論 文 の 内 容 の 要 旨

論文題目　**Study on PRMT5 role in bovine leukemia virus (BLV) infection**
（牛白血病ウイルス(BLV)感染におけるPRMT5の生理的意義に関する研究）

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Introduction

Bovine leukemia virus (BLV), an oncogenic member of the Deltaretrovirus genus, infects cattle worldwide and decreases profitability in the dairy industry. Some 70% of infected cattle, the natural host of BLV, remain asymptomatic, whereas the major portion of the remaining infected cattle develops persistent lymphocytosis (PL), and only 1-5% develop leukemia/lymphoma after a 10-year or longer period of a proviral latency. Because of the limited variation in the BLV genome, host factors are believed to play a crucial role in determining the BLV infection profile. BLV proviral load (PVL) is an important index for disease progression. BLV-infected cattle are classified into two groups based on their BLV proviral load: high proviral load cattle (HPVL) (> 10,000 copies/ 10^5 cells) and low proviral load cattle (LPVL) (≤ 10,000 copies/10^5 cells). BLV infected cattle at the PL stage are known to carry a significantly increased number of proviral load compared with the number of the proviral load in aleukemic cattle. Moreover, a further increase was observed at the lymphoma stage.

Arginine methylation is an important posttranslational modification that plays crucial roles in chromatin regulation, transcription control, RNA processing, nuclear/cytoplasmic shuttling, DNA repair, and some other biological processes. Arginine methylation is catalyzed by the protein arginine-N-methyltransferase (PRMT) family. PRMTs are divided into four types of enzymes. Type I, the most common type of PRMTs, induces asymmetric dimethylation (aDMA); Type II catalyzes the symmetric dimethylation (sDMA); Type III produces monomethyl arginine as their final product, and Type IV is found only in fungi. In the virology field, protein arginine methylation has been determined to play critical roles in the biology of several viruses including hepatitis delta virus (HDV), hepatitis B virus (HBV), human immunodeficiency virus type-1 (HIV-1), HTLV-1, Epstein-Barr virus (EBV), and Kaposi's sarcoma associated herpesvirus (KSHV).

Research Importance and Aim

Until now, studies investigating PRMTs role in BLV biology have not been conducted; therefore, we aim to investigate the role of PRMT5, which is Type II PRMTs, in some aspects of BLV infection *in vivo* and *in vitro*. First, we focused on an investigation of the correlation between PRMT5 expression level and BLV proviral load, which is an index of virus infectivity, in peripheral blood from infected animals with various stages, such as asymptomatic and lymphoma stages. Second,

we revealed the impact of PRMT5 inhibition on BLV gene expression, gp51 glycosylation and syncytium formation. To our knowledge, this is the first study that investigates the PRMT5 role in BLV infection.

Results and Discussion

1. PRMT5 is overexpressed in BLV infected cattle with a high proviral load *in vivo*

BLV proviral load is not only an important index of the risk of BLV transmission and infectivity but also an important index of disease progression. In order to investigate the correlation between PRMT5 expression and BLV PVL, we collected blood samples from 62 cows which were asymptomatic and did not develop symptoms of lymphoma at the time of blood collection. Firstly, we performed the CoCoMo-qPCR-2 for calculation of the BLV PVL and accordingly, the cows were classified into three groups: BLV negative cattle (Uninfected group), BLV-infected cattle with low-proviral load (LPVL group) and BLV-infected cattle with high-proviral load (HPVL group). Next, we evaluated PRMT5 expression at RNA levels by qRT-PCR. The mean fold change of PRMT5 was 1.12±0.62 in the control group, 1.18±0.64 in the LPVL group, and 1.64±0.62 in the HPVL group. PRMT5 expression is significantly higher in HPVL group than that in the uninfected group (p = 0.0014) and in LPVL group (p = 0.012).

2. PRMT5 overexpression starts from the early stage of BLV infection *in vivo*

Our previous results showed that PRMT5 is upregulated only in BLV infected cattle with a HPVL. To further investigate the role of PRMT5 in BLV infection *in vivo*, we examined whether PRMT5 is overexpressed in the early stage of BLV infection. Therefore, we performed an experimental infection of five BLV-negative Japanese black calves carrying susceptible alleles *BoLA-DRB3**1601/1601 that is connected with a high proviral load BLV infection, and we collected blood samples at different time points during the first month of infection (0, 0.5, 1, 2, 3 and 4 weeks). Then we monitored BLV PVL and the PRMT5 expression at each time point. The mean of BLV PVL was 0 copies/10^5 cells before infection (0 week), 34 copies/10^5 cells after 3 days, 1158 copies/10^5 cells after 1 week, 26760 copies/10^5 cells after 2 weeks, 90150 copies/10^5 cells after 3 weeks, and 62440 copies/10^5 cells after 4 weeks. The mean fold change of PRMT5 was 1 before infection, 1.44 after 3 days, 1.48 after 1 week, 2.6 after 2 weeks, 3.7 after 3 weeks, and 2.3 after 4 weeks. The highest expression of PRMT5 occurs during the third week (FC = 3.74, p = 0.0008) then it slightly drops after 4 weeks (FC = 2.3, p = 0.01). Of interest, a strong positive correlation is found between PRMT5 upregulation fold and BLV PVL (r = 0.79). We concluded that PRMT5 expression is upregulated in response to BLV infection, and its upregulation fold positively correlates with BLV PVL. Moreover, PRMT5 upregulation starts from the early stage of BLV infection rather than being established after a long period of proviral latency. Taken together, our data suggest that PRMT5 overexpression is a contributing host factor for developing and keeping BLV infection.

3. PRMT5 overexpression continues to the lymphoma stage of BLV infection *in vivo*

It is well known that PRMT5 plays a role as an oncogene protein; also, PRMT5 upregulation was determined in several human cancers, including B and T cell lymphoma. We have already showed

that PRMT5 is overexpressed in clinically healthy BLV-infected cattle with HPVL. Finally, we investigated whether PRMT5 expression change in BLV infected cattle reaching the lymphoma stage of the disease. We compared PRMT5 expression at the RNA level among 3 groups of cattle: 20 BLV-negative cattle, 42 BLV-infected but clinically normal cattle and 20 BLV-infected cattle with lymphoma. PRMT5 expression fold change was 1.12±0.62 in the uninfected cattle, 1.48± 0.66 in the asymptomatic group and 2.45±1.1 in the lymphoma group. This finding strongly indicates that PRMT5 upregulation continues to the lymphoma stage of BLV.

4. PRMT5 knockdown enhances BLV gene expression *in vitro*

After we found that PRMT5 overexpression might contribute to develop HPVL BLV infection *in vivo*. Next, we examined the impact of PRMT5 inhibition on BLV infection *in vitro*. PRMT5 is a well-known regulator of gene expression either by catalyzing symmetric dimethylarginine of histone proteins to generate repressive histone marks including H2AR3me2s, H3R8me2s, and H4R3me2s or by methylation of non-histone proteins such as transcription factors. To investigate the effect of PRMT5 inhibition on BLV gene expression, we knockdown PRMT5 of two cell lines by siRNA: FLK-BLV, a permanently BLV infected cell line, or PK15-BLV, a stably transfected cell line with CMVΔU3-pBLV-IF2. We measured the mRNA of two viral transcripts: *gag* that is produced un-spliced mRNA, and *tax* that is produced by the alternative splicing. In addition, we measured the protein levels by Western blotting analysis for two viral proteins (Gag p24 and Env gp51). Herein, we determined that PRMT5 knockdown enhances BLV gene expression at the transcription and the protein levels in a dose dependent manner *in vitro*. Additionally, we demonstrated that PRMT5 knockdown does not impair the gp51 expression at the cell membrane. We, thus, concluded that the observed upregulation of BLV viral proteins after PRMT5 knockdown is caused by a higher expression rate rather than an impaired protein trafficking.

These results provide an evidence that PRMT5 works as a negative regulator of BLV gene expression. In addition, it might reveal one mechanism of the BLV proviral latency observed *in vivo*.

5. Selective PRMT5 inhibitor alters gp51 glycosylation processing *in vitro*

To further investigate the role of PRMT5 in BLV infection, we utilized a small molecular PRMT5 inhibitor (CMP5), which developed previously by Alinari et al. By using CMP5, we also confirmed that CMP5 treatment enhances BLV gene expression. Most surprisingly, we noticed that CMP5 treatment alters gp51 electrophoretic mobility over SDS-PAGE and forms gp51 with higher molecular weight than gp51 in the untreated cells. This shift was more evident in PK15-BLV cell line. This observation inspired us to investigate the possible impact of this inhibitor on BLV envelope protein glycosylation. Thus, we used two glycosidase enzymes for further investigation; PNGase F and Endo H. PNGase F removes all types of *N*-linked glycosylation from the glycoprotein: high mannose, hybrid, and complex. PNGase F severs the bond between *N*-acetylglucosamine (GlcNAc) and Asn, liberating the entire sugar chain and converting Asn into Asp. Endo H cleaves only high mannose and hybrid types of *N*-glycans, the complex type remains

resistant to Endo H digestion. Endo H cleaves the bond between the two GlcNAc residues in the core region, leaving one GlcNAc still bound to the protein.

Our data showed that PNGase F treatment leads to the accumulation of approximately 30-KDa product. This corresponds to the calculated molecular weight of the envelope peptide core in the absence of any glycosylation of BLV gp51. Thus, after PNGase F, change in migration pattern of gp51 is disappeared, indicating that CMP5 affects the gp51 glycosylation pattern. Endo H digestion further revealed that CMP5 treatment alters gp51 glycosylation processing. This altering differs among various cell lines; in PK15-BLV cell line, CMP5 treatment enhances gp51 glycosylation processing to form a complex type of *N*-glycan that runs slower in SDS-PAGE. In the FLK-BLV cell line, limited evidence was obtained to reveal the type of irregular *N*-glycan caused by CMP5 treatment.

Next, we examined whether CMP5 treatment affects Gag or Env intracellular localization or Env expression at the cell membrane. FLK-BLV cells were grown on coverslips in the absence (CMP5 –) or presence of 20 μM of CMP5 (CMP5 +) for 48 h; Gag and Env intracellular localization were evaluated by the fluorescence confocal microscope after the permeabilization of cells with 0.5% Triton X-100 for 5 min. Gp51 expression at the cell membrane was evaluated by fluorescence confocal microscope without the permeabilization step and by flow cytometry. We observed a large accumulation of Gag after CMP5 treatment. Additionally, the intracellular Env protein accumulated near to the nucleus after the treatment. In contrast, the cell membrane expression of gp51 was not severely affected as demonstrated by the fluorescence confocal microscopy and by flow cytometry.

6. Selective PRMT5 inhibitor impedes BLV ENV-mediated syncytia formation *in vitro*

It has been shown previously that the perturbation of the N-linked glycan structure of the HIV and HTLV-1 envelope proteins affects the syncytia formation ability. Therefore, we aimed to examine the impact of the PRMT5 inhibitor, CMP5, on the syncytium formation ability. FLK-BLV cells were co-cultured with CC81-GREMG, which is a reporter cell line, in the absence or the presence of different concentrations of CMP5. Interestingly, CMP5 treatment negatively affects the syncytia formation; this was detected by the eye under the fluorescence microscope and also by the automated quantification that shows that the syncytia-counts decrease in a dose-dependent manner, showing significance at the concentrations of 10 μM ($p = 0.017$) and 20 μM ($p = 0.0002$). By contrast, the total cell count during the assay remains unaffected. These facts exclude the toxic effect of CMP5 on FLK-BLV or CC81-GREMG. CMP5 treatment did impair Env expression at the cell membrane. Thus, CMP5 treatment likely impedes BLV-induced syncytium formation via affecting the gp51 glycosylation processing as shown in our study.

In conclusion, the present study provides the first report that determined various roles for PRMT5 in BLV infection *in vivo* and *in vitro*; CMP5 and other PRMT5 inhibitors should be further investigated and evaluated in clinical studies as a novel antiretroviral therapy.