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Histone Deacetylase Inhibitors Induce the Differentiation of Eosinophilic Leukemia EoL-1 Cells into Eosinophils

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Abstract – EoL-1 cells differentiate into eosinophils in the presence of *n*-butyrate, but the mechanism has remained to be elucidated. Because *n*-butyrate can inhibit histone deacetylases, we hypothesized that the inhibition of histone deacetylases induces the differentiation of EoL-1 cells into eosinophils. In this study, using *n*-butyrate and two other histone deacetylase inhibitors, apicidin and trichostatin A, we have analyzed the relationship between the inhibition of histone deacetylases and the differentiation into eosinophils in EoL-1 cells. It was demonstrated that apicidin and *n*-butyrate induced a continuous acetylation of histones H4 and H3, inhibited the proliferation of EoL-1 cells, and induced the expression of markers for mature eosinophils such as integrin β7, CCR1, and CCR3 on EoL-1 cells, while trichostatin A evoked a transient acetylation of histones and induced no differentiation into eosinophils. These findings suggest that the continuous inhibition of histone deacetylases in EoL-1 cells induces the differentiation into mature eosinophils.

Keywords □ EoL-1 cells, eosinophils, differentiation, histone deacetylase inhibitor

INTRODUCTION

Chromatin comprises repeating units of nucleosome core particles and linker DNA (Klug *et al.*, 1980). The particles consist of an octamer of core histone containing two molecules of each histone, H2A, H2B, H3 and H4, around which 145 bp of DNA are wrapped in 1.75 turns (Csordas, 1990; Grunstein, 1997). In gene expression, histones in the nucleosome core particles are acetylated at the ε-amino group of specific lysine residues of the N-terminus by histone acetyltransferases (HATs) such as CBP/p300, GCN5, PCAF and TAFII250 (Grunstein, 1997). It is reported that numerous transcription factors are also acetylated by HATs and the acetylation attenuates their activity (Ishihara *et al.*, 2005). The acetylated lysine residues in histones or transcription factors are deacetylated by histone deacetylases (HDACs) (Sterner and Berger, 2000). Therefore, the acetylation by HATs and the deacetylation by HDACs play important roles in cellular function by controlling the level of gene expression. Recently, natural and synthetic compounds with the ability to inhibit HDAC activity have been reported. These HDAC inhibitors are classified into the following 4 groups; the short-chain fatty acids *n*-butyrate (Riggs *et al.*, 1977) and valproic acid (VPA) (Gurvich *et al.*, 2004), the hydroxamic acid trichostatin A (TSA) (Yoshida *et al.*, 1987), the cyclic tetrapeptide apicidin (Darkin-Rattray *et al.* 1996), and the benzamide MS-27-275 (Saito *et al.* 1999). When HDACs are inhibited by HDAC inhibitors, the acetylation by HATs in sustains, resulting in an accumulation of acetylated histones that induces various biological activities including differentiation, proliferation, and apoptosis via gene expression (Villar-Garea and Esteller, 2004).

It has been reported that *n*-butyrate induces the differentiation of eosinophilic leukemia EoL-1 cells (Saito *et al.*, 1985) and HL-60 clone 15 cells (Fischkoff and Condon, 1985) into mature eosinophils. The development and function of eosinophils under conditions of allergic inflammation such as bronchial asthma have also been studied using these cell lines

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(Izumi *et al.*, 1995; Hara *et al.*, 2001). However, the mechanism by which these cells differentiate into mature eosinophils on exposure to *n*-butyrate has remained to be elucidated for about 20 years. Recently, we have suggested that apicidin and *n*-butyrate induce a continuous acetylation of histone H4 and H3, while TSA induces a transient acetylation, and the continuous inhibition of HDACs induces the differentiation of HL-60 clone 15 cells into mature eosinophils (Ishihara *et al.*, 2004). However, it has not been clarified whether EoL-1 cells also differentiate into mature eosinophils following the continuous inhibition of HDACs. Because *n*-butyrate acts to induce the differentiation of EoL-1 cells into eosinophils, clarification of the mechanism involved may contribute to the development of a novel medication for eosinophilic leukemia and for inhibition of the differentiation of stem cells into mature eosinophils in individuals with allergic diseases. Therefore, in this study, we compared the effect of the three HDAC inhibitors apicidin, TSA and *n*-butyrate on HDAC inhibition and on the differentiation of EoL-1 cells into mature eosinophils.

MATERIALS AND METHODS

Materials

EoL-1 cells were purchased from RIKEN Cell Bank (Tsukuba, Japan). Apicidin was from Calbiochem (San Diego, CA, USA). Trichostatin A (TSA) and *n*-butyrate were obtained from Wako (Osaka, Japan).

Cell culture

EoL-1 cells were maintained in RPMI-1640 medium (pH 7.2) (Sigma, St. Louis, MO, USA) supplemented with 10% FBS (Sigma) at 37°C in a humidified atmosphere of 5% CO_2. For the experiments, the cells (5 x 10^4/ml) were incubated at 37°C for specified periods in RPMI-1640 medium (pH 7.8) containing various concentrations of apicidin, TSA or *n*-butyrate.

Proliferation assay

EoL-1 cells were incubated at 37°C for specified periods in medium containing various concentrations of each drug. After the incubation, the cells were enumerated using a hemocytometer.

Western blotting

EoL-1 cells were incubated at 37°C for specified periods with various concentrations of each drug. After the incubation, the cells were washed three times with phosphate-buffered saline (PBS), suspended at 5 x 10^6 cells/ml in a sample buffer (62.5 mM Tris-HCl, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 0.00125% bromophenol blue, and 5% β-mercaptoethanol), sonicated for 15 s using a Handy Sonic Disruptor (Tomy Seiko, Tokyo, Japan) and incubated for 15 min at 65°C. The cell lysate was subjected to electrophoresis on a 15% acrylamide gel for 2 h at 125 V and the proteins separated in the gels were transferred onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) and blocked in a blocking solution (Block Ace, Dainippon Sumitomo Pharma, Osaka, Japan) for 1 h at room temperature. To detect the acetylated histone H4, an acetylated lysine^{14} residue on histone H3, and p27^{Kip1}, the nitrocellulose membrane was incubated for 12 h at 4°C with rabbit anti-acetylated histone H4 polyclonal antibody (ChiPs grade, Upstate Biotechnology, Waltham, MA, USA), rabbit anti-acetylated histone H3 (K14) polyclonal antibody (Upstate Biotechnology), and rabbit anti-p27^{Kip1} polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively. After 4 washes with Tris-buffered saline (TBS) containing 0.1% Tween 20, the membrane was incubated at 4°C for 4 h with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA). The reaction products were incubated for 30 min at room temperature with Vectastain ABC reagent (Vector Laboratories) and visualized using the Chemiluminescence Detection System (Western Lightning Chemiluminescence Reagent Plus, PerkinElmer Life Sciences, Boston, MA, USA). The membrane was exposed to Kodak X-Omat AR film (Eastman Kodak, Rochester, NY, USA). For the detection of actin as an internal control, the membrane was incubated with goat anti-actin polyclonal antibody (Santa Cruz Biotechnology) and phosphatase-labeled anti-goat IgG (Vector Laboratories) as described above. The reaction products were visualized with BCIP/NBT color substrate (Promega, Madison, WI, USA).

Flowcytometry

EoL-1 cells were incubated at 37°C for specified periods with various concentrations of each drug. The cells were harvested and washed with PBS containing 0.25% BSA (Sigma). For the analysis of viability, the cells were incubated for 30 min at room temperature in 0.1 ml of PBS containing 2 μg of 7-amino-actinomycin D (7-AAD, Sigma). For the detection of CC chemokine receptor (CCR) 1, CCR3, and integrin β7, the cells were incubated with phycoerythrin (PE)-labeled anti-human CCR1 (R&D systems, Oxon, UK), anti-human CCR3 (R&D systems) or anti-human integrin β7 (BD Pharmingen,

San Diego, CA, USA) for 30 min at 4°C, and then washed three times with 0.25% BSA-PBS. The fluorescence of the cells stained with 7-AAD and PE was analyzed with a flowcytometer (FACScan, Beckton Dickinson, San Jose, CA, USA).

Statistical analysis

The statistical significance of the results was analyzed using Dunnett's test for multiple comparisons.

RESULT

Effects of apicidin, TSA, and *n*-butyrate on the growth of EoL-1 cells

To clarify whether apicidin, TSA, and *n*-butyrate inhibit the proliferation of EoL-1 cells, the number of cells after the incubation with these HDAC inhibitors was counted. As shown in Fig. 1A, EoL-1 cells proliferated in a time-dependent manner during 8 days of incubation in the absence of HDAC inhibitors. Apicidin at 100 nM and *n*-butyrate at 500 μM significantly inhibited the proliferation of EoL-1 cells until day 8. On treatment with TSA at 30 nM, significant inhibition of the proliferation of EoL-1 cells was observed on days 2 and 4 but not on days 6 and 8 (Fig. 1A). After 8 days of incubation, no significant inhibition was observed by apicidin at 10 and 30 nM or TSA at 30 nM (Fig. 1B).

Effects of apicidin, TSA, and *n*-butyrate on the viability of EoL-1 cells

EoL-1 cells were incubated for 24 h at 37°C with various concentrations of apicidin, TSA or *n*-butyrate. After the incubation, the viability of EoL-1 cells was determined by flowcytometry using 7-AAD. On treatment with apicidin at 10-100 nM, TSA at 10 and 30 nM, or *n*-butyrate at 500 μM, no significant change in the viability of EoL-1 cells was observed (data not shown). At higher concentrations of apicidin (300 and 1000 nM) and TSA (100 nM), the viability was significantly decreased (data not shown).

Effects of apicidin, TSA, and *n*-butyrate on the acetylation of histones H4 and H3

To clarify whether apicidin, TSA, and *n*-butyrate inhibit HDAC in EoL-1 cells, we determined the levels of acetylated-histones H4 and H3 by Western blotting. As shown in Fig. 2A, treatment of EoL-1 cells with 100 nM of apicidin or 500 μM of *n*-butyrate dramatically induced the acetylation of histone H4. However, the acetylation of histone H4 by TSA at 30 nM reached a peak at 4 h and then declined until 48 h (Fig. 2A and B). The acetylation of histone H4 after 8 days of incubation with apicidin increased in a concentration-dependent manner (Fig. 2C). The acetylation of the lysine[14] residue on histone H3 (histone H3K14) was induced by apicidin at 100 nM and *n*-

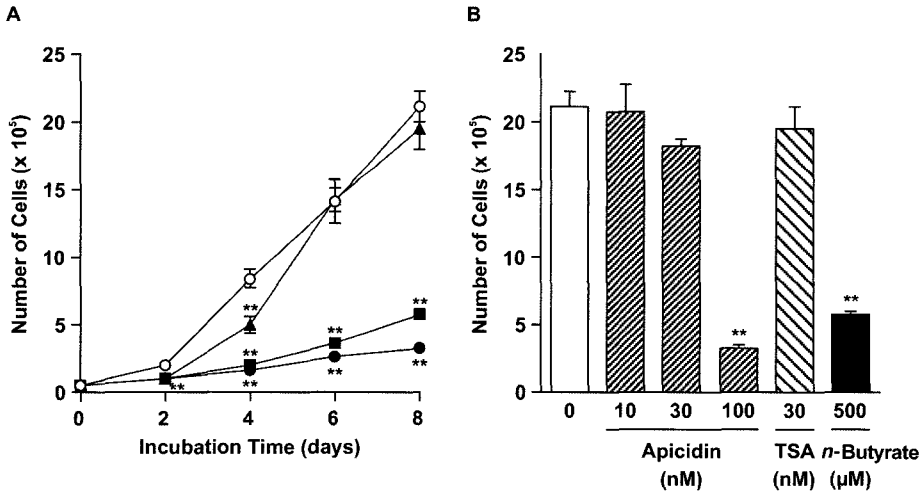

Fig. 1. Effects of apicidin, TSA, and *n*-butyrate on the growth of EoL-1 cells. (A) EoL-1 cells were incubated for the periods indicated at 37°C in the absence (open circles) or presence of 100 nM apicidin (closed circles), 30 nM TSA (closed triangles), or 500 μM *n*-butyrate (closed squares). The cells were then harvested and enumerated. Values are the means from three samples with the S.E.M. Statistical significance: $^{**}P < 0.01$ vs. the corresponding control. (B) EoL-1 cells were incubated for 8 days at 37°C in medium containing the indicated concentration of apicidin, TSA, or *n*-butyrate. The cells were then harvested and enumerated. Values are the means from three samples with the S.E.M. Statistical significance: $^{**}P < 0.01$ vs. the control.

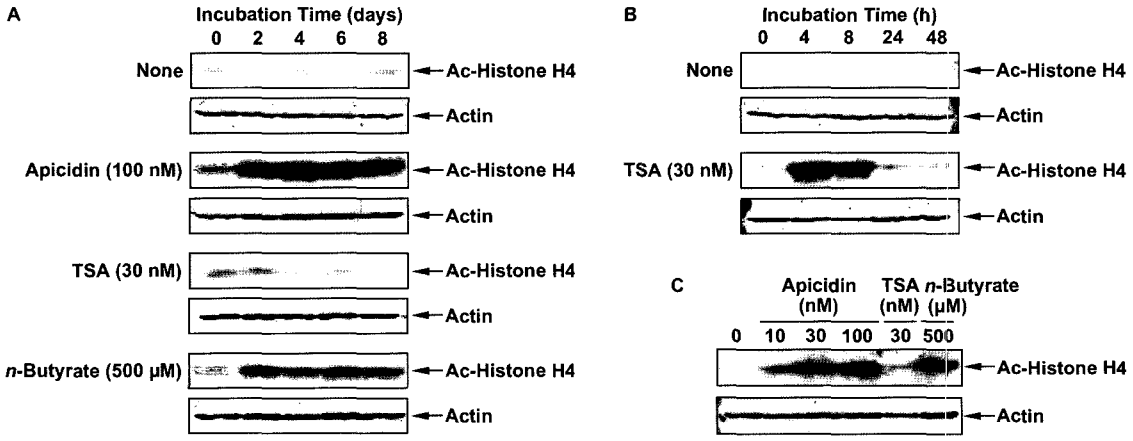

Fig. 2. Effects of apicidin, TSA, and *n*-butyrate on the acetylation of the histone H4. (A, B) EoL-1 cells were incubated for the periods indicated at 37°C in the presence or absence of apicidin (100 nM), TSA (30 nM), or *n*-butyrate (500 μM). (C) EoL-1 cells were incubated for 8 days at 37°C in medium containing the indicated concentration of apicidin, TSA, or *n*-butyrate. The cell lysates were then electrophoresed, and acetylated-histone H4 and actin were detected by Western blotting.

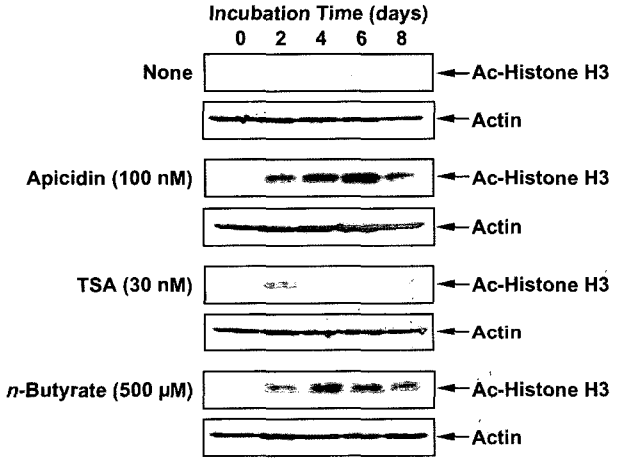

Fig. 3. Effects of apicidin, TSA, and *n*-butyrate on the acetylation of histone H3K14. EoL-1 cells were incubated for the periods indicated at 37°C in the presence or absence of apicidin (100 nM), TSA (30 nM), or *n*-butyrate (500 μM). The cell lysates were then electrophoresed, and acetylated-histone H3K14 and actin were detected by Western blotting.

butyrate at 500 μM on days 2 to 8, while the acetylation of histone H3K14 by TSA at 30 nM was detected only on day 2 (Fig. 3).

Effects of apicidin, TSA, and *n*-butyrate on the expression of p27^{Kip1} in EoL-1 cells

We next examined the effects of apicidin, TSA, and *n*-butyrate on the expression of the cyclin-dependent kinase inhibitor p27^{Kip1} by Western blotting. Apicidin at 100 nM and *n*-butyrate at 500 μM continuously induced the expression of p27^{Kip1} until day 8 (Fig. 4A). In contrast, the expression of p27^{Kip1} induced by TSA at 30 nM reached a maximum at 24 h and decreased gradually (Fig. 4B).

Effects of apicidin, TSA, and *n*-butyrate on the differentiation of EoL-1 cells into eosinophils

After 8 days of incubation, apicidin at 100 nM and *n*-butyrate at 500 μM induced changes in the intracellular structure of EoL-1 cells as determined by measuring side scattering (SSC) (Fig. 5). Furthermore, apicidin at 100 nM and *n*-butyrate at 500 μM induced the expression of the markers of eosinophils, CCR1 and CCR3, on EoL-1 cells (Fig. 5). However, apicidin at 10 and 30 nM and TSA at 30 nM did not induce these changes (Fig. 5). Integrin β7, a marker of eosinophils, was expressed on untreated EoL-1 cells (Fig. 5). Apicidin at 100 nM and *n*-butyrate at 500 μM slightly increased the expression of integrin β7, while apicidin at 10 and 30 nM and TSA at 30 nM had no effect (Fig. 5).

DISCUSSION

EoL-1 cells, which were established from a patient with eosinophilic leukemia in 1985, have been utilized for studies on eosinophilic leukemia (Saito *et al.*, 1985; Cools *et al.*, 2003; Griffin *et al.*, 2003). The development and function of eosinophils under conditions of allergic inflammation such as bronchial asthma have also been studied using EoL-1 cells (Izumi *et al.*, 1995; Hara *et al.*, 2001), because the cells differentiate into mature eosinophils when exposed to *n*-butyrate (Saito *et al.*,

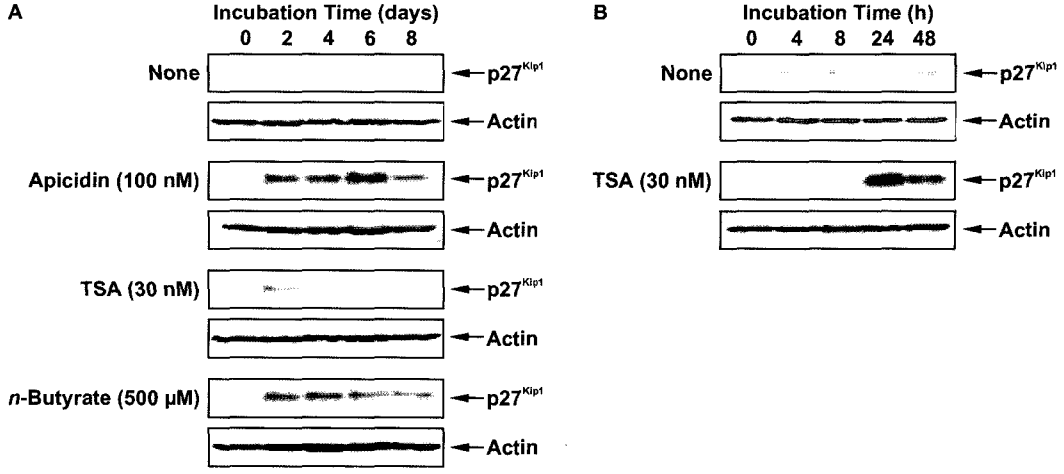

Fig. 4. Effects of apicidin, TSA, and *n*-butyrate on the expression of $p27^{Kip1}$ in EoL-1 cells. (A, B) EoL-1 cells were incubated at 37°C for the periods indicated in the presence or absence of apicidin (100 nM), TSA (30 nM), or *n*-butyrate (500 μM). The cell lysates were then electrophoresed, and $p27^{Kip1}$ and actin were detected by Western blotting.

1985). Clarification of the mechanism behind this differentiation may contribute to the development of a novel treatment for eosinophilic leukemia and for inhibition of the differentiation of stem cells into mature eosinophils in individuals with allergic diseases.

Eosinophils have basic proteins in their granules, and various

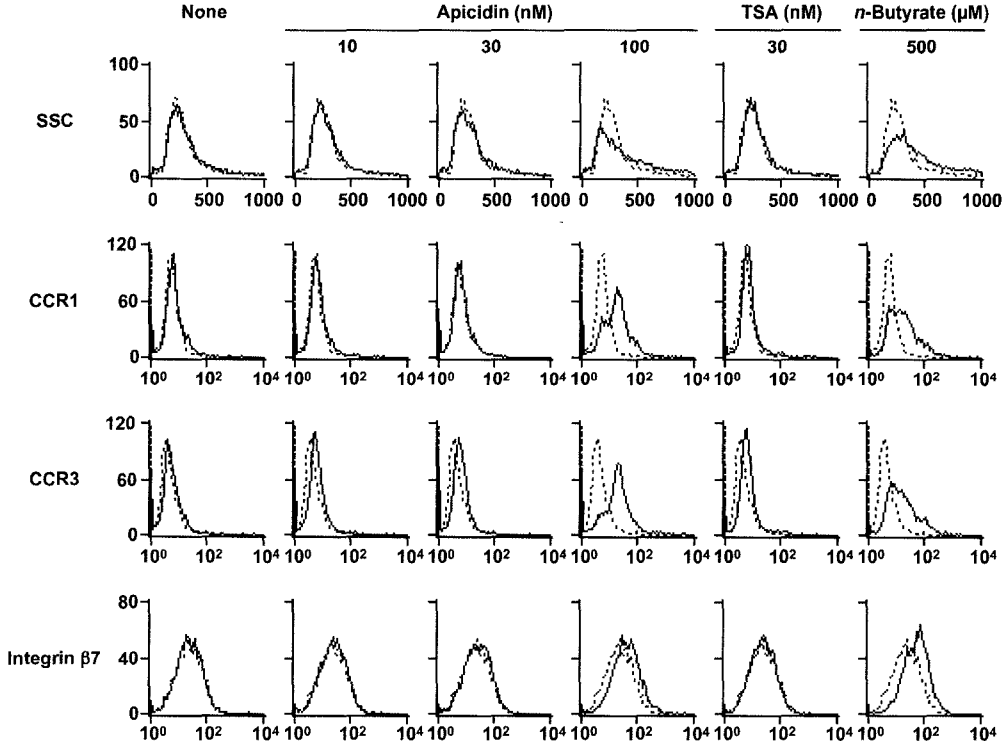

Fig. 5. Effects of apicidin, TSA, and *n*-butyrate on the differentiation of EoL-1 cells into eosinophils. EoL-1 cells were incubated for 8 days at 37°C in medium containing the indicated concentration of apicidin, TSA, or *n*-butyrate. The intracellular structure and the expression of CCR1, CCR3, and integrin β7 were determined by flowcytometry. Dotted and solid lines represent the histogram before and after incubation with the drug, respectively.

receptors and adhesion molecule on their surface (Giembycz and Lindsay, 1999). In this study, we showed the different effects of the HDAC inhibitors apicidin, TSA, and *n*-butyrate on the differentiation of EoL-1 cells into eosinophils. Apicidin at 100 nM suppressed cell proliferation (Fig. 1) without cytotoxicity (data not shown), and induced a change in the intracellular structure as well as the cell-surface expression of the eosinophilic markers integrin β7, CCR1, and CCR3 (Fig. 5), as did *n*-butyrate at 500 μM. In contrast, TSA at 30 nM transiently inhibited the proliferation of EoL-1 cells (Fig. 1), and induced no changes in intracellular structure (Fig. 5) and the cell-surface expression of eosinophilic markers (Fig. 5). The cyclin-dependent kinase inhibitor $p27^{kip1}$, which suppresses cell proliferation as an inhibitor of the cell cycle (Johnson and Walker, 1999), was expressed continuously on exposure to apicidin and *n*-butyrate, but only transiently in the presence of TSA (Fig. 4). As shown in Figs. 3 and 4, continuous acetylation of histone H4 and H3K14 was induced by 100 nM of apicidin or 500 μM of *n*-butyrate until day 8. On the other hand, although 30 nM of TSA acetylated histone H4 at 4 and 8 h (Fig. 2B), and histone H3K14 at 2 days (Fig. 3), the acetylation returned to basal levels thereafter. It is reported that the acetylation of histone H4K8 and H3K9 by HATs such as CBP/p300 and GCN5 results in the phosphorylation of histone H3S10 (Agalioti *et al.*, 2002). The acetylation of histone H3K14 is enhanced by the phosphorylation of H3S10 (Berger, 2002). In the expression of various genes, the binding of activated transcription factors to the promoter region of the target gene leads to the recruitment of HAT, followed by the recruitment of remodeling factors such as SWI/SNF, and then basal transcription factors including TFIID (Agalioti *et al.*, 2000, 2002; Merika and Thanos, 2001). The acetylation of histones is needed to install each component in the promoter region, because these components selectively recognize the acetylated lysine residue in the histone via their bromodomain (Dhalluin *et al.*, 1999; Jacobson *et al.*, 2000; Agalioti *et al.*, 2002). Notably, the acetylation of histone H4K8 regulates recruitment of the SWI/SNF complex, while the acetylation of histone H3 K9 and K14 results in the recruitment of TFIID. These reactions culminate in transcriptional activation (Agalioti *et al.*, 2000, 2002). Therefore, such findings suggest that the continuous acetylation of histones H4 and H3K14 by apicidin and *n*-butyrate contributes to the continuous recruitment. Also the activation of a transcription factor complex leading to the expression of various gene products characterizing eosinophils including CCR1, CCR3 and integrin β7. It is reported that the half-life of TSA in cell cultures is 14.7 h (Komatsu *et al.*, 2001), and the differentiation of HL-60 clone 15 cells into eosinophils was induced by repeated treatment (more than 3 times at an interval for 12 h) with TSA at 30 nM (Ishihara *et al.*, 2004), suggesting that TSA has no activity to induce EoL-1 cells to differentiate into eosinophils due to its short half-life. In this study, we found that 1) apicidin induces the continuous acetylation of histones H4 and H3 and has activity to induce the differentiation of EoL-1 cells into eosinophils, 2) apicidin at 100 nM is as effective as *n*-butyrate at 500 μM, and 3) TSA has no influence on the differentiation of EoL-1 cells into eosinophils due to its transient effect on the acetylation of histones H4 and H3. Therefore, we suggest that the continuous acetylation of histones H4 and H3 induces the differentiation of EoL-1 cells into eosinophils.

We showed that the cyclin-dependent kinase inhibitor $p27^{kip1}$ was expressed continuously in response to apicidin and *n*-butyrate but only transiently in the presence of TSA (Fig. 4). Because it is reported that $p27^{kip1}$ acts as a key regulator of accumulation at G_0-G_1 in the cell cycle (Johnson and Walker, 1999) and participates in cellular differentiation (Liu *et al.*, 1996; McArthur et al., 2002; Bryja et al., 2004), one of the mechanisms by which apicidin and *n*-butyrate suppress the proliferation and induce the differentiation of EoL-1 cells might be the expression of $p27^{kip1}$.

In this study, we showed that EoL-1 cells differentiate into eosinophils when exposed to apicidin or *n*-butyrate. However, these compounds do not induce the differentiation of all types of precursor cells into eosinophils. HDAC inhibitors including apicidin and *n*-butyrate induce a cell type-specific differentiation in many cases by inhibiting HDAC activity: eosinophilic leukemia EoL-1 cells and HL-60 clone 15 cells differentiate into eosinophils in the presence of apicidin or *n*-butyrate (Ishihara *et al.*, 2004), erythroleukemia K562 cells and MEL cells differentiate into erythrocytic cells when exposed to apicidin, MS-275, TSA or *n*-butyrate (Malinin *et al.*, 1980; Yoshida *et al.*, 1987; Witt *et al.*, 2003), myelomonocytic leukemia U-937 cells are induced to differentiate into monocytic cells by VPA (Gurvich *et al.*, 2004), and promyelocytic leukemia HL-60 cells undergo an early differentiation into CD11b-expressing cells in the presence of apicidin or TSA (Hong *et al.*, 2003). However, no expression of CD11b on K562 cells was induced by apicidin and TSA (Hong *et al.*, 2003). The cell type-specific differentiation induced by HDAC inhibitors might be due to the difference in the target proteins acetylated by HAT in each type of cell. In eosinophilic differentiation, the transcription factors GATA1, GATA2, C/EBPα, C/EBPβ C/EBPε, PU.1, and Stat5

cooperatively and synergistically play important roles in the expression of eosinophilic-specific genes (Yamaguchi *et al.*, 1998; Baltus *et al.*, 1999; Du *et al.*, 2002; Hirasawa *et al.*, 2002; Buitenhuis *et al.*, 2003; Gombart *et al.*, 2003). Among them, GATA-1 (Boyes *et al.*, 1998), GATA-2 (Hayakawa *et al.*, 2004), and C/EBPβ (Joo *et al.*, 2004) are acetylated by CBP/p300, PCAF or GCN5, resulting in an increase in DNA-binding activity. Thus, enhanced acetylation of histones and transcription factors in EoL-1 cells exposed to apicidin or *n*-butyrate leads to the differentiation of Eol-1 cells into eosinophils. It is thought that HDAC inhibitors have an ability to induce the differentiation of precursor cells into mature cells, and the maturation could be promoted by changes in the endogenous balance of HATs and HDACs in leukemia cells. Therefore, we also suggest that HDAC inhibitors are applicable to the diagnosis of leukemia, for example, in the detailed identification of the type of leukemia cell. The balance between HAT and HDAC activities is important for the pathogenesis of diseases. In fact, it is reported that the total activity of HDACs and the expression of HDAC2 are reduced in lung macrophages and peripheral lung tissue obtained from patients with chronic obstructive pulmonary disease (COPD), resulting in the insensitivity to glucocorticoid in COPD (Cosio *et al.*, 2004; Ito *et al.*, 2005). Because glucocorticoid receptors deacetylated by HDAC2 inhibit inflammatory gene expression via NF-κB through the interaction with NF-κB, HDAC2 activity plays an important role in the prevention of gene expression via NF-κB (Ito *et al.*, 2006). Therefore, clarification of the mechanism regulating the balance between HAT and HDAC activities would contribute to the development of a novel treatment.

In conclusion, we have demonstrated that apicidin and *n*-butyrate induce the differentiation of EoL-1 cells into eosinophils. *n*-Butyrate acts by continuously inhibiting the histone deacetylases, resulting in a continuous acetylation. HDAC inhibitors such as apicdin and *n*-butyrate would be useful for the treatment of eosinophilic leukemia.

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