Histone Deacetylase Inhibitors Decrease DNA Methyltransferase-3B Messenger RNA Stability and Down-regulate De novo DNA Methyltransferase Activity in Human Endometrial Cells

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Abstract

It is well known that the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) acts synergistically with the DNA methyltransferase (DNMT) inhibitor 5-aza-2'-deoxycytidine (ADC) to reactivate DNA methylation-silenced genes. Moreover, in several studies, TSA was capable of inducing DNA demethylation even in the absence of ADC. Here we describe a mechanism by which HDAC inhibitors affect DNA methylation through their regulation on DNMT3B, a methyltransferase responsible for de novo DNA methylation. Using quantitative real-time PCR and Western blot analysis, we show that TSA down-regulates DNMT3B mRNA and protein expression in human endometrial cancer cells. This decrease in DNMT3B mRNA results in a significant reduction in de novo methylation activities. Further experiments indicated that TSA decreases DNMT3B mRNA stability and reduces its half-life from ~4 to 2.5 hours. We established that protein synthesis is required for posttranscriptional regulation, suggesting the involvement of an RNase and/or key mRNA stabilization factor(s) controlling the DNMT3B mRNA stability. Therefore, TSA may not only modify histone acetylation, but also potentially alter DNA methylation. Since the HDAC inhibitors are frequently used in epigenetic studies and are considered to be promising anticancer drugs, these new findings will have implications in both laboratory and clinical settings. (Cancer Res 2005; 65(7): 2684-9)

Introduction

Two key epigenetic pathways modify chromatin structure and function: DNA methylation and histone acetylation (1–3). Generally, methylated DNA and deacetylated histones are found in "closed" or tightly packed chromatin conformations, which are associated with the suppressed state of transcription (4–9). An accumulating body of evidence supports the concept that methylation is directly involved in X chromosome inactivation (10), epigenetic imprinting (11), and abnormal gene silencing, which is frequently observed during cancer development (12, 13). DNA methylation is carried out by three DNA methyltransferases (DNMT), DNMT1, DNMT3A, and DNMT3B. These enzymes map to 19p13.2, 2p23, and 20q11.2, respectively (14). DNMT1 prefers hemimethylated DNA substrates and is therefore responsible for maintenance methylation, which must occur during DNA replication. DNMT3A and DNMT3B are responsible for de novo methylation and is particularly poignant when considering the aberrant methylation of tumor suppressor genes observed in cancer. Not surprisingly, there is constitutive expression of DNMT1 in proliferating cells. Whereas DNMT3A expression is ubiquitous, DNMT3B is present at low levels except in testes, thyroid, and bone marrow (14).

Two lines of reagents, affecting histone acetylation and DNA methylation, respectively, are frequently used to probe epigenetic regulation. Trichostatin A (TSA; ref. 15) and sodium butyrate (16) are histone deacetylase (HDAC) inhibitors that cause hyperacetylation on histones H3 and H4. 5-Aza-2'-deoxycytidine (ADC) is a DNMT inhibitor that induces DNA demethylation by metabolic incorporation into genomic DNA, thereby covalently arresting DNMT (17). It has been well established that HDAC inhibitors can act synergistically with ADC to reactivate silenced genes (18–20). Indeed, combination treatment with TSA and ADC has been routinely utilized for gene reactivation.

The functional interaction between DNA methylation and histone acetylation has been the current focus of studies on epigenetic gene regulation. These studies have shown that cytosine methylation can influence regional histone acetylation and modulate gene expression (1–4). A group of methylated DNA-binding proteins are able to recognize methylated DNA sequences and recruit HDAC to local chromatin (4, 6, 21). HDAC then catalyzes deacetylation on H3 and H4, ultimately leading to gene silencing. Interestingly, some data support the presence of a reverse regulatory pathway whereby histone acetylation mediates DNA demethylation (22–24). Although in some cases TSA treatment was not found to alter DNA methylation (25–27), several independent studies have suggested otherwise (22, 28–31). Furthermore, TSA has been shown to reactivate methylation-silenced genes even in the absence of ADC (32, 33). In all these cases, however, the mechanism underlying TSA-mediated DNA demethylation was not understood.

In this report, we describe a novel mechanism by which HDAC inhibitors may regulate DNA methylation, therefore explaining the synergy observed between DNMT and HDAC inhibitors in gene reactivation (18–20) and antineoplastic treatment (34). We show that TSA treatment of endometrial cancer cells causes a significant reduction in DNMT3B mRNA and protein levels and de novo DNMT activity. TSA was found to inhibit DNMT3B expression by accelerating DNMT3B mRNA degradation. These findings suggest that HDAC inhibitors may cause DNA hypomethylation by inhibiting DNMT3B activity. Since several HDAC inhibitors are being tested in anticancer trials (35–37), our findings bear clinical significance.
Materials and Methods

Cell lines and reagents. The human endometrial adenocarcinoma Ishikawa cell line was generously provided by Dr. Masato Nishida (Kasumigaura National Hospital, Japan). Human endometrial cancer cell lines KLE and AN3 were obtained from American Type Culture Collection (Rockville, MD). Ishikawa cells were maintained in MEM with L-glutamine (GIBCO, Life Technologies, Grand Island, NY). KLE and AN3 cells were grown and treated with different HDAC inhibitors in 10 cm diameter plastic dishes. Total RNA was isolated using Trizol reagents (Invitrogen, Carlsbad, CA). The mouse monoclonal antibody for proliferating cell nuclear antigen (PCNA) and the rabbit polyclonal antibody for β-actin were purchased from Sigma (St. Louis, MO). HDAC inhibitor-1 is a product of Calbiochem (San Diego, CA). DNMT3B antibody was purchased from Novus Biologicals (Littleton, CO). The mouse monoclonal antibody for DNMT3B was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-acetyl-histone H3 and H4 antibodies were purchased from Upstate (Lake Placid, NY). Purified DNMT3B was purchased from Methylation, Ltd. (Port Orange, FL).

Western blot analysis. Ishikawa, KLE, and AN3 cells were treated with TSA, Valproic acid (VA), or inducing concentrations of HDAC inhibitor-1 for 48 hours at varying concentrations as indicated in the figure legends. Cells were rinsed thrice with cold PBS and harvested with a plastic policeman. Cell extracts were prepared with the lysis buffer containing 20 mmol/L HEPES (pH 7.2), 25% glycerol, 0.42 mol/L NaCl, 1.5 mmol/L MgCl2, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, and 0.5 mmol/L phenylmethanesulfonyl fluoride. Cell lysates were centrifuged at 14,000 × g for 15 minutes at 4°C and the insoluble debris discarded. Protein concentrations were determined using Coomassie protein assay reagent (Pierce, Rockford, IL). Cell extracts (20 μg) were mixed with 5 μL gel-loading buffer [250 mmol/L Tris-HCl (pH 8.0), 20% β-mercaptoethanol, 8% SDS, 1.2 mg/mL bromophenol blue], heated for 5 minutes at 95°C and resolved in SDS polyacrylamide gels (Ready Gel, 4-15% gradient, Bio-Rad Laboratories, Hercules, CA). Proteins were electrically transferred onto an Immun-Blot polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membranes were blocked for 2 hours in PBS buffer containing 0.1% Tween 20 and 10% nonfat dried milk. Antibodies raised against DNMT3B, PCNA, or β-actin were diluted following the manufacturer's recommendations. Primary antibody binding was done at 4°C overnight with constant shaking. The anti-rabbit or anti-mouse antibodies labeled with horseradish peroxidase (Amersham Corp., Arlington Heights, IL) were applied at 1:5,000 dilutions. Secondary antibody binding was carried out at room temperature for 1 hour. Chemiluminescence detection was done with the enhanced chemiluminescence detection system (Amerham Corp., Arlington Heights, IL). The blots were reprobed with β-actin antibody and the results provide loading controls.

RNA isolation, cDNA synthesis, and quantitative real-time PCR. Cells were grown and treated with different HDAC inhibitors in 10 cm diameter dishes. Total RNA was isolated using Trizol reagents (Invitrogen, Carlsbad, CA). cDNA was synthesized from 1 μg RNA with random primers using SuperScript kit (Invitrogen). The 20 μL reverse transcription products were diluted to 100 and 2 μL used for each real-time PCR. PCR reactions were done in 25 μL that contained 140 ng of primers and 12.5 μL SYBR green master mix (Stratagene, Cedar Creek, TX). DNMT3B (166 bp), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 225 bp), and PCNA (220 bp) cDNA were amplified under the following conditions: initial denaturation, 95°C for 10 minutes, followed by 40 cycles with denaturation at 95°C for 30 seconds, annealing at 56°C for 40 seconds, and extension at 72°C for 30 seconds. Primer sequences used for PCR were: DNMT3B-3′, 5′-CCAGCTGAGGCGC- CATGT; DNMT3B-5′, 5′-ATTATTCTGAGGCGGCTT; GAPDH-3′, 5′-GAAGGTGAAGGTCGGAGTTC; GAPDH-5′, 5′-GGAGATGGTGATGGGATTTC; PCNA-5′, 5′-CTCACAATCTTCTGGGTCACAG; PCNA-3′, 5′-GATAAAGG-GACTGCTGGAGAAGG. The threshold cycle number (CT) for each sample was determined in duplicate or triplicate and the experiments were repeated thrice. The CT value for DNMT was normalized against GAPDH or PCNA reference genes, which was expressed as CT<sub>o</sub> = (CT<sub>DNMT</sub> − CT<sub>GAPDH</sub>) or CT<sub>p</sub> = (CT<sub>DNMT</sub> − CT<sub>PCNA</sub>). The CT values were converted to fold (2<sup>-ΔΔCT</sup>) and the relative changes in experimental groups were expressed as a percentage of untreated controls (arbitrarily set at 100%).

Measurement of DNMT3B mRNA stability. Cells were treated for 4 hours with TSA before transcription was blocked with 5 μg/mL actinomycin D. Cells were harvested at 0, 2, 4, and 8 hours post–actinomycin D treatment. RNA isolation, reverse transcription, and real-time PCR were done as described above. The DNMT3B half-life, with or without TSA treatment, was estimated from the plots of DNMT3B mRNA levels as a function of time. To examine the role of protein synthesis in TSA action, cells were pretreated for 2 hours with 10 μg/mL of cycloheximide. Actinomycin D was added after the cells were exposed to TSA for 4 hours. The cells were incubated for 8 hours longer under normal culture conditions and harvested for DNMT3B mRNA measurement. In all these experiments, DNMT3B mRNA levels before actinomycin D addition was determined as the baseline.

Data analysis. All data groups were analyzed by ANOVA to determine if there was significance (P < 0.05) among the groups. For all experimental groups that satisfied the initial ANOVA criterion, individual comparisons were done with the use of post-hoc Bonferroni tests with the assumption of two-tailed distribution and two samples with equal variance at the P < 0.05 level. Statistical significance is indicated by asterisks in the figures.

Results

Trichostatin A treatment decreases DNMT3B mRNA levels. It has been shown that TSA, alone or in combination with ADC, reactivates the expression of methylated genes such as the estrogen and progesterone receptor in endometrial cancer cells (38–40). One possible explanation for this is that TSA may affect DNMT expression. Therefore, we examined the steady state levels of DNMT1, DNMT3A, and DNMT3B mRNA using real-time PCR in endometrial Ishikawa cells (41) following TSA treatment. Although TSA had little effect on DNMT1 and DNMT3A, we found that there was a 2- to 3-fold reduction in DNMT3B mRNA after 48 hours of treatment (Fig. 1A). It is noteworthy that throughout these experiments, the inhibitory effect on DNMT3B mRNA was unchanged when the results were standardized against PCNA (Fig. 1A), a control for cell cycle alterations. To test if the observed TSA effect was specific to Ishikawa cells, we expanded the investigation to assess other endometrial cancer cell lines. DNMT3B mRNA levels decreased after 12 hours and...
continued to decline at 24 and 48 hours following TSA treatment in AN3 (Fig. 1B) and KLE cells (Fig. 1C). We next tested if this was a TSA-specific or a common function shared by other HDAC inhibitors. Significant inhibition in DNMT3B mRNA was also observed using both sodium butyrate and HDAC inhibitor-1 [N-(2-aminophenyl)-4-[(N-[pyridine-3-ylmethoxycarbonyl] amino)methyl]benzamide], a synthetic drug developed for chemotherapy (42). This indicates that the observed reduction in DNMT3B mRNA was specific to HDAC inhibitors, rather than a general cytotoxic effect of TSA (Fig. 1D). Indeed, under the conditions and concentrations applied, cells retained normal morphology and no significant cell death was observed (data not shown).

To confirm the effect of these drugs on histone acetylation, we did Western blot analysis using antihistone and antiacetylated histone antibodies. As expected, treatment of AN3 cells with TSA, sodium butyrate, or HDAC inhibitor-1 induced significant hyperacetylation on both H3 and H4 (Fig. 1E). Histone deacetylase inhibitors decrease DNMT3B protein expression. To examine alterations in DNMT3B protein, Western blot analysis was done using a DNMT3B-specific antibody. These experiments showed that TSA progressively decreases DNMT3B protein levels at 24 and 48 hours after treatment in both AN3 (Fig. 2A) and KLE cells (Fig. 2B). This finding was also present following treatment with sodium butyrate or HDAC inhibitor-1 in AN3 (Fig. 2C), Ishikawa (Fig. 2D), and KLE cells (Fig. 2E). During these experiments, a strong band(s) below the putative DNMT3B band was observed in all the samples examined. To investigate its significance, we did antigen competition experiments. As shown in Fig. 2F, when increasing amounts of purified DNMT3B were added to the primary antibody binding solution, the DNMT3B band was effectively competed away. The “lower” band(s), however, remained mostly unchanged, indicating that these were nonspecific. Thus, consistent with their effects on DNMT3B mRNA levels, diversified HDAC inhibitors were able to reduce DNMT3B protein expression in all cell lines tested, confirming the notion that HDAC inhibitors down-regulate DNMT3B expression.

Histone deacetylase inhibitors decrease de novo DNA methyltransferase activities. We next sought to determine if TSA treatment could alter DNA methylation capacities by the standard in vitro assay using synthetic, unmethylated, CpG-rich oligonucleotide substrates and radioactive S-adenosyl-L-methionine. Figure 3 shows that TSA treatment for 48 hours decreased de novo DNA methylation by 50% in AN3 and KLE cells. These data suggest that TSA not only modifies histone acetylation, but may also contribute to the remodeling of DNA methylation patterns.

Histone deacetylase inhibitors down-regulate DNMT3B mRNA stability. To begin to address the possible mechanisms by which HDAC inhibitors down-regulate DNMT3B expression, we investigated the regulation of DNMT3B transcription by TSA. Although DNMT3B transcription is directed by two alternative promoters (43), the sequence divergence lies in exon 1 and protein translation starts from exon 2. Therefore, the two transcripts
produce the same peptide. We subcloned the two DNMT3B promoters into a luciferase reporter construct and measured their transcriptional activities in endometrial cell lines. TSA treatment was found to increase the activity of both promoters (data not shown). This unexpected result was at odds with our previous findings, suggesting that a mechanism other than transcriptional control accounts for TSA-mediated DNMT3B down-regulation. Consequently, we examined whether TSA alters DNMT3B mRNA stability. Cells were pretreated with TSA for 4 hours before actinomycin D was added to halt RNA synthesis. DNMT3B mRNA degradation curves, with or without TSA treatment, were constructed in AN3 (Fig. 4A) and Ishikawa cells (Fig. 4B). DNMT3B mRNA turnover was relatively rapid, with an average half-life of approximately 3 and 4 hours for AN3 and Ishikawa cells, respectively. Importantly, TSA treatment seemed to destabilize DNMT3B mRNA, reducing its half-life to 1.8 and 2 hours in AN3 and Ishikawa cells,
respective. This result is consistent with the previously observed decrease in DNMT3B mRNA, establishing one mechanism by which TSA inhibits DNA methyltransferase activity.

**Trichostatin A action on DNMT3B mRNA requires protein synthesis.** We next investigated whether protein synthesis was required for TSA to decrease DNMT3B mRNA stability. First, protein synthesis was blocked by cycloheximide pretreatment for 2 hours. TSA and actinomycin D were sequentially added. Figure 5 shows that although TSA treatment destabilized DNMT3B mRNA, this effect was completely abrogated by cycloheximide pretreatment. Therefore, protein synthesis is necessary for the TSA-mediated reduction in DNMT3B mRNA stability.

**Discussion**

As an enzyme responsible for de novo DNA methylation, sufficient DNMT3B levels are crucial for cells to maintain normal DNA methylation patterns (44, 45). DNMT3B disruption results in global as well as gene-specific hypomethylation (46). In cancer cells, antisense-mediated depletion of DNMT3B reactivates methylation-silenced genes (47). In this report, we describe a new pathway that in part explains the synergistic action between DNMT and HDAC inhibitors by reduction in DNMT3B mRNA stability. Although, at present, it is unknown whether TSA-mediated DNMT3B down-regulation leads to DNA demethylation, multiple studies have suggested that this may be the case (30). For example, evidence has been found in Neurospora that TSA was able to greatly reduce DNA methylation in several specific regions (22). Hu et al. (28) have shown that TSA treatment induces a partial relaxation of imprinting as well as demethylation of the IGF2R gene. Cosgrove and Cox (29) found that sodium butyrate administration to several human cell lines resulted in global hypomethylation.

Protein synthesis seems to be crucial for TSA-mediated destabilization of DNMT3B mRNA. Thus, HDAC inhibitors may either activate an RNase(s) entity responsible for DNMT3B clearance (48, 49), or alter a key DNMT3B mRNA-binding protein(s) important for DNMT3B stabilization. Protective protein binding to specific mRNA sequences in the 3′-untranslated region plays an important role in the control of eukaryotic mRNA stability (50, 51). Further investigations characterizing the crucial DNMT3B stabilization sequences and binding factors will be necessary to understand the mechanisms controlling DNMT3B mRNA degragation in this context.

HDAC inhibitors are frequently used to probe the epigenetic mechanisms involved in chromatin remodeling and gene expression. Previous studies have shown that administration of HDAC inhibitors alone was able to induce gene reactivation and demethylation (22, 28–31). Furthermore, "open" chromatin structures containing hyperacetylated histones are often associated with unmethylated DNA (4, 5), suggesting that methylation may be directed by histone acetylation (22–24). The proposed regional, chromatin-specific actions, however, are different from the regulatory mechanism described here. Therefore, there seems to be two parallel pathways initiated by HDAC inhibitors that lead to DNA hypomethylation. It is critical to distinguish the local, chromatin-specific actions, such as DNMT recruitment through protein-protein interactions, from the global regulatory pathway identified in this investigation. For example, it is possible that by inhibiting DNMT3B activity, TSA lowers the threshold for DNMT inhibitors to induce DNA hypomethylation. This mechanism may contribute to the synergy observed between HDAC and DNMT inhibitors with regard to gene reactivation (18–20, 26, 32, 33) and antitumor activity (34).

As for the clinical application of HDAC inhibitors as anticancer drugs (35–37), our findings suggest that these drugs may cause long-term effects on DNA methylation/transcription. Unlike the inhibitory effect on HDACs that is promptly reversed following drug withdrawal, the changes in DNA methylation patterns are relatively stable and inheritable at least in somatic lineages. Studies have shown that the TSA-induced changes in genomic DNA methylation can last at least 60 days (29). These changes may also influence cellular responses to other drugs during combination therapy and should be considered when designing clinical trials utilizing HDAC inhibitors.

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**References**

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