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.

MATERIALS AND METHODS

Colon cancer cell lines were exposed to 1 mmol/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).

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

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 [3]. 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 [3] (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 G1 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 $p21^{WAF1}$ resulting 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

Colon cancer cell lines were exposed to 1 mmol/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).

Treatment of cells with TSA or sodium butyrate

Colon cancer cell lines were exposed to 1 mmol/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 MgCl₂, 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 MgCl₂, 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 p21WAF1 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 bromophenol 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 p21WAF1 (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 p21WAF1 mRNA

mRNA level of p21WAF1 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 Gene-specific TaqMan probes and PCR primers and Colo-320 cells with or without treatment were prepared by Antisense (PE Biosystems, Foster City, CA). The sequence for forward and reverse primers and Fabrazio'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 10×10⁴/T25 flask. Formaldehyde was then added to the cells to a final concentration of 10 mM/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 phenylmethylsulfonfyl 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 then fractionated into 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 NaHCO₃) 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. p21WAF1-specific primers were used to carry out PCR. Sequences of two sets of primers for p21WAF1 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 p21WAF1 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 |
|------|--------------------------|--------------------------|-------|-------------------------|
| p21WAF1 | CTG GAG ACT CTC | GGA TTA GGG CTT | ACG GCG GCA GAC | NM_078467 |
| β-actin | AGG GTC GAA | CCT CTT GGA | CAG CAT GA | |
| | CTG GCA CCC AGC | GGA CAG CGA | ATC ATT GCT | BC016045 |
| | ACA ATG | GGC CAG GAT | CCT CCT GAG | |

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 | GTT GCC ATC TCC | 153 bp 95 °C 5 min 95 °C 1 min, | |
|         | TGG CCG GGA CC | TGC TCG AA | 56 °C 1 min, 72 °C 1 min, 35 cycles | U24170 |
| p21WAF1 (P1) | CGT GGT GGT GGT | CTG TCT GCA | 296 bp 95 °C 5 min 95 °C 1 min, | U24170 |
|         | GAG GTA GA | CCT TCG CTC GT | 58 °C 1 min, 72 °C 1 min, 35 cycles | |
| p21WAF1 (P2) | GGT TGT ATA | CCT TCA CCT CCT | 128 bp 95 °C 5 min 95 °C 1 min, | |
|         | TCA GGG CCG | CTG AGT GC | 58 °C 1 min, 72 °C 1 min, 35 cycles | |
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\textsuperscript{WAF1} protein in Colo-320 cells. Lane 1: Control (PBS); lane 2: Control (alcohol); lane 3: TSA 1 \(\mu\)mol/L, 24 h; lane 4: NaBu 5 \(\mu\)mol/L, 24 h.

**Either TSA or sodium butyrate induced re-expression of p21\textsuperscript{WAF1} mRNA and protein**

To understand the change of p21\textsuperscript{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\textsuperscript{WAF1} mRNA and protein were activated after treatment of TSA and sodium butyrate. In addition, Colo-320 cells had an initial increase in p21\textsuperscript{WAF1} expression level following HDAC inhibitors treatment, we examined accumulation of mRNA and protein in 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\textsuperscript{WAF1} through acetylation of histones H4- and H3-associated p21\textsuperscript{WAF1} promoter.

**DISCUSSION**

Several lines of evidence suggest that histone acetylation plays a role in transcriptional regulation, probably by altering chromatin structure\textsuperscript{[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\textsuperscript{[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\textsuperscript{[16]}. HDAC inhibitors appear to be selective with regard to the genes whose expression is altered\textsuperscript{[17]}. Total cellular histone acetylation is also involved in the regulation of gene expression. Several studies\textsuperscript{[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\textsuperscript{[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-β1 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\textsuperscript{WAF1} transcription was dependent upon acetylation at the level of chromatin, since the level of p21\textsuperscript{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\textsuperscript{WAF1}-associated histones induced by HDAC inhibitors was higher than that in total cellular chromatin, although there was accumulation of...
In summary, this study demonstrated that HDAC inhibitor, butyrate treatment, respectively. Accordingly, the protein level family of proteins[20], which contains a conserved basic region analyzed. The result of our observations suggested that Dulic V Sherr CJ.

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because there is probably no mutation of the of HDAC inhibitors in the treatment of colorectal carcinoma, Our observations support the claim for the therapeutic potential expression is reduced in adenomas and colorectal carcinomas. It has been shown that p21WAF1 expression could be activated by histone acetylation of its promoter region independent of p53 binding sites[24,25].

Also, we showed that the levels of p21WAF1 mRNA and protein in colon cancer cells were very low, even difficult to detect before treatment. In Colo-320, p21WAF1 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 p21WAF1 was elevated. Similar effects were shown in SW116 cells (data not shown). Our data about TSA or sodium butyrate inducing p21WAF1 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 p21WAF1 mRNA and protein, and this increased expression was associated with an accumulation of acetylated histones in total cellular chromatin and the chromatin of p21WAF1 gene in these two colon cancer cell lines. It has been shown that p21WAF1 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 p21WAF1 gene in colorectal cancer.

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