Original Paper

Involvement of Sp1 in Butyric Acid-Induced HIV-1 Gene Expression

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Key Words

HIV-1 • Butyric acid • Sp1 • HDAC

Abstract

Background/Aims: The ability of human immunodeficiency virus-1(HIV-1) to establish latent infection and its re-activation is considered critical for progression of HIV-1 infection. We previously reported that a bacterial metabolite butyric acid, acting as a potent inhibitor of histone deacetylases (HDACs), could lead to induction of HIV-1 transcription; however, the molecular mechanism remains unclear. The aim of this study was to investigate the effect of butyric acid on HIV-1 gene expression. **Methods:** Butyric acid-mediated HIV-1 gene expression was determined by luciferase assay and Chromatin immunoprecipitation assay. Western blot analysis and ELISA were used for the detection of HIV-1. **Results:** We found that Sp1 binding sites within the HIV-1 promoter are primarily involved in butyric acid-mediated HIV-1 activation. In fact, Sp1 knockdown by small interfering RNA and the Sp1 inhibitor mithramycin A abolished the effect of butyric acid. We also observed that cAMP response element-binding-protein (CBP) was required for butyric acid-induced HIV-1 activation. **Conclusions:** These results suggest that butyric acid stimulates HIV-1 promoter through inhibition of the Sp1-associated HDAC activity and recruitment of CBP to the HIV-1 LTR. Our findings suggest that Sp1 should be considered as one of therapeutic targets in anti-viral therapy against HIV-1 infection aggravated by butyric acid-producing bacteria.

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Introduction

Human immunodeficiency virus-1 (HIV-1) is the causative agent of acquired immunodeficiency syndrome (AIDS). It is well established that the extents of its *in vivo* replication and the viral load are well correlated with the rate of disease progression. Current anti-retroviral therapy (ART) protocols consist of combinations of various anti-retroviral agents that can efficiently decrease the viral load to below the detection limit, thus

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reducing the AIDS mortality rate [1-3]. However, despite the potency of ARTs, latent HIV-1 infection can escape from host immune responses and anti-retroviral therapy. These latently infected cells are permanent sources of viral re-activation that can lead to rebound of the viral load after interruption of ART [1, 4]. Therefore, the viral latency is the main obstacle in eradication of HIV-1 from patients. Thus, elucidation of the molecular mechanisms involved in the maintenance and cessation of HIV latency are required to comprehensively illustrate the pathophysiology of HIV-1 infection in order to develop preventive measures and novel therapies.

HIV-1 gene expression is the major determinant of viral replication leading to progression of AIDS. After HIV-1 infection, integrated HIV-1 proviral DNA is incorporated into the host nucleosomes, where the transcriptional activity of viral long terminal repeat (LTR) is under the control of local nucleosomal structure [5-8]. Evidences have indicated that epigenetic modifications of the LTR play regulatory roles in the induction of HIV-1 transcription and viral expression [2, 5, 8, 9]. Previous studies have shown that the presence of histone deacetylases (HDACs) leads to the repression of HIV-1 LTR, whereas inhibition of HDACs by HDAC inhibitor (HDACi) results in up regulation of HIV-1 gene expression [2, 5, 8]. HDAC1 mediates chromatin remodelling resulting in repression of both HIV-1 transcriptional activity and viral replication [8, 10-13]. Negative transcription factors such as ying-yang protein 1-late SV40 factor (YY1-LSF) complex, nuclear factor- κ B (NF- κ B) p50 homodimer, C-promoter-binding factor and the specificity protein 1 (Sp1)-c-Myc complex have been shown to mediate recruitment of HDAC1 to the LTR and, consequently, inhibit transcription from the HIV-1 promoter [10-13]. We also reported that activator protein 4 (AP-4) acts as a transcriptional repressor by recruiting HDAC molecules and is involved in the maintenance of viral latency [14].

On the other hand, many reports have shown that butyric acid, an anaerobic metabolite of certain bacteria [15], is a potent inhibitor of HDAC, which leads to alterations in chromatin structure and diminished HIV-1 gene expression [16-18]. Kashanchi et al. [19] demonstrated augmentation of HIV-1 replication by butyric acid in latently infected primary mononuclear cells from HIV-1 infected individuals as well as HIV-1 latently infected cell lines, such as OM10.1, ACH-2 and U1. We previously demonstrated that butyric acid-producing bacteria in gut, oral cavities and vagina can strongly induce histone acetylation and HIV-1 replication from latently infected cells by inhibiting HDAC [20, 21]; thus, co-infection with anaerobic bacteria is an important risk factor for progression of AIDS among people infected with HIV-1. However, the molecular mechanism of butyric acid activation of HIV-1 gene expression has not been elucidated.

In the present study, we investigated the effect of butyric acid on HIV-1 gene expression and demonstrated that Sp1 plays a key role in butyric acid-induced HIV-1 gene expression.

Materials and Methods

Reagents

Butyric acid was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Human recombinant tumour necrosis factor- α (TNF- α) was purchased from Roche Diagnostics Deutschland GmbH (Mannheim, Germany) and used at a concentration of 3.0 ng/mL for NF- κ B stimulation. Mithramycin A and C646 were purchased from Sigma (St. Louis, MO, USA). Antibodies for Sp1, CBP and the acetylated form of human histone H3 (Ac-H3) were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, USA), anti-HDAC1, c-myc, RNA polymerase II (RNAPII) and normal rabbit anti-IgG were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and anti-AP-4 antibody was obtained as previously described [14].

Cell culture and HIV-1 latently infected human cell lines

ACH-2 is a chronically HIV-1-infected T-cell line derived from the parental cell line A3.01 [22, 23] that was obtained from the AIDS Research and Reference Reagent Program (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA) and maintained at 37°C in Roswell

Park Memorial Institute 1640 medium (Sigma) with 10% heat-inactivated foetal bovine serum (Sigma), penicillin (100 U/mL) and streptomycin (100 µg/mL). To maintain HIV-1 latency in ACH-2 cells, 20 µM azidothymidine (Sigma) was added to the culture medium but was excluded prior to conducting the experiments. The cells (2.0×10^6 cells/2.0 mL medium/well) were treated with butyric acid. HeLa cells and TZM-bl cells, a HeLa-derived cell line expressing surface CD4, CXCR4 and CCR5 containing a chromatin-integrated HIV-1 LTR [24, 25] were grown at 37°C in Dulbecco's modified Eagle's medium (Sigma) with 10% heat-inactivated foetal bovine serum.

Construction of plasmids

Construction of HIV-1 LTR-based luciferase expression plasmid: wild-type (WT CD12-luc) HIV-1 LTR-Luc (containing the HIV-1 LTR U3 and R regions), its 5' truncated mutant CD23-Luc (containing positions -117 to +80 of HIV-1 LTR), CD52-Luc (containing positions -65 to +80 of HIV-1 LTR) and the NF-κB mutant HIV-1 LTR-Luc, where NF-κB sites were mutated in WT HIV-1 LTR-Luc, were prepared as previously described [26-28]. Sp1, AP-4 or LSF mutant HIV-1 LTR-Luc plasmids were generated by PCR using a QuikChange site-directed mutagenesis kit (Stratagene, Inc., La Jolla, CA, USA) as described previously [14, 26]. The following mutant sequences were utilized: Sp1 mutant HIV-1 LTR-Luc, TCG ttA; AP4 mutant HIV-1 LTR-Luc, CAG CTc; LSF mutant HIV-1 LTR-Luc, CAG tcG (consensus sequences of the binding sites are underlined and the mutated sequences are denoted by lower case letters). All constructs were confirmed by dideoxynucleotide sequencing using the ABI PRISM® dGTP BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing kit (PerkinElmer, Inc., Waltham, MA, USA) on an ABI PRISM® 373 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Sp1-Luc [29] was kindly provided by Dr. K. Kohno.

Transfection and luciferase assay

HeLa cells (1×10^5 cells/mL) were cultured in 12-well plates and HIV-1 LTR-based reporter plasmids (20 ng) described above, together with 10 ng of the internal control plasmid pRL-TK (Promega, Fitchburg, WI, USA) expressing *Renilla* luciferase, were transfected with FuGENE 6 transfection reagent (Roche) according to the manufacturer's protocol [14, 26]. After 24 h of transfection, the cells were treated with butyric acid (1.5 mM) or TNF-α (3 ng/mL), as a positive control, for an additional 24 h. These cells were then harvested using Passive Lysis Buffer (Promega), and the extracts were assessed for luciferase activity using the Dual-Luciferase Assay System (Promega). The luciferase activity was normalized by *Renilla* luciferase activity for transfection efficiency. All experiments were performed in triplicate and the data are presented as the fold increase in luciferase activity (mean ± SD) relative to controls.

Western blot analysis

The Western blot protocol was described previously [26, 28]. Briefly, the cells were washed once with ice-cold phosphate-buffered saline and harvested with lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA). Cellular debris was removed by centrifugation at 15000 *g* for 10 min. After centrifugation, the supernatant proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (EMD Millipore Corporation, Billerica, MA, USA). The Pierce Microplate BCA Protein Assay kit-Reducing Agent Compatible kit (Thermo Scientific, Inc., Rockford, IL, USA) was used to standardise the protein concentration in all samples. The membrane was probed with respective antibodies and immunoreactive proteins were visualised using the SuperSignal West Pico enhanced chemiluminescence kit (Thermo Scientific, Inc.). To detect HIV-1 proteins, the cell lysates were subjected to immunoblotting using sera collected from AIDS patients [20, 26].

RNA interference

Short interfering (si) RNAs against Sp1 and control GFP were purchased from Santa Cruz Biotechnology, Inc. and Takara Bio., Inc. (Otsu, Japan), respectively. TZM-bl cells were cultured in 12-well plates and treated with 100 nM siRNAs using Lipofectamine RNAiMAX reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions and incubated for additional 48 h. The cells were then incubated for another 8 h in the presence or absence of butyric acid (1.5 mM). Western blotting was performed with anti-Sp1 antibody to confirm knockdown of the Sp1 protein. The transfected cells were harvested and assessed for luciferase activity.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously using the Magna ChIP assay kit (EMD Millipore Corporation) [20, 26]. Briefly, TZM-bl cells (2×10^6) were fixed with 1% formaldehyde at 37°C for 10 min and the reaction was stopped by the addition of 125 mM glycine. The cells were lysed and the cross-linked chromatin was sheared by sonication 10 times for 30 s each at maximum power with 30 s of cooling on ice between pulses (Bioruptor; Cosmo Bio Co., Ltd., Tokyo, Japan). Sheared chromatin fractions were collected and pre-cleared with salmon sperm DNA and protein G-agarose beads for 1 h followed by immunoprecipitation with the desired antibodies at 4°C overnight with rotation. The precipitated DNA was analysed by PCR (35 cycles) with HotStarTaq Master Mix (Qiagen, Hilden, Germany) and primers for HIV-1 LTR (-176 to +61; forward, 5'-CGA GAC CTG CAT CCG GAG TA-3'; reverse, 5'-AGT TTT ATT GAG GCT TAA GC-3'). PCR products were separated by 2% SDS-PAGE. For each reaction, 10% of the original sheared chromatin DNA was similarly reversed cross-linked and purified, and the recovered DNA was used as the input control.

Measurement of viral p24 antigen

The stimulatory effect of butyric acid in ACH-2 cells was evaluated based on the extent of HIV-1 p24 core antigen production as previously described [20, 21, 28]. Briefly, cells (2×10^6) were incubated with or without butyric acid for 24 h at 37°C. The culture supernatants were then collected and measured for viral p24 antigen concentrations using the Retrotek HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (Cellular Products, Buffalo, NY, USA). All experiments were performed in triplicate and the data are presented as means $\pm$ SD.

Results*Effects of butyric acid on HIV-1 LTR transcription*

As we previously reported, butyric acid could activate HIV-1 replication from latently infected cells in a dose-dependent manner [20, 21]. To determine the mechanism of how butyric acid activates HIV-1 gene expression, we generated various plasmid constructs, as shown in Fig. 1A, and transfected each construct into HeLa cells to determine the level of HIV-1 gene expression using luciferase assay. The wild-type (WT) HIV-1 LTR promoter (CD12-luc) contains binding sites for several transcription factors, including AP-1, Myb, nuclear factor of activated T-cells-1 (NFAT1), cytoplasmic 2, NF- κ B, Sp1, TATA-box binding protein, AP-4 and LSF (Fig. 1A). As shown in Fig. 1B, butyric acid (1.5 mM) stimulated the WT HIV-1 LTR promoter in a dose-dependent manner. TNF- α (3 ng/ml) could also activate HIV-1 gene expression (positive control) but to a much lesser extent. Butyric acid could stimulate the CD23-Luc HIV-1 LTR promoter, although devoid of the upper sequence elements from -117, similarly to WT HIV-1 LTR (Fig. 1C). Therefore, the AP-1, Myb and NF-AT1 binding sites of HIV-1 LTR were not essential for butyric acid-induced HIV-1 gene expression. The activation of NF- κ B binding to the HIV-1 LTR is the most critical step for cytokine-stimulated induction of HIV-1 gene expression [6, 9]. In addition, the NF- κ B-p50 homodimer was shown to mediate HDAC1 recruitment to the LTR and, consequently, inhibited transcription of the viral promoter [12]. Thus, we first examined whether NF- κ B is involved in butyric acid-induced HIV-1 transcription. When NF- κ B binding sites in LTR were eliminated (CD52-Luc), marked transcriptional activation by butyric acid was still observed, whereas it totally abolished the effects of TNF- α (Fig. 1D). To confirm the role of the NF- κ B binding sites in butyric acid-induced HIV-1 gene expression, the HIV-1 LTR construct lacking two NF- κ B binding sites (NF- κ B mut HIV-1 LTR-Luc) was transfected into HeLa cells. When these cells were stimulated with butyric acid, significant induction by butyric acid was still observed (Fig. 1E). These results clearly indicate that transcriptional control elements in adjacent to the HIV-1 mRNA start site within the LTR, but not the NF- κ B binding sites, are responsible for the butyric acid-induced HIV-1 gene expression.

Fig. 1. The effect of butyric acid on HIV-1 LTR transcription. (A) A schematic diagram of the HIV-1 LTR-based reporter constructs used to determine the contribution of various portions of the HIV-1 promoter. CD12-Luc contains the full sequences of the U3 and R regions. CD23-Luc and CD52-Luc encompass the downstream sequence from position -117 (the HIV-1 LTR cap site) and that from position -65, respectively. NF- κ B mutant HIV-1 LTR-Luc was mutated at the NF- κ B binding sites of the WT HIV-1 LTR-Luc. (B–E) Results of luciferase assays using HIV-1 LTRs of various mutants. The HIV-1 LTR luc reporter constructs described in (A) expressing the luciferase gene under the control of HIV-1 LTR at 20 ng were transfected into HeLa cells, which were incubated for 24 h and stimulated with either butyric acid (BA; 1.5 mM) or TNF- α (3 ng/ml) for additional 24 h. The luciferase activity of each cell lysate was then measured. All experiments were performed in triplicate and the data are presented as the fold increase in luciferase activity (means $\pm$ S.D.) relative to the control.

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The Sp1 binding site is required for butyric acid-induced HIV-1 LTR gene expression

Butyric acid inhibits the enzymatic activity of HDAC by competing with the HDAC substrate at the enzyme catalytic centre [18], stimulating transcription of various genes including HIV-1 [15, 20, 21]. Three tandem repeats of putative Sp1-binding sites (GC) (position -69 to -78, position -58 to -67 and position -47 to -56), one AP-4 (position -16 to -21) and two LSF (position +2 to -3 and position +12 to +8) binding sites are located within or near the HIV-1 LTR start site and these transcription factors recruit HDAC1 to the HIV-1 LTR [10, 11, 14]. To examine the role of these *cis*-acting elements and the respective relevant transcription factors in butyric acid-induced HIV-1 gene expression, we created a series of plasmid constructs of HIV-1 LTR with mutations to the three Sp1 binding sites, AP-4

Fig. 2. The Sp1 binding site is essential for butyric acid-induced transactivation of HIV-1 LTR. (A) A schematic diagram of the HIV-1 LTR plasmid constructs with mutations to the Sp1, AP-4 and LSF binding sites. (B) The Sp1 binding site is required for butyric acid-induced HIV-1 LTR gene expression. The HIV-1 LTR promoter constructs described in (A) were transiently transfected in HeLa cells, which were then treated with butyric acid (1.5 mM) for additional 24 h. The cells were then harvested and luciferase activity was measured. The data are presented as means $\pm$ S.D. of triplicate experiments.

Fig. 3. Sp1 is involved in latent HIV-1 LTR gene expression. (A) Confirmation of siRNA knockdown of Sp1. TZM-bl cells were transfected with 100 nM siRNA directed against either Sp1 or GFP (control) mRNAs. After 48 h of transfection, cells were lysed and Sp1 levels were assessed by western blotting using specific antibodies. The blot was stripped and re-probed with anti- β -actin antibody. (B) Inhibition of HIV-1 gene expression by Sp1 depletion. TZM-bl cells were transfected with either Sp1 siRNA or a control. After 48 h of transfection, cells were either left untreated or treated with 1.5 mM of butyric acid and incubated for additional 8 h. Then, the cells were harvested and luciferase activity was measured. (C) Mithramycin A inhibits butyric acid-stimulated HIV-1 replication from latently infected cells. ACH-2 cells were pre-treated with mithramycin A at the indicated concentrations. After 1 h of pre-treatment, cells were stimulated with butyric acid (1.5 mM) and incubated for additional 24 h. The culture lysate was analysed for detection of HIV-1 proteins by western blotting with sera collected from AIDS patients.

binding sites and LSF binding site as described (Fig. 2A). In cells transfected with mutant LTR reporters lacking either AP-4 or LSF binding site, the extent of transcriptional induction by butyric acid was largely unchanged compared with WT LTR (Fig. 2B; compare with Fig. 1B). In contrast, transcriptional activation by butyric acid was abolished when the tandem Sp1 binding sites were mutated. These findings indicate that Sp1 appears to be the crucial cis-regulatory element with LTR for the transcriptional activation of HIV-1 by butyric acid.

Fig. 4. CBP/p300 involvement of butyric acid-induced HIV-1 gene expression. (A) Effects of butyric acid on Sp1 promoter activation. A Sp1-luc reporter construct (20 ng) was transfected into HeLa cells, incubated for 24 h and stimulated with butyric acid (1, 2.5 and 5 mM) or TNF- α (1, 2 and 3 ng/ml) for additional 24 h. The luciferase activity of each cell lysate was then measured. (B) Effects of CBP on butyric acid-induced HIV-1 LTR gene expression. The CBP or mutant CBP (CBP Δ HAT) expression plasmid was co-transfected with a HIV-LTR luc reporter construct into HeLa cells. After 24 h of transfection, the cells were stimulated with butyric acid (1.5 mM) for additional 24 h, and then the luciferase activity of each cell lysate was measured. (C) CBP Δ HAT inhibits butyric acid-induced Sp1 promoter activation. The CBP Δ HAT expression plasmid was co-transfected with a Sp1-luc reporter construct into HeLa cells, which were incubat-

ed for 24 h and stimulated with butyric acid (1.5 mM) for additional 24 h. The luciferase activity of each cell lysate was then measured. (D) Inhibition of HIV-1 re-activation by C646. ACH2 cells were pre-treated with C646 (5 and 30 μ M) for 1 h and then stimulated with butyric acid (1.5 mM) for additional 24 h. The cell culture supernatants were analysed for p24 antigen levels by ELISA (upper panel). Experiments were performed in triplicate and data are presented as means and SDs (pg/mL). Detection of various viral proteins in the cell lysate was performed by western blotting (lower panel).

The effect of Sp1 inhibition on HIV-1 gene expression

To examine the effect of endogenous Sp1, we used siRNA technique to knockdown Sp1 expression and examined HIV-1 LTR transcriptional levels after depletion of endogenous Sp1. We employed TZM-bl cells, containing a stable chromosomally integrated HIV-1 LTR [24, 25], to confirm the effect of butyric acid on HIV-1 transcription. We found that significant depletion of Sp1 by siRNA knockdown (Fig. 3A) resulted in downregulation of the butyric acid-induced transcription from HIV-1 LTR (Fig. 3B) without significant reduction of the cell viability (data not shown). To further confirm the involvement of Sp1 in butyric acid-mediated induction of HIV-1 transcription, we used mithramycin A, a competitive antagonist for the DNA-binding of Sp1 [30], on HIV-1 replication from latently HIV-1 infected cells. In this experiment, we used the human ACH-2 cell line [22, 23], known to harbour latent HIV-1 proviruses and it is derived from human CD4 T cells and the most studied model of post-integration latency. We observed that butyric acid-induced HIV-1 replication in ACH-2 cells was greatly inhibited by mithramycin A (Fig. 3C). The similar result was observed in OM10.1 cells, a macrophage cell line chronically and latently infected with HIV-1 (data not shown).

Fig. 5. ChIP detection of Sp1 and CBP on HIV-1 LTR. (A) Dynamic association/dissociation of Sp1, HDAC1, c-myc, acetylated histone H3 (Ac-H3), RNA polymerase II (RNAPII) and CBP. TZM-bl cells were left untreated or treated with 1.5 mM butyric acid for 90 min, and the ChIP assay was performed using the designated antibodies. The recovered DNA was PCR-amplified using HIV-1 LTR-specific primers and separated on a 2% agarose gel. Input DNA (input) represents 10% of total input chromatin DNA, and immunoprecipitation with IgG serves as a negative control. (B) Sp1 recruits CBP to the HIV-1 LTR. TZM-bl cells were transfected with siRNA targeting Sp1 or GFP, incubated for 48 h, and the association of Sp1 and CBP were analysed using the ChIP assay. The experiments were repeated with reproducible results. Representative results are shown.

These observations suggested the crucial role of Sp1 for the butyric acid-induced HIV-1 replication.

CBP/p300 involvement in butyric acid-induced HIV-1 gene expression

To examine whether the effect of butyric acid on HIV-1 gene expression is solely dependent on Sp1, we utilized a synthetic promoter that is driven by three Sp1 consensus sites only (pSp1-luc) devoid of other cis-regulatory elements on HIV-1 LTR [29]. As shown in Fig. 4, when cells were transfected with pSp1-luc, the Sp1-dependent transcription activity was augmented by butyric acid in a dose-dependent manner. However, this effect was not observed with TNF- α (Fig. 4) confirming the result of Fig. 1. Previous studies have shown that Sp1 sites can recruit transcriptional co-activators such as cAMP response element-binding (CREB)-binding protein (CBP)/p300 as well as repressor factors/complexes [31, 32]. In addition, butyric acid treatment promoted the histone acetylation in latently HIV-1 infected cells [20, 21]. Thus it was suggested that derepression of HDAC activity by butyric acid appears to convert the action of Sp1 from transcriptional repressor to activator through subsequent recruitment of transcriptional co-activators such as CBP/p300 instead of HDAC1.

We then examined the effect of CBP overexpression on the butyric acid-induced HIV-1 gene expression. As shown in Fig. 4B, CBP overexpression dose-dependently augmented the butyric acid-induced HIV-1 LTR gene expression. In contrast, overexpression of a mutant CBP lacking histone acetyltransferase (HAT) activity (CBP Δ HAT) inhibited the butyric acid-induced HIV-1 gene expression (Fig. 4B). CBP Δ HAT also inhibited butyric acid-induced pSp1-luc activity (Fig. 4C). To further investigate the involvement of CBP in butyric acid-mediated activation, we used C646 a specific inhibitor of CBP/p300 HAT [33]. As demonstrated in Fig. 4D, the butyric acid-induced viral production was dramatically downregulated either in the culture supernatant (upper panel) or within the cytoplasm (lower panel) upon treatment of ACH-2 cells with C646. These results suggest that CBP is involved in the butyric acid-induced HIV-1 transcription at least in a part.

ChIP analysis to detect Sp1 and CBP on HIV-1 LTR

The above results suggest that Sp1 is solely responsible for the butyric acid-mediated transcriptional induction from HIV-1 LTR through recruitment of transcriptional coactivators such as CBP. To further confirm the role of Sp1 in the recruitment of CBP, we performed ChIP assay using specific antibodies with TZM-bl cells. When TZM-bl cells were treated with butyric acid to induce HIV-1 transcription, we observed dissociation of HDAC1 and c-Myc from the HIV-1 promoter (Fig. 5A). In contrast, CBP was recruited to the HIV-1 LTR upon

butyric acid treatment concomitantly with the recruitment of Ac-H3 and RNAPII to the viral promoter. Interestingly, the amount of Sp1 recruited to the HIV LTR remained unchanged by butyric acid treatment. To further confirm whether Sp1 is essential for the recruitment of CBP to the HIV-1 promoter, we examined the effect of Sp1 knockdown on the HIV-1 LTR transcription (Fig. 5B). Depletion of Sp1 by siRNA resulted in the abrogation of CBP recruitment and the local H3 acetylation in the vicinity of HIV-1 promoter. These results have indicated that inhibition of Sp1-associated HDAC activity by butyric acid is essential for the CBP recruitment to the HIV-1 promoter and induction of viral transcription.

Discussion

The ability of HIV-1 to establish the latent infection and its occasional re-activation is considered crucial for the progression of HIV-1-associated diseases. Thus, elucidation of the molecular mechanism that regulates the viral gene expression is essential for understanding the HIV-1 latency and for developing novel therapy against HIV-1 infection to prevent disease progression to AIDS. It is now clear that HDACIs could stimulate replication from HIV-1 provirus through transcriptional induction from the proviral LTR associated with histone deacetylation [2, 6, 9]. It was demonstrated that metabolites produced from anaerobic bacteria such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Clostridium cochlearium* are responsible for this HDACI activity [20, 21, 34]. Among the short chain (C2-C5) fatty acids (SCFAs) metabolites produced from anaerobic bacteria, butyric acid demonstrated the most potent activity as an HDACI and exhibited a number of distinctive biological activities when added to live cells [15, 16, 18]. We reported previously that butyric acid is the major SCFA for stimulation of HIV-1 gene expression in the latently infected cells and suggested that microbiological control of infection by such bacteria upon periodontitis or change of gut flora should prevent the HIV-1 disease progression [20, 21, 34]. Here we have provided scientific evidence to further clarify the molecular mechanism by which butyric acid activates latently infected HIV-1.

There are a number of distinct *cis*-regulatory elements within the HIV-1 LTR to which respective trans-acting factors interact and co-ordinately regulate viral transcription. Among the host transcription factors that are known to recruit HDAC proteins to the HIV-1 LTR thus being involved in the maintenance of HIV-1 latency [10-14], we found that the Sp1 sites among other *cis*-regulatory elements were responsible for the butyric acid-induced gene expression (Fig. 1 and 2). This finding was confirmed by the experiment using Sp1-specific promoter (Fig. 4) and Sp1 depletion using the specific siRNA (Fig. 3). In addition, a specific Sp1 inhibitor compound mithramycin A blocked the butyric acid-mediated induction of HIV-1 viral replication from the latently infected cells (Fig. 3). Thus we concluded that Sp1 is primarily responsible for the effect of butyric acid.

Sp1 binding sites, rich in GC sequences, are ubiquitously present in many cellular and viral promoters. In fact, Sp1 binding sites are occupied during the transcription of large number of genes and actively involved in their gene expression [35-37]. Although it is still too premature to conclude that the presence of Sp1 binding sites is prerequisite for the responsiveness to butyric acid, a previous study reported poor butyrate activation of some cellular genes including the p21/WAF1/Cip1 and death receptor 5 genes that lack Sp1 binding sites in the promoter [38, 39]. Choudhary et al. reported that hexamethylbisacetamide could activate HIV-1 expression depending on the presence of the Sp1 site through recruitment of CDK9, a catalytic subunit of P-TEFb that is essential for the Tat-mediated trans-activation of HIV-1, in the absence of NF- κ B binding site [40]. Thus, together with our findings, the butyric acid-induced HIV-1 gene expression is attributable to Sp1. Furthermore, it was reported that butyrate-induced HIV-1 LTR expression does not require *de novo* protein synthesis [17]. Interestingly, Sp1 was required for the transcriptional repression mediated by c-Myc acting as a docking molecule between c-Myc and HDAC1 [11]. In our experiment with ChIP assay, butyric acid administration disrupted the formation of repressor complex by displacing

HDAC1 from the HIV-1 promoter region (Fig. 5). Thus, Sp1 might act as a docking molecule between transcription factors and transcriptional co-regulators either co-activators or co-repressors.

We further found that the presence of transcriptional coactivator CBP/p300 and its HAT activity was indispensable by the experiments using CBP mutant lacking HAT activity (CBP Δ HAT) and a specific inhibitor for CBP/p300 (Fig. 4 and 5). Upon the treatment with butyric acid, the enzymatically active CBP was recruited to the HIV-1 LTR as evidenced by the appearance of the acetylated form of H3 in the HIV-1 promoter (Fig. 5). Furthermore, siRNA-mediated Sp1 depletion resulted in the decreased recruitment of CBP to the promoter (Fig. 5). These observations suggested that CBP competes with HDAC1 binding to Sp1 upon treatment with butyric acid, thus abolishing HDAC1-mediated repression of HIV-1 LTR.

It is well established that HIV-1 gene regulation depends on reversible acetylation of histones and subsequent changes in the chromatin structure. Previous studies demonstrated that Sp1 could act as both positive and negative regulator of gene expression in the context of transcriptional regulatory circuit, wherein Sp1 can directly interact with either HATs, a transcriptional coactivator, or a transcriptional repressor, such as HDAC [31, 32, 36]. In addition, several nuclear HATs, such as CBP/p300 and p300/CBP-associated factor, are positively involved in HIV-1 transcription [6, 9]. Moreover, the minimum LTR promoter (CD52 Luc) and even the Sp1 Luc reporter plasmid were susceptible to the butyric acid-induced transcriptional activation (Fig. 1 and 4). Thus, it is suggested that butyric acid could activate Sp1-dependent transcription by altering chromatin modification via HATs through inhibiting HDAC activity. In addition, previous reports indicated that acetylation of Sp1 influences its transcriptional activity and protein stability [36, 41]. Moreover, Sp1 was shown to contribute to the recruitment of a positive transcription elongation factor complex to the HIV-1 promoter [40]. It is possible that such biochemical modification of general transcription factors or mediators might determine the direction of transcriptional regulation and play a key role in this drastic transcriptional conversion. Thus, Sp1 represents a modular molecule that specifically target HDACs and HATs at the HIV-1 promoter, regulating histone acetylation status. Further studies are warranted to elucidate the molecular mechanism and the direct target molecule of butyric acid-mediated de-acetylation that converts transcriptionally-silent Sp1 to its active form.

The biological and clinical implication of this butyric acid-mediated transcriptional control of HIV-1 replication might explain why periodontitis is strongly correlated with the progression of HIV-1 infection [34, 42, 43]. There are a number of butyric acid-producing bacteria in the gut and vagina, both of which are major sites of HIV-1 transmission and its replication [44-49]. In fact, it is known that infection of butyric acid-producing bacteria is involved in AIDS progression and causally related to the re-activation of the latent HIV-1 provirus [20, 21, 47, 50, 51]. Further elucidation of the molecular mechanism by which butyric acid activates the latent HIV-1 provirus and basic studies of butyrate-producing bacteria should facilitate the development of new measures to prevent the clinical progression of HIV-1 infection though developing specific small molecular compounds or even probiotics approaches.

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Disclosure Statement

The authors confirm that there are no conflicts of interest.

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