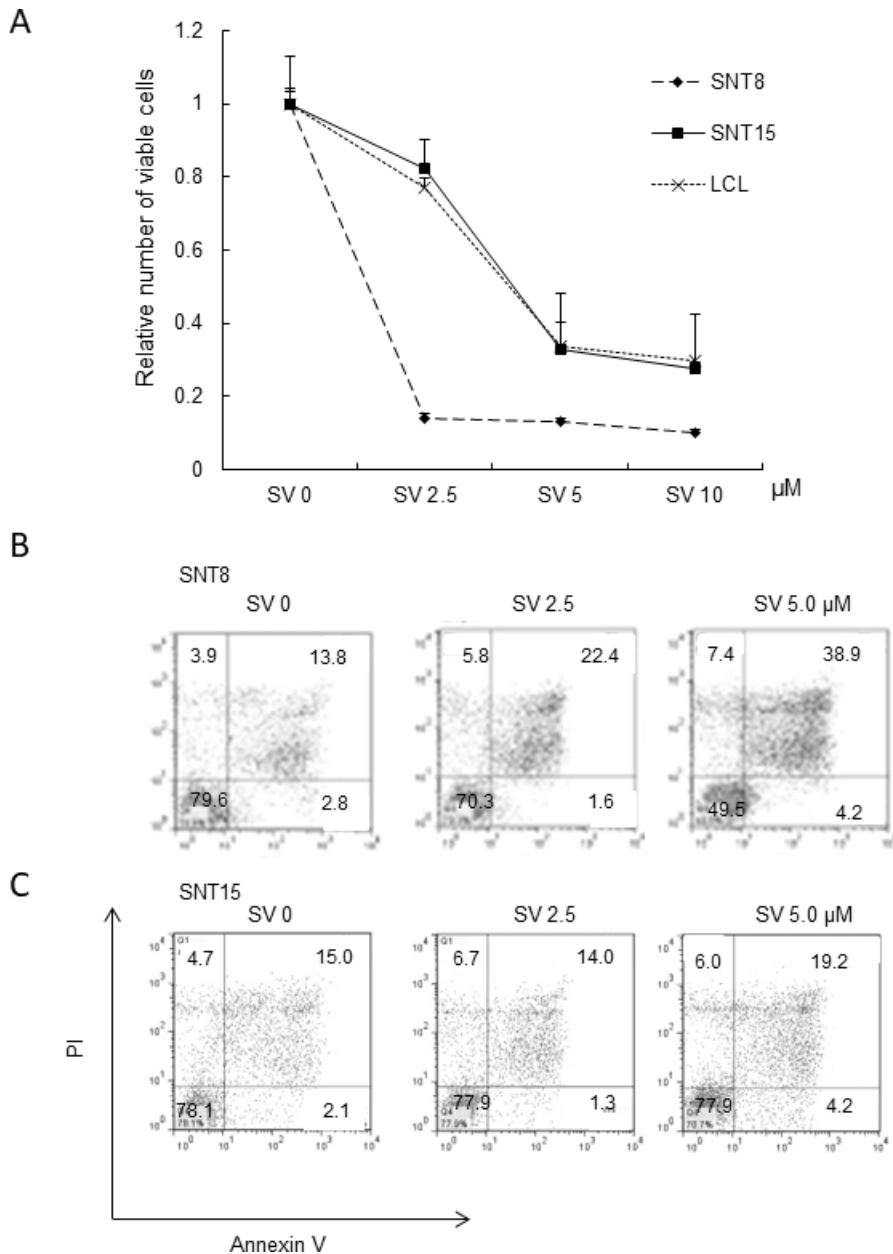


Figure 1. Simvastatin (SV) suppressed survival and induced apoptosis in EBV-positive cell lines from systemic chronic active Epstein-Barr virus infection

(A) Cells from EBV-positive T-cell lines SNT8 and SNT15 were treated with SV for 48 hours, and the number of viable cells was estimated by XTT assay and expressed in arbitrary units. An EBV-negative T-cell line, Jurkat cells and lymphoblastoid cell line (LCL) were used as positive controls. The data represent the mean \pm S.D. of three independent experiments. (B) SNT8 and (C) SNT15 cells were treated with SV for 48 hours and subjected to Annexin V apoptosis assay.

Table 1. Characteristics of Patients

Case	Infected cell	Age (years)	Sex	Symptoms	EBV-DNA (copies/ μ g DNA) of the EBV-infected cells in PB
1	CD4	33	M	HMB, fever, liver dysfunction	1.8×10^4
2	CD4	28	F	HMB, HLH	1.5×10^5
3	CD4	64	F	Fever, liver dysfunction	6.4×10^5
4	CD8	63	F	Fever, liver dysfunction	1.7×10^4
5	CD8	28	M	Fever, liver dysfunction	4.1×10^5
6	CD56	24	M	Fever, liver dysfunction	4.0×10^4

M, male; F, female; HMB, hypersensitivity to mosquito bites; HLH, hemophagocytic lymphohistiocytosis; EBV, Epstein-Barr virus; PB, peripheral blood.

and SNT15 cells treated with simvastatin. These results suggested that simvastatin suppressed the survival of sCAEBV cell lines by inhibiting the mevalonate pathway.

Simvastatin showed in vivo effects on sCAEBV xenograft model

Finally, we validated the results in xenograft models of NOG mice. The models were generated by transplanting PBMCs from two patients with sCAEBV, Cases 4 and 6, whose PBMCs could be obtained for transplantation, to NOG mice as described in the Materials and Methods. Mouse 1 and 2 were generated by the transplantation of PBMCs from Case 4. Mouse 3 to 6 were generated by the transplantation of PBMCs from Case 6. After transplantation of the PBMCs, engraftments were confirmed by detecting the EBV genome in the PBMCs. Simvastatin and ddH₂O were administered orally to Mouse 1 to 3 and Mouse 4 to 6, respectively, as described in the Materials and Methods. As shown in **Figure 4A**, simvastatin significantly reduced the EBV-DNA load in the PB of sCAEBV xenograft models. We were able to obtain histological specimen from Mouse 3 and Mouse 4. **Figure 4B** and **C** show the spleen of the mice. The infiltration of EBV-infected cells was

detected in the spleen of Mouse 4 treated with ddH₂O (**Figure 4B**). However, as shown in **Figure 4C**, the number of EBV-infected cells drastically decreased in Mouse 3 treated with simvastatin. These results indicate that simvastatin had effects on sCAEBV *in vivo*.

Discussion

Our results showed that simvastatin induced growth inhibition and apoptosis in sCAEBV cells, and, moreover, reduced the viral load of EBV-DNA in PB of the xenograft models. We recently reported the effects bortezomib, a proteasome inhibitor, on sCAEBV xenograft models¹³. The mice with significantly decreased EBV-DNA load in PB clearly showed reduced infiltration of EBV-positive cells in the liver. In sCAEBV, EBV-DNA load reflects the status of the disease¹⁴. We also reported that EBV-DNA load in PB of patients with sCAEBV decreases after HSCT¹⁵. From these findings, we assume that EBV-DNA may reflect the status of sCAEBV even in xenograft models. Thus, our results indicate that simvastatin may have anti-tumor effects on sCAEBV not only *in vitro* but also *in vivo*.

Mouse 1 and 2 were generated by transplanting PBMCs from Case 4. Mouse 3 to 6 were generated by transplanting PBMCs from Case 6. Simvastatin

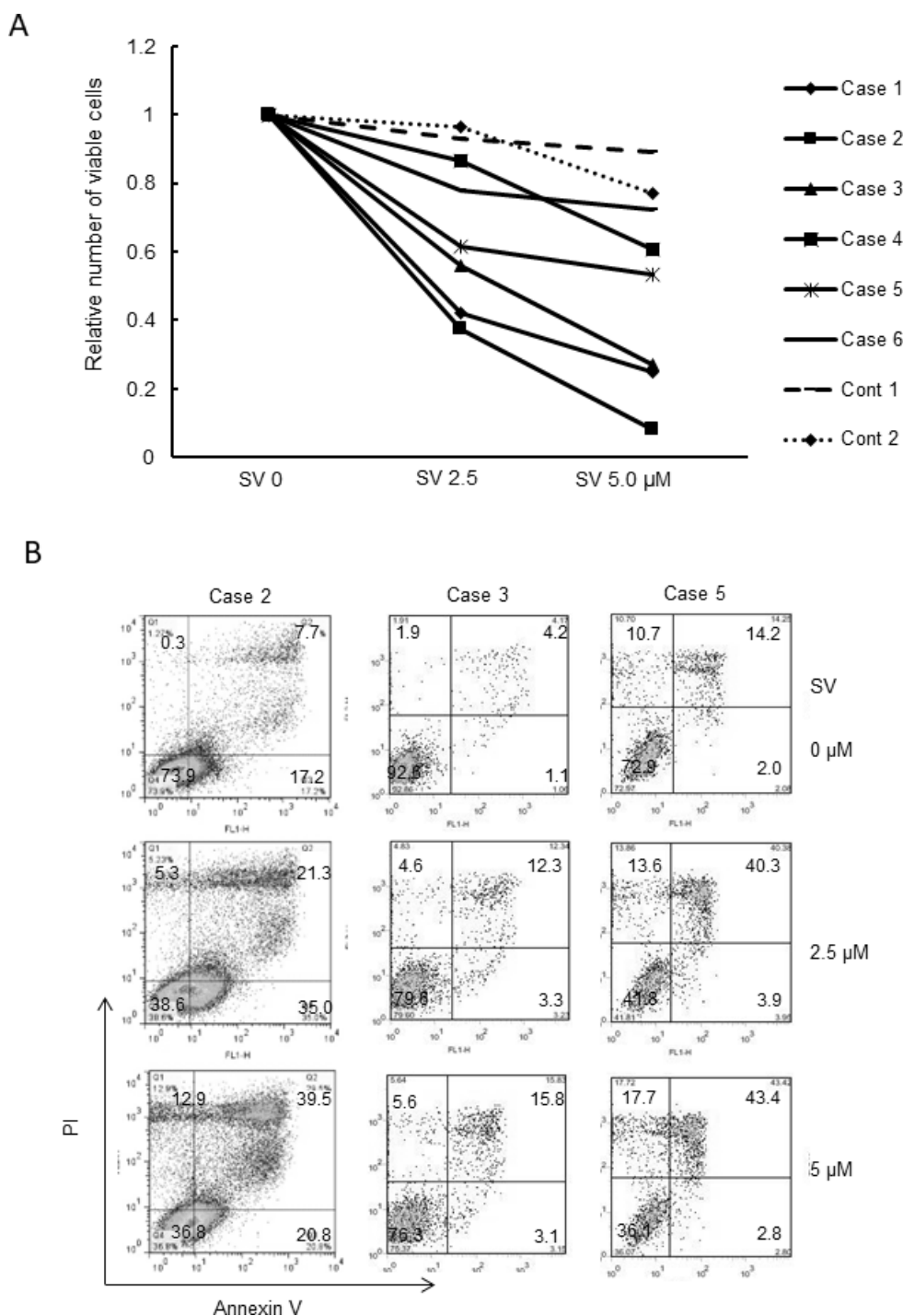


Figure 2. Simvastatin (SV) suppressed survival and induced apoptosis in peripheral blood mononuclear cells (PBMCs) from patients with systemic chronic active Epstein-Barr virus infection (sCAEBV)

(A) PBMCs derived from patients with sCAEBV were treated with SV for 48 hours. The number of viable cells was estimated by XTT assay and expressed in arbitrary units. PBMCs from healthy individuals were used as negative controls. The data represent the mean \pm S.D. of three independent experiments. (B) PBMCs derived from patients with sCAEBV were treated with SV for 48 hours and subjected to Annexin V apoptosis assay.

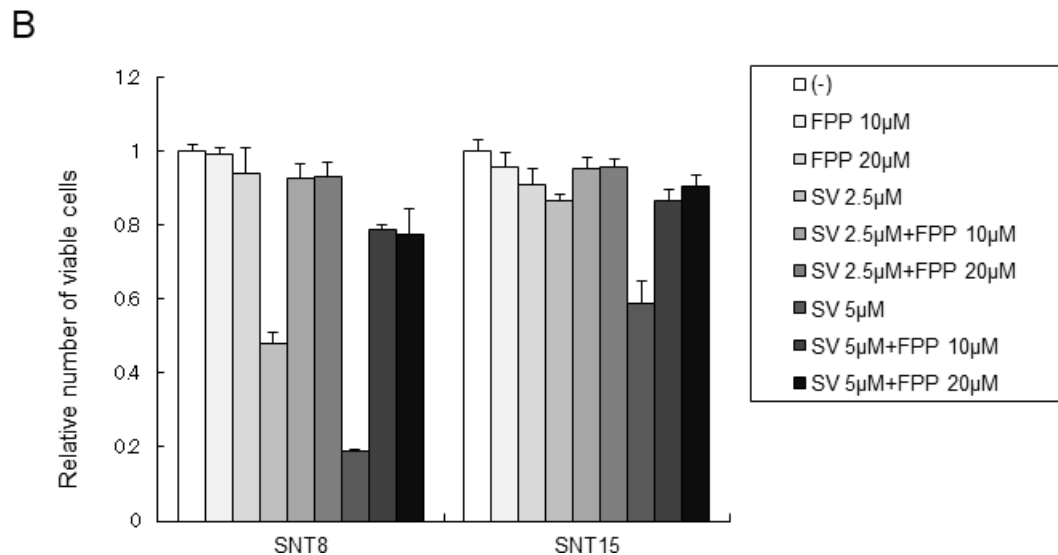
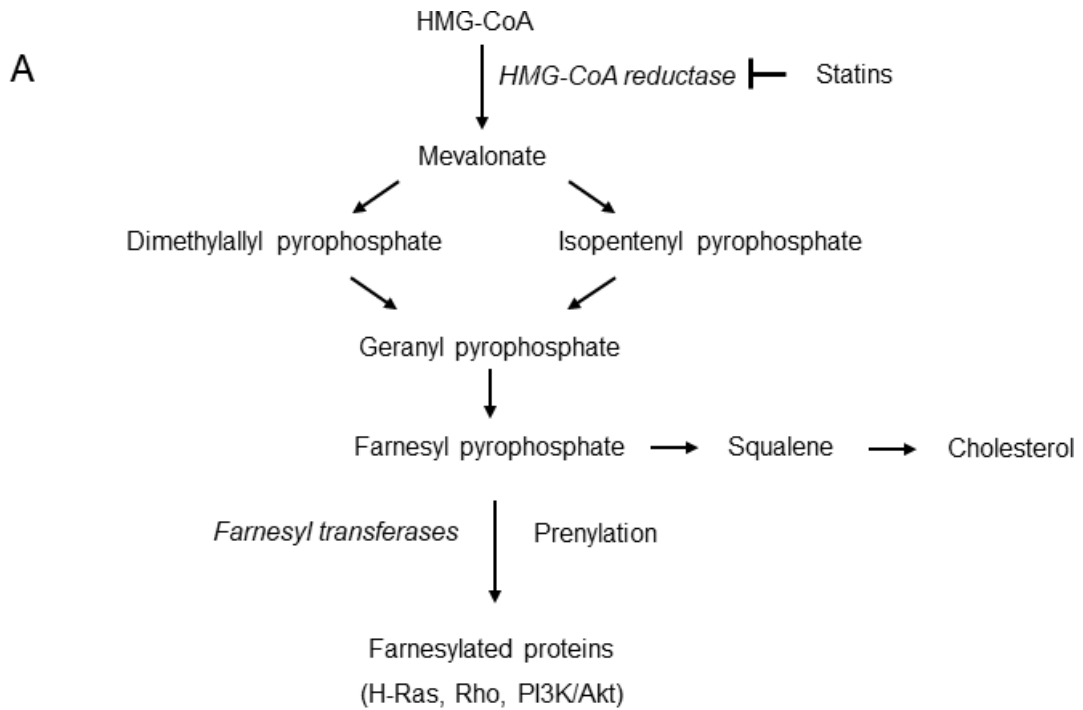
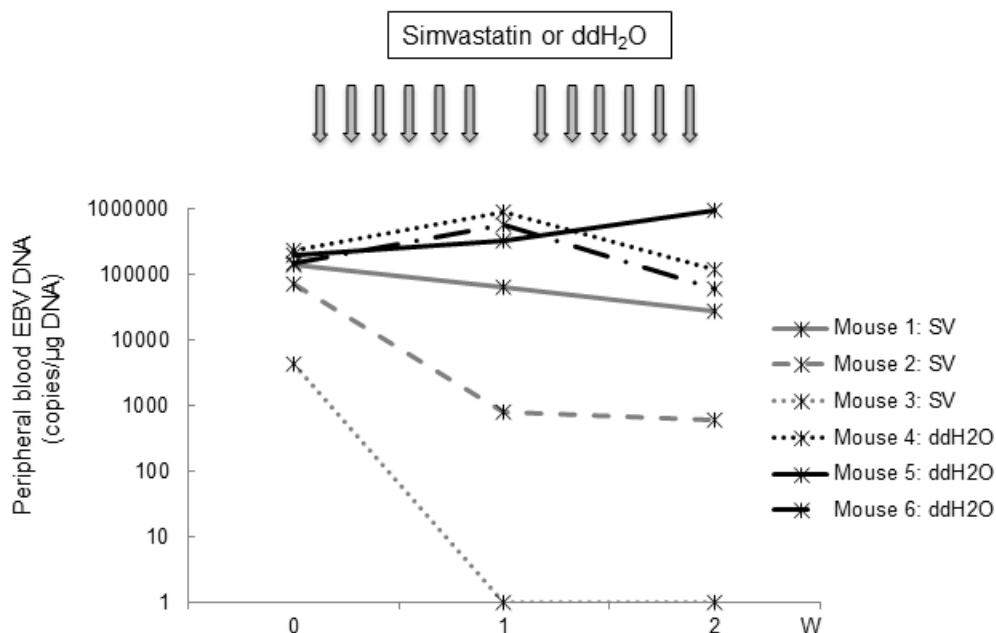


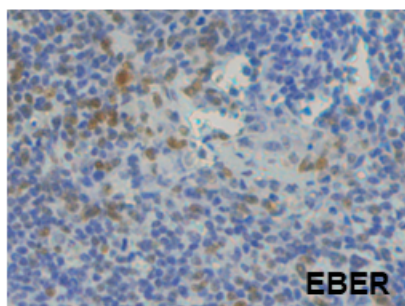
Figure 3. Simvastatin (SV) suppressed the survival of cell lines with systemic chronic active Epstein-Barr virus infection by inhibiting the mevalonate pathway

(A) The representative metabolites in the mevalonate pathway. Cells from EBV-positive T-cell lines SNT8 (B) and SNT15 (C) were treated with SV with or without farnesyl pyrophosphate (FPP) for 48 hours. The viable cell number was estimated by XTT assay and expressed in arbitrary units. The data represent the mean \pm S.D. of 3 independent experiments.

A



B



C

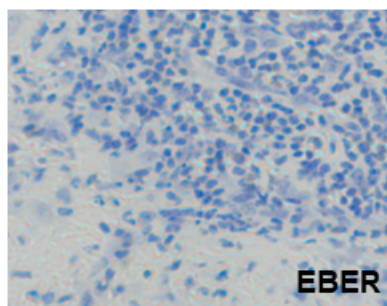


Figure 4. In vivo effect of simvastatin (SV) in xenograft mice with systemic chronic active Epstein-Barr virus (EBV) infection

(A) EBV-DNA load in peripheral blood (PB) of the mice. Mice 1 — 3 and Mice 4 — 6 were treated with SV and ddH₂O, respectively. EBV DNA levels in PB of the mice treated with SV were significantly decreased in comparison to those in the mice treated with ddH₂O. (B) Epstein-Barr virus-encoded small RNA (EBER) in situ hybridization of the spleen of Mouse 4 treated with ddH₂O. EBV-positive cells infiltrated to the spleen. Magnification 400 \times . (C) EBER in situ hybridization of the spleen of Mouse 3 treated with SV. The infiltration of EBV-positive cells to the spleen was drastically suppressed. Magnification 400 \times .

was administered orally to Mice 1 to 3 and ddH₂O to Mice 4 to 6. Ideal analysis is to prepare the same number of mice for case 4 and 6 to administer simvastatin and ddH₂O, but it was not possible because of the limitation of cells obtainable from a patient. Simvastatin reduced the viral load of EBV-DNA in PB of all the three xenograft models. Among the three model mice, EBV-DNA disappeared from blood

in Mouse 3 after two weeks from the administration of statin. The effect was greater compared to Mouse 1 and 2. The greater effect in Mouse 3 may be associated with low EBV-DNA load at the time of statin administration. Or it may depend on the characteristic of graft. We need to further investigate the effects of statin in a larger number of mice.

How does simvastatin induce apoptosis in

sCAEBV? It has been reported that simvastatin has effects on various cancer cell lines, not only of hematopoietic but also of solid tumors such as lung cancer, ovarian cancer, and prostatic cancer¹⁶⁾. In these neoplastic cells, simvastatin suppressed the prenylation of PI3K, Akt and small GTPases such as Rap1 and Ras¹¹⁾. These are survival-promoting molecules that are activated by prenylation through the mevalonate pathway and that are also expressed in lymphocyte. There is a good possibility that they are the target of simvastatin. In addition, Katano et al. reported that simvastatin altered the localization of a viral protein LMP1 in EBV-positive lymphoma cell lines⁴⁾. LMP1 usually exists in lipid rafts of the cell membrane, and localization is important for its transformation through upregulation of survival-promoting molecular signaling. LMP1 recruits TRAF3, which induces NF- κ B activation in a lymphoblastoid cell line¹⁷⁾. LMP1 plays a role in EBV-induced transformation of not only B cells but also T or NK cells¹⁸⁾. Simvastatin may work as an LMP1 inhibitor in EBV-positive T or NK-cells.

This study has some limitations. First, the number of samples is small due to the rarity of the disease, and we could only collect samples from six patients. Moreover, most of the samples were of EBV-positive T cells. To prove the effects of simvastatin on sCAEBV, more clinical samples, especially those of EBV-positive NK cells, are needed. Second, the dosage of simvastatin administered to the mice was high, which resulted in an effective concentration of simvastatin of 2.5 — 5 μ M. In clinical practice, however, simvastatin is usually administered at 10 mg/day, and the estimated plasma concentration is approximately 0.2 μ M. Thus, the effective concentration in this study is more than 10 times higher than the plasma level after 10-mg oral administration in humans. In addition, it is assumed that mice tolerate a higher dose of simvastatin compared to humans because their speed of metabolism of simvastatin is much faster¹⁹⁾. While the safety of high-dose simvastatin for humans needs to be evaluated, we wait for a new reagent with a high titer.

Conclusion

Simvastatin may be an attractive reagent for sCAEBV. To further prove its efficacy, future study with a larger number of patients' cells and model mice will be necessary.

Acknowledgments

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Conflicts of Interest

The authors have nothing to disclose.

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