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Novel strategy for anti-HIV-1 action: selective cytotoxic effect of *N*-myristoyltransferase inhibitor on HIV-1-infected cells

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Abstract ***N*-myristoyltransferase (NMT) is essential for the survival of eukaryotes and the production of infectious human immunodeficiency virus type-1(HIV-1) by the host cell. In this study, we found decreases in the mRNA levels of human NMT isoforms and the NMT activities in the course of HIV-1 infection in the human T-cell line, CEM. Investigating the cytotoxic effect of the novel synthetic NMT inhibitors on the chronic HIV-1 infected T-cell line, CEM/LAV-1, and the uninfected CEM, revealed that the cytotoxic effect was significantly selective for CEM/LAV-1. This was thought to be due to the difference between the NMT levels of the cell lines. In this paper, we propose that NMT may be a candidate target for anti-HIV-1-infected-cell agents.**

Key words: *N*-myristoyltransferase; Myristoylation; Human immunodeficiency virus type-1

1. Introduction

Highly active anti-retroviral therapy (HAART) has been a successful means of markedly reducting viremia in human immunodeficiency virus type-1 (HIV-1)-infected individuals, and has led to a reduction of the mortality rate of patients with acquired immunodeficiency syndrome in recent years [1,2]. However, the therapeutic strategy, which involves blockage of steps of the viral life cycle, cannot completely eradicate the virus from infected individuals. Even though the HIV-1 RNA level in blood becomes undetectable during HAART, the virus is capable of reactivation of productive infection following cessation of the therapy, which indicates that inducible HIV-1 latent reservoirs exist during HAART [3–5]. It has been estimated that the reservoir may be stable for up to 60 years during HAART [6].

N-myristoyltransferase (NMT) (EC 2.3.1.9.7) catalyzes the covalent attachment of myristate to the N-terminal Gly residue of various proteins, the modification of which is called *N*-myristoylation. Since the disruption of NMT expression was found to cause recessive lethality and developmental defects in *Saccharomyces cerevisiae* [7] and *Drosophila* [8], respectively, NMT appears to be essential for the survival of eukaryotes. NMT also plays a very important role in HIV-1 replication, because the N-termini of viral proteins, $p17^{gag}$ and Nef, are *N*-myristoylated [9–12], which is indispensable for conferring infectivity [13,14] and the effective replication of HIV-1 [15,16]. It is therefore thought that human NMT (hNMT) is a potential target of anti-HIV-1 agents, whereas inhibition of hNMT activity may damage the host cell.

Previously, we reported that the expression level of hNMT was decreased in acute and chronic HIV-1-infected human T-cell line CEM cells [17]. In this study, the mRNA levels of hNMT isoforms and the activity of NMT in acute HIV-1-infected CEM cells were measured. Consistent with the profile of the expression level of the protein, both levels decreased partially but significantly during the process of HIV-1 infection. Novel synthetic NMT inhibitors, i.e. serinal derivatives [18], exhibited a significantly selective cytotoxic effect on chronic HIV-1-infected cells, CEM/LAV-1, and the difference in selectivity between CEM and CEM/LAV-1 cells was thought to be due to their NMT expression levels. The strategy of specifically targeting HIV-1-infected cells with an NMT inhibitor may contribute to eradicating the HIV-1 reservoir in these patients.

2. Materials and methods

2.1. Materials

The reagents used in the study were obtained from the following sources: the RPMI-1640 medium and Dulbecco's modified Eagle's medium (DMEM) were from Nissui Seiyaku Co. (Tokyo, Japan); *N*-myristoyl serinal bisulfite, *O*-myristoyl serinal bisulfite, *N*-acetyl-*O*-palmitoyl serinal diethylacetal, and *N*-acetyl-*O*-myristoyl serinal diethylacetal were gifts from Dr. S. Ikeda (Torii and Co., Ltd., Tokyo, Japan).

2.2. Cell culture and HIV-1 preparation

A human T-cell line, CEM, and a chronically HIV-1-infected T-cell line, CEM/LAV-1, were maintained as previously described [19]. A human colon adenocarcinoma cell line, COLO320DM, was cultured at 37°C in DMEM supplemented with 5% fetal calf serum (100 IU/ml penicillin and 100 μg/ml streptomycin) in 5% CO_2.

Preparation of infectious HIV-1 (LAV-1 strain) and titration of infectivity were performed as previously described [20].

2.3. Acute HIV-1 infection

CEM cells (2×10^5 cells/ml, 10 ml) were infected with HIV-1 at a multiplicity of infection (m.o.i.) of 0.25, incubated for 1 h at 37°C in 5% CO_2, washed twice with PBS(–), and resuspended in the RPMI-1640 medium. The cells were incubated for 0, 24, 48, 72 or 96 h at 37°C in 5% CO_2 and subjected to NMT–enzyme-linked immunosor-

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Abbreviations: NMT, *N*-myristoyltransferase; ELISA, enzyme-linked immunosorbent assay; HIV-1, human immunodeficiency virus type-1; RT, reverse transcriptase; HAART, highly active anti-retroviral therapy

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bent assay (ELISA) and reverse transcriptase (RT)-PCR. Cell viability was determined by the trypan blue dye exclusion method.

2.4. Cell lysis and NMT–ELISA

CEM cells (7.5×10^5) were lysed with lysis buffer (10 mM Tris–HCl (pH 7.4), 10 mM EDTA, 0.5% Triton X-100, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 0.1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 0.1 mM *N*-α-*p*-tosyl-L-phenylalanine chloromethyl ketone, 2 μg/ml leupeptin, 2 μg/ml pepstatin A). After centrifugation at 15 000 rpm for 5 min, the supernatants were used for NMT–ELISA as previously described [21].

2.5. Semiquantitative RT-PCR

Total RNA isolation from the cells was performed using a QuickPrep Total RNA Extraction kit (Amersham Pharmacia Biotech UK, Ltd.) in accordance with the manufacturer's instructions. The total RNA (0.5 μg) was reverse transcribed to cDNA using MuLV RT according to the instructions enclosed in the Gene Amp RNA PCR kit (Applied Biosystems, Foster City, CA, USA). Amplification of hNMT1, hNMT2, HIV-1, and β-actin sequences by PCR using Ampli Taq Gold (Applied Biosystems, Foster City, CA, USA) was carried out in a thermal cycler using the following protocol: one cycle at 94°C for 9 min; 33 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and one cycle at 72°C for 5 min. The oligonucleotide primers used for the PCR were as follows: NMT1 sense primer CCGCAGATGATGGAAGGGAA; NMT1 antisense primer CCTCTCTGCTGGCAAAGAGTTCA; NMT2 sense primer GAAGTCCTGGAGGGTATTTG; NMT2 antisense primer CTGCATTGGAACACTGGGATT; SK38 (HIV-1*gag*) ATAATCCACCTATCCCAGTAGGAGAAAT; SK39 (HIV-1*gag*) TTTGGTCCTTGTCTTATGTCCAGAATGC; β-actin CGGAACCGCTCATTGCC; β-actin ACCCACATCGTGCCCATCTA. The PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The density of each product was semiquantified using the luminoimage analyzer LAS-1000 plus (Fuji film). Each RNA value was expressed as percentage of the value of the uninfected CEM cells.

2.6. Fluorography

COLO320DM cells were metabolically labeled for 24 h with 10 μCi/ml [9,10-^{3}H]myristic acid (NEN Research Products) in the presence or absence of serinal derivatives. After radiolabeling, the cells were washed twice with PBS(–) and lysed in lysis buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 50 μg/ml each of antipain, leupeptin, pepstatin A, and aprotinin). The cell lysates were subjected to SDS–PAGE followed by fluorography.

2.7. Cytotoxicity

A cytotoxicity test of serinal derivatives was performed as previously described [18].

3. Results and discussion

The alteration of host gene expression occurs after HIV-1 infection, with several reports on the investigation of the mRNA level [22–24]. Previously, we reported that the expression levels of hNMTs in CEM cells decreased in the course of HIV-1 infection. To investigate the change of the mRNA expression levels of hNMT isoforms, hNMT1 and hNMT2, acute HIV-1 (LAV-1 strain)-infected CEM cells were cultured for 0, 24, 48, 72, or 96 h. The acute HIV-1-infected cells and chronic HIV-1-infected CEM cells, CEM/LAV-1, were harvested and the total RNAs were extracted, followed by reverse transcription to cDNA. Using specific primer pairs for the amplification of cDNA from hNMT1, hNMT2, HIV-1 and actin mRNA, semiquantitative PCR was performed as described in Section 2. As shown in Fig. 1A, the densities of the amplified 289 bp band from β-actin mRNA were similar in the course of infection. The ratio of the density of the band from hNMT1 (214 bp) or hNMT2 (157 bp) mRNA to that of the band from actin mRNA at the corresponding time was expressed as percentage of that of uninfected CEM (at 0 h postinfection). As shown in Fig. 1B, a decrease in both the hNMT1 and hNMT2 mRNA levels was observed in the course of infection. The hNMT1 and hNMT2 mRNA levels of CEM/LAV-1 were approximately half of those of CEM.

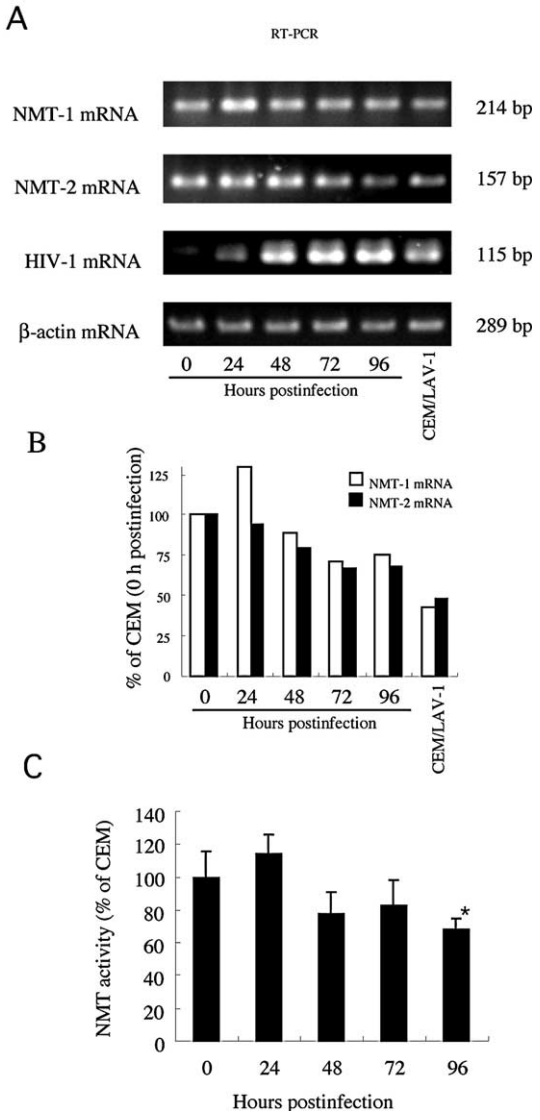

Fig. 1. NMT level in HIV-1-infected CEM. A: CEM cells were infected with HIV-1 (LAV-1 strain) at an m.o.i. of 0.25 and cultured for 0, 24, 48, 72, or 96 h. Acute HIV-1-infected CEM cells and chronic HIV-1-infected CEM cells, CEM/LAV-1, were harvested. The total RNAs were extracted, followed by reverse transcription to cDNA. Using specific primer pairs for the amplification of cDNA from hNMT1, hNMT2, HIV-1 and actin mRNA, semiquantitative PCR was performed as described in Section 2. Representative results are shown. B: The ratio of the density of the band from hNMT1 or hNMT2 mRNA to that of the band from actin mRNA at the corresponding time was expressed as the percentage of that of uninfected CEM cells (at 0 h postinfection). Open column, NMT1 mRNA; closed column, NMT2 mRNA. C: CEM cells were infected with HIV-1 (LAV-1 strain) at an m.o.i. of 0.25 and cultured for 0, 24, 48, 72, or 96 h. The cells were lysed and subjected to NMT–ELISA using the peptide substrate of synthetic biotinylated Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys. Significant differences from CEM as determined by Student's *t*-test are noted as follows: $^*P<0.05$.

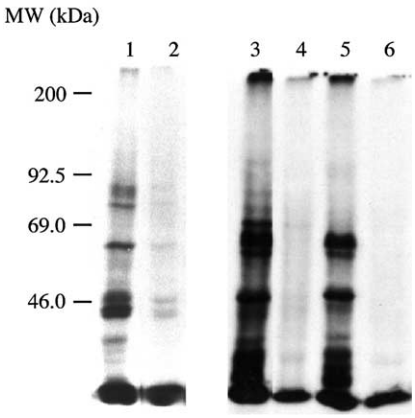

Fig. 2. Blockage of *N*-myristoylation by serinal derivatives in COLO320DM. Human colon adenocarcinoma cell line COLO320DM was metabolically labeled for 24 h with [9,10-^{3}H]myristic acid in the presence (100 μM) or absence of serinal derivatives. The cells were lysed and subjected to SDS–PAGE, followed by fluorography. Lanes 1 and 3, control; lane 2, *N*-myristoyl serinal bisulfite; lane 4, *O*-myristoyl serinal bisulfite; lane 5, *N*-acetyl-*O*-palmitoyl serinal diethylacetal; lane 6, *N*-acetyl-*O*-myristoyl serinal diethylacetal.

On the other hand, the band from HIV-1 mRNA (115 bp) was detected weakly at 24 h postinfection and strongly at 48–96 h postinfection and its density was higher than that of CEM/LAV-1 (Fig. 1A).

Moreover, we evaluated NMT activities of the cells in the course of infection by NMT–ELISA [21]. The assay can detect the NMT activity in CEM lysate quantitatively (data not shown). NMT activity was expressed as percentage of that of uninfected CEM. As shown in Fig. 1C, partially decreased NMT activity was observed from 48 to 96 h postinfection, and was significant ($P < 0.05$) at 96 h postinfection. These results indicate that the expression levels of hNMTs decreased due to HIV-1 infection. It is also strongly suggested that the decrease of NMT expression in levels in both mRNA and protein [17] is closely related to the decrease in the NMT activity. Therefore, it is thought that chronically infected CEM with weak expression of NMT would cause a decrease in NMT activity. The event with HIV-1 infection was of interest to us because NMT is thought to be essential for both HIV-1 and the host. HIV-1 gene products namely, p17^{gag} and Nef, are *N*-myristoylated, which is important for trafficking to and associating with lipid rafts [25] or raft-like membrane microdomains called barge [26]. In the host cell, many *N*-myristoylated proteins participate in the signal transduction pathway, vesicular trafficking, cell survival, and regulation of cytoskeletal assembly [27]. We hypothesized that the decrease in the expression level of NMT due to HIV-1 infection may be related to the mechanism of viral latent replication [17], although it has not been proven yet.

Protein *N*-myristoylation appears to be limited to eukaryotes. Studies of NMT inhibitors as anti-fungal agents are well known [28–30]. It is, therefore, thought to be inevitable that NMT inhibitors induce lethal damage of eukaryotic-cell-containing mammalian cells. Although it is still unknown whether this partial down-regulation of hNMTs with HIV-1 infection is advantageous or disadvantageous for HIV-1 and the host cell, complete or marked suppression of NMT activity by the addition of an NMT inhibitor has a lethal effect on the HIV-1-infected host cell. Furthermore, it is expected that the concentration of the NMT inhibitor that will exert a lethal effect on an infected cell is lower than that for an uninfected cell.

As a candidate NMT inhibitor, serinal derivatives [18] were synthesized. The in vivo inhibitory activity of the serinal derivatives on NMT was tested. The human colon adenocarcinoma cell line COLO320DM was metabolically labeled for 24 h with [9,10-^{3}H]myristic acid in the presence or absence of serinal derivatives. The cells were lysed and subjected to SDS–PAGE, followed by fluorography to detect radiolabeled *N*-myristoylated proteins. As shown in Fig. 2, a number of *N*-myristoylated proteins were detected in control cell lysate (lanes 1 and 3), whereas only a few or no proteins were detected in cell lysates treated with *N*-myristoyl serinal bisulfite (lane 2), *O*-myristoyl serinal bisulfite (lane 4), and *N*-acetyl-*O*-myristoyl serinal diethylacetal (lane 6) at 100 μM. In vitro enzyme assay revealed that these derivatives inhibited NMT activity (data not shown). In the case of treatment with *N*-acetyl-*O*-palmitoyl serinal diethylacetal (lane 5), the inhibitory effect of *N*-myristoylation was not observed. In this assay, the cytotoxicity of serinal derivatives to COLO320DM was not observed.

The cytotoxic effects of the four serinal derivatives on CEM and CEM/LAV-1 cells were evaluated. The cells were cultured in the presence or absence of the derivatives for 96 h. The number of viable cells was determined by the trypan blue dye exclusion method. Cell viability was expressed as percentage of the control. As shown in Fig. 3, the three serinal derivatives with NMT inhibitory effect, *O*-myristoyl serinal bisulfite (Fig. 3A), *N*-acetyl-*O*-myristoyl serinal diethylacetal (Fig. 3B), and *N*-myristoyl serinal bisulfite (Fig. 3C) showed cytotoxic effect on both CEM and CEM/LAV-1 cells, with the effect being significantly intense on CEM/LAV-1 cells, although the selectivity indexes are still low (the values were < 6). On the other hand, the serinal derivative with no inhibitory effect on NMT, *N*-acetyl-*O*-palmitoyl serinal diethylacetal, did not influence the viability of both CEM and CEM/LAV-1 cells. The results suggest that one of the mechanisms of the selective cytotoxicity to CEM/LAV-1 cells of the serinal derivatives is the marked inhibition of NMT activity in CEM/LAV-1 cells in contrast to the partial inhibition of the same in CEM cells at a concentration that exhibit a significant and selective cytotoxic effect.

Now, emergence of a drug-resistant virus could arise with treatment failure [31]. The ultimate objective of HIV-1 therapy should be the eradication of viral reservoirs. The proposal of NMT as a candidate target for the infected-cell-selective cytotoxic effect is therefore very significant, although it remains to be confirmed whether the selective cytotoxic effect can be applied in the case of combination of other kinds of HIV-1 strains and host cells. There are several isoforms of hNMT, including at least the hNMT1 short form, hNMT1 long form [32], and hNMT2 [33]. In addition, there are a number of *N*-myristoylated proteins [27]. It has been unclear which NMT isoform and which NMT substrate is related to cell survival or death in the T-cell line. Clarification of the detailed mechanism would contribute to the development of more effective and more specific anti-HIV-1-infected cell agents.

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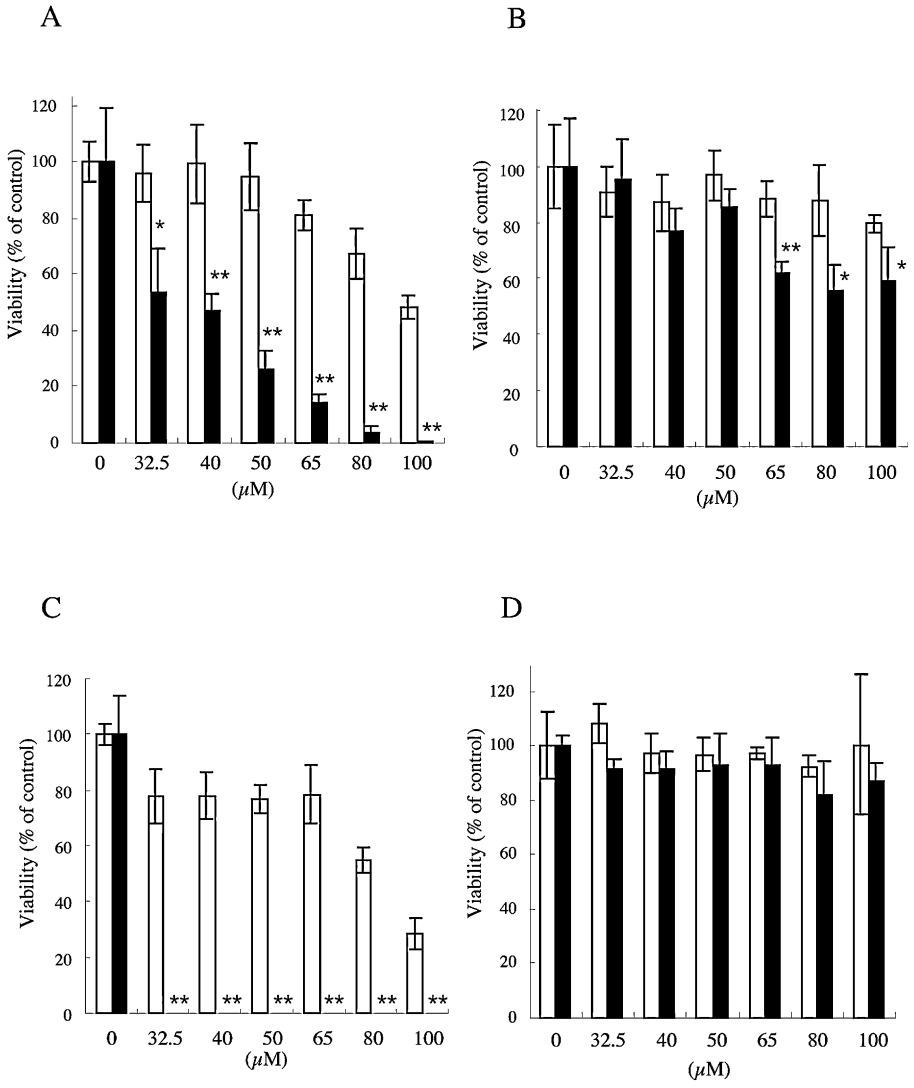

Fig. 3. Cytotoxic effect of serinal derivatives on CEM and CEM/LAV-1. CEM and CEM/LAV-1 (0.5×10^5 cells/ml) were separately cultured for 96 h after a single addition of the test serinal derivatives at the indicated concentration. The viable cells number was determined by the trypan blue dye exclusion method. Cell viability was expressed as percentage of the control. The results shown are the mean values (±S.D.) of three independent assays. Significant differences from CEM as determined by Student's *t*-test are noted as follows: $*P<0.05$; $**P<0.01$. Open column, CEM; closed column, CEM/LAV-1. A: *O*-myristoyl serinal bisulfite; B: *N*-acetyl-*O*-myristoyl serinal diethylacetal; C: *N*-myristoyl serinal bisulfite; D: *N*-Acetyl-*O*-palmitoyl serinal diethylacetal.

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