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The cholesterol, fatty acid and triglyceride synthesis pathways regulated by site 1 protease (S1P) are required for efficient replication of severe fever with thrombocytopenia syndrome virus

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ABSTRACT

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease caused by the SFTS virus (SFTSV), which has a high mortality rate. Currently, no licensed vaccines or therapeutic agents have been approved for use against SFTSV infection. Here, we report that the cholesterol, fatty acid, and triglyceride synthesis pathways regulated by S1P is involved in SFTSV replication, using CHO-K1 cell line (SRD-12B) that is deficient in site 1 protease (S1P) enzymatic activity, PF-429242, a small compound targeting S1P enzymatic activity, and Fenofibrate and Lovastatin, which inhibit triglyceride and cholesterol synthesis, respectively. These results enhance our understanding of the SFTSV replication mechanism and may contribute to the development of novel therapies for SFTSV infection.

1. Introduction

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease caused by the SFTS virus (SFTSV), which has a high mortality rate of 10% [1–3]. SFTSV was originally reported in China in 2011, and classified into the genus *Phlebovirus*, of the family *Phenuiviridae* [1,2]. SFTSV, which is a tick-borne virus, has been detected not only in China but also in Japan and South Korea [3,4]. Other phleboviruses that are phylogenetically related to SFTSV, Heartland virus and Malsoor virus, were also isolated in Missouri, USA, and western India, respectively [5,6].

The antiviral effects of ribavirin (Rib) and interferons on SFTSV replication have been reported [7,8]. In addition, the efficacy of T-705, also known as Favipiravir, against SFTSV replication was recently demonstrated both *in vitro* and *in vivo* [9]. However, no effective vaccines or antiviral agents have yet been approved for the treatment of SFTSV.

Site 1 protease (S1P), also known as subtilisin/kexin-isozyme 1 (SKI-1), is a member of the proprotein convertase (PC) family [10]. S1P cleaves latent ER-membrane bound transcription factors to their active forms, including sterol regulatory element binding protein (SREBP)-1 and −2, which are involved in cholesterol and fatty acid homeostasis [11]. Therefore, S1P is a potential target for treating patients with dyslipidaemia and metabolic syndrome. Accordingly, a small molecule S1P inhibitor, PF-429242, was synthesized and was reported to reduce cholesterol/fatty acid levels *in vitro* and *in vivo* [12,13]. Several lines of evidence showed that S1P is also involved in some viral life cycles, directly or indirectly, which suggests that S1P could be a good target for combating pathogenic viruses, including Lassa virus, Lymphocytic Choriomeningitis (LCM) virus, Junin virus, Lujo virus (all *Arenaviridae*), Andes virus (*Hantaviridae*), Hepatitis C virus (HCV), and Dengue virus (DENV) (*Flaviviridae*) [14–20]. Recent study showed that several proteases, including serine protease, furin, or S1P, were not involved in SFTSV Gn/Gc cleavage, using specific chemical inhibitors AEBSF, PCI, or PF-429242, respectively [21].

In this study, we examined the role of synthesis pathway of cholesterol, fatty acid, and triglyceride on SFTSV propagation, and showed that this pathway is involved in SFTSV replication.

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2. Materials and Methods

2.1. Cells, reagents, viruses, and antibodies

Huh-7 cells and Vero 76 cells were obtained from the Health Science Research Resources Bank (JCRB0403 and JCRB9007), and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). SW13 cells were also maintained in DMEM supplemented with 1% penicillin/streptomycin and 10% FBS. CHO-K1 cells were maintained in DMEM/Ham's F12 1:1 (Sigma; #D6434) supplemented with 1% penicillin/streptomycin and 10% FBS (CHO complete medium). SRD-12B cells were maintained in CHO complete medium supplemented with 0.7% oleic acid-albumin from bovine serum (Sigma; #O3008), 1 mM sodium mevalonate (Sigma; #M4667), and 50 μM cholesterol/lipid (Lipids Cholesterol Rich from adult bovine serum, Sigma; #C7305). Fenofibrate, Lovastatin, MβCD and PF-429242 were obtained from Cayman (#10005368), Adipogen (#AG-CN2-0051), Sigma (#C4555) and Tocris Bioscience (#3354), respectively. The SFTSV YG1 strain, which was isolated in Yamaguchi prefecture, Japan, was obtained from Dr. Maeda (Yamaguchi Univ.) [3], and all the experiments were performed using less than five passages in Vero 76 cells. Anti-SFTSV N antibodies were either obtained from the National Institute of Infectious Diseases, Japan (NIID) or produced by immunization of rabbits with recombinant SFTSV-N (Eurofins Genomics K.K. (Tokyo, Japan)).

2.2. Infection assay

CHO-K1 and SRD-12B cells were seeded on 96 well plate. Following day, cells were infected with SFTSV at multiplicity of infection (moi) = 0.1. After incubation for 1.5 h, media were replaced with fresh one. At 48 h post infection (p.i.), culture supernatants were centrifuged to remove cell debris (13,000 × g, 5 min, 4 °C), and used for infection to fresh Vero 76 cells in 96 well plate, which were seeded one day before the infection. Infected Vero 76 cells were fixed with 4% paraform aldehyde (PFA) at 16 h p.i.. CHO-K1 cells and SRD-12B cells were also fixed when the supernatant was collected. Fixed cells were stained with anti-SFTSV N antibody as described below in the Virus titration section. To examine the virus production from CHO-K1 cells and SRD-12 cells, cells were infected with SFTSV at moi = 0.1, and incubated for 1.5 h, followed by replacement with fresh medium. 24 and 48 h p.i., culture supernatant was collected, centrifuged to remove cell debris, and used to measure the virus titre as described in virus titration section. For the compound treatment, SW13 cells were seeded in 96 well plate, and following day, cells were infected with SFTSV at moi = 0.1. After 1.5 h incubation, culture media was replaced with fresh media containing indicated compounds (PF-429242 (30 μM), Lovastatin (20 μM), Fenofibrate (55 μM), MβCD (10 μM)). At 24 and 48 h p.i., culture supernatant was collected to measure virus titre as described in the virus titration section.

2.3. Immunofluorescent staining

Approximately 10 μg/mL BODIPY® 493/503 was used to stain lipid droplets (LD). SFTSV-N was stained with anti-SFTSV-N rabbit polyclonal antibody, followed by anti-rabbit IgG (Alexa Fluor 647) (Abcam; ab150079). Either SFTSV-Gn or double-stranded RNA (dsRNA) was stained with anti-G1 (Immune Technology Corp.; IT-017-004M6) or anti-dsRNA monoclonal antibody (English & Scientific Consulting Kft.; K1-1301), followed by anti-mouse IgG-TRITC (Sigma; T5393). Nuclei (DAPI) are also shown in merged frames. Samples were observed using an LSM780 microscope (Carl Zeiss).

2.4. Cell viability assay

Cytotoxicity was assessed in SW13 cells using the CellTiter-Glo Luminescent Cell Viability Assay (Promega), which determines the number of viable cells based on cellular ATP. Briefly, 3×10^4 cells were plated on a 96-well plate. The following day, the indicated agents were applied and the cells were allowed to incubate for 24 and 48 h, and CellTiter-Glo reagent was added. Thereafter, the assay was performed according to the manufacturer's recommendations, with a luminometer (Tristar LB941, BERTHOLD). The viability of dimethyl sulphoxide (DMSO)-treated control cells was set at 1.0.

2.5. Virus titration

Vero 76 cells (2×10^4 cells/well) were seeded one day prior to infection in 96 well plate. Cells were infected with 1:10 virus dilutions. After 2 h adsorption, culture medium was replaced with fresh medium and incubation was continued for 14 h at 37 °C, 5% CO_2. Cells were fixed with 4% PFA for 30 min at room temperature (RT), and incubated with PBS-T (0.1% Tween20 in PBS (−)) for 1 h at RT. Blocking with 10% FBS/dilution buffer (3% BSA, 0.3% Triton-X100/PBS (−)) was performed at 4 °C overnight. SFTSV-N protein was detected using anti-SFTSV-N antibody, followed by anti-rabbit IgG-FITC antibody (Abcam; ab6009). Nuclei were stained with DAPI and samples were observed using an AxioVert.A1 microscope (Carl Zeiss). N-positive cells were counted and normalized as fluorescent focus units (FFU)/mL.

2.6. Statistical analysis

Statistically significant differences between groups were determined by the student's *t*-test (*$p < 0.01$).

3. Results

The host factors involved in SFTSV replication have not been elucidated completely. We and others have reported the involvement of S1P in the replication of several virus species [14–17,19,20,22]. In the present study, two approaches were used to examine the role of S1P in SFTSV replication; first using an S1P-deficient cell line and second using an S1P-specific chemical compound inhibitor. Parental CHO-K1 cells and S1P-deficient CHO-K1 cells (SRD-12B) [23], were infected with SFTSV at moi = 0.1. At 48 h p.i., the infected cells were fixed and stained with an anti-SFTSV-N antibody. To quantitate the production of infectious virions, the culture supernatants were also collected and inoculated into cultures of Vero 76 cells. At 16 h p.i., the cells were fixed and then stained with the anti-SFTSV-N antibody (Fig. 1A and B). There were significantly fewer SFTSV-N-positive cells in the infected SRD-12B cells than in the CHO-K1 cells, suggesting that S1P is involved in SFTSV replication (Fig. 1B). Consistent with this observation, only 6.5% as many infectious virions were produced from SRD-12B cells as were produced from CHO-K1 cells (Fig. 1B and C). These results suggested that S1P has important roles in the replication and propagation of SFTSV. To exclude the possibility that the results shown in Fig. 1B and C were due to a difference in growth rates between the CHO-K1 and SRD-12B cell lines, wells were seeded with the two cell lines at equal cell numbers and the cells were counted 24 and 48 h after seeding. As shown in Fig. 1D, the growth rates of these two cell lines did not significantly differ. To confirm and examine SFTSV production in CHO-K1 and SRD-12B cells, both cell lines were infected with SFTSV at moi = 0.1 and the culture supernatants were collected at 24 and 48 h p.i.. Virus titres were measured using serial dilution of the culture supernatant as

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