The methyltransferase inhibitor 5-aza-2-deoxycytidine induces apoptosis via induction of 15-lipoxygenase-1 in colorectal cancer cells

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Abstract

DNA methylation by DNA methyltransferases in CpG-rich promoter regions of genes is a well-described component of epigenetic silencing in human cells. Dysregulation of this process in cancer cells may lead to hypermethylation of promoter CpG islands, thus disabling transcription initiation of certain genes, such as tumor suppressor genes. Reversing epigenetic silencing and up-regulating genes involved in preventing or reversing the malignant phenotype has become a new, important targeted approach for cancer prevention and treatment. Therefore, methyltransferase inhibitors (MTI) have emerged recently as promising chemotherapeutic or preventive agents. The potent MTI 5-aza-2-deoxycytidine (5-Azadc) causes growth arrest, differentiation, and/or apoptosis of many tumor types in vitro and in vivo. The present study shows that low micromolar concentrations of 5-Azadc induce the expression of 15-lipoxygenase-1 (15-LOX-1) in human colorectal cancer cells. The expression of 15-LOX-1 correlates with 5-Azadc-induced increases in 13-S-hydroxyoctadecadienoic acid levels, growth inhibition, and apoptosis in these cells. Furthermore, specific inhibition of 15-LOX-1 by pharmacologic means or small interfering RNA significantly reduced the 5-Azadc-induced effects. These novel findings are the first demonstration of a mechanistic link between the induction of 15-LOX-1 by a MTI and apoptosis in cancer cells. This result has important implications for the study of 5-Azadc and other MTIs in the prevention and therapy of colorectal cancer and supports future investigations of the mechanisms by which MTIs up-regulate 15-LOX-1. [Mol Cancer Ther 2005;4(11):1740–6]

Introduction

Cytosine DNA methylation in CpG-rich promoters is a well-described component of epigenetic silencing in human cells (1). The addition of a methyl group to the fifth carbon position of a cytosine residue occurs frequently in CpG dinucleotides (2). This process is closely associated with modifications of chromatin structure located at gene promoter regions and plays an important role in regulating gene expression in normal cells (2). In cancer cells, dysregulation of this process may lead to hypermethylation of promoter CpG islands of certain genes, such as tumor suppressor genes, disabling their transcriptional initiation and thus silencing them (3, 4). Reversal of this epigenetic process and up-regulation of genes important in preventing or reversing the malignant phenotype has become a promising new targeted approach for cancer prevention and therapy.

The potential anticancer activities of DNA methyltransferase inhibitors (MTI) have been studied extensively in recent years, because DNA hypomethylation induces the reactivation of tumor suppressor genes silenced by methylation-mediated mechanisms. Drugs that modulate DNA methylation are in clinical trials (5) and have been shown to affect gene expression in vivo (6). Several lines of evidence indicate that MTIs, such as 5-azacytidine and 5-aza-2-deoxycytidine (5-Azadc), have chemopreventive activity (7–9). These two closely related drugs have long been used experimentally to inhibit DNA methylation in tissue culture and have been shown to reactivate methylation-silenced genes (9). 5-Azadc is a more potent hypomethylating agent than is 5-azacytidine (the only MTI approved by the Food and Drug Administration for treating myelodysplastic syndromes and chronic myelomonocytic leukemia; ref. 10), has promising preclinical in vivo antitumor activity, and is currently undergoing clinical evaluation (7, 8, 11–15).

15-Lipoxygenase-1 (15-LOX-1) and its primary metabolite, 13-S-hydroxyoctadecadienoic acid (13-S-HODE), play important roles in colorectal carcinogenesis (16–19). 15-LOX-1 is expressed in normal human colorectal tissue (16, 17) and suppressed in colorectal cancer in vitro and in vivo (17). Restoring 15-LOX-1 and 13-S-HODE expressions by various means has been shown to restore and be mechanistically linked to apoptosis in colorectal cancer cells (18, 20, 21). We showed recently that low micromolar concentrations of the histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid induces 15-LOX-1...
expression as a mechanism of apoptosis induction in human colon cancer cells (21), providing the first demonstration of a mechanistic link between HDAC-induced 15-LOX-1 and apoptosis in carcinogenesis.

The epigenetic effects of histone deacetylation have been associated with hypermethylation (22). Furthermore, the 15-LOX-1 promoter region is GC rich and apparently belongs to CpG islands (23), so CpG methylation may also be involved in regulating 15-LOX-1 expression during carcinogenesis. Therefore, we hypothesized that MTIs, such as 5-Azadc, can induce 15-LOX-1 expression leading to suppressed proliferation and restored apoptosis in colorectal cancer cells. We tested this hypothesis in several interrelated experiments in colorectal cancer cell lines, including Caco-2, which is the widely used colon cancer cell line in which we established previously a mechanistic link between HDAC-induced 15-LOX-1 and apoptosis (21).

Materials and Methods

Cell Culture
The human colorectal cell lines Caco-2, HCT-116, and SW-480 were obtained from the American Type Culture Collection (Manassas, VA) and grown at 37°C in a humidified 5% CO2/95% air atmosphere. The Caco-2 cells were grown in 1 × Eagle’s MEM (Life Technologies, Inc., Carlsbad, CA) with l-glutamine supplemented with 15% fetal bovine serum, 1 mmol/L sodium pyruvate (Life Technologies), and 1 mg/100 mL gentamicin (Life Technologies). The HCT-116 and SW-480 cells were grown in RPMI supplemented with 10% fetal bovine serum. Trypsin (Life Technologies) was used to subculture cells.

Treatment with MTIs
5-Azadc (Sigma, St. Louis, MO), 5-azacytidine (Sigma), and zebularine (Calbiochem, San Diego, CA) were dissolved in water. Cells were treated from 0 to 5 days and at the doses indicated. Treatment groups were compared with vehicle controls. Caffeic acid (Sigma), a 15-LOX-1 inhibitor, was used at a concentration of 2.2 μmol/L. The specificity of this concentration has been established previously for inhibiting 15-LOX-1 in colorectal cancer cells (18). Cells were harvested at the various time points indicated.

Western Blot Analysis of 15-LOX-1 Protein
In general, SDS-PAGE and Western blotting techniques were carried out as described previously (19). Briefly, treated and control cells groups were washed twice with ice-cold PBS and lysed in protein lysis buffer containing protease and phosphatase inhibitors. Cells were sonicated thrice for 5 seconds each at 50% power for a total protein preparation. Protein content was quantified by the Bradford method as described previously (19). Aliquots of the protein preparation were heated to 70°C in protein sample buffer (Invitrogen, Carlsbad, CA) and separated by a 4% to 12% gradient gel (Invitrogen) according to the manufacturer’s instructions. Proteins were transferred onto nitrocellulose membrane (Invitrogen). Blots were blocked with 10% nonfat dry milk in 20 mmol/L TBS (pH7.4) containing 0.05% Tween 20 and washed. The blots were then incubated in 1% milk in 20 mmol/L TBS (pH7.4) containing 0.05% Tween 20 with an appropriate primary antibody. Rabbit polyclonal antisemirum to recombinant human 15-LOX-1 was a gift from Mary Mulchins and Elliot Sigal (Roche Bioscience, Palo Alto, CA) and was also generated by Lampire Biological Laboratories (Pipersville, PA). The 15-LOX-1 primary antibody was used at a dilution of 1:2,000. Normal human tracheobronchial epithelial cells, as described previously (19), were used as a positive control for the expression of 15-LOX-1. Actin antibody (Sigma) was used at a dilution of 1:2,000. Actin was analyzed as a control for protein loading. After washing, blots were incubated with an appropriate horseradish peroxidase–linked secondary antibody (Amersham, Piscataway, NJ). After reaction with chemiluminescence reagents (Amersham enhanced chemiluminescence system), bands were detected by exposure to film (Amersham).
Genbank M23892 were used to generate hairpin PCR primers. The primers were as follows: 15-LOX-1 6-29 (AAAAAACCCCAATGGAACGCCGATGCGCCA-
GAGCAAGCTTTCTCTACCAGATCCGGCCTGTC-
CAGTGGGGCGGTTTGTCCTTTTCCACA), 15-LOX-1 956-29 (AAAAAGATGTTGAGGATCTCTGAGGCTAGTCCAGGACGTCTCAG-
CACCTGGACCCACCTGAGTTTGGCTCTTTTCCACA), and 15-LOX-1 1933-29 (AAAAAAACCCCAATGAACACGCGGATGCGGCA-
GATCCCCACCACCTGGGTTTTGCTCTTTTCCACA). A PCR-based strategy was used to clone short hairpin RNA sequences. Short hairpin RNA sequences were converted into a single primer sequence onto which 21 nucleotides were added, which have homology with the human U6 small nuclear RNA promoter. The human U6 small nuclear RNA promoter was used to drive expression of encoded short hairpin RNA. The human U6 promoter in pGEM1 was a generous gift from Dr. Gregory J. Hannon (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The Topo SP6 primer used was PENTR/D-Topo SP6 primer: CACCGATTTAGGTGACACTATAG. PCR conditions were as follows: 1 cycle at 95°C for 3 minutes; 30 cycles at 95°C for 30 seconds, 55°C for 3 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 10 minutes. Two cloning technologies, the directional topoisomerase-mediated cloning kit (Invitrogen) and gateway system kit (Invitrogen), were used to clone the short hairpin RNA according to the manufacturer’s instructions. The resultant vector was then transfected into Caco-2 cells. Transfection of small interfering RNA (siRNA) in Caco-2 cells was carried out using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s specifications. Control cells were similarly transfected without siRNAs (i.e., vehicle only). After transfection, cells were replenished with medium containing G418 (650 μg/mL) and clones were selected in the presence of the G418. Clones were propagated and tested for silencing of 15-LOX-1 by Western analysis following treatment with 5-Azadc (1 μmol/L) for 96 hours or suberoylanilide hydroxamic acid (2.5 μmol/L) for 24 hours. This dose of suberoylanilide hydroxamic acid for 24 hours has been shown previously to induce 15-LOX-1 in Caco-2 cells (21). Clone 14 was generated from the 15-LOX-1 6-29 primer.

Results

MTI Effects on 15-LOX-1 Expression in Colorectal Cancer Cells

The colorectal cancer cell lines Caco-2, SW-480, and HCT-116 did not express 15-LOX-1 before but did express 15-LOX-1 following treatment with the three MTIs 5-Azadc, 5-azacytidine, and zebularine (Fig. 1; data not shown). For all subsequent experiments, we exclusively used Caco-2 cells (in which we established previously a mechanistic link between HDAC-induced 15-LOX-1 and apoptosis; ref. 21) and 5-Azadc (which is a potent hypermethylating agent in vitro and has shown recent promise in clinical trials; refs. 5, 6, 9, 12–15, 24). The induction of 15-LOX-1 by 5-Azadc in Caco-2 cells was both dose and time dependent (Fig. 1A). 15-LOX-1 was expressed with 5-Azadc concentrations as low as 0.1 μmol/L but was expressed strongly at doses from 1 to 10 μmol/L (at 96 hours). 15-LOX-1 results after treatment with 1 μmol/L 5-Azadc for 0 to 120 hours are shown in Fig. 1B. 15-LOX-1 was expressed at 48 hours but was expressed more strongly at later time points. Reverse transcription-PCR indicated that 15-LOX-1 mRNA was strongly expressed at 24 hours and continued to be expressed at later time points (Fig. 1C).

5-Azadc Effects on 13-S-HODE Formation

Treatment of Caco-2 cells with 5-Azadc for 72 hours increased endogenous levels of 13-S-HODE (the primary metabolite of 15-LOX-1) by 2-fold compared with levels in vehicle control Caco-2 cells (Fig. 2). Treatment of Caco-2 cells with 5-Azadc plus the 15-LOX-1 inhibitor caffeic acid at a concentration (2.2 μmol/L) shown to be specific for 15-LOX-1 inhibition (18) decreased 13-S-HODE back to levels similar to those in vehicle control cells.

Effect of 15-LOX-1 Inhibition on Cell Growth

Caco-2 cells were treated with 5-Azadc or 5-Azadc plus caffeic acid for 0 to 96 hours. 5-Azadc reduced the cell count by 73% at 72 hours and by 88% at 96 hours compared with vehicle control cells (Fig. 3). Inhibiting 15-LOX-1 activity with caffeic acid (2.2 μmol/L) attenuated the 5-Azadc-induced cell count reduction. Caffeic acid alone did not affect cell growth (Fig. 3).
Effects of 15-LOX-1 Inhibition on 5-Azadc-Induced Apoptosis

We determined whether 15-LOX-1 plays a role in 5-Azadc-induced apoptosis by assessing DNA fragmentation assays of cells treated with 5-Azadc or 5-Azadc plus caffeic acid. DNA laddering occurred at 96 hours in cells treated with 5-Azadc (Fig. 4A). Inhibiting 15-LOX-1 with caffeic acid blocked 5-Azadc-induced DNA laddering. No laddering was observed in untreated control cells or in cells treated with caffeic acid alone. Similar results occurred at 120 hours (data not shown). We measured caspase-3 activity to confirm 5-Azadc-induced apoptosis and its inhibition by caffeic acid. At 96 hours, caspase-3 activity of 5-Azadc-treated Caco-2 cells increased >2-fold (versus in untreated control cells; Fig. 4B). Inhibiting 15-LOX-1 activity with caffeic acid attenuated the activity of caspase-3. Similar results occurred at 120 hours (data not shown).

siRNA-Silenced 15-LOX-1 Expression Attenuates 5-Azadc-Induced Effects on Cell Growth and Apoptosis

To examine whether 15-LOX-1 plays a direct role in 5-Azadc-induced growth inhibition and apoptosis, we transfected Caco-2 cells with siRNA to silence 15-LOX-1 before treating them with 5-Azadc. Stable clones were obtained and tested for silencing of 15-LOX-1 by treatment with 5-Azadc or suberoylanilide hydroxamic acid, a HDAC inhibitor shown to induce 15-LOX-1 in Caco-2 cells (21). Several tested clones had reduced 5-Azadc-induced 15-LOX-1 expression; none had a complete reduction. Clone 14 had the greatest (~85%) inhibition of 15-LOX-1 expression (versus in 5-Azadc-treated Caco-2 cells), so we chose clone 14 for further experiments (Fig. 5A).

To determine whether 15-LOX-1 plays a direct role in 5-Azadc-induced growth inhibition, Caco-2 or clone 14 cells were treated with 5-Azadc (1 μmol/L) for 0 to 96 hours and then assessed for cell growth inhibition. 5-Azadc reduced counts of Caco-2 cells by 67% at 72 hours and 79% at 96 hours (versus in vehicle-treated Caco-2 cells; Fig. 5B). 5-Azadc reduced the counts of clone 14 cells by the substantially lower percentages of 37% at 72 hours and 26% at 96 hours (versus in vehicle-treated clone 14 cells; Fig. 5B), indicating that siRNA-silenced 15-LOX-1 attenuated growth inhibition by 5-Azadc.

To determine whether 15-LOX-1 also plays a direct role in 5-Azadc-induced apoptosis, DNA fragmentation assays were used to assess apoptosis in Caco-2 or clone 14 cells treated with 5-Azadc (1 μmol/L) or vehicle. At 96 hours, DNA laddering was observed for Caco-2 cells treated with 5-Azadc (Fig. 5C); no laddering was observed for clone 14 cells treated with 5-Azadc, indicating that siRNA-silenced 15-LOX-1 blocked 5-Azadc-induced DNA laddering. No laddering was observed for vehicle-treated Caco-2 or clone 14 control cells. Similar overall results were observed at 120 hours (data not shown).

Discussion

Our present results establish for the first time that MTI-induced apoptosis and 15-LOX-1 expression are linked mechanistically in colorectal cancer cells. The demethylating agents 5-Azadc, 5-azacytidine, and zebularine can induce 15-LOX-1 expression, indicating that methylation may play a role in 15-LOX-1 regulation. 5-Azadc induced 15-LOX-1 expression in a dose- and time-dependent manner and induced growth arrest and apoptosis in Caco-2 colon cancer cells. Inhibiting 15-LOX-1 with caffeic acid (at the 2.2 μmol/L concentration shown to be specific for 15-LOX-1 inhibition) or siRNA attenuated 5-Azadc-induced growth.
inhibition and apoptosis, indicating a direct relationship between 15-LOX-1 and these biological effects. These findings identify a molecular mechanism by which MTIs induce apoptosis and other cellular effects and further elucidate the role of 15-LOX-1 in human colorectal carcinogenesis. Our findings are consistent with results of other studies, which showed a 15-LOX-1-apoptosis link with nonsteroidal anti-inflammatory drugs in colorectal and other cancer cells (18, 25–28). Initiation of apoptosis as a result of MTI-induced 15-LOX-1 could involve peroxisome proliferator-activated receptor activities or other targets within the 15-LOX-1 signal transduction pathway (29, 30).

It is known that 13-S-HODE, the primary metabolite of 15-LOX-1, can regulate peroxisome proliferator-activated receptors, which can lead to growth inhibition and apoptosis (19, 29–32). It will be important to clarify the mechanism(s) by which the modulation of 15-LOX-1 affects cell proliferation and apoptosis. The effects of 5-Azadc on 15-LOX-1 expression and apoptosis in our study support the promise of MTIs for colorectal cancer prevention and therapy.

The combination of demethylating agents with other chemotherapeutics is gaining increased interest as a molecular targeted therapeutic strategy in colorectal carcinogenesis. Combining HDAC inhibitors and demethylating agents is attractive, because histones are connected to DNA by both physical and functional interactions. Although little is known about the effects of regulating methylation and histone acetylation on expression of tumor suppressor genes, proto-oncogenes, and other tumor-associated genes in human colorectal cancer, there are accumulating data showing that combined HDAC and methyltransferase inhibition effectively (and synergistically) induces apoptosis, differentiation, and/or cell growth arrest in human lung, breast, thoracic, and colon cancers and leukemia cell lines (22, 33, 34). It has been suggested that methylation and histone deacetylation may act as layers for epigenetic gene silencing 15-LOX-1 expression and apoptosis in our study support the promise of MTIs for colorectal cancer prevention and therapy.

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Figure 4. 15-LOX-1 inhibition attenuates 5-Azadc-induced apoptosis. A, 5-Azadc-induced apoptosis was blocked by inhibition of 15-LOX-1 in Caco-2 cells as measured by DNA laddering. Cells were treated with 5-Azadc (1 μmol/L), 5-Azadc (1 μmol/L) + caffeic acid (2.2 μmol/L), or vehicle (control) for 96 h, harvested, and assayed for DNA fragmentation. Representative of one of three separate experiments with similar results. Lane 1, control cells; lane 2, 5-Azadc-treated cells; lane 3, cells treated with 5-Azadc + caffeic acid. B, 5-Azadc-induced apoptosis was blocked by the inhibition of 15-LOX-1 in Caco-2 cells as measured by caspase-3 activity. Cells were treated with 5-Azadc (1 μmol/L), 5-Azadc (1 μmol/L) + caffeic acid (2.2 μmol/L), or vehicle (control) for 96 h, harvested, and measured for caspase-3 activity, which is reported in arbitrary units. Representative of one of two separate experiments with similar results. Columns, mean; bars, 95% confidence interval. *P = 0.014, 5-Azadc versus control (96 h); P = 0.022, 5-Azadc versus 5-Azadc + caffeic acid (96 h).

Figure 5. Silencing 15-LOX-1 by siRNA attenuates 5-Azadc-induced effects on cell growth and apoptosis. Western analysis of 15-LOX-1 siRNA-transfected clone 14 cell lysates showed reduced induction of 15-LOX-1 by 5-Azadc (1 μmol/L). Actin is a control for the amount of protein loaded (30 μg total protein/lane). Representative of one of three separate experiments with similar results. A, Western analysis showed that after treatment with 5-Azadc (1 μmol/L) for 96 h decreased expression of 15-LOX-1 was observed in clone 14 cells compared with in Caco-2 cells. Lane 1, 15-LOX-1 standard; lane 2, clone 14 control cells; lane 3, 5-Azadc-treated clone 14; lane 4, Caco-2 control cells; lane 5, 5-Azadc-treated Caco-2 cells. B, 5-Azadc reduced the growth of Caco-2 cells in a time-dependent fashion. Silencing of 15-LOX-1 by siRNA attenuated this effect. Caco-2 or clone 14 cells were treated with 5-Azadc (1 μmol/L) or vehicle (control) for 0 to 96 h and harvested. Representative of one of three separate experiments with similar results. Points, mean proliferation rates (number of attached cells at the time of harvesting); bars, 95% confidence interval. *P = 0.016, 5-Azadc-treated cells versus control Caco-2 cells (72 h); P = 0.012, 5-Azadc-treated clone 14 cells versus control clone 14 cells (72 h). C, 5-Azadc-induced apoptosis was blocked by silencing of 15-LOX-1 in Caco-2 cells as measured by DNA laddering. Caco-2 or clone 14 cells were treated with 5-Azadc (1 μmol/L) or vehicle (control) for 96 h, harvested, and assayed for DNA fragmentation. Representative of one of three separate experiments with similar results. Lane 1, Caco-2 control cells; lane 2, 5-Azadc-treated Caco-2; lane 3, clone 14 control cells; lane 4, 5-Azadc-treated clone 14 cells.
silencing (22). To achieve maximal gene reactivation, it may be necessary to block the processes essential to both the formation and the maintenance of transcriptionally repressed chromatin (i.e., both DNA methylation and histone deacetylation). Inhibition of HDAC activity can induce gene expression without a large-scale change from repressive to accessible chromatin (35–38). Other studies have suggested that chromatin structure is the dominant force in methylation-associated gene silencing (39–43). For example, Cameron et al. (22) showed that the HDAC inhibitor trichostatin A failed to reactivate expression of transcriptionally silenced genes (MLH1, tissue inhibitor of metalloproteinase 3, and CDKN2A) in a colorectal carcinoma cell line. However, after partial demethylation with 5-AzadC, the addition of trichostatin A resulted in robust expression of MLH1, tissue inhibitor of metalloproteinase 3, and CDKN2A (22). Cameron et al. suggest that DNA methylation may be the dominant component for maintenance of silencing at CpG island promoters. Therefore, the function of DNA methylation may be to “lock” genes into a silenced chromatin state (44). The roles that methylation and histone deacetylation may play in the regulation of 15-LOX-1 remain to be elucidated. Future studies should also evaluate the potential effects of combined HDAC inhibitors and demethylating agents on 15-LOX-1 and apoptosis and other cellular functions during carcinogenesis.

In conclusion, the present study shows that the induction of 15-LOX-1 expression is involved mechanistically in MTI-induced growth arrest and apoptosis in colorectal cancer cells. Establishing this mechanistic link emphasizes the importance of future investigations of the mechanisms by which MTIs, such as 5-AzadC, up-regulate 15-LOX-1. Further mechanistic studies of 15-LOX-1 regulation in colorectal cancer cells potentially will advance the development of effective agents for colorectal cancer prevention and therapy.

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