Interleukin-6 Regulation of the Human DNA Methyltransferase (HDNMT) Gene in Human Erythroleukemia Cells

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Methylation of mammalian DNA by the DNA methyltransferase enzyme (dnmt-1) at CpG dinucleotide sequences has been recognized as an important epigenetic control mechanism in regulating the expression of cellular genes (Yen, R. W., Vertino, P. M., Nelkin, B. D., Yu, J. J., el-Deiry, W., Cumaraswamy, A., Lennon, G. G., Trask, B. J., Celano, P., and Baylin, S. B. (1992) Nucleic Acids Res. 20, 2287–2291; Ramchandani, S., Bigey, P., and Spyropoulos, D. D., Pharr, P. N., Lavenburg, K. R., Jackers, P., Papas, T. S., Ogawa, M., and Watson, D. K. (1998) Mol. Cell. Biol. 15, 5643–5652). The data suggest that inflammatory cytokines such as IL-6 may exert many epigenetic changes in cells via the regulation of the methyltransferase gene. Furthermore, IL-6 regulation of transcription factors like Fli-1, which can help to direct cells along opposing differentiation pathways, may in fact be reflected in part by their ability to regulate the methylation of cellular genes.

The transfer of a methyl group to the cytosine portion of the CpG dinucleotide by dnmt-1 permits or enables the binding of methyl-specific DNA-binding proteins to the methylated CpG site (1, 2, 4, 5). The binding of methyl-specific proteins such as MeCP1 and MeCP2 to regulatory elements represses transcription by blocking the binding of other positive acting transactivation factors (6). Methyllytosine-DNA-binding proteins can attract histone deacetylases to the sites, which remodel chromatin into highly repressed states (7). Thus, DNA methylation can result in permanent epigenetic alteration of genes and is important in promoting or guiding the differentiation of cells and the establishment of tissue-specific gene expression patterns (8).

The inflammatory cytokine IL-62 is able to induce the maturation and differentiation of cells (9). Treatment of the human erythroleukemia cell line K562 with IL-6 induces the expression of megakaryocytic markers and the silencing of certain globin genes (10). Derived from an acute erythroblastic leukemia, K562 cells are multipotent in that they can be directed into two separate differentiation pathways (11). K562 cells express low levels of both erythrocytic- and megakaryocytic-specific genetic markers and can be induced to differentiate along one of these two major pathways depending upon the external stimuli applied to the cells (12, 13). This ability suggests some form of epigenetic control over the differentiation process. The ETS family of transcription factors represent a large family of differentially expressed, positive and negative regulators of transcription and are involved in cell differentiation (3). Here we show that when K562 cells are induced to enter the megakaryocytic differentiation pathway by IL-6, an increase in Fli-1 expression occurs, which results in the trans-activation of the human methyltransferase-1 gene expression.

EXPERIMENTAL PROCEDURES

Cell Culture—COS-1 cells were obtained from the American Type Culture Collection (CRL-1650) and maintained in Dulbecco’s modified Eagle’s medium high glucose supplemented with 10% FBS, glutamine, and penicillin-streptomycin solutions. Human erythroleukemia K562 cells (ATCC CCL-240) were maintained in RPMI 1640 medium supplemented with 10% FBS, glutamine, and penicillin-streptomycin solutions. Recombinant interleukin-6 (catalog number 200-06) was purchased from Pepro-Tech Inc. (Rocky Hill, NJ). For IL-6 stimulation, K562 cells were collected by centrifugation, resuspended in phosphate-buffered saline, pH 7.4, then resuspended in RPMI 1640 medium supplemented with glutamine, penicillin-streptomycin, and 0.05% FBS for 48 h, then treated with IL-6.

Methylation Assay—Cell nuclear pellets were freeze-thawed three times and centrifuged to remove debris. Clarified lysates were mixed with an equal volume of Chelex-100 resin (50%w/v) to remove DNA and RNA from the sample. For each replicate, 5 μg of the protein lysate was added to 200 μl of an assay mixture consisting of 20 μM Tris-HCl, pH 7.4, 5 mM EDTA, 25% glycerol, 0.5% Triton X-100, 1 μM dithiothreitol, 0.2 mM phenylmethylsulfonlfuriode, 5 μCi of S-adenosyl-L-[methyl-3H]methionine (12 Ci/mmol), 4 μg of poly(dI-dC), and 200 μg/ml bovine serum albumin and incubated at 37 °C for 2 h. Incorporated label was assessed by scintillation counting.

Reverse Transcription-PCR Assays—cDNAs for each gene were prepared from Trizol (Life Technologies, Inc.) extracted total RNAs. Reverse transcription reactions were run on 2 μg of total RNA. Following reverse transcription reactions, PCR reactions were run to the midpoint of each PCR fragment’s linear synthesis curve. Glyceraldehyde-3-phos-
We examined the effect of IL-6 treatment on methyltransferase activity by using K562 cells in a rested state in RPMI 1640 medium supplemented with 0.05% FBS for 48 h. The cells were rinsed twice in serum-free RPMI 1640 and then treated with IL-6 (100 ng/ml). After 8 h incubation, the cells were harvested, and methyltransferase activity assays were performed as described previously (14) to determine the relative levels of activity following IL-6 treatment. Lanes 1 and 2 in Fig. 1 show the results obtained from control reactions utilizing only cell lysates with no poly(dI-dC)poly(dI-dC) substrate added and poly(dI-dC)poly(dI-dC) substrate with no cell lysates added, respectively. Lane 3 represents the basal level of methylation activity obtained from rested K562 cells, while lane 4 shows a 3.2-fold increase in activity following treatment with IL-6. Based on these observations, treatment with IL-6 appears to increase overall methylation activity.

To determine whether treatment with IL-6 activates the hdnmt-1 promoter, we generated a series of deletion constructs as shown in Fig. 2A. The constructs were sequenced and used to transfect K562 cells, which were rested prior to stimulation with IL-6 as described above. Sequential deletion of increasing amounts of the wild-type promoter as shown in lane 1 (ΔMT1), lane 2 (ΔMT2) (−1214 to +71 bp), lane 3 (ΔMT3) (−815 to +71 bp), and lane 4 (ΔMT4) (−474 to +71) did not abrogate the IL-6-induced activity. The results shown in Fig. 2B, lane 5, indicate that IL-6-induced promoter activity is localized to the ΔMT5 segment (−243 to +71 bp), which encodes several potential ETS family recognition sites. Fig. 2C, lane 1, shows the results of transfecting wild-type ΔMT5 and then stimulating with IL-6. Unstimulated wild-type ΔMT5 reporter levels are represented in lane 2. Fig. 2C, lanes 3 and 4, show the activity levels of a ΔMT5 triple-mutant reporter stimulated with IL-6 and unstimulated, respectively. The ETS site-mutated ΔMT5 reporter shows a markedly suppressed response, as the loss of the three Fli-1 binding sites in ΔMT5 abrogated the IL-6-mediated response.

To determine whether IL-6 induced increased expression of hdnmt-1 and Fli-1 mRNA, K562 cells were incubated in RPMI 1640 medium supplemented with 0.05% FBS for 48 h. The cells were collected at 0, 1, 2, 4, 6, 8, 12, and 24 h post-treatment with IL-6. Total cellular RNA was prepared at each time point and stored at −70 °C. Ultraviolet spectroscopy was used to quantitate equally each RNA sample, and final working dilutions for each time point were rechecked following initial dilution to a concentration of 100 ng/μl. The adjusted total RNA preparations were then used to create cDNA, on which PCR reactions were performed. Using primers specific for hdnmt-1 and Fli-1, PCR was performed for each time point to determine the relative expression level of each gene. The temporal expression pattern of hdnmt-1 is shown in Fig. 3A. The expression of hdnmt-1 begins to appear at 6 h post-treatment, reaching a peak between 8 and 12 h. At 24 h, hdnmt-1 mRNA is still expressed, but the level is considerably diminished. The double-banded PCR product seen for the hdnmt-1 is due to an intronic insertion, and the PCR primers were chosen to amplify this region to determine whether both possible gene products are affected equally by IL-6 (15). In K562 cells, no difference in expression levels between the two possible hdnmt-1 mRNA products were noted.

The ETS family transcription factor, Fli-1, which is known to be expressed in megakaryocytic lineages as a mediator of differentiation (16), begins to be expressed at −4 h post-treatment (Fig. 3B) and continues to increase throughout the sampling period. The expression pattern of the prototypic ETS family member, Ets-1, did not show a response to IL-6 when cDNA from the rested K562 cells was analyzed in parallel reactions with hdnmt-1 and Fli-1, and Ets-1 did not produce a discernible band when the reactions were analyzed at the midpoint of the amplification curve (data not shown.) Fig. 3C shows equivalent expression levels of the GAPDH gene cDNA control at each time point.

Analysis of the ΔMT5 promoter elements reveals three potential Fli-1 binding sites at −194, −170, and −60 base pairs (17). A series of singular and multiple point mutations of the ΔMT5 constructs, shown in Fig. 4a, were co-transfected into COS-1 cells with pSG5Fli-1 expression plasmid to determine the authenticity of each potential ETS binding site. Fig. 4b shows the strongest activation with all three potential Fli-1 binding sites left intact. The intact promoter construct
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Fig. 2. A, *hdnmt-1* promoter constructs in pGL-3 Basic luciferase reporter vector. Methyltransferase I (HDNMT-1) promoter region spanning nucleotides 248–1950 from the published complete genomic clone sequence. Nucleotides encoding restriction endonuclease sites for *NheI* and *HindIII* were included in the 5′ ends of PCR primers used to generate various promoter deletion constructs. Primers used to construct promoter deletions are described under “Experimental Procedures.” B, activation of *hdnmt-1* promoter by IL-6 stimulation of K562 cells. Cells were transfected with 2 μg of plasmid by Fugene-6 reagent and allowed to incubate overnight. Cells were then rinsed in PBS, pH 7.4, and resuspended in resting medium as described. After 48 h, IL-6 was added, and cells were harvested the following morning (16 h). Lane 1, ΔMT1; lane 2, ΔMT2 (−1214 to +71 bp); lane 3, ΔMT3 (−815 to +71 bp); lane 4, ΔMT4 (−474 to +71 bp); lane 5, ΔMT5 (−243 to +71 bp). IL-6-induced promoter activity is localized to the ΔMT5 (−243 to +71 bp) segment. C, transactivation of *hdnmt-1* ΔMT5 reporter in IL-6-stimulated K562 cells. K562 cells were stimulated with IL-6 24 h post-transfection with wild-type and mutated MT-5 luciferase reporter plasmid. Lanes 1 and 2, transfected with wild-type ΔMT5 plasmid: lane 1, treated with IL-6; lane 2, no IL-6. Lanes 3 and 4, transfected with *Fli-1* binding site triple-mutated ΔMT5 plasmid: lane 3, treated with IL-6; lane 4, no IL-6.

![Diagram](http://www.jbc.org/)

Fig. 3. Reverse transcription-PCR analysis of IL-6-treated K562 cells. A, *hdnmt-1* 22 cycles; B, *Fli-1* 20 cycles; C, GAPDH 18 cycles; at 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 45 s.

ΔMT5-WT (lane 1) is transactivated nearly 29-fold over the triple mutant ΔMT5–25 (lane 8), which shows merely 2-fold activation over background. The other point-mutated ΔMT5 constructs showed varying degrees of activity, which suggests an additive effect for each site, with the constructs containing only single site mutations ΔMT5–20 (lane 3), ΔMT5–22 (lane 5), and ΔMT5–23 (lane 6), showing the most activity when compared with those constructs receiving two combined site mutations ΔMT5–19 (lane 2), ΔMT5–21 (lane 4), and ΔMT5–24 (lane 7). The mutation of the double ETS binding sites (−194 and −170) in the ΔMT5–24 construct (lane 7) produced nearly the same effect as the triple mutant, with a 5-fold increase in activity compared with 2-fold for the triple mutant.

These results provide evidence of a novel mechanism of IL-6 cytokine-mediated alteration, via the *Fli-1* transcription factor, of methyltransferase gene expression. Previously, it was shown that IL-6 activation of the immediate-early gene junB occurred through an ETS family protein, in cooperation with a CREB-ATF factor (18). An analogous situation exists in *fos* transformed cells, in which the expression of DNA methyltransferase is three times that of normal levels (19). Thus, it has been proposed that *fos* transformation is mediated by increased methyltransferase expression. Therefore, by analogy, even slight alterations in methyltransferase expression resulting from chronic exposure to IL-6 could, over time, result in abnormal patterns of cellular DNA methylation, similar to those caused by transformation of *fos*. Indeed, the importance of *dnmt-1* activity in the establishment and propagation of neoplastic growth has emerged as an important diagnostic factor (20). Methylated CpG dinucleotides are susceptible to spontaneous deamination of 5-methylcytosine to uracil and are believed to be responsible for approximately one-third of C-T transition mutations found in human genetic diseases and tumors (21). Hypermethylation of tumor suppressor genes such as p53 (22), retinoblastoma (Rb) (23), and p16ink (24) occur in many different tumors types, serving to promote tumor growth by rendering these genes inactive. The effects of promiscuous methylation of important tumor suppressor and cell cycle regulatory genes potentially resulting from prolonged exposure to
inflammatory cytokines are probably cumulative in nature, remaining latent until sufficient insult to the cell permits it to transform into a neoplastic growth (25).

We demonstrate here that IL-6, an inflammatory cytokine capable of mediating cellular differentiation, is capable of increasing DNA methytransferase expression and activity. The data suggest that one of the normal molecular consequences of the biological activity of IL-6 may be mediated by DNA methyltransferase activity modifying gene expression. Additionally, IL-6 has been implicated in numerous cancer models, including multiple myeloma and prostate carcinoma (26,27). However, in a few cell lines, IL-6 has shown inhibitory effects on tumorigenesis (28) and anti-inflammatory capacity (29). Notwithstanding these pleiotropic characteristics, chronic exposure of cells to inflammatory cytokines such as IL-6 may have serious consequences by altering the normal levels, or time of expression of many genes.

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Fig. 4. a, site-specific mutations introduced into hdnmt-1 promoter region containing putative Fit-1 binding sites. Mutations are shown in bold text for each site. b, identification of putative Fit-1 binding sites by site-specific mutagenesis. Lane 1, ΔMT5, wild-type; lane 2, ΔMT5–19; lane 3, ΔMT5–20; lane 4, ΔMT5–21; lane 5, ΔMT5–22; lane 6, ΔMT5–23; lane 7, ΔMT5–24; lane 8, ΔMT5–25, triple mutant. c, depicts luciferase activity (-fold activation) of corresponding mutations (shown as black bars) in putative Fit-1 binding sites.
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