Molecular Cancer Research

Upregulation of DNA Methyltransferase–Mediated Gene Silencing, Anchorage-Independent Growth, and Migration of Colon Cancer Cells by Interleukin-6

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Abstract

Inflammatory bowel disease is characterized by chronic inflammation which predisposes to colorectal cancer. The mechanisms by which inflammation promotes tumorigenesis are not fully known. We aimed to investigate the links between colonic inflammation and tumorigenesis via epigenetic gene silencing. Colon cancer specimens were assessed for the expression of DNA methyltransferase-1 (DNMT-1) using immunohistochemistry. Colorectal carcinoma cell lines were assessed for DNMT1 expression, methylcytosine content, promoter methylation, gene expression, and tumorigenesis in response to interleukin (IL)-6. DNMT1 was expressed at higher levels in both the peritumoral stroma and tumor in inflammatory bowel disease–associated cancers compared with sporadic colon cancers. IL-6 treatment of colon cancer cells resulted in an increase in DNMT1 expression, independent of *de novo* gene expression. IL-6 increased the methylation of promoter regions of genes associated with tumor suppression, adhesion, and apoptosis resistance. Expression of a subset of these genes was downregulated by IL-6, an effect that was prevented by preincubation with 5-azadeoxycytidine, a DNMT1 inhibitor. Anchorage-independent growth and migration of colon cancer cells was also increased by IL-6 in a 5-azadeoxycytidine–sensitive manner. Our results indicate that DNMT-mediated gene silencing may play a role in inflammation-associated colon tumorigenesis. *Mol Cancer Res; 8(4); 471–81. ©2010 AACR.*

Introduction

Chronic inflammation of gastrointestinal organs elevates the risk of carcinomas developing in the affected organ. The relationship between inflammation and cancer has been extensively studied in patients with inflammatory bowel disease (IBD) who have a 2- to 3-fold greater lifetime risk of developing colorectal cancer (CRC) compared with the general population (1). Duration and extent of disease, as well as severity of colonic inflammation, are risk factors for colon carcinogenesis in patients with IBD (2). Specific preventive or therapeutic strategies for colitis-associated cancer have not yet been developed because of the absence of a clear understanding of the molecular pathogenesis of this disease complication.

Ulcerative colitis and Crohn's disease are characterized by a cytokine-driven mixed inflammatory infiltrate in the intestinal mucosa (3). The accumulation of pathogenic $CD4^+$ T-lymphocytes at sites of inflammation is mediated in part by interleukin (IL)-6 (4). IL-6 is a pleiotropic cytokine secreted by many cell types which exerts proinflammatory effects in IBD (5-7). High concentrations of IL-6 are associated with disease severity, and increased serum IL-6 levels during remission are predictive of disease relapse (6). Treatment with anti–IL-6 receptor antibody ameliorated disease in the $CD45RB^{high}$/SCID adoptive transfer model of colitis (8), and the results of a small clinical trial in Crohn's disease suggested possible therapeutic efficacy (9). Other studies have recorded high IL-6 serum levels in patients with certain cancers (breast, lung, and lymphoma; refs. 10-12), and have correlated elevated serum IL-6 levels with poor prognosis in colorectal cancers (10). These findings and the results from experiments in an animal model of colitis-associated cancer point to the possible involvement of IL-6 in the pathogenesis of inflammation-associated cancer (11-13).

The mechanisms by which IL-6 might promote carcinogenesis are not well understood. One study reported that overexpression of IL-6 in cholangiocarcinoma cells increased the expression of DNA methyltransferase-1 (DNMT1; ref. 14). This suggests that IL-6, acting in an autocrine manner, might interact with the CpG island methylator mechanism of carcinogenesis. The hypermethylation of CpG islands, and concomitant loss of gene expression, is the best characterized epigenetic change to occur in tumors and is catalyzed by DNMT1, DNMT3a,

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and DNMT3b (15, 16). Classically, DNMT1 is considered a maintenance methyltransferase whereas DNMT3b may be more important for *de novo* CpG methylation, although this distinction is not absolute (17, 18). DNMT1 overexpression has been observed in cancers (19), but whether cytokines expressed during chronic inflammation have any role in regulating its expression is unclear.

In this study, we investigated whether IL-6 might link colonic inflammation with carcinogenesis through the regulation of epigenetic tumor suppressor gene silencing. Our results suggest that IL-6, a component of the inflammatory tumor microenvironment, might promote colonic tumorigenesis through DNMT1-mediated tumor suppressor gene silencing.

Materials and Methods

Patient Selection

The Institutional Review Board of the Mayo Clinic approved this research protocol. IBD-CRC cases were selected from the Mayo Clinic diagnostic index (1976-2006). Sporadic-CRC controls were identified through the Mayo pathology index. Data with regard to age, gender, and duration of IBD were abstracted from the records.

Reagents and Media

McCoy's 5A, DMEM, RPMI 1640, and fetal bovine serum (FBS) were from Life Technologies. IL-6, LY294002, triciribine, SP600125, and curcubitacin were from Calbiochem. Antibodies to DNMT1 were from Santa Cruz Biotechnology, Inc. Antibodies to AKT, STAT3, and ERK were from Cell Signaling. Anti-Lamin B1 was from Abcam. Unless otherwise stated, reagents were obtained from Sigma (Sigma Aldrich).

Immunohistochemistry

Individual 5-μm sections were processed following standard protocols using DNMT1 antibody (AB19905, dilution 1:300; Abcam) and CD68 (dilution 1:100; DAKO). Slides were scored and determined by two pathologists (T. Smyrk and M. Garrity-Park) using an ordinal 0-3 scale for staining intensity, which was multiplied by the percentage of positive cells to give an overall abundance score which ranges from 0 to 300.

Cell Lines

HT29, HCT15, and HCT116 cell lines were used (American Type Culture Collection). HeLa Tet-off cells were from Clontech. HCT116 cells were cultured in McCoy's 5A medium with 10% FBS. HT29 cells were cultured in DMEM with 10% FBS. HCT15 cells were cultured in RPMI 1640 with 10% FBS. HeLa Tet-Off cells were maintained in DMEM with 10% FBS and 100 μg/mL of G418.

Western Blotting

Nuclear proteins were extracted using the NE-PER nuclear extraction kit (Pierce). Nuclear proteins (10 μg) were resolved on 6% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Membranes were incubated overnight at 4°C in the appropriate primary antibody. Membranes were washed and incubated with goat anti-rabbit horseradish peroxidase or goat anti-mouse horseradish peroxidase secondary antibodies (Pierce). A G:BOX chemi-system (Syngene) was used to capture luminescent signals.

Real-time PCR

RNA was extracted from cells using kits (Qiagen) and cDNA was prepared using Superscript II (Invitrogen). Primer sequences were designed using the Primer 3 algorithm (available on request). Real-time PCR was done using Power SYBR Green master mix on a 7500 Real-time PCR system (Applied Biosystems). GAPDH was used as an internal control, and relative expression levels were calculated using the ΔCT method (20).

Promoter Analysis

Luciferase assays were done on P1, P2, P3, and P4 of the DNMT1 promoter. D1P1 contained 1,270 bp of the promoter sequence preceding exon 1 of DNMT1. D1P1x was constructed by deleting the 5′ 786 bp of D1P1. D1P2F and D1P2R consisted of the 1,656 bp fragment encompassing promoters P2 to P4 cloned into pT81luc in forward (D1P2F) or reverse (D1P2R) orientation. These constructs were gifts from Prof. Dr. Wolfgang Schulz (21). For DNMT1 promoter experiments, cells were cotransfected with Renilla and either DNMT1-P1, DNMT1-P1x, DNMT1-P2-P4-F, or DNMT1-P2-P4-R in a 96-well plate at a 1:10 ratio. IL-6 was added 24 h posttransfection, and cells were incubated for a further 24 h. Luciferase assays were done using the Dual-glo luciferase assay (Promega).

DNMT1-V5 Tet-off System

pTRE-tight/DNMT1-V5 was constructed as follows: the stop codon in pORF9-hDNMT1 (Invivogen) was removed by site-directed mutagenesis, and hDNMT1 was excised, purified, and subcloned into pcDNA3.1-V5 (Invitrogen). DNMT1-V5 was excised and subcloned into the pTRE-tight vector (Clontech). The construct was electroporated into HeLa Tet-off cells. Twenty four hours later, doxycycline (2 μg/mL) was added to the medium in the presence or absence of IL-6 (100 ng/mL). In certain experiments, cells were pretreated with 5 μmol/L of triciribine, 50 μmol/L of SP600125, 1 μmol/L of curcubitacin, or vehicle control (DMSO) for 30 min. Cells were then treated with 2 μg/mL of doxycycline, 100 ng/mL of IL-6, or both together.

5-Methylcytosine Assay

Cells were treated with 5 aza-CdR or IL-6 for 72 h, and genomic DNA was obtained using a kit (Qiagen). One microgram of DNA was digested using *Mcr*BC (New England Biolabs) for 2 h. DNA was resolved on a DNA 12000 chip using an Agilent 2100 Bioanalyzer (Agilent

Technologies). DNA between 500 and 12,000 bp was quantified as an index of 5-methylcytosine content using ImageJ software.

Promoter Methylation Array

Promoter methylation was assessed in HCT116 cells using the TransSignal Methylation Array (Panomics). Genomic DNA from control cells or cells treated with IL-6 for 48 h was digested with *Mse*I (New England Biolabs), incubated with methylation-binding protein, and separated. The methylated DNA was biotin-labeled using PCR, and the denatured PCR product was hybridized with the methylation array. The membranes were incubated with streptavidin-horseradish peroxidase before detection. Quantitation of spot densities was done using GeneTools software (Synoptics). The membranes were developed and a G:BOX chemi-system (Syngene) was used to capture luminescent signals. Using GeneTools software, each spot was outlined and the density was compared with the corresponding control spot (14). For each promoter, the average of two methylation spot densities was compared between control and IL-6–treated samples.

Proliferation Assays

HCT116 cells were treated with 50 ng/mL IL-6 or 4 μmol/L 5-aza-CdR as indicated, and seeded into 96-well plates. Plates were incubated for 24, 48, 72, and 96 h, and proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (22).

Invasion Assays

Invasion assays were carried out using Matrigel inserts (BD Biosciences). HCT116 cells suspended at a density of 5×10^5 cells/mL in McCoy's medium + 0.5% FBS. McCoy's medium (0.75 mL) containing 20% FBS, ±100 ng/mL of IL-6 was added to 24-well plates, and the invasion inserts were transferred into the wells. HCT116 cells (0.5 mL) were added to the top chamber of the assay and plates were incubated for 24 h. Invasion membranes were fixed, stained, and mounted. Five fields were counted per treatment.

Anchorage-Independent Growth Assays

HCT116 cells were treated with IL-6 (100 ng/mL) and/or 5-aza-CdR (4 μmol/L) for 24 h prior to seeding. Cells were trypsinized and suspended at a density of 1×10^4 cells/mL in McCoy's 5A medium supplemented with 20% FCS and 0.3% Noble agar. The cell suspension was added to 96-well plates containing a base of McCoy's medium with 0.6% Noble agar. The plates were overlaid with medium containing 20% FCS and 0.6% agar. Colonies were photographed after 14 d.

Statistical Analyses

For analysis of immunohistochemical studies, the abundance of staining was estimated as the product of a 0-3 intensity scale and the percentage of positive cells for the DNMT1 tumor, DNMT1 peritumor, and CD68 variables. The Pearson correlation coefficient was used to assess the degree of linear relationship between abundance variables and age. The two-sample t test was used to compare mean age between the patients with IBD and sporadic disease. A general linear model was fitted to compare mean abundance between IBD and sporadic patients with age included as a covariate. The underlying assumptions for the model were checked using suitable residual plots. All analyses were conducted using Minitab 15. All analyses relating to real-time PCR data comparisons were done using ANOVA with Tukey and Dunnet's multiple comparisons procedures. A significance level of 0.05 was used for all analyses.

Results

DNMT1 Is Overexpressed in IBD-CRC Compared with Sporadic-CRC

As an initial step towards assessing a role for increased DNA methylation in the causation of colitis-associated cancer, we assessed the expression of DNMT1 and macrophage marker CD68 in 36 IBD-CRC and 44 sporadic-CRC tumor specimens. We focused on exploring the effects of inflammation on the expression of DNMT1 rather than other DNMTs because prior work emphasized the importance of DNMT1 in colon cancer cells (18). Patient gender and tumor location were similar between the two groups (data not shown). Immunohistochemistry revealed a mostly nuclear pattern of DNMT1 staining both in peritumoral stroma and in tumors, whereas CD68 staining was seen only in peritumoral stroma (Fig. 1A). A matrix scatterplot of abundance scores for each of the three variables (tumor DNMT1, peritumor DNMT1, and CD68) and age by IBD presence is given in Fig. 1B. In each case, the line of best fit is included as a suitable summary of the underlying pairwise relationships between the variables. The difference in age distribution is clear where the mean age was significantly lower in IBD-CRC than sporadic-CRC ($P < 0.001$). Based on the 95% confidence of the difference in mean age, patients with IBD-CRC tended to be between 11 and 23 years younger than patients with sporadic-CRC. There was evidence of higher mean abundance in IBD-CRC than sporadic-CRC for each variable in which patients with IBD had a significantly greater mean tumor DNMT1 ($P < 0.001$), peritumor DNMT1 ($P < 0.001$), and CD68 ($P < 0.001$). There was evidence of a significant positive correlation between tumor DNMT1 and peritumor DNMT1 ($r = 0.45$, $P = 0.009$) and between tumor DNMT1 and CD68 ($r = 0.28$, $P = 0.03$) among patients with IBD-CRC. There was a borderline significant positive correlation between tumor DNMT1 and CD68 ($r = 0.29$, $P = 0.06$) in the patients with sporadic-CRC. Age was a useful explanatory variable when comparing tumor DNMT1 ($P = 0.024$) and peritumoral DNMT1 levels ($P = 0.017$). The residual plots suggested that a model using the natural log of peritumor DNMT1 and CD68 abundance as transformed response variables might be more suitable. When fitting this model to the transformed response variables, the final conclusions were identical, in terms of

identifying significantly higher mean peritumor DNMT1 and CD68 for the patients with IBD compared with the patients with sporadic disease ($P < 0.001$ in both cases). These observations show that DNMT1 is overexpressed both in the tumors and in the peritumoral stroma of IBD-associated colon cancers to a greater extent than in sporadic cancers. The findings of higher DNMT1 expression in inflamed colon cancers, and the positive correlation between CD68 and tumor DNMT1, support the hypothesis that DNA methylation might be increased by inflammatory mediators.

Treatment with IL-6 Induces DNMT1 Expression in Colorectal Cancer Cells

Next, we tested the possibility that proinflammatory cytokines might be responsible for the increased DNMT1 expression found in IBD-CRC. Preliminary experiments were conducted using a number of proinflammatory cytokines and the most interesting results were obtained using IL-6. Western blots of nuclear extracts showed a progressive increase in DNMT1 protein expression following treatment with IL-6 in HCT116 cells (Fig. 2A). Similar increases in DNMT1 expression were found with HCT15 and HCT29 cells (data not shown). We next analyzed DNMT1 mRNA expression using real-time PCR. Results showed that IL-6 induced an approximate doubling of DNMT1 mRNA expression after 6 or 12 hours, but that levels had decreased to baseline values by 24 hours (Fig. 2B). DNMT3b mRNA levels were also elevated in response to IL-6 treatment albeit at much lower abundance than DNMT1 (data not shown). As DNMT3b protein levels were difficult to detect by Western blotting in colon cancer cells, we focused on IL-6–induced changes in DNMT1 expression only.

To determine if IL-6 controlled DNMT1 levels by regulation of gene expression, we transfected HCT116 cells with a series of DNMT1 promoter luciferase constructs and measured the effect of IL-6 on luciferase activity 24 hours after stimulation (Fig. 2C). The P1 region but not the P2 to P4 region–containing plasmids stimulated significant baseline DNMT1 luciferase expression. However, no change in promoter activity was observed following treatment with IL-6 at any dose administered (1 and 10, data not shown; or 100 ng/mL). Taken together, these results indicate that IL-6 significantly upregulates DNMT1 expression, but the relatively minor effect on mRNA expression and lack of effect on DNMT1 promoter activation indicate that the effect of IL-6 is not transcriptionally regulated.

Increased DNMT1 Protein Levels Are Not Due to Increased Cycling through S Phase or *De novo* Protein Synthesis

As DNMT1 is an S phase–regulated protein, we examined whether the increase in DNMT1 expression we observed was S phase–regulated. Cells were pretreated with

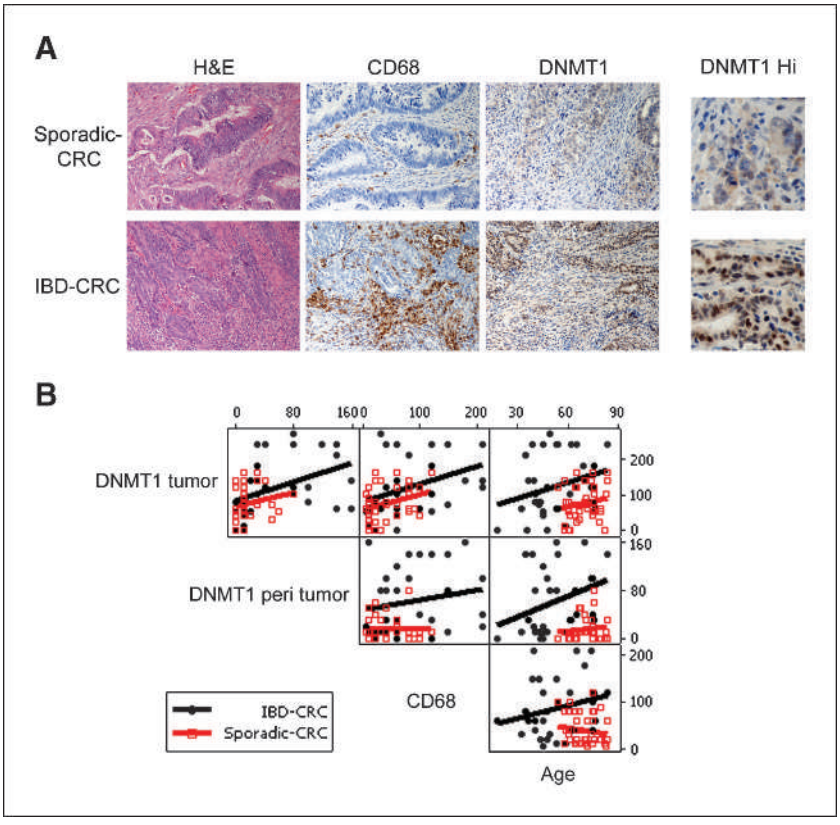

FIGURE 1. Greater expression of CD68 and DNMT1 in IBD-associated than in sporadic colon cancers. A, H&E-stained, anti-CD68–stained, and anti-DNMT1–stained sections of sporadic and IBD-associated CRCs. B, matrix scatterplot showing the abundance scores of the immunohistochemical staining for DNMT1 in tumors and peritumoral areas, and for CD68, and age. The lines represent best-fit linear regressions of the three variables for IBD-CRC (black) and sporadic CRC (red).

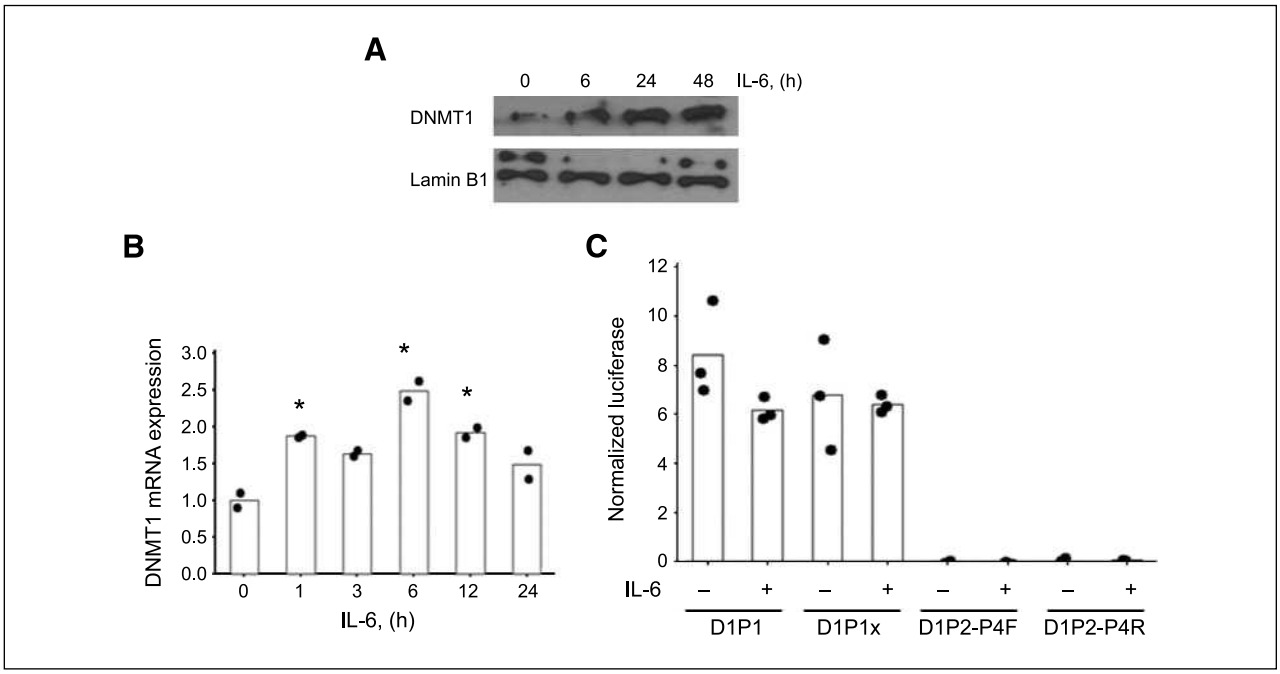

FIGURE 2. IL-6 increases DNMT1 expression in HCT116 colorectal carcinoma cell lines, independent of transcriptional activation. A, HCT116 cells were treated with 100 ng/mL of IL-6 for the time points indicated and DNMT1 levels were assessed by Western blot. B, HCT116 cells were treated with IL-6 for the time points indicated. mRNA levels were assessed by reverse transcription-PCR. C, HCT116 cells were cotransfected with an internal control plasmid and either DNMT1-P1, DNMT1-P1x, DNMT1-P2-P4-F, or DNMT1-P2-P4-R. IL-6 (100 ng/mL) was added 24 h posttransfection, and luciferase expression was measured after an additional 24 h.

lovastatin (10 μmol/L) for 24 hours to block cell cycle progression, before the addition of 100 ng/mL of IL-6 for 48 hours. No effect of lovastatin on IL-6–induced DNMT1 expression was observed (Fig. 3A), but lovastatin did prevent cell cycle progression into S phase as assessed by propidium iodide staining and flow cytometry (data not shown). To determine whether the increased levels of DNMT1 expression observed following treatment with IL-6 required increased protein synthesis, HCT116 cells were treated with cycloheximide (10 μg/mL) for 30 minutes prior to treatment with IL-6 for 24 hours. No change in the effect of IL-6 on DNMT1 expression was observed in the presence of cycloheximide, indicating that increased DNMT1 levels were not due to *de novo* protein synthesis in HCT116 cells (Fig. 3B).

Expression of Epitope-Tagged DNMT1 Is Stabilized by Incubation with IL-6

We next investigated whether the increase in DNMT1 levels we observed was due to stabilization of protein expression. These experiments were conducted using a tetracycline-regulated system of expression of epitope-tagged DNMT1 in HeLa Tet-off cells, which were chosen for this experiment because they stably express the tetracycline repressor. HeLa Tet-off cells were transfected with a plasmid, pTRE-tight/DNMT1-V5, in which doxycycline shuts off the expression of V5 epitope-tagged DNMT1 (Fig. 4A). Twenty-four hours after the transfection of pTRE-tight/DNMT1-V5 into HeLa Tet-off cells, doxycycline was added in the presence or absence of IL-6. Western blot analysis showed that DNMT1-V5 levels gradually decreased following the addition of doxycycline to the medium of cells incubated without IL-6. However, the decrease in expression of DNMT1-V5 was inhibited by the addition of IL-6 to the medium, indicating that IL-6 stabilizes the exogenous DNMT1-V5 protein, an effect that persisted at least 24 hours (Fig. 4B). Densitometry of Western blots showed that the ratio of DNMT1-V5 protein to untreated (no doxycycline) controls in IL-6–treated cells is ~10-fold higher than that of cells treated with doxycycline alone after 24 hours.

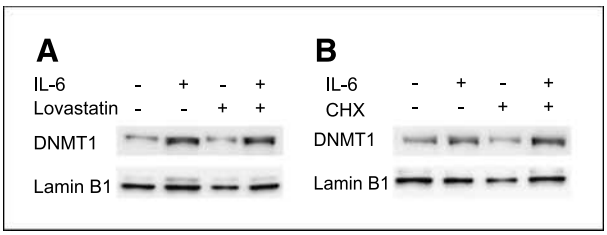

FIGURE 3. Increased DNMT1 expression following IL-6 is independent of new protein synthesis and cell cycle progression. A and B, HCT116 cells were treated with cycloheximide (CHX; 10 μg/mL) or lovastatin (10 μmol/L) for 24 h prior to treatment with IL-6 (100 ng/mL) for 24 h. DNMT1 expression was assessed by Western blotting.

IL-6–Stimulated DNMT1 Stabilization is Mediated by AKT

We next sought to determine which pathways were involved in IL-6–induced stabilization of DNMT1. We used HeLa Tet-off cells and transiently transfected them with pTRE-tight/DNMT1-V5, followed by incubation for 24 hours. Treatment of cells with IL-6 led to activation of the Jak-STAT pathway as assessed by Western blot for pSTAT3, the Jnk pathway as assessed by pERK and the Akt pathway as assessed by pAKT and inhibitors of these pathways, curcubitacin (Jak-STAT), SP600125 (Jnk) and triciribine (AKT), blocked their activation by IL-6 (Fig. 4C). These inhibitors or vehicles were added 30 minutes before the addition of doxycycline and IL-6 to HeLa cells transfected with pTRE-tight/DNMT1-V5. Pretreatment with curcubitacin and SP600125 had little effect on the stabilization of DNMT1 induced by IL-6 (Fig. 4D). However, pretreatment with triciribine abrogated the stabilizing effect of IL-6, indicating that stabilization of exogenous DNMT1 was mediated through AKT (Fig. 4D).

IL-6 Treatment of HCT116 Cells Raises Methylcytosine Levels

Because IL-6 increased DNMT1 expression, we next examined the effect of IL-6 on total genomic methylcytosine abundance. Methylcytosine content was quantified in control or IL-6–treated HCT116 cells by measuring DNA fragmentation following incubation with *Mcr*BC, which cleaves DNA at methylcytosine sites. Genomic methylcytosine levels were significantly increased in IL-6–treated cells compared with resting controls ($P < 0.03$; Fig. 5A). In untreated and IL-6–treated cells, *Mcr*BC-generated DNA fragmentation was decreased in the presence of DNMT1 inhibitor 5-azadeoxycytidine (*5-aza-CdR*). These findings indicate that 5-aza-CdR decreases methylcytosine content and are consistent with the ability of 5-aza-CdR to induce generalized demethylation of DNA. These results are also consistent with a mechanism by which IL-6 induces DNA cytosine methylation in colon cancer cells via an action involving *DNMT1*.

IL-6 Alters Methylation Levels at Gene Promoter Regions

Our results show that IL-6 resulted in increased cytosine methylation in colon cancer cells. We therefore sought to determine if IL-6 regulated the CpG island methylation of tumor suppressor genes, a key mechanism of colon carcinogenesis. We examined the methylation status of HCT116 cells in a panel of genes that are known to be regulated by CpG island methylation using a promoter

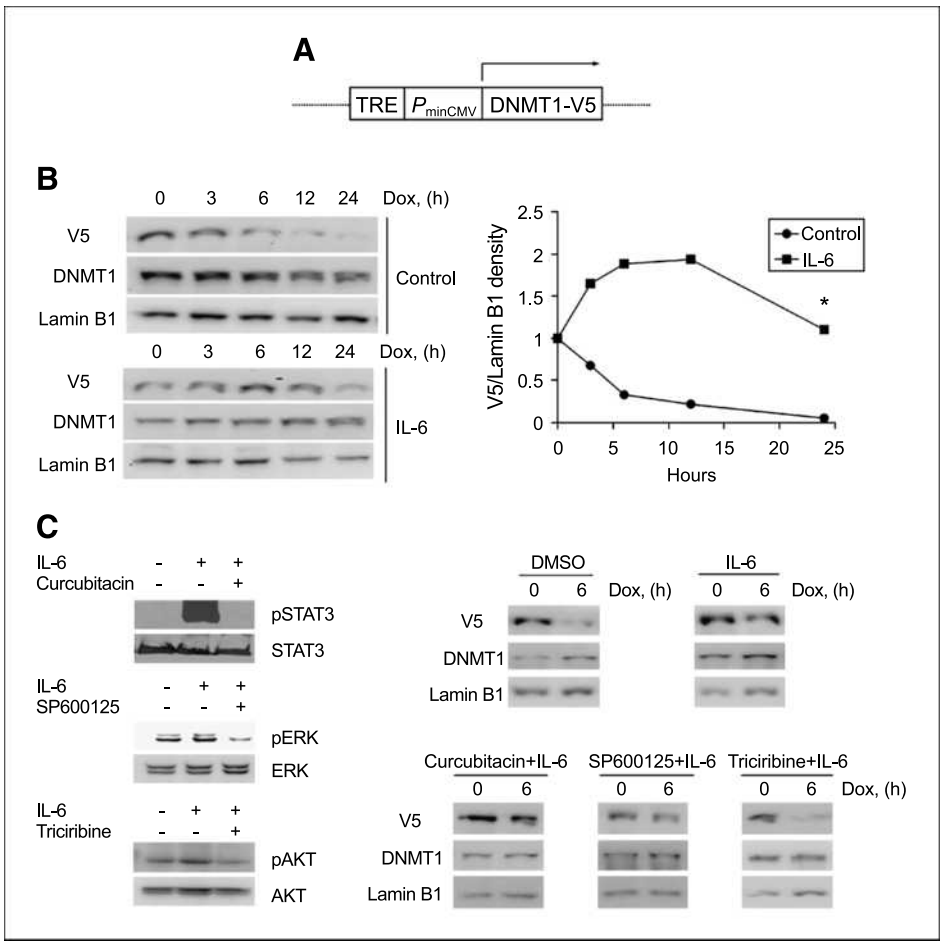

FIGURE 4. Increased DNMT1 expression due to stabilization of DNMT1 protein via Akt. A, diagram of pTRE-tight/DNMT1-V5 (TRE, tetracycline response element). B, left, HeLa Tet-off cells were transfected with pTRE-tight/DNMT1-V5 and incubated for 24 h to allow DNMT1-V5 protein expression. Doxycycline (Dox; 2 μg/mL) was added to the medium in the presence or absence of IL-6 (100 ng/mL). After the addition of doxycycline, cells were harvested at the time points indicated and subjected to Western blot for DNMT1 and V5 epitope. Right, densitometric analysis of Western blotting data (*, $P < 0.01$). C, HeLa Tet-off cells were pretreated with 5 μmol/L of triciribine, 50 μmol/L of SP600125, 1 μmol/L of curcubitacin or vehicle control for 30 min. Cells were then stimulated with 100 ng/mL of IL-6, and 30 min later, extracts were prepared for Western blotting using the indicated antibodies. D, HeLa-tet off cells were transfected with pTRE-tight/DNMT1-V5 and pretreated with curcubitacin, SP600125 or triciribine for 30 min prior to the addition of 2 μg/mL of doxycycline with or without 100 ng/mL of IL-6. Cells were harvested at the time points indicated, and nuclear extracts were subjected to Western blotting with the indicated antibodies.

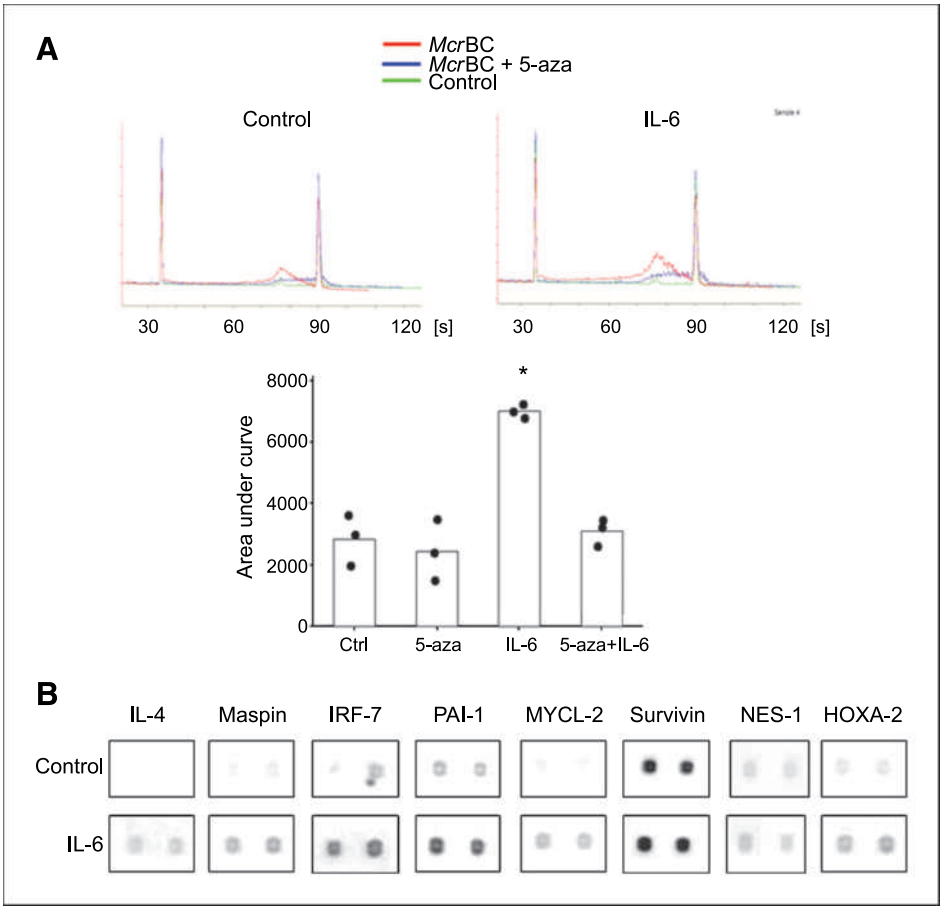

FIGURE 5. Treatment with IL-6 increases methylcytosine levels and promoter CpG island methylation in HCT116 cells. A, top, HCT116 cells were treated with 4 μmol/L of 5-aza-CdR or 100 ng/mL of IL-6. Genomic DNA was harvested and digested with *Mcr*BC before resolution on a DNA 12000 chip using an Agilent 2100 Bioanalyzer. Representative electrophoretograms showing cells treated with IL-6 or vehicle control (red line), 5-aza-CdR (blue line), or enzyme-free controls (green line). Bottom, the area under the curve for each treatment was calculated using ImageJ software (*, $P < 0.01$). B, the TransSignal Methylation array was used to assess promoter methylation in HCT116 cells treated with IL-6 (100 ng/mL) or vehicle control for 48 h.

methylation array. Our results indicated that the majority of these genes were not methylated in HCT116 cells (data not shown). However, several promoter regions were variably methylated (Fig. 5B), and treatment with IL-6 was found to significantly alter the methylation levels of a subset of these promoter regions, to different degrees. Thus, the induction of DNMT1 expression by IL-6 not only induces an elevation of genomic DNA cytosine methylation, but is also accompanied by CpG island methylation of a number of specific target genes.

IL-6 Suppresses the Expression of Methylation Target Genes via DNMT

To confirm that promoter methylation of these genes was functionally significant, we selected four of these targets for expression analysis using real-time reverse transcription-PCR. Expression levels of PAI-1, IL-4, Maspin, and IRF-7 were found to be decreased up to 5-fold following treatment with IL-6 for 48 hours (Fig. 6A). In contrast, Mcl-1 expression was increased by IL-6, consistent with it's upregulation through the Jak-STAT pathway (23). To determine whether the decrease in mRNA levels of PAI-1, Maspin, and IRF-7 detected following treatment with IL-6 was due to increased methylation, cells were incubated with DNMT inhibitor 5-aza-CdR for 24 hours before stimulation with 100 ng/mL of IL-6 for 48 hours. Pretreatment with 5-aza-CdR alone significantly increased the expression of these three genes, an effect consistent with their regulation by promoter CpG island methylation (Fig. 6B). Notably, the inhibitory effect of IL-6 on expression of these genes was abrogated by treatment with 5-aza-CdR. This indicates that the suppressive effect of IL-6 on the expression of these genes is through DNMT-mediated gene silencing.

IL-6 Increases Anchorage-Independent Growth and Invasion of HCT116 Cells via DNMT

Finally, we sought to assess the functional effect of IL-6–stimulated DNMT-mediated methylation on the phenotype of HCT116 cells. First, we examined the growth curves of HCT116 cell cultures. After 72 and 96 hours of treatment, 5-aza-CdR lowered cell density, which suggests that DNMT activity stimulates HCT116 cell growth (Fig. 7A). IL-6 had no effect on cell growth under these conditions. Second, we assessed the effect of 5-aza-CdR and IL-6 on Matrigel invasion, an indicator of the ability of cells to migrate through the extracellular matrix. IL-6 treatment resulted in a 3-fold to 4-fold elevation of the number of invading cells. Pretreatment with 5-aza-CdR lowered the number of invading cells, and completely blocked the ability of IL-6 to stimulate Matrigel invasion

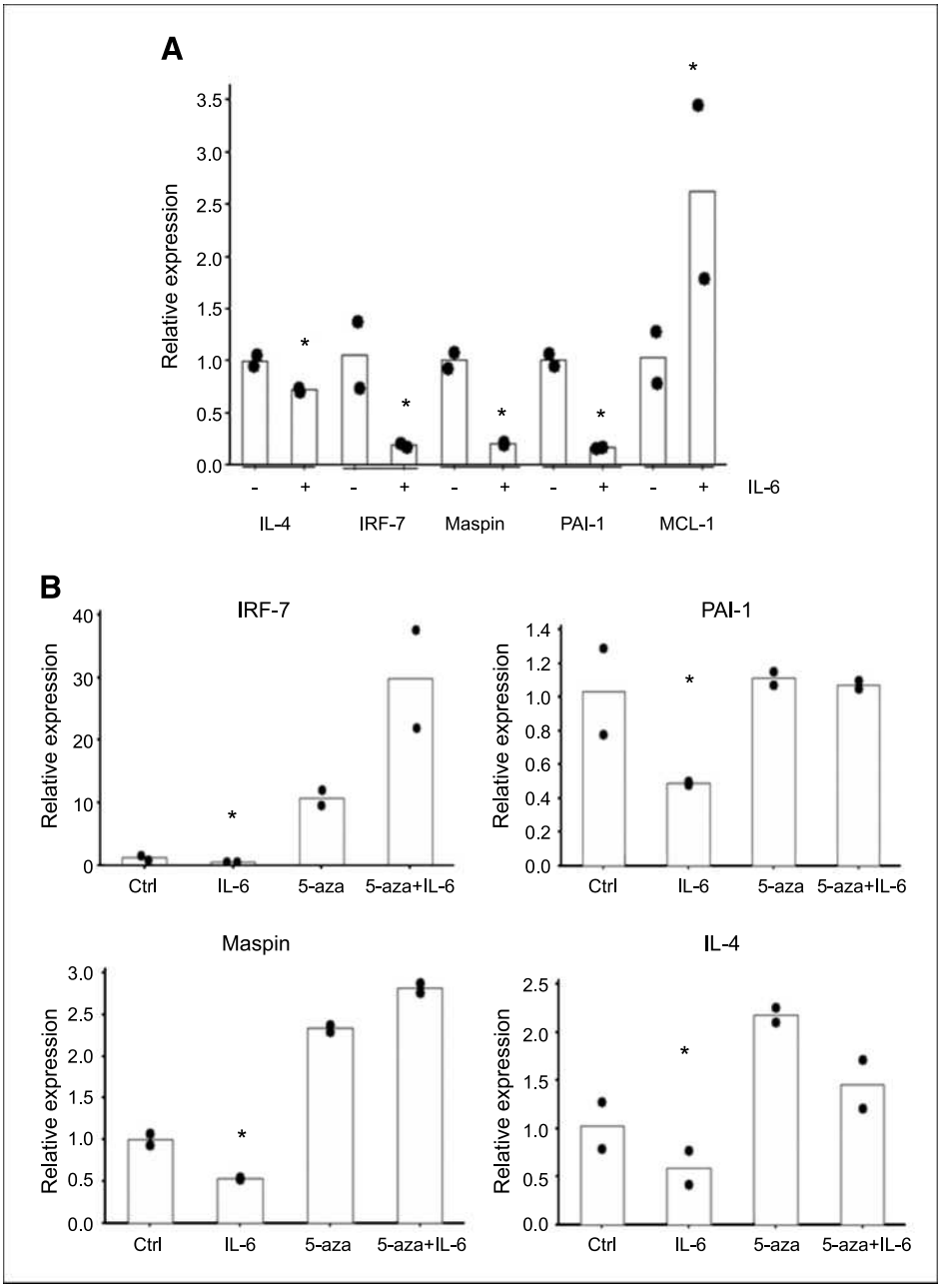

FIGURE 6. IL-6 decreases mRNA levels in a subset of genes in a DNMT-dependent manner. A, the expression levels of PAI-1, IRF-7, IL-4, Maspin, and MCL-1 were assessed by real-time reverse transcription-PCR. Cells were treated with IL-6 (100 ng/mL) or vehicle control for 48 h (*, $P < 0.05$). B, 5-aza-CdR rescues the IL-6–induced decrease in mRNA levels. HCT116 cells were pretreated with 4 μmol/L of 5-aza-CdR prior to treatment with 100 ng/mL IL-6 for 48 h (*, $P < 0.05$).

(Fig. 7B). Third, we assessed the effects of 5-aza-CdR and IL-6 on HCT116 anchorage-independent growth by quantifying colony formation in soft agar. Analogous to the effects observed in the invasion assays, IL-6 stimulated colony growth, which was completely blocked by 5-aza-CdR (Fig. 7C). Together, these results indicate that IL-6–stimulated DNA methylation specifically regulates functionally important cancer cell properties of invasion and anchorage-independent growth. These findings are consistent with a model in which IL-6 acts as an extracellular regulator of nuclear function which has important consequences for colon cancer cell behavior.

Discussion

The pathophysiology of inflammation-associated carcinogenesis in the gastrointestinal tract is complex. Multiple factors contribute to neoplastic transformation of epithelia and progression at different stages. Because chronic inflammation is mediated predominantly by immune cells of myeloid and lymphoid lineage, it is likely that substances derived from those cells in the tumor microenvironment are responsible for inducing carcinogenic changes in the epithelium. Recent work has established the critical roles of macrophages in inflammation-associated carcinogenesis

(24, 25). Our work has extended prior findings on the role of macrophages and one of their key proinflammatory cytokines, IL-6, in colitis-associated carcinogenesis (11-13). We have identified a previously unrecognized mechanism by which IL-6 promotes the neoplastic phenotype of colonic epithelial cells via the CpG island methylation pathway of carcinogenesis. These findings link chronic inflammation with an established pathway of colon carcinogenesis and provide a potential molecular explanation for how an inflammatory tumor microenvironment in the colon induces an increasingly neoplastic phenotype in epithelial cells.

Immunohistochemical evaluation of DNMT1 in colon cancers revealed its overexpression, which is similar to the results obtained in several other cancers (26, 27). DNMT1 expression was higher in IBD-CRC than in sporadic-CRC, and was also detected at higher levels in nonneoplastic peritumoral stroma. Moreover, both in the IBD-CRC and in the sporadic-CRC, CD68 staining in the peritumoral areas was positively associated with the intensity of tumor DNMT1 immunoreactivity. The biological significance of DNA methylation in peritumoral stromal cells is not known but represents an area with great potential for discovery (28-30). The results of the DNMT1 immunohistochemical studies provided support to the idea that DNA methylation could be involved in inflammation-associated carcinogenesis in the colon.

Relatively little is understood about how DNA methylation is controlled, but some recent attention has focused on regulated expression of DNMT1. Studies have reported that DNMT1 gene expression was increased via transcription factor Fli-1 in erythroleukemia cells (31), that DNMT1 expression is controlled by protein degradation in breast cancer cell lines via a destruction domain which is essential for ubiquitination (32), and that AKT activity inhibited DNMT1 degradation in multiple cell lines (33). Our results indicate that in colon cancer cells, DNMT1 levels are controlled by extracellular factors. Using HeLa cells, we found that IL-6 stabilized DNMT1 protein expression, acting via AKT but not other IL-6-induced pathways. This contrasts with a prior report in which IL-6 increased DNMT1 expression through the Jak-STAT pathway, which suggests that cell type–specific IL-6 responses exist (34). Given these limitations of our work, along with the fact that IL-6 seemed to increase DNMT1 levels to a greater extent in HCT116 cells than in HeLa

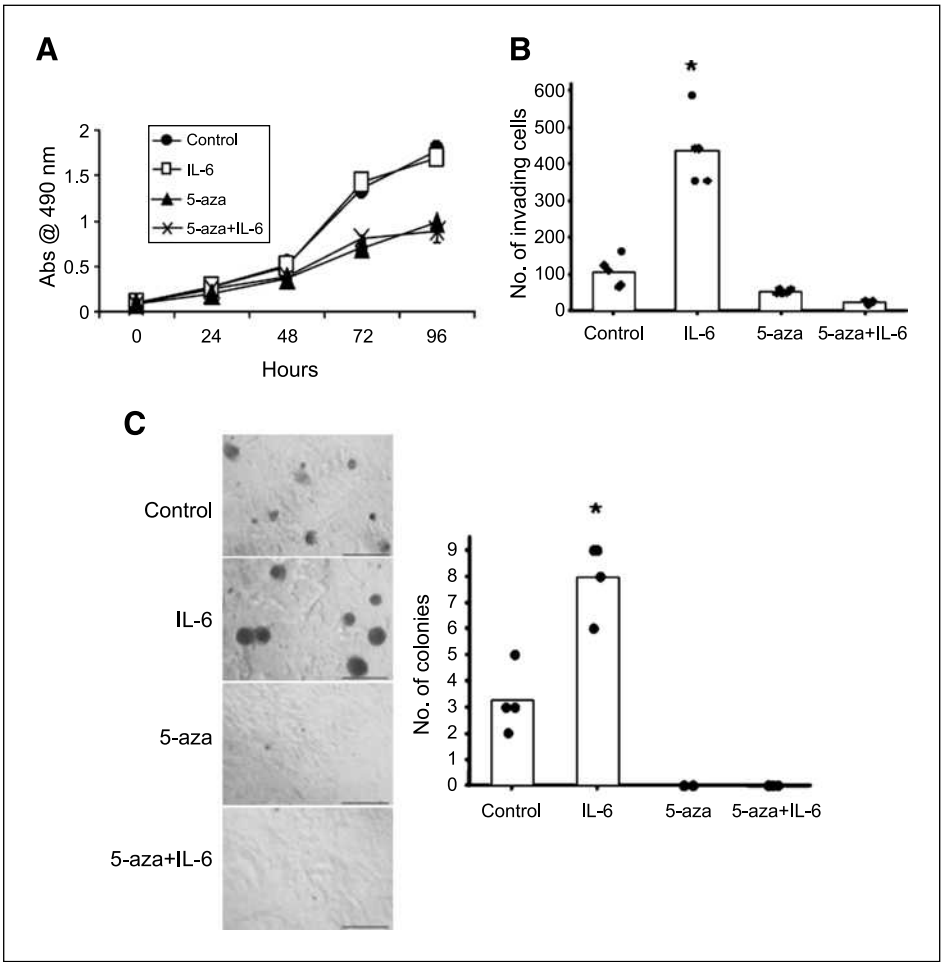

FIGURE 7. IL-6 stimulated invasion and anchorage-independent growth in HCT116 cells, in a DNMT-dependent manner. A, the effects of IL-6 and 5-aza-CdR on the growth of HCT116 cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. B, the effects of IL-6 and 5-aza-CdR on HCT116 cell migration was assessed using a Matrigel invasion assay (*, $P < 0.01$). C, the effects of IL-6 and 5-aza-CdR on the anchorage-independent growth of HCT116 cells were assessed using colony formation assays in soft agar. Bars, 200 μm (*, $P < 0.01$).

cells, further analysis of the mechanisms of DNMT1 protein stabilization by IL-6 in different cell types including colon cancer cells is needed. Those results may allow the testing of novel strategies to reverse this effect.

In the pathogenesis of colitis-associated colon cancer, IL-6–induced STAT signaling has been shown to promote the survival of the neoplastic colon epithelial cells (12, 13). However, the precise molecular pathway responsible for this effect is not known. In our experiments, we highlighted the importance of IL-6–induced DNMT1 overexpression in colon cancer cells by showing the DNMT-dependent effects of IL-6 on DNA methylation, tumor suppressor gene expression, and neoplastic phenotype of colon cancer cells. IL-6 induced both global DNA methylation and selective CpG island methylation, which was accompanied by lower target gene expression, consistent with the concept that methylated CpG islands silence genes. Our work focused on the effects of IL-6 on DNMT1 expression, which is classically considered a maintenance methyltransferase (35), but which could also catalyze *de novo* DNA methylation (18). Nevertheless, our results do not exclude the possibility that other methyltransferases such as DNMT3b, which is important in *de novo* DNA methylation, may also play a role in inflammation-associated CpG methylation.

Although these finding establish the principle that an extracellular factor could regulate DNA modifications to control gene expression, much work remains to be done to understand the kinetics and potential reversibility of IL-6–induced DNA methylation. The other limitations of our work include the fact that DNMT1 protein stabilization by IL-6 was shown in HeLa, not colon cancer cells, and that we conducted most experiments in HCT116 cells, which are known to possess quite extensive DNA methylation. Our work in this report did not extend to the specific identification of the gene(s) whose IL-6–induced silencing is responsible for the effects on cell migration and anchorage-independent growth. The role of DNA methylation as a relevant mechanism of IL-6–promoted colon carcinogenesis *in vivo* also requires further validation.

Clinically, the linking of IL-6 signaling to the regulation of gene expression via an epigenetic mechanism is an important observation. IL-6 is known to play a role in the development of colitis-associated cancers (36). In a mouse model of colitis-associated cancer, transforming growth factor-β inhibited the formation of cancer via inhibition of IL-6 *trans*-signaling (11). IL-6 receptor has been detected in both human samples of colorectal cancer, and in samples of inflamed colon, and patients with colorectal cancer have increased serum levels of IL-6 compared with controls (37). Aberrant promoter methylation in patients with ulcerative colitis has also been reported (38) and estrogen receptor methylation was also predictive of increased risk of neoplasia in patients with ulcerative colitis (39). The blockade of IL-6 signaling pathways has been the focus of several clinical trials in recent years (40) and a neutralizing anti–IL-6 receptor antibody has been licensed for the treatment of rheumatoid arthritis. However, the therapeutic potential of the IL-6 pathway in IBD is only beginning to be discovered. This report adds to the growing body of work that identifies IL-6, a major effector cytokine of inflammation, as a contributing factor in inflammation associated cancer. Our results suggest IL-6–induced DNMT1 overexpression and CpG island methylation as potential preventive or therapeutic targets in colitis-associated carcinogenesis.

Disclosure of Potential Conflicts of Interest

P.J. Limburg, consultant, Genomic Health, Inc. The other authors disclosed no potential conflicts of interest.

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References

1. Bernstein CN, Blanchard JF, Kliewer E, Wajda A. Cancer risk in patients with inflammatory bowel disease: a population-based study. Cancer 2001;91:854–62.
2. Itzkowitz SH, Harpaz N. Diagnosis and management of dysplasia in patients with inflammatory bowel diseases. Gastroenterology 2004; 126:1634–48.
3. Bamias G, Cominelli F. Immunopathogenesis of inflammatory bowel disease: current concepts. Curr Opin Gastroenterol 2007;23: 365–9.
4. Mudter J, Neurath MF. IL-6 signaling in inflammatory bowel disease: pathophysiological role and clinical relevance. Inflamm Bowel Dis 2007;13:1016–23.
5. Rose-John S, Scheller J, Elson G, Jones SA. Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer. J Leukoc Biol 2006;80:227–36.
6. Van Kemseke C, Belaiche J, Louis E. Frequently relapsing Crohn's disease is characterized by persistent elevation in interleukin-6 and soluble interleukin-2 receptor serum levels during remission. Int J Colorectal Dis 2000;15:206–10.
7. Reimund JM, Wittersheim C, Dumont S, et al. Increased production of tumour necrosis factor-α interleukin-1β, and interleukin-6 by morphologically normal intestinal biopsies from patients with Crohn's disease. Gut 1996;39:684–9.
8. Yamamoto M, Yoshizaki K, Kishimoto T, Ito H. IL-6 is required for the development of Th1 cell-mediated murine colitis. J Immunol 2000; 164:4878–82.
9. Ito H, Takazoe M, Fukuda Y, et al. A pilot randomized trial of a human anti-interleukin-6 receptor monoclonal antibody in active Crohn's disease. Gastroenterology 2004;126:989–96; discussion 47.
10. Esfandi F, Mohammadzadeh Ghobadloo S, Basati G. Interleukin-6 level in patients with colorectal cancer. Cancer Lett 2006;244:76–8.
11. Becker C, Fantini MC, Schramm C, et al. TGF-β suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. Immunity 2004;21:491–501.

12. Bollrath J, Phesse TJ, von Burstin VA, et al. gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. Cancer Cell 2009;15:91–102.
13. Grivennikov S, Karin E, Terzic J, et al. IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. Cancer Cell 2009;15:103–13.
14. Wehbe H, Henson R, Meng F, Mize-Berge J, Patel T. Interleukin-6 contributes to growth in cholangiocarcinoma cells by aberrant promoter methylation and gene expression. Cancer Res 2006;66: 10517–24.
15. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415–28.
16. Bestor TH. The DNA methyltransferases of mammals. Hum Mol Genet 2000;9:2395–402.
17. Fatemi M, Hermann A, Gowher H, Jeltsch A. Dnmt3a and Dnmt1 functionally cooperate during *de novo* methylation of DNA. Eur J Biochem 2002;269:4981–4.
18. Jair KW, Bachman KE, Suzuki H, et al. *De novo* CpG island methylation in human cancer cells. Cancer Res 2006;66:682–92.
19. Jones PA, Laird PW. Cancer epigenetics comes of age. Nat Genet 1999;21:163–7.
20. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(–ΔΔC(T)) method. Methods 2001;25:402–8.
21. Kimura F, Seifert HH, Florl AR, et al. Decrease of DNA methyltransferase 1 expression relative to cell proliferation in transitional cell carcinoma. Int J Cancer 2003;104:568–78.
22. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 1986;89:271–7.
23. Meng F, Yamagiwa Y, Ueno Y, Patel T. Over-expression of interleukin-6 enhances cell survival and transformed cell growth in human malignant cholangiocytes. J Hepatol 2006;44:1055–65.
24. Kim S, Takahashi H, Lin WW, et al. Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. Nature 2009;457:102–6.
25. Popivanova BK, Kitamura K, Wu Y, et al. Blocking TNF-α in mice reduces colorectal carcinogenesis associated with chronic colitis. J Clin Invest 2008;118:560–70.
26. Ding WJ, Fang JY, Chen XY, Peng YS. The expression and clinical significance of DNA methyltransferase proteins in human gastric cancer. Dig Dis Sci 2008;53:2083–9.
27. Girault I, Tozlu S, Lidereau R, Bieche I. Expression analysis of DNA methyltransferases 1, 3A, and 3B in sporadic breast carcinomas. Clin Cancer Res 2003;9:4415–22.
28. Floess S, Freyer J, Siewert C, et al. Epigenetic control of the foxp3 locus in regulatory T cells. PLoS Biol 2007;5:e38.
29. Janson PC, Marits P, Thorn M, Ohlsson R, Winqvist O. CpG methylation of the IFNG gene as a mechanism to induce immunosuppression [correction of immunosuppression] in tumor-infiltrating lymphocytes. J Immunol 2008;181:2878–86.
30. Im SH, Hueber A, Monticelli S, Kang KH, Rao A. Chromatin-level regulation of the IL10 gene in T cells. J Biol Chem 2004;279: 46818–25.
31. Hodge DR, Xiao W, Clausen PA, Heidecker G, Szyf M, Farrar WL. Interleukin-6 regulation of the human DNA methyltransferase (HDNMT) gene in human erythroleukemia cells. J Biol Chem 2001; 276:39508–11.
32. Agoston AT, Argani P, Yegnasubramanian S, et al. Increased protein stability causes DNA methyltransferase 1 dysregulation in breast cancer. J Biol Chem 2005;280:18302–10.
33. Hodge DR, Cho E, Copeland TD, et al. IL-6 enhances the nuclear translocation of DNA cytosine-5-methyltransferase 1 (DNMT1) via phosphorylation of the nuclear localization sequence by the AKT kinase. Cancer Genomics Proteomics 2007;4: 387–98.
34. Zhang Q, Wang HY, Woetmann A, Raghunath PN, Odum N, Wasik MA. STAT3 induces transcription of the DNA methyltransferase 1 gene (DNMT1) in malignant T lymphocytes. Blood 2006; 108:1058–64.
35. Jeltsch A. On the enzymatic properties of Dnmt1: specificity, processivity, mechanism of linear diffusion and allosteric regulation of the enzyme. Epigenetics 2006;1:63–6.
36. Fantini MC, Pallone F. Cytokines: from gut inflammation to colorectal cancer. Curr Drug Targets 2008;9:375–80.
37. Chung YC, Chang YF. Serum interleukin-6 levels reflect the disease status of colorectal cancer. J Surg Oncol 2003;83:222–6.
38. Wang FY, Arisawa T, Tahara T, et al. Aberrant DNA methylation in ulcerative colitis without neoplasia. Hepatogastroenterology 2008; 55:62–5.
39. Tominaga K, Fujii S, Mukawa K, et al. Prediction of colorectal neoplasia by quantitative methylation analysis of estrogen receptor gene in nonneoplastic epithelium from patients with ulcerative colitis. Clin Cancer Res 2005;11:8880–5.
40. Naugler WE, Karin M. The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. Trends Mol Med 2008;14:109–19.