p15^{INK4b} in HDAC inhibitor-induced growth arrest

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Abstract Histone deacetylase (HDAC) inhibitors arrest human tumor cells at the G1 phase of the cell cycle and activate the cyclin-dependent kinase inhibitor, p21$^{WAF1/Cip1}$. However, several studies have suggested the existence of a p21$^{WAF1/Cip1}$-independent molecular pathway. We report here that HDAC inhibitors, trichostatin A (TSA) and sodium butyrate, activate the p15^{INK4b} gene, a member of the INK4 gene family, through its promoter in HaCaT cells. Furthermore, we show that up-regulation of p15^{INK4b} by TSA is associated with cell growth inhibition of HCT116 p21 (−/−) cells. Our findings suggest that p15^{INK4b} is one of the important molecular targets of HDAC inhibitors.

Key words: p15^{INK4b}; HDAC inhibitor; Trichostatin A; Butyrate; G1 arrest; Promoter; p21$^{WAF1/Cip1}$

1. Introduction

Cell cycle progression is regulated by proteins called cyclins and cyclin-dependent kinases (CDKs) that associate with each other [1]. The process of cyclin-dependent activation of CDKs is counterbalanced by CDK inhibitors [2]. Two families of CDK inhibitors have already been identified in mammalian cells. One family, the CIP/KIP family, is comprised of p21$^{WAF1/Cip1}$, p27^{Kip1} and p57^{Kip2}, and each member inhibits cyclin E/A-CDK2 complexes [3]. Another family is also called INK4 family proteins. The members of this family, designated p15^{INK4b}, p16^{INK4a}, p18^{INK4c} and p19^{INK4d}, bind directly to CDK4/6 and are specific inhibitors of the cyclin D-dependent kinases [4–6].

Histone deacetylase (HDAC) inhibitors have been reported to arrest the cell cycle at the G1 or G2 phase [7] or to induce differentiation and apoptosis [8,9]. These agents also inhibit the growth of cancer cells in animal models [10]. Clinical applications have been started in a phase II study testing depsipeptide, one of the HDAC inhibitors, in patients with T-cell lymphoma [11].

We previously demonstrated that HDAC inhibitors, such as sodium butyrate (butyrate) and trichostatin A (TSA), inhibit cellular proliferation and induce the expression of the p21$^{WAF1/Cip1}$ gene through its promoter in a p53-independent manner [12,13]. However, several recent reports have suggested the existence of a p21$^{WAF1/Cip1}$-independent pathway of growth arrest by HDAC inhibitors [14–16]. Our results demonstrate that HDAC inhibitors activate p15^{INK4b}, a member of INK4 family proteins, through its promoter in human immortalized keratinocyte HaCaT. Furthermore, to exclude the influence of p21$^{WAF1/Cip1}$ on TSA-mediated cell growth inhibition, we investigated the effect of TSA on p21$^{WAF1/Cip1}$-deleted human colorectal carcinoma cell line HCT116 p21 (−/−) cells.

2. Materials and methods

2.1. Cell culture and reagents

HaCaT cells (a kind gift from Dr. N.E. Fusenig, German Cancer Research Center, Heidelberg, Germany) and HCT116 p21 (−/−) cells (a kind gift from Dr. B. Vogelstein, Johns Hopkins University School of Medicine, Baltimore, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere of 5% CO_2. TSA (Wako, Osaka, Japan) was dissolved in ethanol and sodium butyrate (Wako, Osaka, Japan) in water.

2.2. Analysis of cell cycle progression

Unsynchronized HaCaT or HCT116 p21 (−/−) cells were exposed to HDAC inhibitors for 24 h. HaCaT cells were treated with Triton X-100. HCT116 p21 (−/−) cells were fixed in 70% ethanol and treated with RNase. The nuclei were stained with propidium iodide before the DNA content was measured using a Becton Dickinson FACS Calibur (Becton Dickinson, Mountain View, CA, USA). At least 10 000 cells were counted. The ModFit LD V2.0 software package (Becton Dickinson, Franklin Lakes, NJ, USA) was used to analyze the data.

2.3. Cloning of the 5′-flanking region of the human p15^{INK4b} gene

Genome screening was performed using a human genomic leukocyte library in the IPS1 phage (Mo Bi Tec, Gottingen, Germany) as described previously [17]. The probe encoding a part of the human p15^{INK4b} gene was amplified by PCR based on the data in GenBank accession no. AC000049, with the forward (5′-TAAATAGGTAAGAGTGCAAACAAAG-3′) and the reverse (5′-ATTGCTTCTGGGAAAAAGCGCCTAG-3′) primers.

2.4. Plasmid preparation

An approximately 7.8 kb fragment derived from the positive phage DNA was subcloned into the luciferase reporter plasmid pGVB2 (Nippon Gene, Tokyo, Japan). Human p15^{INK4b} expression vector (pCD15) and its empty vector (pCD0) were gifts from C.W. Miller, UCLA School of Medicine, Los Angeles, CA, USA [18]. These vectors encode neomycin resistance gene.

2.5. Protein isolation and Western blot analysis

The cells were lysed by RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate) for p15^{INK4b} detection or lysis buffer (50 mM Tris–HCl, pH 7.5, 0.1% SDS) for detection of the retinoblastoma gene (RB) product. The protein extract was boiled for 5 min and loaded onto a 12% (for p15^{INK4b} detection) or 7% (for RB detection) polyacrylamide gel, electrophoresed, and transferred to a nitrocellulose membrane. A rabbit polyclonal antibody to p15^{INK4b} (C-20 from

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Santa Cruz Biotechnology, CA, USA) or to RB (PM-14001A from Pharmingen, NJ, USA) was used as the primary antibody. The signal was then developed with the enhanced chemiluminescence system (Amersham Pharmacia Biotech, UK Limited).

2.6. RNA isolation and Northern blot analysis

Total RNA was isolated using a Sepasol RNA isolation kit (Nacalai Tesque Inc., Kyoto, Japan), and poly(A)$^+$ mRNA was separated from 100 μg of total RNA using an Oligotex™-dT30 < Super > mRNA Purification kit (Takara Bio Inc., Shiga, Japan). Exon 1 of *p15* cDNA was used as a probe. Northern blot analysis was performed using standard methods [19]. The mRNA level was determined using a Fuji Image Analyzer Bas 2000 (Fujix, Tokyo, Japan).

2.7. Transient transfection and luciferase assay

HaCaT cells (5×10^4 cells) were seeded in 12-well plates, and 1.0 μg of reporter plasmid was transfected using a CellPhect transfection kit (Amersham Pharmacia Biotech, UK). After 24 h, HDAC inhibitors were added, and 48 h after transfection, the cells were harvested. The luciferase activity of each cell lysate was measured using the Luciferase Reporter Plasmid System (Promega, WI, USA). The luciferase activity was normalized for the amount of protein in each cell lysate. Each experiment was repeated at least three times. Data were analyzed using the two-tailed Student's *t*-test and differences were considered significant from controls when $P<0.05$.

2.8. Colony assay

HaCaT cells in a six-well plate were cultured for 1 day, and 2.0 μg of pCD15 or pCD0 was transfected using lipofectamine and plus reagent (Invitrogen, CA, USA). After transfection, cells were maintained in DMEM with 10% fetal bovine serum for 5 days. G418 (Nacalai Tesque Inc., Kyoto, Japan) was added to the medium to a final concentration of 800 μg ml^{-1}. Medium containing G418 was exchanged twice a week. After 10 days, resistant colonies were scored by the method of formalin fixation and crystal violet staining.

3. Results

3.1. HDAC inhibitors induce growth arrest at the G1 phase in cell cycle progression in HaCaT cells

We first examined the effect of HDAC inhibitors, TSA and butyrate, on the proliferation of human immortalized keratinocyte HaCaT cells. TSA inhibited the proliferation of HaCaT cells in a dose-dependent manner and 180 nM TSA had a cytostatic effect (Fig. 1A). To investigate the effect of TSA on the cell cycle progression, the DNA content of the cell nuclei was measured by flow cytometric analysis. The treatment with TSA increased the percentage in the G1 phase, and decreased that in the S phase (Fig. 1B). Butyrate also inhibited the proliferation in a dose-dependent manner, and had cytostatic effect at 2.4 mM (data not shown). The treatment with butyrate increased the percentage of the G1 phase from 29.4% to 81.9%, and decreased the S phase from 45.2% to 6.7% (data not shown). These data demonstrate that HDAC inhibitors arrest the cell cycle of HaCaT cells at the G1 phase.

3.2. HDAC inhibitors increase p15^{INK4b} mRNA and protein levels in HaCaT cells

In this study, we investigated whether HDAC inhibitors affect p15^{INK4b} gene expression in HaCaT cells. We found that TSA stimulates the expression of p15^{INK4b} mRNA in a dose-dependent manner (Fig. 2A). This data is consistent with the result that TSA inhibited the growth of HaCaT cells in a dose-dependent manner (Fig. 1A). The time course study indicated that the p15^{INK4b} mRNA was up-regulated 3 h after treatment with TSA (Fig. 2B).

Next, we tried to elucidate whether the expression of p15^{INK4b} protein could also be induced by treatment with

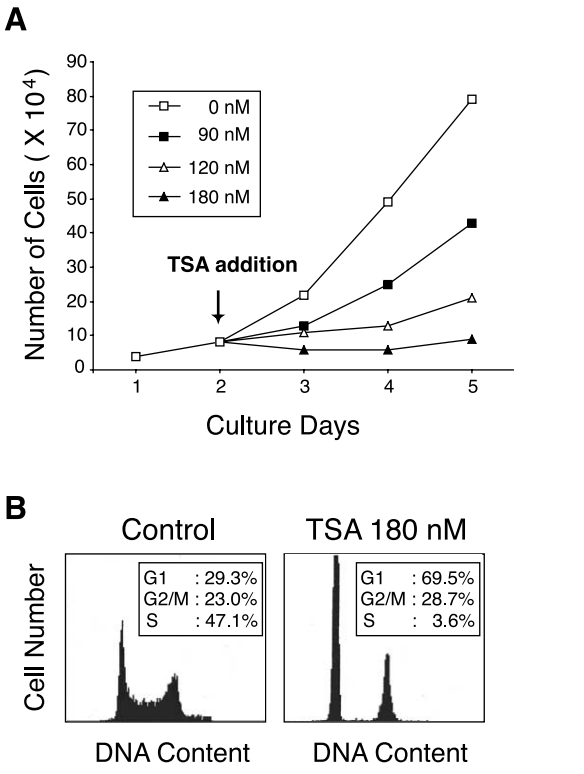

Fig. 1. Effect of TSA on the growth and cell cycle of HaCaT cells. A: One day after inoculation of HaCaT cells, TSA at 90, 120, or 180 nM was added, and cell growth was compared with control culture with equivalent ethanol. The number of viable cells was counted by trypan blue dye exclusion test. B: Unsynchronized cells were incubated in the presence of either ethanol or 180 nM TSA for 24 h, and the DNA content of the cells was determined by flow cytometry. Data represent means of duplicate experiments.

TSA in HaCaT cells. We found that the treatment with TSA stimulated the expression of p15^{INK4b} protein in a dose-dependent manner (Fig. 2C). The time course study showed that the expression of p15^{INK4b} protein was significantly increased 3 h after the treatment (Fig. 2D). Butyrate also activated the expressions of p15^{INK4b} protein and mRNA in a dose-dependent manner (data not shown).

p15^{INK4b} is a specific inhibitor of the cyclin D-dependent kinases, and the subsequent dephosphorylation of RB protein causes G1 arrest [4]. Therefore, we examined whether TSA can alter the phosphorylation status of RB protein in HaCaT cells by Western blotting. A hyperphosphorylated form of RB protein began to be converted into a hypophosphorylated form from 12 h after the treatment (Fig. 2E).

Taken together, these results indicate that HDAC inhibitors up-regulate the p15^{INK4b} at mRNA and protein levels and, subsequently, a hyperphosphorylated form of the RB protein is converted into a hypophosphorylated form in HaCaT cells.

3.3. HDAC inhibitors activate the p15^{INK4b} promoter activity in HaCaT cells

Having demonstrated that the p15^{INK4b} mRNA expression is induced by HDAC inhibitors in HaCaT cells, we investigated whether HDAC inhibitors activate the promoter activity of the p15^{INK4b} gene. We cloned and sequenced a 7.8 kb fragment containing the 5′-flanking region of the human p15^{INK4b} gene. The 5′-flanking region of the human p15^{INK4b} gene was ligated to an empty luciferase reporter plasmid, pGVB2.

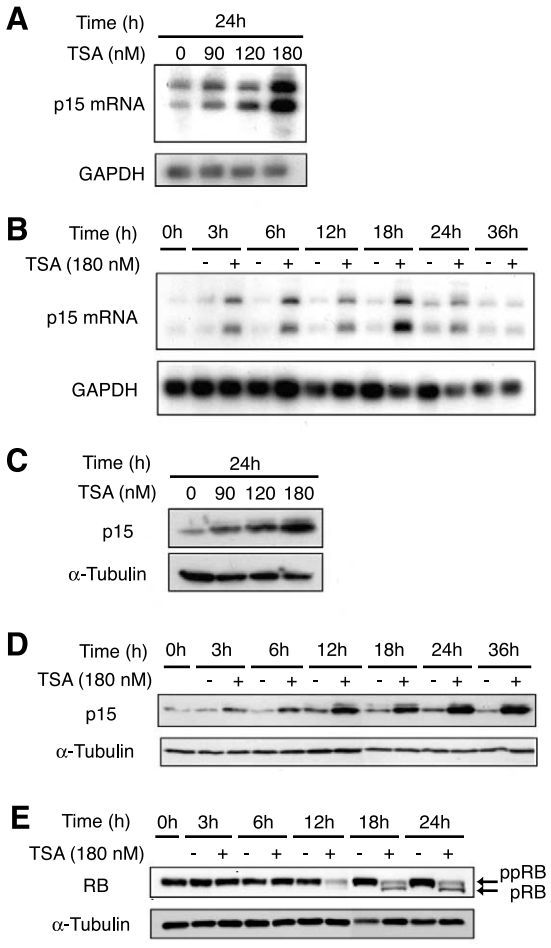

Fig. 2. Northern blot analysis of $p15^{INK4b}$ mRNA and Western blot analysis of $p15^{INK4b}$ or RB protein in HaCaT cells. A,B: HaCaT cells were treated in the presence of various concentrations of TSA for 24 h (A), or in the presence of 180 nM TSA for the indicated times (B), and the expression of $p15^{INK4b}$ mRNA was examined. The mRNA level of $p15^{INK4b}$ was standardized by that of glyceraldehyde-3-phosphate dehydrogenase. C: HaCaT cells were treated with various concentrations of TSA. The expression of $p15^{INK4b}$ protein was examined after 24 h exposure to TSA. α-Tubulin (Oncogene Research Product, CA, USA) was chosen as a loading control in all Western blotting. D,E: HaCaT cells were exposed to either ethanol alone (−) or 180 nM TSA (+) for the indicated times. The expressions of $p15^{INK4b}$ protein (D) and RB protein (E) were detected.

Treatment with TSA activated the promoter activity of $p15^{INK4b}$ in a dose-dependent manner (Fig. 3). Butyrate also increased the $p15^{INK4b}$ promoter activity (data not shown). These results indicate that HDAC inhibitors stimulate the $p15^{INK4b}$ promoter activity in HaCaT cells.

3.4. Overexpression of $p15^{INK4b}$ inhibits cell growth in HaCaT cells

We examined the effect of $p15^{INK4b}$ overexpression on cell growth of HaCaT cells by selecting for Geneticin resistance after transfection with expression vectors encoding the $p15^{INK4b}$ gene and neomycin resistance gene. The results are expressed as percent recovery compared to the empty vector (Fig. 4A). The recovery of antibiotic-resistant HaCaT cells was markedly inhibited by the overexpression of $p15^{INK4b}$. Transfection with $p15^{INK4b}$ yielded 0% of the resistant colonies seen with the control. Furthermore, we showed the expression of $p15^{INK4b}$ protein significantly increased 24 h after transfection in HaCaT cells (Fig. 4B). These results demonstrate that the overexpression of the $p15^{INK4b}$ gene inhibits cell growth in HaCaT cells, and suggest that the activation of the $p15^{INK4b}$ gene may contribute to the growth arrest induced by HDAC inhibitors.

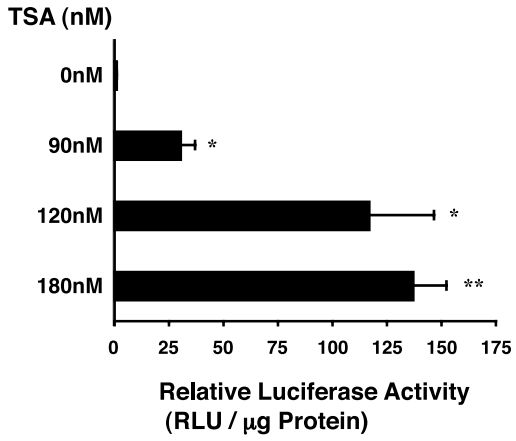

Fig. 3. Activation of $p15^{INK4b}$ promoter activity in HaCaT cells by treatment with TSA. HaCaT cells were transiently transfected with $p15^{INK4b}$-luciferase fusion plasmid, and luciferase activities were measured after incubation with medium containing various concentrations of TSA for 24 h. Data are shown as means ± S.D. ($n = 3$). $^{*}P < 0.03$; $^{**}P < 0.01$.

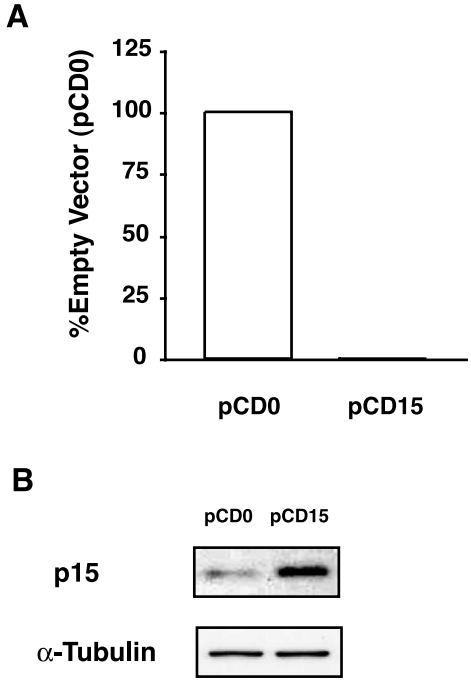

Fig. 4. Effect of $p15^{INK4b}$ overexpression on cell growth in HaCaT cells. A: The bars represent relative recovery of cells after transfection with the empty vector (pCD0) or expression vector (pCD15) encoding $p15^{INK4b}$. The results were normalized by pCD0 at 100%. Data are shown as means of triplicate experiments. B: After transfection with pCD0 or pCD15, the exogenous expression of $p15^{INK4b}$ protein was examined by Western blotting.

3.5. HDAC inhibitors inhibit the cell growth in HCT116 p21 (−/−) cells, and activate the expression of the $p15^{INK4b}$ gene

We previously demonstrated that HDAC inhibitors inhibit cellular proliferation and induce the expression of the $p21^{WAF1/Cip1}$ gene through its promoter in a p53-independent manner [12,13]. However, several studies have recently suggested the existence of a p21-independent pathway of the G1 phase arrest induced by HDAC inhibitors [14–16]. Therefore, to exclude the influences of $p21^{WAF1/Cip1}$ on TSA-mediated cell growth inhibition, the p21-deleted human colorectal carcinoma cell line HCT116 p21 (−/−) was used. The treatment with 120 nM TSA had a cytostatic effect on the cell growth, and caused the G1 and G2 arrest in the cell cycle (data not shown). The treatment with TSA stimulated the expression of $p15^{INK4b}$ protein in a dose- and time-dependent manner (data not shown), and a hyperphosphorylated form of RB protein was converted into a hypophosphorylated form (data not shown).

4. Discussion

Our results indicate that HDAC inhibitors stimulate the $p15^{INK4b}$ promoter activity and up-regulate the $p15^{INK4b}$ mRNA and protein levels, and then a hyperphosphorylated form of the RB protein is converted into a hypophosphorylated form in HaCaT cells. Furthermore, HDAC inhibitors arrest the cell cycle of HaCaT cells at the G1 phase and inhibit cell growth. In addition, the overexpression of $p15^{INK4b}$ completely suppressed the colony formation of HaCaT cells. Taken together, our results indicate that HDAC inhibitors activate the expression of the $p15^{INK4b}$ gene through its promoter, and suggest that its activation may partially contribute to growth arrest induced by HDAC inhibitors at the G1 phase in HaCaT cells.

We previously demonstrated that HDAC inhibitors inhibit cellular proliferation and induce the expression of the $p21^{WAF1/Cip1}$ gene through its promoter in a p53-independent manner. Furthermore, it has been previously shown that $p21^{WAF1/Cip1}$ is involved in butyrate-mediated growth inhibition of HCT116 cells [20]. However, several studies have recently suggested the existence of a $p21^{WAF1/Cip1}$-independent pathway of the G1 phase arrest induced by HDAC inhibitors [14–16]. We demonstrated that TSA activated the expression of the $p15^{INK4b}$ gene in HCT116 p21 (−/−), accompanied by cell growth inhibition. This result suggests that the activation of the $p15^{INK4b}$ gene may be important in $p21^{WAF1/Cip1}$-independent cell growth inhibition by TSA.

Inactivation of $p16^{INK4a}$ has been extensively reported in most human malignant tumors [21]. As a member of the INK4 family, $p15^{INK4b}$ is known to possess a function similar to that of $p16^{INK4a}$. This functional similarity to $p16^{INK4a}$ suggests that $p15^{INK4b}$ may function as a replacement for $p16^{INK4a}$ in case $p16^{INK4a}$ is inactivated. Therefore, transcriptionally regulated agents of the $p15^{INK4b}$ gene may contribute to new strategies for the prevention or therapy of malignancies, which we have termed 'gene-regulating chemoprevention or chemotherapy' [22,23]. In that sense, butyrate, which activates $p15^{INK4b}$ gene expression, would be representative of gene-regulating chemopreventive agents. Butyrate is one of the most abundant short chain fatty acids in the large intestine generated by bacterial fermentation of dietary fibers [24]. Butyrate has anti-tumor properties in vivo and in vitro at physiological concentrations, suggesting that butyrate may have preventive effects against large bowel cancer [25–27].

U. Aytac et al. [18] have recently reported an interesting result showing that $p15^{INK4b}$ inhibits cell proliferation independent of RB. Ectopic overexpression of $p16^{INK4a}$ can inhibit the growth of cell lines depending on RB status. However, in the case of $p15^{INK4b}$, cell proliferation was inhibited in cells lacking RB as well as in cells with wild-type RB expression. Therefore it is suggested that the agents activating the $p15^{INK4b}$ gene may have growth inhibitory effects on a broad spectrum of tumors regardless of RB status.

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