Trichostatin A activates p18^{INK4c} gene: differential activation and cooperation with p19^{INK4d} gene

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Abstract We have reported that histone deacetylase (HDAC) inhibitors activate a member of the INK4 family, the p19^{INK4d} gene, causing G1 phase arrest. We report here that HDAC inhibitor, Trichostatin A, activates another member of the INK4 family, the p18^{INK4c} gene, through its promoter in Jurkat cells. Interestingly, the activation patterns of the p18^{INK4c} gene were different from those of p19^{INK4d}. Furthermore, mouse embryo fibroblasts lacking p18^{Ink4c} or p18^{Ink4c}/p19^{Ink4d} were resistant to the growth inhibitory effects of TSA as compared to their wild-type counterpart. Our findings suggest that p18^{INK4c} is involved in TSA-mediated cell growth inhibition and cooperates with p19^{INK4d}.

Keywords: Histone deacetylase inhibitor; TSA; p18^{INK4c}; p19^{INK4d}; Promoter activation

1. Introduction

The histone deacetylase (HDAC) inhibitors are a new class of cytostatic agents that inhibit the proliferation of tumor cells in culture and in vivo by inducing cell cycle arrest at G1 or G2/M phase [1], differentiation and apoptosis [2,3]. Several compounds are currently in early phase of clinical applications as one of the most attractive and promising anti-tumor agents for solid and hematological cancers [4].

Cell-cycle progression is regulated by cyclin-dependent kinases (CDKs) that form complexes with cyclin in a phase-specific manner during the cell cycle [5]. Cyclin D-CDK4/6 and cyclin E-CDK2 play important roles in promoting the transition from the G1 phase to the S phase of the cell cycle by phosphorylating the tumor suppressor retinoblastoma protein (pRB) [5,6]. Activation of cyclin/CDKs is counterbalanced by CDK inhibitors (CKIs). The first family of CKIs, referred to as the Cip/Kip family, consists of related proteins known as p21$^{WAF1/Cip1}$, p27^{Kip1} and p57^{Kip2}, and each member inhibits a broader spectrum of cyclin/CDK complexes [7,8]. Previously, we demonstrated that HDAC inhibitors, such as sodium butyrate and Trichostatin A (TSA), inhibit cellular proliferation and induce the expression of the p21$^{WAF1/Cip1}$ gene in a p53-independent manner [9,10]. However, several reports strongly suggest that HDAC inhibitors induced-growth arrest can be mediated by a p21$^{WAF1/Cip1}$-independent molecular pathway [11,12].

The second family of CDK inhibitors is called the INK4 family (for inhibitors of Cdk4) proteins. The four members of the INK4 family, designated as p16^{INK4a}, p15^{INK4b}, p18^{INK4c} and p19^{INK4d}, specifically and directly bind to CDK4/6 and inhibit their activities [7,13–15]. Previously, we reported that HDAC inhibitors activate the p15^{INK4b} gene through its promoter in human immortalized keratinocyte HaCaT [16]. Furthermore, we demonstrated that treatment of Jurkat cells with TSA significantly activated the p19^{INK4d} gene through its promoter [17]. However, it remains to be elucidated whether the p18^{INK4c} gene is involved in G1 phase arrest induced by HDAC inhibitors. Here, we demonstrated that TSA activates the p18^{INK4c} gene and compared the activation patterns of the p18^{INK4c} gene by TSA with those of the p19^{INK4d} gene. Furthermore, to examine the involvement of p18^{INK4c} in TSA-induced growth arrest, we tested the sensitivity of mouse embryo fibroblasts lacking p18^{Ink4c} or p18^{Ink4c}/p19^{Ink4d} [18] to the growth inhibitory effects of TSA.

2. Materials and methods

2.1. Cell Culture, cell proliferation studies and treatment with Trichostatin A

The human T lymphoblastic T cell line, Jurkat (a kind gift from Dr. J.I. Fujisawa, Kansai Medical University) was maintained in RPMI 1640 medium with 10% fetal bovine serum and incubated at 37 °C in a humidified atmosphere of 5% CO_2. Wild type, p18^{Ink4c}-null and p18^{Ink4c}/p19^{Ink4d} double-null mouse embryo fibroblasts (MEFs) (a kind gift from Dr. M.F. Roussel, St. Jude Children's Research Hospital) were maintained in DMEM supplemented with 10% fetal bovine serum as previously described [19]. For the cell proliferation studies, cells were plated at a density of 5×10^4 cells in 35 mm-diameter dishes. Twenty-four hours after cell plating, 5 nM TSA (Wako, Osaka, Japan) was added to the culture medium. From the first day to the fifth day after plating, the number of viable cells was counted by a Trypan-blue dye exclusion test. This cell growth study was carried out in triplicate and repeated at least three times.

2.2. RNA isolation and Northern blot analysis

Jurkat cells were treated with various concentrations of TSA. Total RNA was isolated from cells grown in a 100-mm diameter dish using a Sepasol RNA isolation kit (Nacalai Tesque Inc., Kyoto, Japan), and 10 μg of total RNA per lane was used for Northern blot analysis. The *p18^{INK4c}* cDNA and *p19^{INK4d}* cDNA used for the probe were described previously [17,20]. Northern blot analysis was performed using standard methods [21]. Expression of mRNA was quantified by Quantity

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Abbreviations: HDAC, histone deacetylase; TSA, Trichostatin A

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One 4.3.0 Quantitation Software (BIO RAD) and normalized by 28S ribosomal RNA quantified by Quantity One 4.3.0 Quantitation Software. Each experiment was repeated at least three times.

2.3. 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% SDS, and 1% sodium deoxycholate) for $P19^{INK4d}$ and $p18^{INK4c}$ detection or Lysis buffer (50 mM Tris–HCl, pH 7.5, and 0.1% SDS) for detection of the retinoblastoma (RB) gene products. The protein extract was boiled for 5 min and loaded onto a 12% (for $p18^{INK4c}$ and $p19^{INK4d}$ detection) or 7% (for pRB detection) polyacrylamide gel, electrophoresed, and transferred to a nitrocellulose membrane. A rabbit polyclonal antibody to human p18 (N-20, Santa Cruz Biotechnology, CA, USA), a mouse monoclonal antibody to human p19 (DCS-100 from NeoMarkers, Union City, CA, USA) or pRB (PM-14001A from Pharmingen, Becton-Dickinson Franklin Lakes, NJ, USA) was used as the primary antibody. The signal was then developed with the enhanced chemiluminescence system (Amersham Pharmacia Biotech, UK). Expression of the proteins was quantified by Quantity One 4.3.0 Quantitation Software and normalized by α-Tubulin. Each experiment was repeated at least three times.

2.4. Plasmid preparation, transient transfection and luciferase assay

As previously reported, an approximately 3.8-kb fragment of 5′-flanking region of human $p19^{INK4d}$ gene was subcloned into the luciferase reporter plasmid pGVB2 (Nippon Gene), which was termed p19 (−3814/−2) [22]. Similarly, an approximately 5.2-kb fragment of 5′-flanking region of human $p18^{INK4c}$ gene was subcloned into pGVB2, which was termed p18 (−5786/−514) [20]. Jurkat cells were transiently transfected with $p18^{INK4c}$ or $p19^{INK4d}$-luciferase fusion plasmid, by the DEAE-dextran method as described previously [23]. Twenty-four hours after transfection, various concentrations of TSA were added, and 48 h after the transfection, the cell lysates were collected for a luciferase assay. Cells were washed twice with PBS and resuspended in 100 μl of 0.25 M Tris–HCl (pH 7.5). Five cycles of freeze–thawing were performed in liquid nitrogen/water at 37 °C. Luciferase activities were normalized for the amount of protein in the cell lysates. Data were analyzed using the Student's *t* test. A *P* value less than 0.05 was considered significant. Each experiment was repeated at least three times.

3. Results

3.1. TSA increases $p18^{INK4c}$ mRNA levels – differential activation pattern of $p18^{INK4c}$ and $p19^{INK4d}$ mRNA by TSA

Among the INK4 family, both $p18^{INK4c}$ and $p19^{INK4d}$ are normally expressed, whereas $p16^{INK4a}$ and $p15^{INK4b}$ are homozygously deleted in Jurkat cells [24,25]. We have already reported that treatment of Jurkat cells with TSA activates $p19^{INK4d}$ gene, resulting in G_1 phase arrest [17]. In this study, we investigated whether TSA activates $p18^{INK4c}$ gene in Jurkat cells. We found that $p18^{INK4c}$ mRNA was upregulated 18 h after treatment with TSA (Fig. 1A). These data are consistent with our previous results showing that TSA inhibits the proliferation of Jurkat cells and that 120–240 nM TSA had cytostatic effects [17].

Interestingly, the activation patterns of the $p18^{INK4c}$ mRNA were different from those of $p19^{INK4d}$ mRNA. The $p18^{INK4c}$ mRNA was maximally activated by treatment with 120 nM TSA, whereas $p19^{INK4d}$ mRNA is stimulated in a dose-dependent manner up to 240 nM TSA (Fig. 1A). Time course studies showed that $p18^{INK4c}$ mRNA was upregulated 12–18 h after treatment with 120 nM TSA, whereas $p19^{INK4d}$ mRNA was upregulated rapidly 3 h after treatment with TSA and was maximally activated about 6 h after treatment (Fig. 1B). Taken together, $p18^{INK4c}$ mRNA was upregulated by treatment with TSA, but maximal activation was seen with a lower dose and a longer treatment as compared to $p19^{INK4d}$ mRNA.

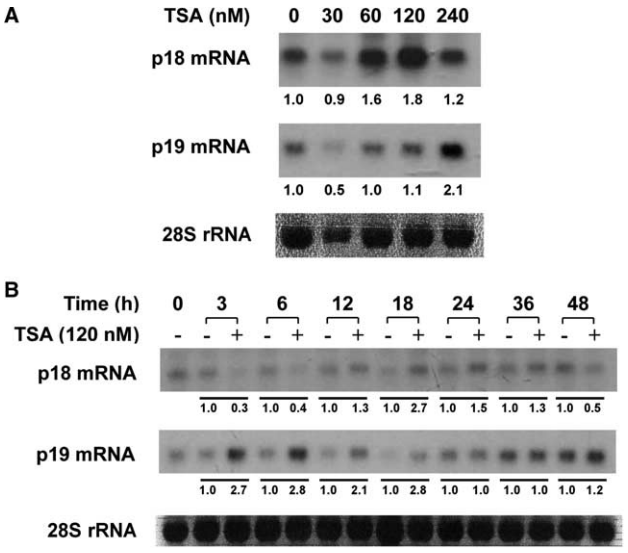

Fig. 1. Northern blot analysis of $p18^{INK4c}$ and $p19^{INK4d}$ mRNA in Jurkat cells. Jurkat cells were treated, (A) in the presence of various concentrations of TSA for 18 h, or (B) in the presence of 120 nM TSA for the indicated times, and the expressions of $p18^{INK4c}$ and $p19^{INK4d}$ mRNA were examined. Data shown below the blots represent fold induction in RNA expression normalized by 28S ribosomal RNA as described in Section 2, and each value was compared with that of the control without TSA which was estimated as 1.0.

3.2. TSA upregulates the $p18^{INK4c}$ protein – differential activation pattern of the $p18^{INK4c}$ and $p19^{INK4d}$ protein by TSA

Next, Western blot analysis showed that treatment with different concentrations of TSA for 24 h induced the expression of the $p18^{INK4c}$ protein as well as the $p19^{INK4d}$ protein (Fig. 2A).

Time course studies indicated that the $p18^{INK4c}$ protein was significantly increased from 12 to 18 h after the treatment with 120 nM TSA, whereas the $p19^{INK4d}$ protein was significantly increased 3 h after the treatment with TSA, and that strong induction of the $p19^{INK4d}$ protein was observed from 6 to 12 h after exposure to TSA compared with the treatment with solvent of TSA alone (Fig. 2B). The results of time course study of the $p18^{INK4c}$ and $p19^{INK4d}$ induction patterns seem to be well correlated with those of $p18^{INK4c}$ and $p19^{INK4d}$ mRNA activation pattern.

Because overexpression of $p18^{Ink4c}$ and $p19^{Ink4d}$ induces G1 phase arrest by blocking Cdk4/6 kinase activities and pRB phosphorylation [13,15], we tested by Western blotting whether TSA altered the phosphorylation of pRB protein. We found that about 24 h after treatment, hyperphosphorylated form of pRB protein began to be converted into a hypophosphorylated form (Fig. 2C).

Collectively, the activation patterns of the $p18^{INK4c}$ gene were different from those of $p19^{INK4d}$.

3.3. TSA stimulates $p18^{INK4c}$ promoter activity – differential activation pattern of the $p18^{INK4c}$ and $p19^{INK4d}$ promoter by TSA

Because $p18^{INK4c}$ mRNA expression is induced by TSA in Jurkat cells, we investigated whether TSA could transactivate the $p18^{INK4c}$ promoter, using a wild-type $p18^{INK4c}$ promoter-luciferase fusion reporter plasmid, p18 (−5786/−514). Follow-

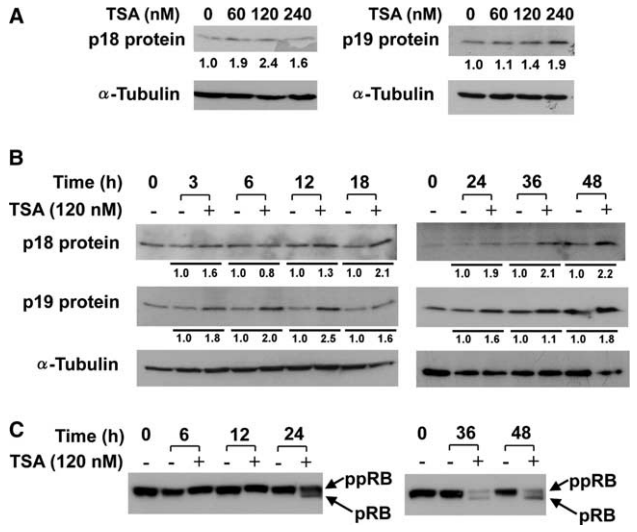

Fig. 2. Western blot analysis of the $p18^{INK4c}$, $p19^{INK4d}$ and the pRB protein. (A) Jurkat cells were treated with various concentrations of TSA for 24 h and the expressions of the $p18^{INK4c}$ and $p19^{INK4d}$ protein were examined α-Tubulin (Oncogene Research Product, CA, USA) was chosen as a loading control in all blots. (B, C) Jurkat cells were exposed to either DMSO alone (−) or 120 nM TSA (+) for the indicated times. The expressions of the $p18^{INK4c}$ and $p19^{INK4d}$ (B) and the pRB protein (C) were analyzed. Data shown below the blots represent fold induction in the protein expression normalized by α-Tubulin as described in Section 2, and each value was compared with that of the control without TSA which was estimated as 1.0.

ing a 24-h exposure with various concentrations of TSA, the $p18^{INK4c}$ promoter was maximally activated by treatment with 120 nM TSA (Fig. 3A), while the $p19^{INK4d}$ promoter is stimulated in a dose-dependent manner up to 240 nM TSA (Fig. 3B).

These results suggest that TSA transactivates the $p18^{INK4c}$ promoter as well as the $p19^{INK4d}$ promoter, but that their activation patterns are remarkably different from one another.

Furthermore, the results of the promoter activation of $p18^{INK4c}$ and $p19^{INK4d}$ seem to be also well correlated with those of both mRNA activations. Therefore, a possible molecular basis for such differential activation patterns of both genes may be explained by differences in the transcriptional regulation.

3.4. Effect of TSA on the growth of wild type, $p18^{Ink4c}$-null or $p18^{Ink4c}/p19^{Ink4d}$ double-null MEFs

We previously demonstrated that MEFs lacking $p19^{Ink4d}$ were resistant to the growth inhibitory effects of TSA and concluded that the activation of $p19^{INK4d}$ is involved in growth inhibition induced by HDAC inhibitors [17]. In this study, to further analyze the physiological relevance of the induction of $p18^{INK4c}$ in TSA-induced growth arrest, we examined the effects of TSA on cell proliferation of wild type and $p18^{Ink4c}$-deficient [p18 (−/−)] MEFs. p18 (−/−) MEFs grew more rapidly than wild-type MEFs in the absence of TSA (Fig. 4A). As expected, $p18^{Ink4c}$-null MEFs were more resistant to the growth inhibitory effects of TSA than were their wild type counterparts on days 3–5, further supporting a model in which the endogenous $p18^{Ink4c}$ gene is involved in TSA-mediated cell growth inhibition (Fig. 4A and B). Moreover, $p18^{Ink4c}/p19^{Ink4d}$ double-null MEFs were also resistant to the growth inhibitory effects of TSA on days 3–5 (Fig. 4A and B).

Taken together, these results suggest that the activation of $p18^{INK4c}$ is involved in growth inhibition induced by TSA.

4. Discussion

The results of the present study indicate that the $p18^{INK4c}$ gene, in addition to the $p19^{INK4d}$ gene, is transcriptionally activated by TSA in Jurkat cells. Interestingly, the activation patterns of the $p18^{INK4c}$ gene were different from those of $p19^{INK4d}$. Our findings further suggest that $p18^{INK4c}$ is involved in TSA-mediated cell growth inhibition and cooperates with $p19^{INK4d}$.

It was reported that treatment of $p18^{Ink4c}$ null and heterozygous mice with a chemical carcinogen causes tumor at an accelerated rate, and that the remaining wild-type allele of $p18^{Ink4c}$ is normal in tumors derived from heterozygotes [26]. Recently, we demonstrated that activation of protein kinase C induces cell growth through suppression of human $p18^{INK4c}$ expression [20]. Furthermore, leukemogenesis by an

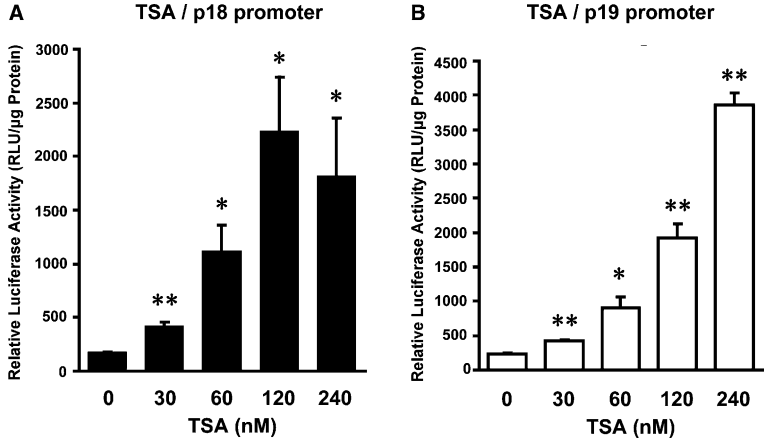

Fig. 3. Activation of $p18^{INK4c}$ and $p19^{INK4d}$ promoter activity in Jurkat cells by TSA. Jurkat cells were transiently transfected with $p18^{INK4c}$ or $p19^{INK4d}$-luciferase fusion plasmid, and luciferase activities were measured after incubation with medium containing various concentrations of TSA for 24 h. Data are shown as means (*bars*, SD) ($n = 3$), $^{*}P < 0.05$; $^{**}P < 0.01$.

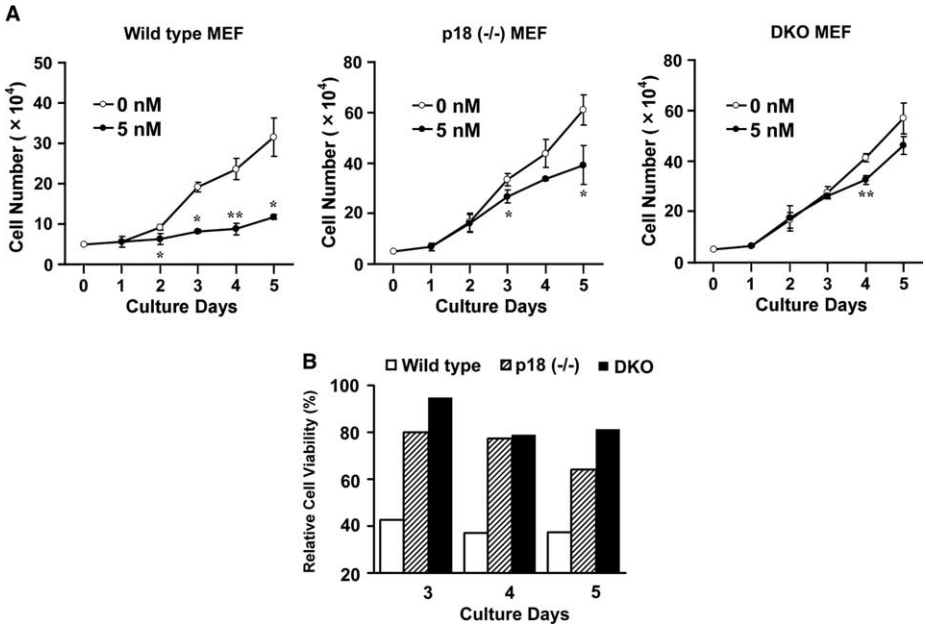

Fig. 4. Effect of TSA on the growth of wild type, $p18^{Ink4c}$-null or $p18^{Ink4c}/p19^{Ink4d}$ double-null MEFs. (A) One day after inoculation of both MEFs, TSA at 5 nM (●) was added, and cell growth was compared with control culture (○). Data represent means of triplicate experiments. Data are shown as means (*bars*, SD) ($n = 3$), $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Relative cell viability of wild type (□), $p18^{INK4c}$-null (▨) or $p18^{Ink4c}/p19^{Ink4d}$ double-null (■) MEFs treated with 5 nM TSA versus those that were untreated.

aberrant ARG kinase involves the suppression of $p18^{INK4c}$ expression and suppression of ARG kinase activity by STI571 induces cell cycle arrest through upregulation of $p18^{INK4c}$ [27]. These reports establish $p18^{Ink4c}$ as a haploinsufficient tumor suppressor and suggest that its quantitative decrease may be involved in human tumorigenesis [26]. On the other hand, so far there was no report that loss of $p19^{INK4d}$ plays a critical role in tumorigenesis in humans. Therefore, activation of the $p18^{INK4c}$ gene, which plays some part in human tumorigenesis, may be of great significance in the therapy or the prevention of malignant tumors.

Inactivation of $p16^{INK4a}$ has been extensively reported in most human malignant tumors [15,28], whereas genetic alterations of $p18^{INK4c}$ are rare events [29]. Recently, however, it was reported that the $p18^{INK4c}$ gene was epigenetically silenced through its promoter hypermethylation in some tumor types [30]. These observations suggest that chemotherapy with single use of HDAC inhibitors may be potentially limited in tumors with hypermethylation in the $p18^{INK4c}$ promoter; however, cotreatment with HDAC inhibitors and demethylating agents could restore $p18^{INK4c}$ expression and be effective in these tumors. In fact, DNA demethylation and HDAC inhibition act synergistically in re-expression of the hypermethylated genes such as $p16^{INK4a}$ [31].

Because $p18^{INK4c}$ possesses functional similarity to $p16^{INK4a}$ as a member of the INK4 family [32], $p18^{INK4c}$ may be able to compensate for $p16^{INK4a}$ loss commonly found in human tumors. Therefore, transcriptional activation of the $p18^{INK4c}$ gene by chemotherapeutic agents may contribute to a novel approach for chemotherapy or chemoprevention against cancers that harbor a deletion or mutations in $p16^{INK4a}$, which we have termed "gene-regulating chemotherapy" or "gene-regulating chemoprevention" [33,34].

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