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• COLORECTAL CANCER •

Histone acetylation regulates *p21^{WAF1}* expression in human colon cancer cell lines

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Abstract

AIM: To investigate the effect of histone acetylation on regulation of *p21^{WAF1}* gene expression in human colon cancer cell lines.

METHODS: Two cell lines, Colo-320 and SW1116 were treated with either trichostatin or sodium butyrate. Expressions of *p21^{WAF1}* mRNA and protein were detected by real-time RT-PCR and Western blotting, respectively. Acetylation of two regions of *p21^{WAF1}* gene-associated histones and total cellular histones were examined by chromatin immunoprecipitation assay and Western blotting.

RESULTS: Trichostatin or sodium butyrate re-activated *p21^{WAF1}* transcription resulted in up-regulated *p21^{WAF1}* protein level in colon cancer cell lines. Those effects were accompanied by an accumulation of acetylated histones in total cellular chromatin and *p21^{WAF1}* gene-associated region of chromatin.

CONCLUSION: Histone acetylation regulates *p21^{WAF1}* expression in human colon cancer cell lines, Colo-320 and SW1116.

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INTRODUCTION

Cell cycle progression is controlled by various cyclin-dependent kinases (CDKs), whose activation is carefully regulated at multiple levels including the induction and degradation of cyclin protein, CDKs phosphorylation by cyclin-activating kinase, and the induction of CDK inhibitors (CDKIs)[1]. CDKI *p21^{WAF1}* was first cloned and characterized as an important effector that acts to inhibit CDK activity in p53 mediated cell cycle arrest in response to various agents[2]. Increased expression of *p21^{WAF1}* may play a crucial role in the G_1/S phase arrest induced in transformed cells, and may prevent the progression of neoplasia[3].

Histone acetylation is emerging as a major regulatory mechanism thought to modulate gene expression by altering the accessibility of transcription factors to DNA and recent studies suggest that these alterations may also be important in the process of neoplasia formation[4]. The level of histone acetylation depends on the activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs). An important approach that has been used to study the function of chromatin acetylation is the use of specific inhibitors of HDAC. Trichostatin A[5,6] (TSA, a hybrid polar compound of specific inhibitor) and sodium butyrate[4] (a short chain fatty acid produced in human colon by bacterial fermentation of carbohydrate) were reported to inhibit HDAC activity.

Previously it was revealed that acetylation of gene-associated histone or total cellular histone alone regulated *p21^{WAF1}* expression in colon cancer cell lines[7,8]. We have shown[9] that TSA or sodium butyrate induced G_1 phase cell cycle arrest was linked to increased expression of *p21^{WAF1}*. However, little is known about the regulation of acetylation of both gene-associated histone and total cellular histone on *p21^{WAF1}* expression in human colon cancer. It is as yet not clear about the effect of histone acetylation on *p21^{WAF1}* protein in Colo-320 and SW1116 cell lines. Therefore, in the present study, we further investigated whether TSA and sodium butyrate induced overexpression of *p21^{WAF1}* resulted from hyperacetylation of gene-associated histones and histones in total cellular chromatin in two human colon cancer cell lines, Colo-320 and SW1116.

MATERIALS AND METHODS

Cell culture

Human colon cancer-derived cell lines Colo-320 and SW1116 were obtained from Shanghai Institute of Biochemistry and Cell Biology, SIBS, China and Shanghai Second Medical University Ruijin Hospital, respectively. Colo-320 and SW1116 cells were maintained in RPMI 1640 supplemented with 100 mL/L heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a 50 mL/L CO_2 incubator.

Treatment of cells with TSA or sodium butyrate

Colon cancer cell lines were exposed to 1 μmol/L TSA or 5 mmol/L sodium butyrate (Sigma, St. Louis, MO) alone for 24 h, as described by Siavoshian *et al*.[10]. The control cultures were treated simultaneously with phosphate-buffered saline (PBS) or alcohol (control for TSA treatment, because TSA can only be dissolved in alcohol).

Western blotting of acetylated histones and p21^{WAF1}

Colo-320 cells were cultured as described below with or without treatment. Cells were recovered by centrifugation, washed twice with ice-cold PBS, and resuspended for lysis in 1 mL buffer A (10 mmol/L HEPES, pH 7.4, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.5 mmol/L DTT, 0.2 mmol/L PMSF, 1 μg/mL protease inhibitors, 0.25 g/L NP40) for 15 min with rotation at 4 °C and the nuclear pellet was resuspended in 100 μL buffer B (20 mmol/L HEPES, pH 7.4, 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA,

250 mL/L glycerol, 0.5 mmol/L DTT, 0.2 mmol/L PMSF, 1 μg/mL protease inhibitors) for 30 min, then the soluble nuclear protein was collected by centrifugation. Fifty μg (for acetylated histone H3 and H4) or 150 μg (for $p21^{WAF1}$ protein) of nuclear extracts was boiled in loading buffer (125 mmol/L Tris-HCl, pH 6.8, 40 g/L SDS, 200 g/L glycerol, 0.05 g/L bromphenol blue) for 5 min and then loaded onto a 150 g/L SDS-polyacrylamide gel. After electrophoresis, proteins were transferred onto nitrocellulose membrane (0.45 μm). The following antibodies were used: rabbit polyclonal antibody against acetylated histone H3 or H4 (Upstate Biotechnology, Lake Placid, NY) and goat polyclonal antibody against $p21^{WAF1}$ (C19, Santa Cruz, California). The bindings of antibodies were detected using ECL-system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and membranes were then exposed to Kodak BioMax film for 1 min. Antibody against β-actin (Sigma) in Western blot was used as a control for protein concentration.

Real-time RT-PCR for $p21^{WAF1}$ mRNA

mRNA level of *$p21^{WAF1}$* was measured using a real-time quantitative PCR system. Total RNA samples from SW1116 and Colo-320 cells with or without treatment were prepared by TriZol Reagent. Gene-specific TaqMan probes and PCR primers were designed using Primer Express software (PE Biosystems, Foster City, CA). The sequence for forward and reverse primers and the probe are shown in Table 1. Triplicate PCR reactions were prepared for each cDNA sample. PCR consisted of 40 cycles of 95 °C denaturation (15 s) and 60 °C annealing/extension (60 s). Thermal cycling and fluorescent monitoring were performed using an ABI 7 700 sequence analyzer (PE Biosystems). The point at which the PCR product is first detected above a fixed threshold, termed cycle threshold (Ct), was determined for each sample, and the average Ct of triplicate samples was calculated. To determine the quantity of gene-specific transcripts present in treated cells cDNA relative to untreated cells, their respective Ct values were first normalized by subtracting the Ct value obtained from the β-actin control (△Ct = Ct FAM-Ct VIC). The concentration of gene-specific mRNA in treated cells relative to untreated cells was calculated by subtracting the normalized Ct values obtained with untreated cells from those obtained with treated samples (△△Ct = △Ct of treated cells-DCt of untreated cells), and the relative concentration was determined ($2^{-\triangle\triangle Ct}$). Altered mRNA expression was defined as 3-fold difference in the expression level in cells after the treatment relative to that before treatment[11].

Chromatin immunoprecipitation (ChIP) assay

A ChIP assay kit from Upstate Biotechnology was used according to the manufacturer's protocol and Richon's report[12]. Colo-320 cells that were either treated with 1 μmol/L TSA or 5 mmol/L sodium butyrate for 24 h or untreated were plated at a density of 10×10^6 /T25 flask. Formaldehyde was then added to the cells to a final concentration of 10 g/L, and the cells were incubated at 37 °C for 10 min. The medium was removed, and the cells were suspended in 1 mL of ice-cold PBS containing protease inhibitors [1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL aprotinin and 1 μg/mL pepstatin A, Boehringer Mannheim]. Cells were pelleted, resuspended in 0.2 mL of SDS lysis buffer, and incubated on ice for 10 min. Lysates were sonicated. The majority of DNAs ranged from 200 bp to 1 000 bp. Debris was removed from samples by centrifugation for 10 min at 15 000 *g* at 4 °C in a microcentrifuge. An aliquot of the chromatin preparation (200 μL) was set aside and designated as the input. Supernatants were 10-fold diluted in ChIP dilution buffer containing the protease inhibitors as above, and 80 μL of a salmon sperm DNA/protein A-agarose beads was added and incubated for 30 min at 4 °C with rocking. Beads were pelleted by centrifugation, and supernatants were placed in tubes with 10 μg of antibody against acetylated histone H3 or H4, or normal rabbit IgG, and incubated overnight at 4 °C with rotation. Salmon sperm DNA/Protein A-agarose beads (60 μL) was added, and samples were rocked for 1 h at 4 °C. Protein A complexes were centrifuged and washed 5 times for 5 min each with low salt buffer, high salt buffer, LiCl buffer and TE buffer, respectively. Immune complexes were eluted twice with 250 μL of elution buffer (10 g/L SDS/0.1 mol/L $NaHCO_3$) for 15 min at room temperature. NaCl (5 mol/L, 20 μL) was added to the combined eluate, and the samples were incubated at 65 °C for 4 h. EDTA, Tris-HCl, pH 6.5, and proteinase K were then added to the samples at a final concentration of 10 mmol/L, 40 mmol/L, and 0.04 μg/μL, respectively, and the samples were incubated at 45 °C for 1 h. Immunoprecipitated DNA (both immunoprecipitation samples and input) was recovered by phenol/chloroform extraction and ethanol precipitation and analyzed by PCR. *$p21^{WAF1}$*-specific primers were used to carry out PCR. Sequences of two sets of primers for *$p21^{WAF1}$* PCR and PCR condition are shown in Table 2. The first set primer was used to amplify -576 to -293 and the second set primer was used to amplify -51 to +77 of *$p21^{WAF1}$* promoter and exon 1, which contained the transcription factor E2A binding sites.

RESULTS

HDAC inhibitors resulted in accumulation of acetylated core histones H3 and H4

Western blotting showed that before incubation with TSA or

Table 1 Sequence of primers and probes for real-time PCR

Gene	Primer (forward) (5'→3')	Primer (reverse) (5'→3')	Probe	GenBank accession number
$p21^{WAF1}$	CTG GAG ACT CTC AGG GTC GAA	GGA TTA GGG CTT CCT CTT GGA	ACG GCG GCA GAC CAG CAT GA	NM_078467
β-*actin*	CTG GCA CCC AGC ACA ATG	GGA CAG CGA GGC CAG GAT	ATC ATT GCT CCT CCT GAG	BC016045

Table 2 Sequence of primers and program of PCR for ChIPs

Primers	Sense (5'→3')	Antisense (5'→3')	Size of product and PCR condition	GenBank accession number
γ-*actin*	GGA CCT GGC TGG CCG GGA CC	GTG GCC ATC TCC TGC TCG AA	153 bp 95 °C 5 min 95 °C 1 min, 56 °C 1 min, 72 °C 1 min, 35 cycles	
$p21^{WAF1}$ (P1)	CGT GGT GGT GGT GAG CTA GA	CTG TCT GCA CCT TCG CTC CT	296 bp 95 °C 5 min 95 °C 1 min, 58 °C 1 min, 72 °C 1 min, 35 cycles	U24170
$p21^{WAF1}$ (P2)	GGT TGT ATA TCA GGG CCG	CTC TCA CCT CCT CTG AGT GC	128 bp 95 °C 5 min 95 °C 1 min, 58 °C 1 min, 72 °C 1 min, 35 cycles	U24170

sodium butyrate, the levels of acetylated H3 and H4 in colo-320 cells were low. Incubation with HDAC inhibitors resulted in the accumulation of acetylated histones H3 and H4 (Figure 1).

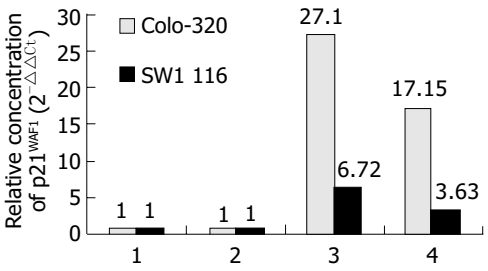


Figure 1 Western blotting of acetylated histones H3, H4 and $p21^{WAF1}$ protein in Colo-320 cells. Lane 1: Control (PBS); lane 2: Control (alcohol); lane 3: TSA 1 μmol/L, 24 h; lane 4: NaBu 5 mmol/L, 24 h.

Either TSA or sodium butyrate induced re-expression of $p21^{WAF1}$ mRNA and protein

To understand the change of *$p21^{WAF1}$* expression level following HDAC inhibitors treatment, we examined accumulation of mRNA and protein by RT-PCR and Western blotting. As shown in Figures 1 and 2, *$p21^{WAF1}$* mRNA and protein were activated after treatment of TSA and sodium butyrate. In addition, Colo-320 cells had an initial increase in *$p21^{WAF1}$* expression to a higher level than that in SW1116 cells.

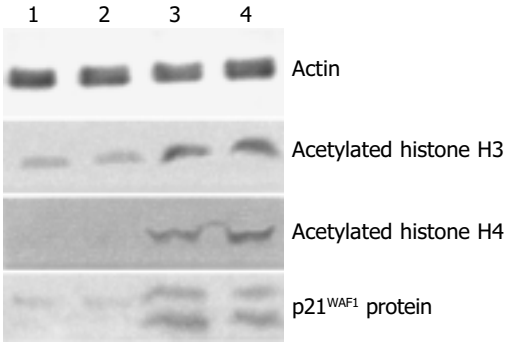
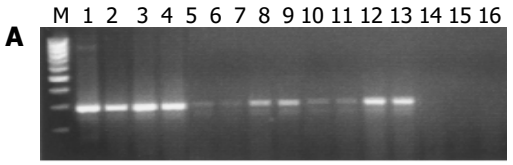


Figure 2 RT-PCR showed either TSA or sodium butyrate induced overexpression of $p21^{WAF1}$ mRNA in human colon cancer cell lines Colo-320 and SW1116. Lane 1: Control (PBS); lane 2: Control (alcohol); lane 3: TSA 1 μmol/L, 24 h; lane 4: NaBu 5 mmol/L, 24 h.



C

Drugs	IP.DNA/Input DNA	
	TSA	NaBu
1st set primers fold increase		
Anti-H3	1.80	2.01
Anti-H4	2.17	2.53

Figure 3 HDAC inhibitors induced accumulation of acetylated histones H3 and H4 in chromatin associated with $p21^{WAF1}$ gene. A: PCR products for the 1st set primer of $p21^{WAF1}$; B: PCR products for the 2nd set primer of $p21^{WAF1}$; C: The figure in A and B was scanned and quantified by using IMAGE analysis software. The ratio between input DNA and precipitated DNA was calculated for each treatment and primer set. The increase after treatment with either TSA or sodium butyrate was calculated from indicated ratios. PCR products of the 2 nd primer were not visualized on agarose gel before treatment with HDAC inhibitor, so it could not be calculated. Input DNA, lanes 1-4: Soluble chromatin was immunoprecipitated with antiacetylated histone H3 antibody; lanes 5-8: Soluble chromatin was immunoprecipitated with antiacetylated histone H4 antibody; lanes 9-12: Normal rabbit serum as negative control; lanes 13-16, 1, 5, 9, 13: PBS control; lanes 2, 6, 10, 14: Alcohol control; lanes 3, 7, 11, 15: TSA 1 μmol/L, 24 h; lanes 4, 8, 12, 16: NaBu 5 mmol/L, 24 h.

$p21^{WAF1}$ gene-associated histone H3 was hyperacetylated in colon cancer cells treated with TSA or sodium butyrate

To determine whether histone acetylation reflected *$p21^{WAF1}$* transcription and the functional interaction between *$p21^{WAF1}$* and TSA or sodium butyrate treatment, ChIPs-PCR was performed. As shown in Figure 3, the densities of bands of *$p21^{WAF1}$* gene-associated acetylated histones H4 and H3 were higher in chromatin extracted from Colo-320 cells treated with either TSA or sodium butyrate than that from cells mock treated, either the first or the second set PCR primer.

Taken together, TSA or sodium butyrate activated the transcription of *$p21^{WAF1}$* through acetylation of histones H4- and H3-associated *$p21^{WAF1}$* promoter.

DISCUSSION

Several lines of evidence suggest that histone acetylation plays a role in transcriptional regulation, probably by altering chromatin structure[13]. Acetylation of core nucleosomal histones is regulated by the opposing activities of HATs and HDACs. The latter catalyze the removal of an acetyl group from the ε-amino group of lysine side chains of histones H2A, H2B, H3 and H4, thereby reconstituting the positive charge in lysine. Transcriptionally silent chromatin is composed of nucleosomes in which the histones have low levels of acetylation of lysine residues at their amino-terminal tails[14,15]. Acetylation of histone neutralizes the positive charge in lysine residues and disrupts nucleosome structure, allowing unfolding of the associated DNA, access by transcription factors, and changes in gene expression. Chromatin fractions enriched in actively transcribed genes are also enriched in the more highly acetylated isoforms of the core histones[16]. HDAC inhibitors appear to be selective with regard to the genes whose expression is altered[17].

Total cellular histone acetylation is also involved in the regulation of gene expression. Several studies[18] indicated that the effect of HDAC inhibitors on gene transcription was associated with an increased accumulation of acetylated histones H3 and H4 in total cellular chromatin. However, Lee's group[19] showed an accumulation of acetylated histones H3 and H4 in total cellular chromatin after treatment with HDAC inhibitor (MS-275), but no change in the level of histone acetylation in chromatin-associated TGF-β I receptor gene. Therefore, we wanted to know whether HDAC inhibitor affected the acetylation level of histones in both gene-associated and total cellular chromatin. The data from ChIP and Western blotting suggested that *$p21^{WAF1}$* transcription was dependent upon acetylation at the level of chromatin, since the level of *$p21^{WAF1}$* promoter amplified from acetylated histone H3- or H4-associated chromatin was greater in chromatin isolated from HDAC inhibitor-treated cells than that from untreated cells. Accumulation of acetylated *$p21^{WAF1}$*-assocaited histones induced by HDAC inhibitors was higher than that in total cellular chromatin, although there was accumulation of

acetylated histones H3 and H4 in total cellular chromatin.

It is noteworthy that, the level of acetylated histones H3 and H4 at the domain containing the transcriptional start site in *p21*^{WAF1} promoter and the binding sites of E2A was significantly higher than that at another domain or total cellular chromatin analyzed. The result of our observations suggested that *p21*^{WAF1} expression could be activated by histone acetylation of its transcription start domain in promoter. Therefore, a possible mechanism involved the binding of transcription factor E2A to *p21*^{WAF1} promoter at transcription start site of acetylated *p21*^{WAF1} gene-associated histones H3 and H4, and the enhancement of *p21*^{WAF1} gene transcription. It is known that histone acetylation can be targeted to specific promoters by gene-specific activator. E2A transcription factor belongs to the basic helix-loop-helix family of proteins[20], which contains a conserved basic region responsible for DNA binding and a helix-loop-helix domain for dimerization[21]. E2A binds *p21*^{WAF1} at the domain nearby TATA box in the promoter. TATA box-independent transcription of the *p21*^{WAF1} promoter has been previously reported[22]. The proximal *p21*^{WAF1} promoter contains a TATA box[23]. Some reports indicated that *p21*^{WAF1} was up-regulated by E2A binding to HTLV-1-infected T cells[20]. Moreover, overexpression of E2A proteins, such as E47 has been shown to induce *p21*^{WAF1} promoter activity independent of p53 binding sites[24,25].

Also, we showed that the levels of *p21*^{WAF1} mRNA and protein in colon cancer cells were very low, even difficult to detect before treatment. In Colo-320, *p21*^{WAF1} mRNA was increased by 27.1-fold and 17.15-fold after 1 μmol/L TSA and 5 mmol/L sodium butyrate treatment, respectively. Accordingly, the protein level of *p21*^{WAF1} was elevated. Similar effects were shown in SW1116 cells (data not shown). Our data about TSA or sodium butyrate inducing *p21*^{WAF1} mRNA and protein expression are consistent with previous reports[12].

In summary, this study demonstrated that HDAC inhibitor, TSA or sodium butyrate, activated the expressions of *p21*^{WAF1} mRNA and protein, and this increased expression was associated with an accumulation of acetylated histones in total cellular chromatin and the chromatin of *p21*^{WAF1} gene in these two colon cancer cell lines. It has been shown that *p21*^{WAF1} expression is reduced in adenomas and colorectal carcinomas. Our observations support the claim for the therapeutic potential of HDAC inhibitors in the treatment of colorectal carcinoma, because there is probably no mutation of the *p21*^{WAF1} gene in colorectal cancer.

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