Interleukin-6 Contributes to Growth in Cholangiocarcinoma Cells by Aberrant Promoter Methylation and Gene Expression

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Abstract
The association between chronic inflammation and the development and progression of malignancy is exemplified in the biliary tract where persistent inflammation strongly predisposes to cholangiocarcinoma. The inflammatory cytokine interleukin-6 (IL-6) enhances tumor growth in cholangiocarcinoma by altered gene expression via autocrine mechanisms. IL-6 can regulate the activity of DNA methyltransferases, and moreover, aberrant DNA methylation can contribute to carcinogenesis. We therefore investigated the effect of chronic exposure to IL-6 on methylation-dependent gene expression and transformed cell growth in human cholangiocarcinoma. The relationship between autocrine IL-6 pathways, DNA methylation, and transformed cell growth was assessed using malignant cholangiocytes stably transfected to overexpress IL-6. Treatment with the DNA methylation inhibitor 5-aza-2'-deoxycytidine decreased cell proliferation, growth in soft agar, and methylcytosine content of malignant cholangiocytes. However, this effect was not observed in IL-6-overexpressing cells. IL-6 overexpression resulted in the altered expression and promoter methylation of several genes, including the epidermal growth factor receptor (EGFR). EGFR promoter methylation was decreased and gene and protein expression was increased by IL-6. Thus, epigenetic regulation of gene expression by IL-6 can contribute to tumor progression by altering promoter methylation and gene expression of growth-regulatory pathways, such as those involving EGFR. Moreover, enhanced IL-6 expression may decrease the sensitivity of tumor cells to therapeutic treatments using methylation inhibitors. These observations have important implications for cancer treatment and provide a mechanism by which persistent cytokine stimulation can promote tumor growth. (Cancer Res 2006; 66(21): 10517-24)

Introduction
Cholangiocarcinomas are malignancies arising from biliary tract epithelia that are typically associated with a poor prognosis related, in part, to both a lack of understanding of their underlying pathogenetic mechanisms and refractoriness of these cancers to current therapies. The predisposition of chronic biliary tract inflammation to cholangiocarcinoma is well characterized (1). The inflammation-associated cytokine interleukin-6 (IL-6) has been identified as contributing to hepatic epithelial changes during hepatic inflammation. IL-6 has pleiotropic effects with both mitogenic and cytoprotective effects in biliary tract epithelia (2–5). IL-6 is increased in the blood and bile of patients with cholangiocarcinoma and in cholangiocarcinoma cells in vitro (6). The presence of autocrine mechanisms by which IL-6 contributes to tumor growth is supported by in vitro and in vivo studies (7, 8). Experimentally enforced expression of IL-6 enhances the growth of malignant cholangiocyte xenografts in athymic mice. Thus, targeting IL-6-mediated cell processes may be therapeutically useful for cholangiocarcinoma. However, the mechanisms by which sustained exposure to IL-6 enhances tumor growth are poorly understood, and moreover, it remains unknown if overexpression of IL-6 per se can promote cancer formation.

To elucidate potential mechanisms by which overexpression of IL-6 may contribute to tumorigenesis or tumor growth, we evaluated epigenetic mechanisms of regulation of gene expression. DNA methylation can result in epigenetic regulation of gene expression and is associated with altered expression of genes that may be involved in carcinogenesis or tumor growth, such as oncogenes, tumor suppressor genes, and DNA repair genes or other genes. Aberrant promoter methylation of several genes, such as p16, TIMP-3, hMLH1, RASSF1A, and others, has been reported in cholangiocarcinoma (9–13). Generally, methylation serves to modulate gene expression and represents a powerful mechanism that may contribute to tumor growth by altered expression of genes involved in tumor cell behavior. Methylation patterns in mammalian cells are regulated by a complex interplay of at least three independently encoded DNA methyltransferases (DNMT). IL-6 has been shown to regulate the promoter of DNMT (dnmt-1) and its resulting enzyme activity (14). dnmt-1 transfers a methyl group to the cytosine portion of the CpG dinucleotide, which allows for the binding of methyl-specific DNA-binding proteins to the methylated CpG site. The binding of methyl-specific proteins, such as MeCP1 or MeCP2, to regulatory elements of the gene represses transcription by blocking the action of transactivation factors. These binding proteins can attract histone deacetylases, which then remodel chromatin into highly repressed states.

The use of inhibitors of DNA methylation, such as 5-aza-2'-deoxycytidine (5-aza-CdR), or histone deacetylase inhibitors to modulate methylation-dependent epigenetically regulated genes involved in tumor growth is gaining momentum, with several agents being evaluated in clinical trials (15). 5-aza-CdR has been used extensively to modulate the expression of individual genes in experimental studies of the cancer epigenome (16). However, the use of these agents for cholangiocarcinoma is unknown. Our hypothesis was that overexpression of IL-6 could enhance tumor formation or behavior in cholangiocarcinoma by modulating gene
expression via mechanisms involving DNA methylation. Thus, we sought to evaluate the effect of IL-6 overexpression on DNA methylation, to identify methylation-dependent IL-6-regulated genes that may be involved in cholangiocarcinoma growth and to explore the potential use of methylation inhibitors as therapeutic agents for cholangiocarcinoma. We asked the following questions: Does inhibition of methylation modulate transformed cell growth of malignant cholangiocytes? Can IL-6 overexpression alter DNA methylation, and if so, can IL-6 alter gene promoter methylation of genes? Are there any genes that can be regulated by either IL-6 overexpression or inhibition of methylation? If so, can these participate in tumor growth or survival? Our results indicate that IL-6 can epigenetically modulate the expression of growth-regulatory pathways, such as those involving the epidermal growth factor receptor (EGFR), and identify a novel and important mechanism by which overexpression of IL-6 can contribute to cholangiocarcinoma growth.

Materials and Methods

Cell lines and culture. Mz-ChA-1 cells derived from metastatic gall bladder cancer were obtained as described previously (17). Cells were stably transfected with full-length IL-6 to generate a cell line that overexpressed IL-6 under basal conditions (Mz-IL-6) as described previously (7). Cells were cultured in CMRL 1066 medium with 10% fetal bovine serum (FBS), 1% l-glutamine, and 1% antimycotic-antibiotic mix. To assess 5-aza-CdR effects, Mz-ChA-1 and Mz-IL-6 cells were grown to ~75% confluency on 100-mm culture dishes and then treated with 5 μmol/L 5-aza-CdR for 24 hours at 37°C. Following treatment, cells were washed twice with cold 1× PBS before harvesting for isolation genomic DNA or total protein.

Cell proliferation. Mz-ChA-1 and Mz-IL-6 cells were grown to 75% confluency on 100-mm culture dishes and then treated with 5-aza-CdR for 24 hours at 37°C. Following conditioning overnight at 37°C, cells were treated with 5-aza-CdR at 0, 1, 5, 10, or 50 μmol/L for 4 days. Every 24 hours, cell viability was measured using a colorimetric assay (CellTiter 96 AQueous, Promega Corp., Madison, WI). A proliferation index was expressed as a percentage of control.

Growth in soft agar. To assess the effect of anchorage-independent growth, cells were grown in soft agar as described previously (18). Mz-ChA-1 and Mz-IL-6 cells were seeded in 96-well plates (10,000 per well) in CMRL medium with 20% FBS with 0 or 5 μmol/L 5-aza-CdR. The final concentration of the bottom and top feeder layers of the agar system was 0.6% and the cell suspension layer was 0.4%. Cells were incubated for 21 to 24 days in a humidified incubator at 37°C, after which the total number of colonies was counted. Studies of anchorage-independent transformed cell growth was determined using two complementary assays to assess anchorage-independent growth. First, 1 × 10^5 cells were plated in soft agar in 96-well plates and growth was quantitated by determining Alamar Blue fluorescence after 7 days. A longer-term clonogenic assay in soft agar was also done in which Mz-ChA-1 cells (1 × 10^3) treated with 5-aza-CdR or diluent controls were plated in soft agar in 35-mm plates, and the total number of colonies formed was determined after 21 to 28 days. 5-aza-CdR decreased transformed cell growth in Mz-ChA-1 cells in both assays.

Figure 1. The methylation inhibitor 5-aza-CdR decreases growth of malignant cholangiocytes. A, cells were treated with 5-aza-CdR at the indicated concentrations for 4 days or with 5 μmol/L 5-aza-CdR for the indicated times. At each time point, the proliferation index was determined using a viable cell assay and expressed as a percentage of the control. The effect of 5-aza-CdR on Mz-ChA-1 cells on cell growth was assessed using a viable cell assay and determining the proliferation index. B, Mz-ChA-1 cells were incubated with 5 μmol/L 5-aza-CdR or diluent controls for 48 hours. The effect on transformed cell growth was determined using two complementary assays to assess anchorage-independent growth. First, 1 × 10^5 cells were plated in soft agar in 96-well plates and growth was quantitated by determining Alamar Blue fluorescence after 7 days. A longer-term clonogenic assay in soft agar was also done in which Mz-ChA-1 cells (1 × 10^3) treated with 5-aza-CdR or diluent controls were plated in soft agar in 35-mm plates, and the total number of colonies formed was determined after 21 to 28 days. 5-aza-CdR decreased transformed cell growth in Mz-ChA-1 cells in both assays. C, effects of incubation with the methylation inhibitor 5-aza-CdR (5 μmol/L) on the growth of IL-6-overexpressing Mz-IL-6 cells were assessed by determining anchorage-dependent growth, short-term anchorage-independent growth, and colony formation in soft agar. In all assays, the growth-inhibitory effects of 5-aza-CdR were decreased by IL-6 overexpression.
growth were also done in a short-term assay in which cells were incubated for 7 days (19). The total number of colonies was quantified as a direct proportion of fluorescence. Alamar Blue (Biosource International, Camarillo, CA) was added to the sample wells, and fluorescence was measured using a CytoFluor multiwell plate reader (excitation 530/25 nm; emission 580/50 nm).

Microarray analysis. Microarray analysis was done using Affymetrix U133A plus 2 chips (Affymetrix, Santa Clara, CA). Genes were considered to be altered in expression if they exhibited a minimum 1.5-fold difference in expression relative to control and they were recorded as present. Data were analyzed using GeneSpring 7.0 Software (Silicon Genetics, Redwood City, CA). Expression analysis using Gene Ontology and pathway mapping was done using the Gene Map Annotator and Pathway Profiler (GenMAPP; refs. 20, 21).

5-Methylcytosine assay. Cells were treated with 5-aza-CdR as described above. Genomic DNA was obtained from the cells using a commercially available DNA extraction kit (Chemicon, Temecula, CA), and subsequently, 1 μg was digested using 10 units McrBC (New England Biolabs, Ipswich, MA) for 2 hours. Resulting DNA (20 ng) was then resolved on a DNA 12000 chip using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). The amount of DNA sized between 500 and 12,000 bp was quantitated as an index of 5-methylcytosine content.

Promoter methylation array. Promoter methylation was assessed in Mz-ChA-1 and Mz-IL-6 cells using the TransSignal Methylation Array (Panomics, Redwood City, CA) following the manufacturer’s instructions. In brief, 2 μg of genomic DNA were digested with MseI (New England Biolabs). The methylated DNA was incubated with methylation binding protein and separated using the provided spin column. The methylated DNA was incubated with methylation binding protein and then hybridized with the methylation array. The membranes were incubated with streptavidin-horseradish peroxidase (HRP) followed by incubation with detection buffer. Chemiluminescence was detected by overlaying the membranes with Kodak BioMax MR film (New Haven, CT) for 1 hour. The film was developed and the image was then scanned for quantitation purposes. Quantitation was done using the Storm 840 imaging system (Amersham Biosciences, Piscataway, NJ).

Western blotting. Cells were treated with 5-aza-CdR as described above. Total protein was extracted with 0.5 mL lysis buffer containing protease inhibitors. Equivalent amounts of protein samples were mixed with 4× sample buffer and separated on 4% to 12% gradient polyacrylamide gels (Novex, San Diego, CA) and then transferred to nitrocellulose membrane (Millipore, Bedford, MA). The membranes were blocked with 50% nonfat dry milk in TBS (pH 7.4) containing 0.05% Tween 20 (TBST) for 1 hour and then incubated overnight at 4°C with the respective anti-human primary antibody (1:1,000). The membrane was washed thrice for 5 minutes with TBST and then incubated with IRDye 700–labeled (Molecular Probes, Eugene, OR) and IRDye 800–labeled (Rockland, Inc., Gilbertsville, PA) secondary antibodies (1:2,000) for 30 minutes. LI-COR Odyssey IR Imaging System (LI-COR Biosciences, Lincoln, NE) was used to visualize and measure the target protein expression. Relative expression was determined by probing against β-actin (1:2,000 primary antibody; 1:4,000 secondary antibody).

Methylation-specific PCR. Briefly, bisulfite modification of DNA converts unmethylated cytosines to uracil but does not alter those that are methylated. The altered DNA can then be amplified and the methylation status of the CpG sites can be analyzed. Cells were treated with 5-aza-CdR as indicated, and genomic DNA was collected as described above. To create ssDNA, 1 μg of genomic DNA in a volume of 50 μL was treated with NaOH (final concentration, 0.2 mol/L) for 10 minutes at 50°C. To the DNA, we added 30 μL of 10 mmol/L hydroxquinone and 520 μL of 3 mol/L sodium bisulfite at pH 5.5, both prepared fresh and mixed well with the DNA samples. A layer of mineral oil (50 μL) was added, and the samples were incubated
at 50°C for 16 hours. Modified DNA was purified using 1 mL of Wizard DNA purification resin (Promega) according to the manufacturer. The modified DNA was then treated with NaOH (final concentration, 0.3 mol/L) at 37°C for 10 minutes followed by ethanol precipitation with 1 A L glycogen as a carrier and eluted with 20 A L water. PCR amplification was then done using primers specific for the human EGFR promoter. The primer sequences used were 5'-TGTTTTGTTTTTTTGTGTTTTGGTTTGTGT-3' (sense) and 5'-CATCCAAATCTAAAAACACAACAACACCACA-3' (antisense) for unmethylated DNA and 5'-TGTTTTTTCCCTCGTTTCCGTCCGCGCC-3' (sense) and 5'-CGATCGATCGATCGATCGATCGATCG-3' (antisense) for methylated DNA (22). The PCR mixture contained 1/2 PCR SuperMix High Fidelity Buffer (Invitrogen, Carlsbad, CA) containing a mixture of Taq and Pyrococcus GB-D DNA polymerase, Mg2+ and deoxynucleotide triphosphate, primers (200 nmol/L final concentration of each), and bisulfite-modified DNA (~300 ng) in a final volume of 100 A L. Amplification was done on a Techne Genius Thermal Cycler (Techne, Inc., Princeton, NJ). Reactions were hot started at 95°C for 10 minutes and then amplified for 40 cycles [45 seconds at 95°C, 45 seconds at 45°C (first 20 cycles) and 50°C (last 20 cycles), and 45 seconds at 72°C] followed by a final 5-minute extension at 72°C. Controls without DNA were done for each set of primers. Both primer pairs amplified an ~190-bp product. Each PCR (1 A L) was then resolved on a DNA 500 chip and analyzed on an Agilent 2100 Bioanalyzer.

**Materials.** Cell culture medium and supplements and primers were from Invitrogen, Carlsbad, CA containing a mixture of Taq and Pyrococcus GB-D DNA polymerase, Mg2+ and deoxynucleotide triphosphate, primers (200 nmol/L final concentration of each), and bisulfite-modified DNA (~300 ng) in a final volume of 100 A L. Amplification was done on a Techne Genius Thermal Cycler (Techne, Inc., Princeton, NJ). Reactions were hot started at 95°C for 10 minutes and then amplified for 40 cycles [45 seconds at 95°C, 45 seconds at 45°C (first 20 cycles) and 50°C (last 20 cycles), and 45 seconds at 72°C] followed by a final 5-minute extension at 72°C. Controls without DNA were done for each set of primers. Both primer pairs amplified an ~190-bp product. Each PCR (1 A L) was then resolved on a DNA 500 chip and analyzed on an Agilent 2100 Bioanalyzer.

**Statistics.** Data are expressed as the mean ± 95% confidence limits from at least three separate experiments. The difference between groups was analyzed using a double-sided Student’s t test. Statistical significance was considered as P < 0.05.

**Results**

The DNA methylation inhibitor 5-aza-CdR decreases cholangiocarcinoma cell growth. The effect of 5-aza-CdR on growth of cholangiocarcinoma cells in vitro was assessed to evaluate the potential use of methylation inhibitors for cholangiocarcinoma. Incubation of Mz-ChA-1 cells with 5-aza-CdR decreased cell proliferation in a concentration- and time-dependent manner (Fig. 1A). Next, we assessed the effect of 5-aza-CdR on phenotypic changes of transformed cells by assessing anchorage-independent growth. Incubation with 5-aza-CdR decreased colony formation in soft agar over a longer period (>3 weeks), indicating that the effects of methylation inhibition were sustained (Fig. 1B). These studies indicate that transformed cell growth in cholangiocarcinoma cells involves methylation-dependent processes and suggest the possibility of using methylation inhibitors as therapeutic agents.

**Overexpression of IL-6 decreases the effects of 5-aza-CdR on transformed cell growth.** Autocrine mechanisms have been implicated in cholangiocarcinoma growth, and we have previously shown that enforced expression of IL-6 increases transformed cell growth in malignant cholangiocytes both in vitro and in vivo (7).
Despite the obvious clinical relevance of these findings, the mechanisms involved are unknown. The ability of IL-6 to modulate DNMT expression and activity raised the possibility that methylation-dependent processes could contribute to these effects. Thus, we evaluated the effects of 5-aza-CdR in Mz-IL-6 cells, which overexpress IL-6. In contrast to the effects of 5-aza-CdR on Mz-ChA-1 cells, there was no significant change in either proliferation or anchorage-independent growth in Mz-IL-6 cells (Fig. 1C). These observations suggest that increased IL-6 expression can counter the effects of inhibition of methylation in decreasing transformed cell growth.

IL-6 increases 5-methylcytosine content. We next evaluated whether the abrogation of the antiproliferative response to 5-aza-CdR could reflect alterations in methylation by IL-6. In humans, DNA methylation occurs preponderantly at CpG sites (i.e., cytosines that are followed by a guanine). The 5-methylcytosine content in genomic DNA provides a measure of global DNA methylation. Methylcytosine content was quantitated in both Mz-ChA-1 and Mz-IL-6 cells by assessing DNA fragmentation following cleavage by the endonuclease McrBC, which cleaves DNA at methylcytosine sites (Fig. 2). In Mz-ChA-1 cells, McrBC-generated DNA fragmentation was decreased in the presence of 5-aza-CdR. These data indicate that 5-aza-CdR decreases methylcytosine content and are consistent with the ability of 5-aza-CdR to induce generalized global demethylation. In Mz-IL-6 cells, however, addition of 5-aza-CdR did not alter methylcytosine content, indicating that IL-6 overexpression can overcome the effect of 5-aza-CdR on global demethylation. These findings are consistent with the reported effects of IL-6 on DNMT expression.

IL-6 overexpression alters methylation at gene promoter regions. Although we have shown that IL-6 can alter global methylation, the relevance of this finding to modulation of gene expression was unknown. To determine if IL-6 could selectively regulate gene expression by a mechanism involving promoter methylation, we screened for changes in the basal level of methylation of several gene-specific promoters caused by overexpression of IL-6. Although altered methylation was not observed for the majority of promoters, several gene promoters were identified for which there was altered promoter methylation (Fig. 3). Both increased promoter methylation of genes, such as CASP8, Survivin, or HoxA2, as well as decreased promoter methylation of genes, such as TFF1 and Tastin, were observed in Mz-IL-6 cells under basal conditions. These findings indicated that the enforced expression of IL-6 could result in altered gene expression by epigenetic reprogramming in response to alterations in basal promoter methylation. Notably, though, IL-6 overexpression did not alter the relative methylation of many genes that are regulated by methylation and involved in the pathogenesis of cancer, such as the tumor suppressor Rb, cell cycle regulator p21, or oncogenes, such as K-ras. These studies suggest that overexpression of IL-6 is more likely to contribute to tumor progression rather than to tumor initiation.

Changes in gene expression by IL-6 overexpression and 5-aza-CdR. Although both transcriptional and translational regulation of gene expression by IL-6 are well recognized (5, 23), our observations indicate the possibility of a novel pathway by which IL-6 can alter gene expression via modulation of DNA methylation. To identify candidate genes that may be regulated in a methylation-dependent manner by IL-6, we did genome-wide microarray analysis of basal gene expression in IL-6-overexpressing cells and controls to identify IL-6-regulated genes. We then compared these with methylation-dependent genes identified by microarray analysis of gene expression in the presence of 5-aza-CdR. Although 5-aza-CdR-induced loss of methylation may increase gene expression by reactivation of methylation-silenced genes, experimental studies have shown both increased and decreased gene expression in response to this agent (16). Thus, we included genes that showed either an increase or a decrease in expression for each condition (Table 1). In 5-aza-CdR-treated cells, there were 1,589 genes identified that showed a greater than or less than 1.5-fold difference in expression (Fig. 4). Comparison of gene expression also identified 300 genes that were increased or decreased by 1.5-fold in Mz-IL-6 cells relative to Mz-ChA-1. Altered expression of genes involved in diverse cellular processes was observed. For example, Mcl-1, a Bcl-2 family protein that promotes cell survival

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<th>Table 1. Genes significantly altered in expression by 5-aza-CdR or by enforced expression of IL-6 in malignant cholangiocytes</th>
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and suppresses apoptosis in cholangiocarcinoma, was increased >2-fold in Mz-IL-6 cells as we have previously observed and verified by real-time PCR (7).

A subset of 92 genes that were altered in expression by both enforced expression of IL-6 and by the methylation inhibitor 5-aza-CdR was identified. By analysis of Gene Ontology classifications, this group included several genes involved in cell proliferation (e.g., EGFR, IRS-2, and IGFBP-1), cell cycle regulation (CCNG2), or DNA replication (e.g., MCM5, NFIC, and PRIM2A). In contrast, only one gene each was identified involved in either apoptosis (YARS) or DNA repair (NTHL-1). IL-6-activated mitogen-activated protein kinase (MAPK) signaling pathways have been shown to play a critical role in cholangiocarcinoma growth. To examine the possibility that IL-6 could epigenetically modulate mitogen growth factor signal transduction pathways, we mapped these genes on biological pathways involved in cell proliferation using GenMAPP. The EGFR and the MAPK kinase 2 were identified as being regulated by both IL-6 overexpression and inhibition of methylation.

Methylation-dependent regulation of the EGFR by IL-6. Our findings suggested that EGFR-mediated signaling could be epigenetically modulated. The expression of the EGFR was increased >2.5-fold in Mz-IL-6 cells compared with Mz-ChA-1 cells. Continuous EGFR activation promotes the growth of many cancer cells, including cholangiocarcinoma (24, 25). Altered methylation of the EGFR may be associated with the development of many solid tumors. Thus, we further examined regulation of expression of EGFR by IL-6 and assessed the role of methylation in regulation of the EGFR. A 2-fold increase in EGFR protein levels was noted in Mz-IL-6 cells compared with Mz-ChA-1 cells (Fig. 5). Moreover, EGFR protein expression was significantly decreased in cells treated with 5-aza-CdR, indicating that the constitutive expression of EGFR was methylation dependent. Using methylation-specific PCR, we analyzed the methylation status of the EGFR promoter. Compared with MzChA-1 cells, EGFR methylation was decreased to 60.9 ± 15.5% in Mz-IL-6 cells (average ± 95% confidence intervals from four experiments). Thus, IL-6 overexpression is associated with decreased methylation of the EGFR promoter as well as enhanced EGFR protein expression. Collectively, these results indicate that IL-6 can selectively regulate the expression of genes involved in cholangiocarcinoma growth, such as EGFR, by manipulation of gene promoter methylation.

Discussion

IL-6 is an important mediator of inflammation and is prominently increased in hepatic epithelia in response to inflammatory mediators and stimuli, such as endotoxins or tumor necrosis factor (TNF)-α. Although cellular changes in response to acute stimulation with IL-6 are well characterized, there is a paucity of information about the effect of chronic exposure to IL-6 in hepatic epithelia. Altered expression of IL-6 is associated with growth of hepatic epithelia and has been associated with several cancers. Understanding the mechanism by which IL-6 can contribute to
IL-6 Modulates Methylation and Gene Expression

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Studies during acute stimulation with IL-6, and their relevance to
representative immunoblot along with quantitative data of EGFR/
isolation of total protein, and immunoblot analysis was done for EGFR protein
expression is increased by overexpression of IL-6. Mz-ChA-1
expression at a single allele for genes in which the other allele is
mutated. Similarly, dysregulated expression of growth-regulatory or
survival genes can contribute to tumor cell behavior, such as
enhanced proliferation and resistance to therapy.

Dysregulation of methylation by overexpression of IL-6 may
reflect direct modulation of expression and activity of dnmt-1, an
enzyme involved in the methylation at cytosine residues in Cpg
dinucleotides (14). Increased DNMT activity is associated with
cancer cells and may contribute to tumorogenesis or tumor
progression (26). In addition, IL-6 may indirectly influence
methylation by effects on the expression or activity of other
enzymes, such as demethylases, or expression of proteins, such as
histones, which are involved in the regulation of gene methylation
and transcription. Deregulation of gene expression by methylation
may be relevant to other cellular processes in which IL-6 plays a
role, such as development, maturation, differentiation, and cellular
responses to injury in the liver as well as other tissues and organs.
Potential gene targets regulated by IL-6 include p53 and the
nucleotide excision repair gene hHR23B (27, 28). These have been
studied during acute stimulation with IL-6, and their relevance to
tumor pathogenesis or behavior is thus particularly relevant to
cancers associated with chronic inflammation and increased IL-6
expression, such as cholangiocarcinoma. Enforced expression of IL-
6 in malignant cholangiocytes can promote tumor growth (7).
Herein, we show that IL-6 overexpression can alter promoter
methylation and gene expression of several genes, including the
EGFR that has been implicated in cancer growth. The observation
that prolonged exposure to IL-6 can contribute to transformed cell
behavior in cholangiocarcinoma by epigenetic regulation of gene
expression has several important biological implications for this
and other epithelial cancers. Enhanced IL-6 expression could
potentially contribute to tumor initiation by inactivation of
expression at a single allele for genes in which the other allele is
mutated. Similarly, dysregulated expression of growth-regulatory or
survival genes can contribute to tumor cell behavior, such as
enhanced proliferation and resistance to therapy.

The EGFR is identified as a candidate target of epigenetic
regulation by IL-6 in malignant cholangiocytes. The involvement
of EGFR-mediated signaling pathways in growth of human cholan-
giocarcinoma is supported by several studies (24, 25, 29).
Modulation of EGFR-mediated signaling holds promise as a
potential therapy for cholangiocarcinoma. Indeed, the use of
drugs, such as imatinib mesylate, to selectively inhibit tyrosine
kinase activity and decrease EGFR expression may be effective for
human cholangiocarcinoma (30). Most reported studies have
focused on the activation of EGFR-mediated signaling by ligand
binding or receptor activation by transactivation. The identifica-
tion of a novel epigenetic mechanism by which EGFR expression
could be regulated thus provides another potential target for the
manipulation of EGFR-mediated signaling for the therapy of
cholangiocarcinoma.

Pharmacologic inhibition using 5-aza-CdR may be expected to
enhance the gene expression by reversal of epigenetically silenced
promoters. However, 5-aza-CdR decreased expression of EGFR in
both MzChA-1 and Mz-IL-6 cells. Although 5-aza-CdR may
potentially regulate gene expression by mechanisms other than
modulation of methylation, we note that enhanced expression of
EGFR in Mz-IL-6 cells was associated with demethylation of the
EGFR promoter, suggesting an indirect effect mediated by a
methylation-dependent intermediate. Several genes were also
down-regulated by 5-aza-CdR, and similar results showing
decreased gene expression by 5-aza-CdR have been observed in
microarray studies by others (16). Although some of these changes
may reflect secondary changes due to altered expression of
regulatory proteins, a primary effect of 5-aza-CdR cannot be
excluded. Microarray-based studies and analysis of gene expression
changes in response to pharmacologic or genetic modulators of
methylation have identified novel sites for aberrant methylation in
several gastrointestinal cancers (31–34). Studies that have reported
only genes that are increased in expression in response to
methylation inhibitors, such as 5-aza-CdR, may therefore have
potentially missed recognizing biologically relevant changes.

There are several important clinical implications of our work.
Gene promoter methylation has become an attractive target for
developing strategies for molecular screening for early detection
and diagnosis of gastrointestinal cancers, including cholangiocar-
cinoma. Techniques for the evaluation of changes in biliary
epithelial cells in bile have been developed. However, IL-6 levels
in blood and bile can be increased in the presence of biliary tract
inflammation or infection and could confound efforts at using
methylation marker profiling for either screening or diagnosis of
biliary cancers. Similarly, the modulation of expression of growth-
regulatory genes, oncogenes, or tumor suppressor genes by
methylation is an attractive therapeutic target as these changes
can contribute to tumor growth. 5-aza-CdR has been widely
studied as a DNA methylation inhibitor for the treatment of
hematologic diseases, and low doses are now being studied for the
treatment of solid tumors (35). The potential use of methylation
inhibitors, such as 5-aza-CdR, in the treatment of cholangiocarci-
noma is attractive because of the lack of other effective therapeutic
agents for this cancer and is supported by our data showing
alterations in transformed cell growth in vitro. Although 5-aza-CdR
may have limited efficacy in the presence of increased IL-6, we

Figure 5. EGFR expression is increased by overexpression of IL-6. Mz-ChA-1 or
Mz-IL-6 cells were incubated with 5 μmol/L 5-aza-CdR for 24 hours before
isolation of total protein, and immunoblot analysis was done for EGFR protein
expression. The blots were stripped and reprobed for antibodies for β-actin.
Representative immunoblot along with quantitative data of EGFR/β-actin
expression relative to expression in Mz-ChA-1 cells. Columns, mean of four
separate experiments; bars, 95% confidence intervals. *, P < 0.05, compared
with Mz-ChA-1 control; #, P < 0.05, compared with Mz-IL-6.
expect that the sensitivity to 5-aza-CdR may be improved by strategies to simultaneously target IL-6 expression. These observations also suggest a mechanism by which other cancers may resist therapy with 5-aza-CdR that should be further investigated for other cancers.

Ablerrant hypomethylation and hypermethylation of CpG dinucleotides are observed in the genomes of many cancers, and epigenetic regulation of gene expression by deregulated gene promoter methylation has become recognized as an important mechanism involved in cancer development. The demonstration that an inflammation-associated cytokine can modulate gene expression of growth-regulatory pathways involved in epithelial carcinogenesis and tumor growth raises several important biological and clinically relevant questions. Can other cytokines modulate gene expression in a similar manner? How is gene selective expression achieved? What is the relevance to therapy with methylation inhibitors that are currently undergoing clinical trials? Can therapies targeting inflammatory responses enhance treatment responses? By providing evidence for the role of cytokine stimulation in tumor progression and a mechanism by which repeated exposure to the inflammatory cytokine IL-6 contributes to tumor growth, these studies emphasize the role of cytokines in modulating gene expression and tumorigenesis.

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