DNA-hypomethylating agent, 5'-azacytidine, induces cyclooxygenase-2 expression via the PI3-kinase/Akt and extracellular signal-regulated kinase-1/2 pathways in human HT1080 fibrosarcoma cells

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Abstract. The cytosine analogue 5'-azacytidine (5'-aza) induces DNA hypomethylation by inhibiting DNA methyltransferase. In clinical trials, 5'-aza is widely used in epigenetic anticancer treatments. Accumulated evidence shows that cyclooxygenase-2 (COX-2) is overexpressed in various cancers, indicating that it may play a critical role in carcinogenesis. However, few studies have been performed to explore the molecular mechanism underlying the increased COX-2 expression. Therefore, we tested the hypothesis that 5'-aza regulates COX-2 expression and prostaglandin E2 (PGE2) production. The human fibrosarcoma cell line HT1080, was treated with various concentrations of 5'-aza for different time periods. Protein expressions of COX-2, DNA (cytosine-5)-methyltransferase 1 (DNMT1), pAkt, Akt, extracellular signal-regulated kinase (ERK), and phosphorylated ERK (pERK) were determined using western blot analysis, and COX-2 mRNA expression was determined using RT-PCR. PGE2 production was evaluated using the PGE2 assay kit. The localization and expression of COX-2 were determined using immunofluorescence staining. Treatment with 5'-aza induces protein and mRNA expression of COX-2. We also observed that 5'-aza-induced COX-2 expression and PGE2 production were inhibited by S-adenosylmethionine (SAM), a methyl donor. Treatment with 5'-aza phosphorylates PI3-kinase/Akt and ERK-1/2; inhibition of these pathways by LY294002, an inhibitor of PI3-kinase/Akt, or PD98059, an inhibitor of ERK-1/2, respectively, prevents 5'-aza-induced COX-2 expression and PGE2 production. Overall, these observations indicate that the hypomethylating agent 5'-aza modulates COX-2 expression via the PI3-kinase/Akt and ERK-1/2 pathways in human HT1080 fibrosarcoma cells.

Introduction

Fibrosarcoma is a malignant cancer that originates in the connective tissue found at the ends of bones of the legs or arms, and then spreads to the surrounding soft tissues. Soft tissues include joint tissue, blood vessels, fat, muscles, tendons, and fibrous tissue. Although fibrosarcoma-related morbidity is rare, patient survival rates are low (1).

During the last few decades, epigenetics has been one of the emerging fields in cancer research (2). Epigenetics affects the transcription of cells, thereby regulating gene expression. Abnormal epigenetic changes can have adverse effects on the organism. Methylation of cytosine-phosphate-guanine (CpG) islands in the promoter region of a gene has now been strongly linked to gene silencing.

DNA methylation is regulated by DNA methyltransferases (DNMT1, DNMT3A and DNMT3B), which catalyze the transfer of a methyl group from S-adenosyl-l-methionine (SAM) to the cytosine of a CpG dinucleotide (adjacent within a single DNA strand), immediately following replication (3). DNMTs are classified as maintenance or de novo methyltransferases. Maintenance DNMT1 binds methyl groups to methylated DNA during replication, whereas de novo DNMT3A and DNMT3B add methyl groups to CpG dinucleotides of unmethylated DNA. Previous reports have shown that some anticancer cascades abnormally activate the DNMT1, enzyme that maintains the DNA methylation pattern (3).

When the levels of DNMT1 decrease, as is the case following azacytidine or decitabine treatment, daughter strands are less likely to undergo maintenance to restore full methylation; thus, with each replication, CpG pairs become unmethylated, rendering their promoter regions more accessible to transcription factors.

DNA damage induced by 5'-azacytidine (5'-aza) is reversible, since the drug does not influence de novo DNMT synthesis (4-6). 5'-aza has been used clinically for treating diverse diseases such as acute myelogenous leukemia, hemotological malignancies, gastrointestinal, lung, ovarian, prostate, breast, and head and neck cancers, melanoma, and malignant...
mesothelioma (7). Global hypomethylation is a hallmark of cancer (8). It was believed that global hypomethylation principally targets repetitive sequences, but some genes involved in metastasis were also previously indicated to be hypomethylated in cancers (9).

There are three major isoforms of cyclooxygenase (COX), COX-1, COX-2, and COX-3. COX-1, a constitutive isoform, plays a role in modulating physiologic activities in tissues. COX-2 is an inducible isoform of the enzyme that responds to specific stimuli such as growth factor, hormones, endotoxins, and cytokines (10,11). The third isoform, COX-3, an inactive protein, is an alternative splice variant of COX-1 (12).

Overexpression of COX-2 in tumors is linked to the overproduction of the pro-inflammatory prostaglandin Eα (PGEα) (13,14). It may also trigger the acquisition of essential cancer traits (15), including inhibition of apoptosis (16,17), immunosuppression (18), continued proliferation (19), invasion (20), angiogenesis (21,22), and metastasis (23,24).

COX-2 is usually associated with inflammation and is markedly upregulated in various types of cancer, as well as in other diseases (25-28).

The methylation status of the COX-2 promoter was shown in several cancers, and some research showed that the transcriptional silencing of COX-2 is involved in the methylation status of the CpG pairs of the COX-2 gene (29,30). However, the effects of 5'-aza on COX-2 expression in human fibrosarcoma HT-1080 cells have not been reported.

In this study, we examined whether 5'-aza regulates COX-2 expression in human fibrosarcoma HT-1080 cells. We showed that 5'-aza increases COX-2 expression and PGEα production, and identified the signaling pathways involved in these mechanisms.

Materials and methods

Reagents and chemicals. 5'-Azacytidine was purchased from Sigma-Aldrich (St. Louis, MO, USA). PD 98059 was purchased from Calbiochem (San Diego, CA, USA) and LY 294002 was purchased from Tocris (Bristol, UK). Cell culture medium and fetal bovine serum (FBS) were obtained from Invitrogen (Gaithersburg, MD, USA). PCR primers were purchased from Genotek Co., (Daejeon, Korea).

Cell culture and experimental conditions. HT1080 cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cell line was cultured in RPMI-1640 containing 10% (v/v) FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin (Sigma-Aldrich) at 37°C in a humidified incubator containing 5% CO2. The medium was refreshed daily over a period of 2 days. Treatment with drugs was performed as indicated in the figure legends.

Western blot analysis. Cells were washed with PBS and lysed using RIPA buffer containing 50 mM Tris-HCl; pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% sodium dodecyl sulfate (SDS), supplemented with inhibitors for proteases and phosphatases. Then the lysates were clarified by centrifugation (1,300 rpm, 10 min, 4°C) and collected. Proteins were size-fractionated using SDS-PAGE and transferred to a nitrocellulose membrane (Whatman Schleicher and Schuell, Dachen, Germany). Antibodies against DNMT-1, COX-2, pAkt, Akt, pERK, ERK, and β-actin were used (Cell Signaling Technology, Beverly, MA, USA). The nitrocellulose sheet was blocked with 5% non-fat dry milk in Tris-buffered saline for 1 h. Proteins were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies, followed by enhanced chemiluminescence (ECL). Blots were developed using an image-Quant LAS 4000 (Amersham Biosciences Corp, Piscataway, NJ, USA).

Prostaglandin Eα (PGEα) assay. Cells (2x10⁴ cells/well) were seeded onto 96-well plates. At 24 h after treatment, conditioned medium was harvested, and PGEα concentrations were determined using an ELISA assay kit according to instructions supplied by the manufacturer (Assay Designs, Ann Arbor, MI, USA). Samples were assayed in triplicate in each of three independent experiments. PGEα levels were calculated against a standard PGEα curve.

Immunofluorescence (IF) staining. Cells were treated for 24 h with or without 5'-aza in the presence of inhibitor, PD 98059 or LY 294002. Cells were harvested, fixed with ice-cold 3.5% paraformaldehyde (Sigma-Aldrich) for 15 min, and washed with ice-cold phosphate-buffered saline (PBS). Cells were then permeabilized with 0.1% Triton X-100 for 15 min, washed with ice-cold PBS, and stained with antibodies against COX-2. Cell nuclei were stained with 4i,6-diamidino-2-phenylindol (DAPI, Molecular Probes/Invitrogen, Carlsbad, CA, USA) and were observed using a fluorescence microscope (Olympus, Tokyo, Japan) with peak excitation wavelengths at 570 and 460 nm.

Data analysis and statistical analyses. Values are presented as the mean ± Standard deviation (SD) of at least three independent experiments. Data were evaluated using the one-way ANOVA. P<0.05 was considered to indicate statistically significant differences between values.

Results

This study aimed to determine whether 5'-aza regulates COX-2 expression in HT1080 cells. HT1080 cells treated with 5'-aza
exhibited augmented COX-2 expression in comparison with untreated cells (Fig. 1A). A concentration-independent increase in COX-2 expression was observed (Fig. 1A). Stimulation of cells with 5'-aza markedly increased COX-2 expression, which was evident within 6 h, and reached a maximum at 24 h (Fig. 1C). Densitometric evaluation of a representative western blot experiment was performed in triplicate (Fig. 1A and C, right panels).

To assess the effect of 5'-aza on COX-2 activity, we quantified the production of PGE\(_2\) in HT1080 cells untreated and treated with 5'-aza for 24 h (Fig. 1B and D). A significant increase in PGE\(_2\) synthesis was verified in HT1080 cells treated with 5'-aza. The increase in PGE\(_2\) production and COX-2 expression induced by 5'-aza, was similar (Fig. 1B and D).

COX-2 expression is highly regulated via both transcriptional and post-transcriptional mechanisms, depending on its activator and the cell type (31,32). To verify the 5'-aza-induced COX-2 expression at the mRNA level, we treated the HT1080 cells with 5'-aza. RNA was extracted from the cells, and COX-2 mRNA levels were detected using RT-PCR. COX-2 expression increased in a dose- and time-dependent manner in cells treated with 5'-aza (Fig. 2). To correct differences in loading, the signal density of each COX-2 band was divided by the signal density of the GAPDH band (Fig. 2).

Several studies have shown the involvement of the PI3K/ Akt and ERK-1/2 pathways in the regulation of COX-2 expression and PGE\(_2\) synthesis (33,34).

In this study, we found that 5'-aza phosphorylated ERK-1/2 and Akt. This observation was evident within 1 h of treatment.
Figure 3. Treatment of 5′-aza activates the PI3K/Akt and ERK1/2 pathways. (A) Chondrocytes were treated with and without various concentrations of 5′-aza for 12 h. (B) Chondrocytes were treated with and without 10 µM 5′-aza for the indicated time periods. (A and B) Activation of pAkt, Akt, pERK, and ERK was analyzed using western blot analysis. Actin was used as a loading control.

Figure 4. Inhibition of PI3K/Akt and ERK1/2 pathways prevents COX-2 expression and PGE2 production induced by 5′-aza. Chondrocytes were treated with and without various concentrations of 5′-aza in the absence and presence of 10 µM PD98059 (PD) and 10 µM LY294002 (LY). (A) Expression of COX-2, DNMT-1, pAkt, Akt, pERK, ERK and actin was detected using western blot analysis. Actin was used as a loading control. (B) The expression level of COX-2 was determined using RT-PCR. GAPDH was used as a loading control. (C) Secreted PGE2 was quantified using the PGE2 assay. (D) The expression of COX-2 was determined using immunofluorescence staining. The nuclei of cells were stained with 4′-6-diamidino-2-phenylindole (DAPI). The results represent three independent experiments. The data represent three similar experiments.

Figure 5. SAM inhibits 5′-aza-induced COX-2 expression. (A and B) Chondrocytes were treated with and without 10 µM 5′-aza in the absence and presence of 20 µg/ml SAM. (A) Expression of COX-2, DNMT-1, and actin was detected using western blot analysis. Actin was used as a loading control. (B) The expression level of COX-2 was determined using RT-PCR. GAPDH was used as a loading control. The data represent three similar experiments.
with phosphorylation levels reaching a maximum at 12 h, and then decreasing (Fig. 3B). The total ERK-1/2 and Akt remained consistent through the duration of the experiments (Fig. 3).

To determine whether ERK-1/2 and PI3K/Akt are involved in 5'-aza-induced COX-2 expression and PGE2 synthesis, cells were treated with the ERK-1/2 inhibitor, PD 98059, and the PI3K/Akt inhibitor LY 294002 (Fig. 4).

Pre-treatment with PD 98059 or LY 294002 followed by stimulation with 5'-aza resulted in inhibition of 5'-aza-induced effects on COX-2 mRNA expression and protein levels (Fig. 4A and B), and PGE2 synthesis (Fig. 4C). Immunofluorescence microscopy further indicated that treatment with 5'-aza dramatically increased COX-2 expression, but these effects were inhibited by treatment with PD 98059 and LY 294002 (Fig. 4D). Taken together, these results indicate that the ERK-1/2 and Akt pathways participate in the 5'-aza-induced increase in COX-2 expression and PGE2 production (Fig. 4).

SAM is a methyl donor and an inhibitor of DNA methylation (35). We therefore examined and observed that SAM could inhibit the 5'-aza-induced COX-2 expression (Fig. 5A). Similar to COX-2 protein expression levels, COX-2 mRNA levels were also altered by SAM (Fig. 5B). These data demonstrate that COX-2 expression induced by 5'-aza is regulated by alterations in DNA methylation (Fig. 5).

**Discussion**

COX-2 is transcriptionally modulated in normal tissues by various factors including pro-inflammatory cytokines, tumor necrosis factor (TNF)-α (36), interleukin (IL)-1β (37), interferon (IFN)-γ (38), and bacterial endotoxin (36).

Overexpression of COX-2 promotes cell growth, but at the same time leads to cell cycle arrest in diverse cell types (39,40). In other words, the upregulated COX-2 expression may inhibit proliferation in the short-term, but ultimately allows cancer growth in the long-term. PGE2 is a major metabolite derived from arachidonic acid and its production is mediated by the action of COX-2. PGE2 inhibits cell growth in melanocytes (41), gastric cancer cells (42), colonic epithelial cells (43), and neuroblastoma cells (44).

In contrast, it has been reported that increased levels of PGE2 accelerates cell motility, alters morphology (45), and has growth-promoting activity via the epidermal growth factor (EGF) receptor (46).

Therefore, increased COX-2 expression and upregulated PGE2 production lead to various responses in carcinogenesis. Treatment with COX-2 inhibitors is known to reduce proliferation and cause apoptosis of cancers, but our previous results indicate that there is no relationship between COX-2 expression and proliferation of HT1080 cells (47). Although various studies on COX-2 regulation are available, the molecular basis of COX-2 expression in cancer has not been established yet.

DNA methylation is an epigenetic mechanism of gene silencing, with no effects on chromatin structure (48). Global hypomethylation at repetitive sequences causes genomic instability. Aberrant hypomethylation of CpG islands in promoter region of a gene has been implicated in the transcriptional silencing of various genes in carcinogenesis (48).

Studies have indicated that COX-2 promoter methylation may be an additional regulator of COX-2 expression in some cancer cell lines (29,49).

In this study, we therefore examined whether COX-2 expression in human HT1080 fibrosarcoma cells is regulated through DNA methylation upon treatment with 5'-aza. We demonstrated that COX-2 expression and PGE2 production were regulated by the methylation status of the COX-2 promoter. Although overexpression of COX-2 has been observed in many cancers, concomitant increases in COX-2 expression may not be detected in HT1080 cells. However, after treatment with 5'-aza, increased COX-2 expression became detectable, consistent with the increased COX-2 mRNA levels (Figs. 1 and 2).

These findings suggest that methylation of the COX-2 promoter may be associated with transcriptional down-regulation of the gene. We also examined the possibility that the opposing effects of DNA demethylation might be addressed by combining a DNA-demethylating agent with a DNA-methylating agent. SAM is synthesized in humans from the methyl donors present in the diet, and is a natural compound that is a cofactor of methylation reactions in vivo. We therefore tested whether SAM antagonizes the effect of 5'-aza (Fig. 5). We demonstrated that SAM inhibits the global hypomethylation caused by 5'-aza and antagonizes the effect of 5'-aza on COX-2 expression.

Promoter hypomethylation by 5'-aza upregulated COX-2 expression and PGE2 production (Figs. 1 and 2), while hypermethylation by SAM resulted in the opposite effect (Fig. 5).

Many reports indicate that mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways are involved in regulating COX-2 expression and PGE2 production (50,51). We assessed the role of MAPKs and PI3K activation in 5'-aza-induced COX-2 expression and PGE2 production (Figs. 3 and 4). 5'-aza activated ERK-1/2 and Akt, but not p38 and c-Jun N-terminal kinase (JNK) in HT1080 cells (data not shown). Therefore, another major finding of our study is that 5'-aza activates the ERK-1/2 and PI3K/Akt pathways (Fig. 3).

In conclusion, our data suggest that the expression of COX-2 in HT1080 cells is regulated by DNA methylation status, and these effects are regulated by ERK-1/2 and PI3K/Akt pathways.

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