Published in final edited form as:

FASEB J. 2008 June ; 22(6): 1981–1992.

15-Lipoxygenase-1 transcriptional silencing by DNA methyltransferase-1 independently of DNA methylation

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Abstract

Methylation of promoter DNA contributes to transcriptional silencing of various tumor-suppressor genes in cancer. Transcriptional silencing of 15-lipoxygenase-1 (15-LOX-1) promotes tumorigenesis. Methylation of 15-LOX-1 promoter DNA occurs in some cancers, but its mechanistic role in 15-LOX-1 transcriptional silencing is unclear. We examined the mechanistic role of 15-LOX-1 promoter DNA methylation in 15-LOX-1 transcriptional regulation in human colorectal cancers. 15-LOX-1 promoter methylation occurred in colorectal cancer cells in vitro, in 36% of tumor tissue samples of colorectal cancer patients, and in virtually no normal colonic mucosa samples of 50 human subjects with no history of colorectal cancer or polyps. 15-LOX-1 promoter DNA methylation levels, however, did not correlate with 15-LOX-1 expression levels (Spearman's $r = .21$; $P = 0.38$). We employed siRNA knockdown and genetic disruption models of DNA methyltransferases (DNMTs) to study the effects of this methylation on 15-LOX-1 expression in colon cancer cells. 15-LOX-1 promoter demethylation was insufficient to reestablish 15-LOX-1 expression. 15-LOX-1 transcription was activated by the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) only after DNMT-1 dissociation from the 15-LOX-1 promoter and without altering 15-LOX-1 promoter DNA methylation. DNMT-1 protein hypomorphism impaired DNMT-1 recruitment to the 15-LOX-1 promoter, which allowed 15-LOX-1 transcription activation by SAHA. DNMT-1 has a direct suppressive role in 15-LOX-1 transcriptional silencing that is independent of 15-LOX-1 promoter DNA methylation.

Keywords

colon cancer; promoter DNA methylation; transcriptional regulation

INTRODUCTION

Promoter DNA methylation is a well-recognized mechanism for inactivating various tumor suppressor genes in cancer (1). 15-Lipoxygenase-1 (15-LOX-1) is the rate limiting-step enzyme for the conversion of linoleic acid to 13-*S*-hydroxyoctadecadienoic acid (2,3) and also is involved in the production of anti-inflammatory lipid signaling mediators from arachidonic acid (*e.g.*, lipoxins (4)) and docosahexaenoic acid (*e.g.*, protectin D1 (5)). Overexpression of 15-LOX-1 inhibits inflammation in animal models (4,6). 15-LOX-1 is down-regulated in human colon (7–9), esophageal (10), breast (11), and pancreatic cancers (12). 15-LOX-1 re-expression via pharmaceutical agents (*e.g.*, nonsteroidal anti-inflammatory drugs, histone deacetylase inhibitors [HDACIs]) or transfection with plasmid or adenoviral vectors induces apoptosis and inhibits tumorigenesis in cancer cells (8–10,13–19).

The 15-LOX-1 promoter contains CpG islands (20,21) and is methylated in lymphoma, lung, epidermoid, cervical, and prostate cancer cell lines (21,22). Whether 15-LOX-1 promoter DNA methylation mechanistically contributes to 15-LOX-1 transcriptional suppression in human cancers, however, has remained controversial. Conflicting data have shown that the hypomethylating agent 5-aza-2-deoxycytidine (5-aza-dc) has both induced (23) and failed to induce (24) 15-LOX-1 expression in colon cancer cell lines and has both facilitated 15-LOX-1 transcriptional activation by interleukin-4 or HDACIs (in the L428 lymphoma cell line (21)) and inhibited 15-LOX-1 gene transcription (in prostatic cancer cell lines (22)). These conflicting data have clouded the role of 15-LOX-1 promoter methylation in 15-LOX-1 transcriptional silencing in cancer cells. In the present study, we tested whether 15-LOX-1 promoter DNA methylation is associated with 15-LOX-1 transcriptional suppression in patients with colorectal cancers, which are well known to have down-regulation of 15-LOX-1 (7–9); we also investigated the mechanistic contribution of 15-LOX-1 promoter methylation to the suppression of 15-LOX-1 transcription in colorectal cancer cells.

MATERIALS AND METHODS

Acquisition of clinical samples

We collected colonic biopsy specimens during colorectal endoscopic procedures after obtaining written informed consent from participating patients. Study patients were selected from among patients seen at outpatient gastrointestinal clinics at The University of Texas M. D. Anderson Cancer Center and other hospitals within the Texas Medical Center (the Gastroenterology section at Baylor College of Medicine, an outpatient gastrointestinal endoscopy unit affiliated with St. Luke's Hospital, and the Michael E. DeBakey VA Medical Center) for colorectal cancer screening and for the follow-up and management of colorectal cancers. This study was approved by the institutional review board at each participating institution.

Our study involved a total of 100 patients divided into two groups of 50 patients each. The first group included patients with colorectal cancers in whom samples were obtained from the colorectal cancers and from normal-appearing mucosa at least 10 cm from the tumor. The second group included subjects who had a normal complete colonoscopic examination at the time of sample procurement and no personal history of colorectal cancer or polyps. In this normal-colon group, two sets of samples of the colonic mucosa were obtained—one from the left and one from the right colon.

Subjects in both groups were required to be between 45 and 85 yr old, to have no history of hereditary colon cancer (familial colorectal polyposis syndrome, hereditary nonpolyposis colon cancer syndrome, or family history of one or more first-degree relatives with colon cancer), and to be U.S. citizens or permanent residents (to reduce the potential for large

variability in risk factors such as dietary habits if international patients were included (25, 26)). Patients were excluded if they had a history of inflammatory bowel disease, had received chemotherapy within 4 wk prior to the colonoscopy, had participated in a chemopreventive study during the month prior to the colonoscopy, had a history of bleeding diathesis, had a history of another active cancer within 5 yr prior to enrollment (except for non-melanoma skin cancer), were taking warfarin, or were taking anti-inflammatory medications (*e.g.*, nonsteroidal agents, aspirin, sulfasalazine) within 1 wk of the colonoscopies. Samples were collected between 2001 and 2006. All tissue samples were fresh frozen and stored at -80°C until the time of laboratory analyses.

Cells, antibodies, and reagents

We obtained Caco-2 and SW480, LOVO, SW620, HT29, and DLD1 human colon carcinoma cell lines from the American Type Culture Collection (Manassas, VA, USA) and RKO human rectal carcinoma cells from Dr. Michael Brattain (The University of Texas, San Antonio, TX). The HCT-116 parental colorectal cell line, DNMT-1 HM [HCT-116 cells with DNA methyltransferase 1 genetic disruption resulting in DNMT-1 hypomorph protein (DNMT-1 HM)], DNMT-3B KO (HCT-116 cells with DNMT-3B knockout), and DKO (HCT-116 cells with both DNMT-1 HM and DNMT-3B KO) cells were gifts from Dr. Bert Vogelstein (The Johns Hopkins University School of Medicine, Baltimore, MD).

We purchased anti-human DNMT-1 and anti-human DNMT-3B antibodies from Upstate Cell Signaling Solutions (Lake Placid, NY, USA); siGENOME SMARTpool and ON-TARGET plus siRNA for DNMT-1, DNMT-3A, and DNMT-3B and a nonspecific control siRNA (siGLO RISC-Free siRNA) were obtained from Dharmacon, Inc. (Lafayette, CO, USA). Normal human-derived tracheobronchial epithelial cells cultured in a multilayered, pseudostratified, and highly differentiated *in vitro* model that closely resembles the epithelial tissue of the respiratory tract were obtained from MatTek Corporation (Ashland, MA, USA). Rabbit polyclonal antiserum to recombinant human 15-LOX-1 was obtained as described previously (14); 5-aza-dc was purchased from Sigma-Aldrich (St. Louis, MO, USA), and suberoylanilide hydroxamic acid (SAHA) was provided by the National Cancer Institute's Chemical Carcinogen Reference Standards Repository. Other reagents, molecular-grade solvents, and chemicals were obtained from commercial manufacturers or as specified.

Cell culture

The HCT-116 parental colorectal cell line and DNMT-1 HM, DNMT-3B KO, and DKO cells were grown in McCoy's 5A medium; SW480, LOVO, SW620, HT29, RKO, and DLD1 cells were grown in RPMI 1640 medium; and Caco2 cells were grown in MEM medium (Cambrex, East Rutherford, NJ, USA) with added L-glutamine in a humidified atmosphere containing 5% CO_2 at 37°C . Media contained 10% fetal bovine serum and were supplemented with 1% penicillin–streptomycin as described previously (14).

siRNA transfection

HCT-116 and DNMT-1 HM cells were cultured to 40%–50% confluence and then transfected with 100 nmol/L of a pooled mixture of either four SMART-selected or ON-TARGET plus siRNA duplexes for the targeted gene (Dharmacon) or with a nonspecific control siRNA (siGLO RISC-Free siRNA; Dharmacon) using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA).

Bisulfite treatment of genomic DNA

To examine DNA methylation patterns in the CpG island of the 15-LOX-1 gene promoter, we extracted genomic DNA from patient samples and the cell lines using a standard phenol–

chloroform method. In a subset of patients in whom both 15-LOX-1 promoter DNA methylation and 15-LOX-1 RNA expression were examined (19 colon cancer patients), both total RNA and genomic DNA were extracted from the same sample by using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). Each biopsy specimen was homogenized in 0.5 ml of TRI Reagent. The homogenates were separated into aqueous and organic phases by adding bromochloropropane and then by centrifugation. Total RNA was precipitated from the aqueous phase, and the DNA was precipitated from the phenol and interphase. The isolated genomic DNA was treated with sodium bisulfite (Sigma, St. Louis, MO, USA) as described previously (27). Bisulfite-treated DNA was stored at -20°C until it was used for bisulfite cloning sequencing or pyrosequencing assays.

Bisulfite pyrosequencing

Bisulfite-treated DNA was amplified using a pyrosequencing method. PCR amplified 150 bp of the 15-LOX-1 promoter region (Fig. 1A) that contains three CpG sites (located between +13 and +22 bp) using 10 pM of forward primer (GGGTTT TAGGTTGGGTTATTTA), 1 pmol of reverse primer with universal overhang (GGGACACCGCTGATCGTTTAACCAACCACAACCTACACCTAATTAT), and 10 pmol of universal biotinylated primer (GGGACACCGCTGATCGTTTA). The PCR conditions were as follows: 95°C for 5 min, followed by 50 cycles of 95°C for 30 s, 50°C for 45 s, and 72°C for 45 s, and a final incubation at 72°C for 4 min. Biotin-labeled PCR product was isolated with streptavidin sepharose HP (Amersham Biosciences, Uppsala, Sweden) and subjected to pyrosequencing as described previously (27). The methylation levels at three different CpG sites within the 15-LOX-1 promoter sequence were averaged to represent the degree of methylation in the 15-LOX-1 promoter.

Genomic DNA cloning and sequencing

A region of the 15-LOX-1 promoter that contains 10 CpG sites (Fig. 1A) was PCR amplified from bisulfite-treated genomic DNA of four colorectal cancer cell lines and sequenced to confirm the pyrosequencing results. The PCR step amplified the targeted 244-bp region of the 15-LOX-1 promoter using 2 μl of bisulfite-treated DNA and the following oligonucleotide primers: 5'-GATAGTGGTTTTTATTTTTTGTGTTT-3' (sense primer) and 5'-AACCCATCTTACTCAAAAATATTTTC-3' (antisense primer). The PCR conditions were 94°C for 3 min and then 94°C for 20 s, 60°C for 30 s, and 71.5°C for 70 s for 35 cycles. The PCR products were purified and subcloned into pCRII-TOPO vector (Invitrogen). Plasmid DNA was extracted from individual clones using a QIAprep Spin Miniprep Kit (QIAGEN, Inc., Valencia, CA, USA) and sequenced at the SeqWright Company (Houston, TX, USA). Five clones were sequenced for each cell line.

RNA extraction and real-time PCR

Total RNA was extracted from cells using TRI Reagent. One microgram of total RNA from each sample was reverse transcribed in a 20- μl reaction using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR was carried out in triplicate with 25 μl of each reaction containing 1 μl of cDNA (50 ng/ μl), 12.5 μl of 2 $\times$ TaqMan Universal PCR Master Mix (Applied Biosystems, Inc., Foster City, CA, USA), and 1.25 μl of primer and probe mixture (Applied Biosystems) using a 7300 real-time PCR system (Applied Biosystems) with the following program: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s and at 60°C for 1 min. A sequence-detection program calculated a threshold cycle number (C_t) at which the probe cleavage-generated fluorescence exceeded the background signal (28). We calculated the relative RNA expression level using a comparative threshold cycle method ($\Delta\Delta C_t$), as previously described (18).

Western blotting

Western blot analyses for 15-LOX-1 protein expression were performed as described previously (14).

Chromatin immunoprecipitation assays

HCT-116, Caco2, HT29, and SW480 cells were treated with 5 μ M SAHA or DMSO for 4 h and then cross-linked by adding formaldehyde to the culture medium to a final concentration of 1% for 10 min at 37°C. Chromatin immunoprecipitation (ChIP) assays were carried out using a commercial assay kit according to the manufacturer's protocol (Upstate Cell Signaling Solutions). Chromatin was immunoprecipitated using anti-human DNMT-1 and anti-human DNMT-3B (Upstate Cell Signaling Solutions). Real-time PCR was used to assess ChIP DNA of the 15-LOX-1 promoter region between -150 nt and -300 nt relative to the ATG site. The PCR reaction consisted of 1 μ l of immunoprecipitated DNA (20 ng/ μ l), 12.5 μ l of 2 $\times$ TaqMan Universal PCR Master Mix (Applied Biosystems), and 1.25 μ l specific primer and probe mixture (Applied Biosystems). The PCR reaction was performed using the following program: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s and at 60°C for 1 min in a 7300 real-time PCR system (Applied Biosystems). For each experiment, the threshold bar was set within the linear range of PCR amplification. To assess the relative binding of a protein (*e.g.*, DNMT-1) to the 15-LOX-1 promoter region, we used the comparative C_t method to calculate the relative sequence abundance of the ChIP DNA fraction to the input sample (% of input) according to the following formula: % input = $[2^{C_t(\text{input})-C_t(\text{ChIP})}] \times 100$. The C_t value of the ChIP DNA sample is denoted by $C_t(\text{ChIP})$ and that of the input by $C_t(\text{input})$ (29,30).

Statistical analyses

We used Pearson's correlation coefficient for correlating the methylation levels from the pyrosequencing and cloning sequencing methods. We used the *t* test for two-group comparisons (*e.g.*, methylation levels between cancerous and normal mucosa in patients with colorectal cancer). Chi-square and Kruskal-Wallis tests were used to compare clinical characteristics between the colon cancer group subsets (*i.e.*, highly methylated *versus* nonmethylated cancer tissue subsets). Spearman's correlation method was used to correlate the methylation levels with 15-LOX-1 mRNA levels. For analyses involving the simultaneous consideration of two factors, we performed two-way analysis of variance (ANOVA).

Our analyses proceeded as follows: We first tested the interaction effect, and if it was significant, we subsequently performed specific comparisons to investigate which differences were driving this effect, using the Bonferroni correction to adjust for the multiple testing problem. This means that if we performed *k* comparisons, an individual comparison would not be considered significant unless its *P* value was less than 0.05/*k*. If the interaction effect was not significant, we tested the individual main effects. Then, if those were significant, we determined which pairwise comparisons were significant, again adjusting for multiplicities using the Bonferroni correction. For analyses involving single factors, we performed a one-way ANOVA. If the overall ANOVA test was significant, then we performed pairwise comparisons, adjusting for multiplicities using the Bonferroni correction. All tests were two sided and conducted at the $P \leq 0.05$ level. All quantitative analyses (sample size < 35) were done on the log-transformed data because we found that the log transformation decoupled the relationship between the mean and variance and accommodated the normal-distributional assumptions underlying the methods. Data were analyzed using SAS software (SAS Institute, Cary, NC, USA).

RESULTS

15-LOX-1 promoter methylation levels in colorectal cancer *in vitro*

The results for the 15-LOX-1 promoter methylation levels of the cloning sequencing assay highly correlated with the ones from the pyrosequencing assay (Pearson's correlation coefficient, $r = .999$, $P = 0.0006$). HCT-116 cells with both DNMT-1 and DNMT-3B KO (*i.e.*, DKO cells) had very low methylation density (2%) (Fig. 1B). A high methylation level was defined as a methylation density of $>10\%$ (*i.e.*, two times higher than the bisulfite/pyrosequencing assay background) (27). All eight tested colorectal cancer cell lines had high methylation levels (Fig. 1C). Methylation densities were $>50\%$ in seven of the eight colorectal cancer cell lines (Fig. 1C), and only Caco2 cells had a level $<50\%$ (14%).

15-LOX-1 promoter methylation levels in subjects with colorectal cancer and those with normal colons

The demographic and clinical characteristics of subjects enrolled in the study are shown in Table 1. The anatomic distribution for the colorectal cancer was as follows: cecum 2%, ascending colon 2%, transverse colon 4%, descending colon 4%, sigmoid colon 22%, and rectum 66%.

The cancerous mucosa had high methylation levels on average, but the levels were highly variable ($14\% \pm 13.75\%$, mean $\pm$ SD). High methylation levels ($>10\%$ methylation density) were detected in a subset of patients (36%) (methylation levels $28\% \pm 14\%$) (Fig. 1D). The remaining portion of the cancer patients had methylation levels in the colonic mucosa of $6\% \pm 1.86\%$. The cancer patients with high methylation levels were statistically significantly younger than those with normal methylation levels (Table 2). The two cancer subsets had no significant differences in sex, tumor stage, or location distribution (Table 2).

The paired normal tissues from cancer patients had low methylation levels ($6\% \pm 2\%$). Within this group, only two of 50 adjacent normal tissues had levels $>10\%$; in both cases, the levels were 11%. In specimens obtained from colonic biopsies from patients with normal colons, the methylation levels were low and similar for left and right sides ($6\% \pm 2\%$; $P = 0.22$). Only three of 100 mucosal samples from patients with normal colons had levels $>10\%$. The levels in those three samples were 11% (right side), 13% (left side), and 17% (left side). The matched samples from the other side of these subjects' colons were $<10\%$ (6% [left side], 6% [right side], and 7% [right side], respectively). Methylation levels were statistically higher in the cancer tissues than they were in the paired normal tissues from the same patients and in the samples from patients with normal colons ($P < 0.0001$). Methylation levels were not statistically different between normal colonic mucosa from colorectal cancer patients and normal mucosa from patients with normal colons ($P = 0.36$).

Correlation between 15-LOX-1 promoter methylation and 15-LOX-1 expression *in vitro* and *in vivo*

We correlated the levels of 15-LOX-1 expression and 15-LOX-1 promoter DNA methylation to examine the relevance of 15-LOX-1 promoter DNA methylation to the loss of 15-LOX-1 expression in cancer cells. 15-LOX-1 mRNA expression levels in colorectal cancer cell lines had a weak inverse correlation with 15-LOX-1 promoter DNA methylation levels that failed to reach statistical significance ($n = 8$; Spearman's $r = -.5$; $P = 0.21$; Fig. 2A). 15-LOX-1 RNA expression levels were lower in the cancerous than in the paired normal mucosa ($P = 0.006$; data not shown). In the subset of 19 colon cancer patients whose DNA and RNA were simultaneously extracted from the same samples, the correlation between the 15-LOX-1 mRNA and DNA promoter methylation level was, however, nonsignificant ($n = 19$; Spearman's $r = .21$; $P = 0.38$; Fig. 2B).

Effects of 15-LOX-1 promoter demethylation on 15-LOX-1 expression

We further studied the mechanistic importance of 15-LOX-1 promoter DNA methylation to 15-LOX-1 transcriptional silencing by examining the effects of reversing 15-LOX-1 promoter methylation on 15-LOX-1 transcriptional suppression in cells that had high DNA methylation of the 15-LOX-1 promoter. We used the HCT-116 colon cancer cell line, which we found to have a high level of 15-LOX-1 methylation (Fig. 1B, C) and which has been used extensively in the past to study DNA methylation. We used three approaches to demethylate the 15-LOX-1 promoter: (a) siRNA to rapidly down-regulate the expression of DNMT-1, -3A, and -3B, (b) treatment with 5-aza-dc, a well-known DNA demethylating agent, and (c) combined DNMT-1 and -3B genetic disruption in HCT-116 cells.

DNMT-1 siRNA reduced DNMT-1 mRNA levels by 78%–80% in cells transfected with DNMT-1 siRNA compared with those transfected with nonspecific siRNA ($P < 0.0001$; Fig. 3A). DNMT-3A siRNA transfection reduced DNMT-3A mRNA levels by 41% in cells transfected with DNMT-3A siRNA compared with those transfected with nonspecific siRNA ($P < 0.0001$; Fig. 3B). DNMT-3B siRNA transfection reduced DNMT-3B mRNA levels by 60%–64% in cells transfected with DNMT-3B siRNA compared with those transfected with nonspecific siRNA ($P < 0.0001$; Fig. 3C). 15-LOX-1 promoter methylation levels were reduced in cells transfected with DNMT-1 and -3B siRNA or with DNMT-1, -3A, and -3B siRNA by 42% and 37%, respectively; however these differences failed to reach statistical significance ($P = 0.264$; Fig. 3D).

5-Aza-dc treatment reduced 15-LOX-1 promoter methylation levels for the two concentrations tested, 1 μM and 2.5 μM , by 51% and 38%, respectively ($P = 0.0545$; Fig. 3D). DNMT-1 and -3B siRNA transfection with and without -3A siRNA increased 15-LOX-1 mRNA levels by onefold compared with nonspecific siRNA ($P < 0.0001$; Fig. 3E). 5-Aza-dc treatment also increased 15-LOX-1 mRNA by 30% ($P = 0.09$) for the 1 μM concentration and 79% ($P = 0.0126$) for the 2.5 μM concentration (Fig. 3E). Neither DNMT-1, -3A, or -3B siRNA down-regulation nor 5-aza-dc treatment was able to induce protein expression of 15-LOX-1 in HCT-116 cells (Fig. 3F). Combined DNMT-1 and -3B genetic disruption in HCT-116 cells (DKO) resulted in significant reduction of the average methylation scores, from 91% to 2.5% ($P < 0.0001$; Fig. 3G). 15-LOX-1 expression levels were, however, lower in DKO cells than they were in the parental HCT-116 cells ($P = 0.0002$; Fig. 3H).

15-LOX-1 transcriptional activation through specific inhibition of DNMT-1

We examined whether 15-LOX-1 promoter DNA methylation might play a secondary role in 15-LOX-1 transcriptional silencing by investigating the effects of reversing DNA methylation on 15-LOX-1 transcriptional activation by the HDACI, SAHA. Treatment of HCT-116 cells with the hypomethylating agent 5-aza-dc resulted in a small increase in 15-LOX-1 mRNA expression (0.6-fold); SAHA treatment resulted in a higher increase of 15-LOX-1 mRNA expression (3.22-fold; $P < 0.0001$; Fig. 4A). The combination of 5-aza-dc and SAHA resulted in a markedly enhanced increase in 15-LOX-1 mRNA (8.8-fold; $P < 0.0045$; Fig. 4A). As was the case with 5-aza-dc, DNMT-1 and -3B combined genetic disruption in HCT-116 cells (*i.e.*, in DKO cells) markedly increased 15-LOX-1 expression by SAHA from 3.22- to 11.74-fold compared with that in parental HCT-116 cells ($P < 0.0001$; Fig. 4B). DKO cells were generated from HCT-116 cell line that had homozygous genetic disruption of DNMT-3B (DNMT-3B KO) and DNMT-1 (DNMT-1 HM) (31). DNMT-1 genetic disruption by deleting exons 3, 4, and 5 of the DNMT-1 protein produced a truncated hypomorph protein (DNMT-1 HM) that retained the c-terminal catalytic domain for DNA methyltransferase activity (32). SAHA induced 15-LOX-1 mRNA to a level similar to that in the DKO cells (Fig. 4C) in DNMT-1 HM but not DNMT-3B KO cells (DKO *versus* DNMT-1 HM [$P = 0.12$], DKO *versus* DNMT-3B [$P < 0.0001$]). DNMT-1 HM and DKO cells but not DNMT-3B KO cells

had 15-LOX-1 protein expression induction by SAHA (Fig. 4D). DNMT-1 siRNA transfection significantly reduced the expression of DNMT-1 in HCT-116 cells compared with that in mock-transfected or nonspecific siRNA-transfected cells ($P < 0.0001$; Fig. 4E). DNMT-1 siRNA down-regulation of DNMT-1 by SAHA treatment resulted in a marked increase in 15-LOX-1 expression levels in HCT-116 cells compared with those in cells treated identically with SAHA but transfected with nonspecific siRNA ($P = 0.03$; Fig. 4F). The increase in 15-LOX-1 expression with the combination of DNMT-1 down-regulation (by siRNA) and SAHA treatment in the parental HCT-116 cells was similar to that achieved in DNMT-1 HM cells treated with SAHA and transfected with nonspecific siRNA ($P = 0.06$; Fig. 4F).

Mechanisms of DNMT-1 suppression of 15-LOX-1 transcription

Although 15-LOX-1 promoter methylation levels were significantly lower in DKO cells than they were in parental HCT-116 cells ($P < 0.0001$), the methylation levels were not significantly different between parental HCT-116, DNMT-1 HM, and DNMT-3B KO cells; SAHA had no significant effect on methylation levels in all cell lines ($P = 0.662$; Fig. 5A). SAHA treatment markedly increased 15-LOX-1 mRNA expression levels in three of the colon cancer cell lines (Caco-2, HT-29, and SW-480) compared with that in the HCT-116 cell line ($P < 0.0001$; Fig. 5B), although it failed to change the 15-LOX-1 promoter DNA methylation levels significantly in the HCT-116, Caco-2, SW-480, or HT-29 cell lines ($P = 0.724$; Fig. 5C). SAHA markedly reduced DNMT-1 recruitment to the 15-LOX-1 promoter at 4 h in DNMT-1 HM, Caco-2, HT-29, and SW-480 ($P < 0.0001$ for all four cell lines) but not the HCT-116 colon cancer cells ($P = 0.09$; Fig. 5D). SAHA failed to reduce DNMT-3B binding to the 15-LOX-1 promoter in Caco-2, SW-480, or HT-29 cell lines ($P = 0.08$; data not shown).

DISCUSSION

The major findings of this study demonstrate that DNMT-1 has a direct repressive role in 15-LOX-1 transcriptional suppression independently of 15-LOX-1 promoter DNA methylation. We found that despite its being limited to colorectal cancer cells, 15-LOX-1 promoter DNA methylation had no correlation with 15-LOX-1 mRNA expression levels, and its demethylation failed to reestablish 15-LOX-1 expression. In contrast, the dissociation of DNMT-1 from the 15-LOX-1 promoter was necessary to activate 15-LOX-1 transcription in colon cancer cells.

The evidence for the specific and direct contribution of DNMT-1 to the suppression of 15-LOX-1 transcription included 1) the genetic disruption of DNMT-1 but not DNMT-3B was necessary for SAHA activation of 15-LOX-1 transcription in HCT-116 cells lines, 2) the down-regulation of DNMT-1 by siRNA in HCT-116 cells allowed SAHA to induce 15-LOX-1 transcription to levels similar to those of DNMT-1 HM (HCT-116 cells with the genetic disruption of DNMT-1), and 3) SAHA activation of 15-LOX-1 transcription in various colon cancer cell lines (HCT-116, Caco-2, SW-480, and HT-29) was associated with a reduction in DNMT-1 but not DNMT-3B recruitment to the 15-LOX-1 promoter. These new findings demonstrate the importance of the effects of HDACIs on DNMT-1 to activate gene transcription in cancer cells, as in the case of 15-LOX-1. This study focused on examining this mechanism in relation to 15-LOX-1 transcription activation, which contributes to the apoptotic effects of the HDACIs (16). This mechanism is likely, however, to contribute to the transcription activation of some other important genes in cancer cells. A recent report showed that HDACIs reduce DNMT-1 binding to the estrogen receptor promoter during estrogen receptor transcription activation in the MDA-MB-231 human breast cancer cell line (33). With the use of quantitative ChIP/real-time PCR and various models of DNMT gene disruption and down-regulation, our current findings demonstrate how HDACIs can activate a gene transcription by inhibiting DNMT-1 recruitment to the gene promoter in cancer cells.

Our results indicate that the suppressive effect of DNMT-1 on 15-LOX-1 transcription was independent from 15-LOX-1 promoter methylation. First, the responsiveness of colon cancer to SAHA activation of 15-LOX-1 transcription occurred independently of 15-LOX-1 promoter methylation. For example, methylation levels were similar among HCT-116, DNMT-1 HM, and DNMT-3B KO cells; SAHA, however, activated 15-LOX-1 transcriptional suppression in DNMT-1 HM but not HCT-116 or DNMT-3B KO cells. Second, SAHA activated 15-LOX-1 transcription in the responsive colon cancer cell lines (DNMT-1 HM, DKO, Caco-2, SW-480, and HT-29) without altering the 15-LOX-1 promoter DNA methylation levels. DNMT-1 methyltransferase function is essential for maintenance of normal chromosomal stability and cell survival (34,35). However, DNMT-1 has other active domains, besides its methyltransferase domain, that interact with various transcriptional repressors (*e.g.*, HDAC1, HCAC2, the histone methyltransferase SUV39H1, and the polycomb group protein EZH2) (36–40) and, thus, it has been speculated that DNMT-1 acts as a corepressor independently of its methyltransferase function (41). The significance of this direct DNMT-1 corepressor function was questioned in a recent study showing that the expression of DNMT-1 protein with an inactivated methyltransferase domain (*via* a specific point mutation) fails to rescue DNMT-1 KO mouse embryonic stem cells from the lethal effects of DNMT-1 gene loss (42). Dissecting the significance of DNMT-1 repressor function from its methyltransferase function is difficult, however, when totally depriving the cell of the DNMT-1 methylation function, which is essential for cell survival. In our current study, we evaluated these other DNMT-1 repressive functions using DNMT-1 HM cells, which have a genetically truncated DNMT-1 protein with retained methylation function (31,32). We found that this truncated DNMT-1 protein had a markedly reduced ability to be recruited to the 15-LOX-1 promoter and to be susceptible to the effects of SAHA in dissociating it from the 15-LOX-1 promoter, thus leading to the activation of 15-LOX-1 transcription. SAHA, as expected for an HDACI, failed to alter 15-LOX-1 promoter methylation levels during these events, indicating that the transcriptional activation that occurred by truncating the DNMT-1 protein was independent of 15-LOX-1 methylation status.

15-LOX-1 promoter DNA methylation was specific to colorectal cancer cells: it was observed in all the colorectal cancer cells tested *in vitro* and in a substantial subset of colorectal cancer patients (18 of 50; 36%). In contrast, 15-LOX-1 promoter DNA methylation levels in the normal colorectal mucosal specimens were below the established limits for a high methylation level (>10%), apart from very few questionable exceptions. These questionable exceptions included two normal mucosa samples from the same 50 cancer patients (both had a borderline level, 11%) and three of 100 samples from 50 subjects with normal colons (all three levels were only moderately >10%, and the matched normal mucosa from the contralateral side of the same colon had methylation levels <10%). These new findings show that 15-LOX-1 DNA methylation was thus limited to the cancerous mucosa. We believe that this investigation is the first to include sufficiently large numbers of cancer patients and control subjects for meaningful comparisons of 15-LOX-1 promoter DNA methylation levels. The only previous study to make such comparisons involved five cancer-free control subjects and 43 prostate cancer patients (22). Our study, however, included 50 individuals who were carefully matched prospectively to the cancer patients to address potential confounding factors (history of prior colon cancer or polyps, hereditary or familial colorectal cancers, or use of chemotherapeutic or chemopreventive agents) and thus to provide an accurate comparison of 15-LOX-1 DNA promoter methylation between normal and cancerous mucosa.

15-LOX-1 promoter DNA methylation is unlikely to play a primary role in the transcriptional suppression of 15-LOX-1 in cancer cells. This conclusion is based on our following findings: 1) 15-LOX-1 promoter methylation levels had no significant correlation with 15-LOX-1 mRNA expression levels in either the colorectal cancer cell lines or in the patients' colorectal tumor specimens, 2) 15-LOX-1 DNA promoter hypomethylation by 5-aza-dc or by siRNA

down-regulation of either DNMT-1 and -3B or DNMT-1, -3A, and -3B failed to significantly increase 15-LOX-1 transcription, and 3) complete 15-LOX-1 promoter demethylation by genetic disruption of both DNMT-1 and -3B failed to activate 15-LOX-1 transcription. These data clarify the mechanistic role of 15-LOX-1 DNA methylation in the transcriptional silencing of 15-LOX-1 in cancer cells. Prior investigators have examined this mechanistic role using only a pharmacologic approach via 5-aza-dc and have reported conflicting results, ranging from 5-aza-dc inducing 15-LOX-1 expression by itself (23), facilitating transcriptional activation by IL-4 or HDACIs (21), having no effects (24), to inhibiting 15-LOX-1 transcriptional activation (22). However, we used a more specific and direct approach *via* siRNA and genetic disruption of DNMTs and were able to achieve complete 15-LOX-1 promoter demethylation instead of the partial reversal of methylation achieved with 5-aza-dc. Nevertheless, 15-LOX-1 promoter demethylation was insufficient to activate 15-LOX-1 transcription. Promoter DNA methylation is a well-recognized mechanism that significantly contributes to tumorigenesis by being a primary event in the silencing of various tumor suppressor genes (1). Our data, however, provide evidence that DNA methylation might not always be the primary event for the transcriptional repression maintenance of some genes during tumorigenesis, as in the case of 15-LOX-1.

The mechanistic link between inflammation and cancer is increasingly being supported by emerging data (43), especially in the case of colonic tumorigenesis (44). This link between cancer and inflammation could suggest that the 15-LOX-1 expression loss during colonic tumorigenesis [current manuscript, (7–9)] might potentially contribute to tumorigenesis through the loss of the anti-inflammatory products of 15-LOX-1, including lipoxins (4) and the other recently identified potent anti-inflammatory products of the omega-3 polyunsaturated fatty acid docosahexaenoic acid, resolvins, and protectins (45). Conversely, inflammation might influence 15-LOX-1 transcription, as the inflammatory cytokine IL-6 up-regulates DNMT1 expression (46,47), thus suggesting that inflammation suppresses 15-LOX-1 transcription through DNMT-1 modulation during tumorigenesis, leading to decreased production of the 15-LOX-1 pro-apoptotic product, 13-*S*-hydroxyoctadecadienoic acid (18, 48). Further studies are warranted to further explore these potential roles for 15-LOX-1 transcriptional silencing in the emerging link between inflammation and cancer. The new knowledge gained from studying the mechanisms for silencing 15-LOX-1 transcription through DNMT-1 in tumorigenesis might also provide insight into the mechanisms behind the effects of 15-LOX-1 in various other noncancerous diseases (*e.g.*, atherosclerosis, glomerulonephritis) through its anti-inflammatory role (49,50).

Our new findings thus indicate that in addition to its essential function as a methyltransferase, DNMT-1 also contributes to transcriptional suppression in cancer cells via mechanisms that are independent of DNA methylation. Identification of these mechanisms is important for better understanding the role of DNMT-1 in the transcriptional repression of tumor suppressor genes. It will also be important for developing novel molecular targeting studies in which we can selectively target the non-methyltransferase suppressor functions of DNMT-1 in cancer cells without compromising its methyltransferase function, which is essential to the survival of normal cells.

Acknowledgements

We are indebted to Drs. Madhukar Kaw, Harish Gagneja, Sandeep Lahoti, Patrick Lynch, William Ross, Norio Fukami, F. Lyone Hochman, Robert S. Bresalier, Gulchin Ergun, Rhonda Cole, and David Graham for assistance with clinical sample accrual. We also thank Karen Phillips from the Department of Scientific Publications at The University of Texas M. D. Anderson Cancer Center for editing the manuscript. In addition, we acknowledge the technical assistance of Xiu L. Yang. This project was performed in part using a compound (SAHA) provided by the National Cancer Institute's Chemical Carcinogen Reference Standards Repository operated under contract by Midwest Research Institute, No. N02-CB-07008. I. Shureiqi was supported by the National Cancer Institute, NIH, Department of Health and Human Services K07 grant CA86970 and R01 grant CA106577, by the American Cancer Society Scholar Award

RSG-04-020-01-CNE, by the Caroline Wiess Law Endowment for Cancer Prevention, and by the National Colorectal Cancer Research Alliance.

Nonstandard abbreviations used

5-aza-dc	5-aza-2-deoxycytidine
DKO	DNMT double knockout
DNMT	DNA methyltransferase
HDACI	histone deacetylase inhibitor
15-LOX-1	15-lipoxygenase-1
SAHA	suberoylanilide hydroxamic acid

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Figure 1.

DNA methylation of the 15-LOX-1 promoter in colorectal cancers *in vitro* and *in vivo*. **A)** Schematic illustration of human 15-LOX-1 promoter (–650 bp ~ +50 bp). Each vertical line depicts a single CpG site. The arrow indicates the putative transcription initiation site of the 15-LOX-1 gene. Thick bars indicate the location of regions amplified by PCR after bisulfite treatment with either cloning sequencing or pyrosequencing. **B)** Bisulfite cloning sequencing analyses of DNA methylation of 15-LOX-1 promoter in four colorectal cancer cell lines. A row represents the sequencing results of 10 CpG sites from an individual clone for each cell line, solid circles indicate methylated CpG sites, and open circles indicate unmethylated CpG sites. Values are the percentages of methylated CpG sites from among the total number of CpG sites. **C)** Scatter plot of DNA methylation levels of the 15-LOX-1 promoter in eight colorectal cancer cell lines. DNA methylation levels of the 15-LOX-1 promoter were quantified using pyrosequencing. Each open circle represents the methylation density of a CpG site measured by pyrosequencing. **D)** Scatter plot of DNA methylation levels of the 15-LOX-1 promoter in the colorectal tissues, as quantified using pyrosequencing. Normal-left, normal tissue biopsy specimens from the left colon of patients with no history of colonic cancer or polyps; normal-right, normal tissue biopsy specimens from the right colon of patients with no history of colonic cancer or polyps; cancer-cancer, cancer tissue specimens from the colorectal cancer lesions; cancer-normal, normal tissue specimens from 10 cm away from colorectal cancer lesions. Values represent the average methylation density of three CpG sites measured by pyrosequencing for each tissue sample.

Figure 2.

Correlation between 15-LOX-1 expression and 15-LOX-1 promoter DNA methylation in colorectal cancer tissues and colorectal cancer cell lines. *A*) Scatter plot of 15-LOX-1 mRNA expression in relation to 15-LOX-1 promoter methylation levels in colorectal cancer cell lines. 15-LOX-1 promoter DNA methylation and 15-LOX-1 mRNA levels were measured by bisulfite pyrosequencing and real-time PCR in eight different colorectal cancer cell lines and then correlated. The relative expression levels were calculated as the values relative to that of a calibrator sample (differentiated normal human tracheobronchial cells). Values shown are the means $\pm$ SDs of triplicate experiments. *B*) Scatter plot of 15-LOX-1 expression level in colorectal cancer tissues. 15-LOX-1 promoter methylation and 15-LOX-1 mRNA levels were quantified by bisulfite pyrosequencing and real-time PCR in colorectal cancer tissue samples from 19 patients and then correlated. The expression levels were calculated as the values relative to that of a calibrator sample (average from paired normal colonic mucosa of 12 patients). Values shown are the means $\pm$ SDs of triplicate measurements.

Figure 3.

Effects of 15-LOX-1 promoter demethylation on 15-LOX-1 transcription in HCT-116 cells. (A) Effects of DNMT-1, -3A, and -3B siRNA on DNMT-1 mRNA expression in HCT-116 cells. HCT-116 cells were transfected with a pool of four SMART-selected siRNA duplexes for DNMT-1 with DNMT-3B or DNMT-3B and -3A, a nonspecific siRNA sequence (nonspecific siRNA), or transfection medium only (control). Cells were transfected with the indicated siRNA every 72 h three times. After the third transfection of siRNA for 48 h, the cells were harvested for measurement of DNMT-1 mRNA expression by real-time RT-PCR. The expression levels were calculated as the values relative to that of the calibrator sample (control). Values shown are the means $\pm$ SDs of triplicate measurements; repeated experiments showed similar results. (B, C) Effects of DNMT-1, -3A, and -3B siRNA on DNMT-3A and -3B expression levels. Experiments were performed as described in panel A; DNMT-3A (B) and -3B (C) were similarly measured by real-time PCR. (D) Effects of DNMT-1, -3B, and -3A down-regulation by siRNA and 5-aza-dc treatment on 15-LOX-1 promoter methylation level in HCT-116 cells. HCT-116 cells were either transfected with DNMT-1, -3B, and -3A siRNA (as described in panel A) or treated with 5-aza-dc (AZA) at 1 μ M or 2.5 μ M concentration for 120 h. Cells were harvested, and 15-LOX-1 promoter methylation levels were measured by using bisulfite pyrosequencing. Each open circle represents the methylation density of a CpG site within the 15-LOX-1 promoter. (E, F) Effects of DNMT-1, -3B, and -3A down-regulation by siRNA and 5-aza-dc treatment on 15-LOX-1 mRNA and protein expression levels in HCT-116 cells by real-time PCR (E) and Western blotting (F). Experiments were performed similarly to those described in panel D. Values for panel E are the means $\pm$ SDs of triplicate measurements. For panel F, + indicates the positive control (HCT-116 cells transfected with 15-LOX-1 expression vector). (G) Effects of DNMT-1 and -3B KO on 15-LOX-1 promoter methylation. 15-LOX-1 promoter methylation levels were measured by bisulfite pyrosequencing in parental HCT-116 cells (HCT-116) and DNMT-1 and -3B KO cells (DKO).

Each open circle represents the methylation density of a CpG site within the 15-LOX-1 promoter. *H*) 15-LOX-1 mRNA levels were measured in DKO and parental HCT-116 cells. The expression levels were calculated relative to that of a calibrator sample (differentiated normal human tracheobronchial cells); repeated experiments showed similar results.

Figure 4.

15-LOX-1 transcriptional activation through specific DNMT-1 inhibition. *A*) Effects of 5-aza-de (AZA) on 15-LOX-1 induction by SAHA in HCT-116 cells. HCT-116 cells were treated with 1 μ M 5-aza-de for 96 h and then with 5 μ M SAHA for 24 h. Cells were harvested, and 15-LOX-1 mRNA levels were measured using real-time PCR. The expression levels were calculated as the values relative to that of a calibrator sample (HCT-116 treated with vehicle control [DMSO] only). Values are the means $\pm$ SDs of triplicate measurements; repeated experiments showed similar results. *B*) Effects of DNMT-1 and -3B genetic disruption on 15-LOX-1 induction by SAHA. Parental HCT-116 cells and HCT-116 cells with genetic disruption of DNMT-1 and -3B (DKO) were treated with either 5 μ M SAHA or vehicle solvent (DMSO, control) for 24 h. The cells were harvested, and 15-LOX-1 mRNA levels were measured as described in panel *A*. Repeated experiments showed similar results. (*C*, *D*) Effects of DNMT-1 and -3B genetic disruption on 15-LOX-1 mRNA and protein expression by SAHA. *C*) Parental HCT-116, DKO, and HCT-116 cells with either DNMT-1 or DNMT-3B genetic disruption (DNMT-1 HM and DNMT-3B KO cells) were treated with 5 μ M SAHA for 24 h, and 15-LOX-1 mRNA was measured as described in panel *B*. Repeated experiments showed similar results. *D*) Experiments were similar to those described in panel *C* except that 15-LOX-1 protein expression was measured using Western blot analysis. + indicates positive control (HCT-116 cells transfected with a 15-LOX-1 expression vector). Repeated experiments showed similar results. *E*) Effects of DNMT-1 siRNA transfection on DNMT-1 expression in HCT-116 cells. HCT-116 cells were transfected with a pool of four ON TARGET–selected siRNA duplexes for DNMT-1, with a nonspecific siRNA sequence (nonspecific siRNA), or with transfection medium only (Mock) for 72 h. DNMT-1 mRNA expression was measured by using real-time PCR. The expression levels were calculated as the values relative to that of the mock (DMSO) treated. Values shown are the means $\pm$ SDs of triplicate measurements; repeated experiments showed similar results. *F*) Effects of DNMT-1 knockdown on 15-LOX-1

expression by SAHA. Parental HCT-116 and DNMT-1 HM cells were transfected with siRNA for 48 h as indicated in panel *E* and then treated with 5 μ M SAHA for 24 h. Cells were harvested and processed for the measurement of 15-LOX-1 mRNA expression as in panel *E*. Values shown are the means $\pm$ SDs of triplicate measurements; repeated experiments showed similar results.

Figure 5.

Mechanisms of DNMT-1 suppression of 15-LOX-1 transcription. *A*) Effects of DNMT-1 and -3B genetic disruption and SAHA treatment on 15-LOX-1 promoter DNA methylation levels. Parental HCT-116, DKO, DNMT-1 HM, and DNMT-3B KO cells were treated with SAHA for 24 h. 15-LOX-1 promoter methylation levels were measured by bisulfite pyrosequencing. SAHA treatment is represented by solid squares, and vehicle solvent by open circles. Each value represents the methylation density of a CpG site within the 15-LOX-1 promoter. *B*) Effects of SAHA on 15-LOX-1 expression in colon cancer cells. HCT-116, Caco-2, SW-480, and HT-29 cells were treated with 5 μ M SAHA for 24 h, and then the cells were harvested for measurement of 15-LOX-1 mRNA expression using real-time PCR. The expression levels were calculated as the values relative to that of a calibrator sample (HCT-116 treated with vehicle control [DMSO] only). Values are the means $\pm$ SDs of triplicate measurements; repeated experiments showed similar results. *C*) Effects of SAHA treatment on 15-LOX-1 promoter methylation levels. HCT-116, Caco-2, SW-480, and HT-29 cells were treated with SAHA for 24 h as in panel *B*, and 15-LOX-1 promoter methylation levels were measured by bisulfite pyrosequencing. Solid squares depict values for SAHA-treated cells, and open circles depict those for vehicle solvent treatment (control). Each value represents the methylation density of a CpG site within the 15-LOX-1 promoter. *D*) Effects of SAHA on DNMT-1 binding to the 15-LOX-1 promoter. HCT-116, DNMT-1 HM, Caco-2, SW-480, and HT-29 cells were treated with SAHA or DMSO for 4 h. Cells were subjected to ChIP assays using DNMT-1 antibody. ChIP DNA was quantified by real-time PCR and is presented as a percentage of the respective input genomic DNA for the 15-LOX-1 promoter. Values are the means $\pm$ SDs of triplicate measurements; repeated experiments showed similar results.

TABLE 1

Characteristics of patients with and without colon cancer

Characteristic	Normal Colon (<i>n</i> = 50)	Colon Cancer (<i>n</i> = 50)	<i>P</i>
Age, years			0.036
Mean	59.6	62.6	
Median	58.5	64.0	
Range	50–75	45–80	
Sex (%)			0.017
Male	38	60	
Race (%)			0.085
White	88	76	
Black	10	10	
Other	2	14	
NSAID use (%) ^a	24	16	0.32
Mean body mass index (± SD)	28 ± 5.9	26.8 ± 6.6	0.26

^aNSAID, nonsteroidal anti-inflammatory drug.

TABLE 2

Characteristics of patients with colon cancer and their association with 15-LOX-1 promoter DNA methylation status

Characteristic	Methylation Scores		<i>P</i>
	High (<i>n</i> = 18)	Normal (<i>n</i> = 32)	
Age, years			0.036
Mean	58.8	64.4	
Median	57.5	64.5	
Range	45–74	46–80	
Sex (%)			0.76
Male	44	38	
Disease stage (%)			0.69
0–1	6	16	
2	39	32	
3	22	25	
4	28	25	
Tumor location (%)			0.10
Right colon	17	3	
Left colon	83	97	