Promoter hypermethylation silences cyclooxygenase-2 (*Cox-2*) and regulates growth of human hepatocellular carcinoma cells

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Cyclooxygenase-2 (COX-2) upregulation is recognized to confer advantage in progression in a wide variety of cancers, with colorectal cancer most intensively investigated. Epidemiologically, chemopreventive effects of COX-2 inhibitors have been proven on numerous cancers, but not on hepatocellular carcinoma (HCC). Although the antiapoptotic feature of COX-2 generally supports cancer cell growth, previous reports have shown that COX-2 expression, upregulated in early HCC, is downregulated in advanced HCC. Therefore, COX-2 downregulation may be somehow advantageous and specific for HCC development. However, its mechanism remains unclear. Since promoter hypermethylation often silences the gene expression, we hypothesized that the epigenetic mechanism might regulate COX-2 expression in HCC. We examined the methylation status of the *Cox-2* promoter in six human HCC cell lines (Hep3B, HepG2, SK-Hep1, HuH7, PLC, and FLC-7 cells) using methylation-specific PCR. The promoter was remarkably hypermethylated in Hep3B and FLC-7 cells and moderately in HepG2 and SK-Hep1 cells, but not in HuH7 and PLC cells. In Hep3B cells, coincubation with 5-aza-2'-deoxycytidine, a demethylator, demethylated the promoter and upregulated COX-2 expression as well as prostaglandin E₂ production dose dependently. On the other hand, no such effects were observed in HuH7 cells. Additionally, the methylator suppressed growth of Hep3B cells dose dependently, accompanied by cyclin D1 downregulation, and the growth suppression was abrogated by potent COX-2 inhibition with a COX-2 selective inhibitor celecoxib, but these responses were not found in HuH7 cells. These results indicated that cell growth was largely retarded by *Cox-2* upregulation via promoter demethylation, rather than the potentially reactivated genes concurrently demethylated by 5-aza-2'-deoxycytidine. In conclusion, promoter hypermethylation transcriptionally silences *Cox-2* in HCC cells. Epigenetic alteration of *Cox-2*, at least in part, modulates the growth of HCC cells.

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Genetic changes, such as mutations and translocations, and epigenetic changes, without a change in DNA sequence, play a major role in carcinogenesis. DNA methylation is one of epigenetic changes and hypermethylation of CpG islands in the promoter region is a powerful mechanism for the suppression of gene activity by transcriptional silencing.^{1–3}

In normal cells, methylation of CpG islands generally occurs only in two circumstances: on the inactive X chromosome in female somatic cells and on silenced alleles of parentally imprinted genes.^{4,5} Alterations of global DNA methylation have been found more often in a variety of cancers than in normal postnatal tissues,^{1,6,7} and aberrant DNA methylation is implicated in tumorigenesis.^{8,9} Especially, aberrant hypermethylation of the promoter of tumor suppressor genes can contribute to the formation and progression of cancer.^{1,2} This inappropriate gene silencing affects virtually every step in tumor progression.³

Cyclooxygenase (COX) is a rate-limiting enzyme in the production of prostaglandins (PGs) and thromboxanes, and COX-2 is inducible by a variety

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of cytokines, hormones, and tumor promoters.^{10–13} Upregulation of COX-2 has been demonstrated in various tumor tissues such as colorectal,¹⁴ gastric,¹⁵ esophageal,¹⁶ pancreatic,¹⁷ and lung cancers.¹⁸ COX-2 overexpression harbors antiapoptotic¹⁹ and pro-angiogenic²⁰ properties as well as high metastatic potentials.^{21,22} COX-2 upregulation has also been reported in hepatocellular carcinoma (HCC) tissue and cell lines^{23–28} and in non-tumorous liver tissue.^{24,28,29}

Interestingly, early HCC expresses COX-2 more frequently and strongly than advanced HCC.^{24,26–28} Meanwhile, we have previously reported that COX-2 is overexpressed in advanced gastric cancer rather than early cancer.¹⁵ Therefore, the generally accepted idea that COX-2 is overexpressed along cancer development is not directly applied to HCC, and COX-2 overexpression may play a major role in the oncogenic process in HCC but have a different function in advanced HCC.

COX-2 upregulation allows advantages for tumor cells to grow, but at the same time causes cell cycle arrest in a variety of cells.^{30,31} In other words, COX-2 upregulation may suppress proliferation in the short term, but ultimately supports tumor growth in the long term. PGE₂ is a major metabolite of arachidonic acid and its production is catalyzed by COX-2. E series PGs inhibit cell growth in melanoma,³² neuroblastoma,³³ gastric cancer,^{34,35} and colonic epithelial cells.^{36,37} In contrast, it has also been reported that PGE₂ increases cell motility and alters colony morphology,³⁸ and has growth-promoting activity via transactivation of EGF receptor.³⁹ Therefore, COX-2 upregulation and increased PGE₂ production exert diverse actions in carcinogenesis, and clarification of the regulation of COX-2 expression is inevitable to better understand the mechanism of cancer development. Given that COX-2 expression changes over HCC progression, it is undoubtedly important to elucidate the mechanism of COX-2 regulation.

COX-2 inhibitors or nonsteroidal antiinflammatory drugs (NSAIDs) are known to reduce clinically the risk of fatal cancer such as esophageal, gastric, and colorectal cancer.^{40–42} Unfortunately, epidemiological evidence is lacking as to the growth-inhibitory effects of COX-2 inhibitors on HCC, and whether or not COX-2 inhibitors harbor chemopreventive properties on HCC has yet to be investigated.

Recently, *Cox-2* promoter hypermethylation in colorectal and gastric cancer cell lines has been reported.^{43–45} In cases of HCC, a variety of genes are abnormally methylated, including *Cox-2* promoter.⁴⁶ However, it has yet to be investigated whether hypermethylation of the *Cox-2* promoter indeed regulates its gene expression and modulates growth of HCC cells.

In this study, we explored the significance of the *Cox-2* promoter hypermethylation in HCC cells using methylation-specific PCR (MSP). In addition, we investigated the effect of treatment with a

demethylating agent 5-aza-2'-deoxycytidine (5-Aza-CdR) on HCC cells. This study provided new evidence that hypermethylation of the *Cox-2* promoter silenced the gene expression and the mechanism might contribute to the alteration of cell growth. To our knowledge, this is the first report to demonstrate the regulatory mechanism of *Cox-2* by promoter hypermethylation and implicate its significance in the growth modulation of HCC cells.

Materials and methods

Cell Culture

Experiments were performed on six human HCC cell lines: Hep3B, HepG2, SK-Hep1, HuH7, PLC, and FLC-7 cells. HepG2 cells were established from a human hepatoblastoma, and the remaining five cell lines were derived from human HCC arising in adults. HepG2, SK-Hep1, and HuH7 cells do not have detectable hepatitis B viral sequences, while Hep3B and PLC cells have integrated HBV sequences.⁴⁷ Hep3B cells have a partial deletion of the *p53* gene while HuH7 cells have a point mutation in *p53*.⁴⁸ They were obtained from the American Type Culture Collection, and grown in DMEM (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 0.5% Antibiotics-Antimycotic[®] (Invitrogen Corp., Carlsbad, CA, USA) at 37°C in 5% CO₂ atmosphere.

Methylation-Specific PCR

DNA methylation patterns in the *Cox-2* promoter were determined by MSP as essentially described by Kane *et al.*⁴⁹ Briefly, cells were cultured to subconfluence and harvested to extract genomic DNA using the QIAamp DNA blood mini kit (Qiagen, Germany). DNA (1 µg) was incubated at 37°C for 24 h in a 50-µl reaction for digestion containing 20 U of *MspI*, 20 U of *HpaII* (New England Biolabs Inc., Beverly, MA, USA), or no enzyme. PCR was carried out in a 25-µl reaction mixture containing 2 µl of each digest and 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). Three sets of primers were designed to amplify the *Cox-2* promoter sequence containing one CpG site each: –431 nucleotides (nt) (A), –372 nt (B), and –138 nt (C) from the transcription starting site to produce 159, 101, and 111 bp amplicons, respectively. Primer sequences were: (A) 5'-TTCTTAACCTTACTCGCCC-CAG-3' (sense) and 5'-AGAAGGACACTTGGCTTCC TC-3' (antisense); (B) 5'-AGGAAGCCAAAGTGTCTCT-3' (sense) and 5'-TTCTTCTTCGCAGCTTTTGC-3' (antisense); and (C) 5'-TAAAGAAACCTGCCCCCA-3 (sense) and 5'-GCCCATGTGACGAAATGACT-3' (antisense). After 10 min of initial heat denaturing at 95°C, DNA was amplified by 30 cycles of denaturing at 95°C

for 15 s and annealing and extension at 58°C for 60 s, followed by 7 min at 72°C. To ensure the reproducibility, the same experiments were performed three times.

RT-PCR

RNA was extracted from cultured cells using the RNeasy Mini kit (Qiagen). Reverse transcription was carried out with 1 µg of total RNA as a template with ReverTra Ace-α- (Toyobo, Japan) according to the manufacturer's instructions. Then, cDNA was amplified by PCR with primers specific for *Cox-2* and *GAPDH* as described by Takahashi *et al.*⁵⁰ RT-PCR for *Cox-2* and *GAPDH* was carried out in a solution containing 1 µl of cDNA, 1 × PCR buffer, 200 µM of each deoxynucleotide triphosphate, 0.4 µM of each primer, and 1 U of *Taq* DNA polymerase (Qiagen).

Western Blot Analysis

After washing the cells with PBS (9.57 mM, pH=7.35–7.65), HCC cells were harvested and lysed in RIPA buffer (1 × PBS/1% Nonidet P-40/0.5% sodium deoxycholate/0.1% SDS) containing 0.1 mg/ml of PMSF and 51 µg/ml of aprotinin as final concentrations, followed by incubation on ice for 1 h and centrifugation at 4°C for 20 min at 12 000 g. Protein concentrations of the cell lysates and supernatants were determined by the DC Protein Assay kit (Bio-Rad Laboratories, CA, USA). Then, 50 µg of protein samples was resolved on 10% SDS-polyacrylamide gels and transferred to PVDF membranes. After blocking with 5% nonfat powdered milk in TBS containing 0.1% Tween 20 (TBS-T) for 1 h, the membrane was incubated overnight at 4°C with anti-human COX-2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-cyclin D1 antibody (Santa Cruz), or GAPDH (Chemicon, Temecula, CA, USA). The membrane was washed with TBS-T three times and incubated with anti-rabbit immunoglobulin G (DAKO, Denmark) for 1 h. After washing with TBS-T again, protein was visualized by the SuperSignal[®] chemiluminescence system (Pierce, Rockford, IL, USA).

Treatment with 5-Aza-CdR and Celecoxib

Cells were seeded at a density of 1×10^5 cells/100 mm-dish and allowed to attach over 24 h. To the medium, 5-Aza-CdR (Sigma) was added at a final concentration of 1, 5, and 10 µM, and cells were cultured for 96 h. The medium was replaced every 24 h with a newly prepared medium containing 5-Aza-CdR.

To examine the effect of COX-2 inhibition, celecoxib (Pharmacia, St Louis, MO, USA), a COX-2-selective inhibitor, was added at a final concentration of 1 µM and cells were cultured for 96 h with

different concentrations of 5-Aza-CdR. Then, the supernatant was collected for the measurement of PGE₂.

PGE₂ Measurement

After cells were treated with 5-Aza-CdR at different concentrations for 96 h, the supernatant was collected for PGE₂ measurement by an ELISA kit (Cayman Chemical, Ann Arbor, MI, USA). The concentrations of PGE₂ were expressed as pg/µg cell protein.

Cell Number

Cells were seeded at a density of 2×10^3 /100 µl/well on 96-well plastic plates (about 20% confluence) and were cultured at 37°C in DMEM containing 10% FBS and different concentrations of 5-Aza-CdR. After incubation for 96 h, WST-8 assay was carried out. After 10 µl of the cell count reagent SF (Nacalai Tesque Inc., Japan) was added to each well and the cells were incubated for 3 h at 37°C, the absorbance was measured at 450 nm with a reference wavelength at 630 nm.

Lactate Dehydrogenase Assay

Lactate dehydrogenase (LDH) was measured to examine the cell viability after 5-Aza-CdR treatment. The culture medium was collected and centrifuged at 800 g for 10 min to obtain a cell-free supernatant. The supernatant was frozen for storage and sent to SRL Inc. (Tokyo, Japan) for measurement of LDH.

Statistical Analysis

The results were shown as mean ± s.d. The statistical significance was determined by using Student's *t*-test. A *P*-value less than 0.05 was considered significant.

Results

Cox-2 Promoter Hypermethylation in HCC Cell Lines

The methylation status of the *Cox-2* promoter was analyzed in six human HCC cell lines (Hep3B, HepG2, HuH7, PLC, FLC-7, and SK-Hep1). The *Cox-2* promoter region contains 51 methylatable CpG sites spanning from –590 to +186 nt in relation to the transcription starting site⁴⁴ (Figure 1). This satisfies the established definition of a CpG island (776 bp fragments with a G + C content > 0.57 and an observed/expected presence of CpG > 0.8).^{44,51,52} The methylation status was analyzed at three CpG sites, –431 nt (A), –372 nt (B), and –138 nt (C) by MSP. Genomic DNA was digested with a methylation-sensitive restriction enzyme *HpaII*, which could

Figure 1 CpG sites in *Cox-2* promoter and targeted sites for MSP. The *Cox-2* promoter contains 51 CpG sites. Three sets of primers were designed to amplify the *Cox-2* promoter sequence containing one CpG site each: -431 nt (a), -372 nt (b), and -138 nt (c) from the transcription starting site. Transcription factor binding sites are also depicted.

Figure 2 *Cox-2* promoter hypermethylation in HCC cells. Hyper-methylation of the *Cox-2* promoter was examined by MSP in six HCC cell lines. At the three CpG sites of (a), (b), and (c), MSP produced 159, 101, and 111 bp amplicons, respectively. "C", "M", and "H" represent the amplification of the control (no restriction enzyme), *MspI* (methylation insensitive), and *HpaII* (methylation sensitive) digest, respectively. The presence of a band in the "H" column indicates that the CpG site is methylated. To confirm the cleavable CpG site without mutation, *MspI* digest was subject to MSP. The demonstrated figure was a representative result of three independent experiments that provided essentially similar data.

cleave a CpG site when it was not methylated. To ensure that the CpG site was not mutated and cleavable, the same DNA was digested with a methylation insensitive restriction enzyme *MspI*, which could cleave the CpG site irrespective of the methylation status. In short, when the CpG site was methylated, amplification of the *HpaII* digest should produce a band similar to the one in the control batch (without any restriction enzyme).

As shown in Figure 2, the methylation status at the three sites tended to be similar in each cell line. The promoter in HuH7 and PLC cells was unmethylated at these three sites. In contrast, the promoter in Hep3B and FLC-7 cells was remarkably hypermethylated, while that in HepG2 and SK-Hep1 cells was moderately hypermethylated. Some CpG sites such as (A) in HepG2, and (C) in Hep3B, FLC-7, and SK-Hep1 cells seemed to be half methylated.

5-Aza-CdR Treatment Reactivated the Silenced COX-2

Hep3B cells with the hypermethylated *Cox-2* promoter and HuH7 cells with the unmethylated promoter were treated for 96 h with different concentrations of a demethylating agent 5-Aza-CdR. As shown in Figure 3a, although 5-Aza-CdR treatment at 1 μ M had little effect, the agent demethylated the promoter dramatically at 5 and 10 μ M at all the CpG sites examined in Hep3B cells.

Figure 3 Demethylation by 5-Aza-CdR treatment. Hypermethylation in the *Cox-2* promoter was examined by MSP after 96 h of treatment with 5-Aza-CdR at 1, 5, and 10 μ M in Hep3B cells (a) and HuH7 cells (b). At all the three CpG sites, 5-Aza-CdR significantly demethylated the promoter in Hep3B cells in a dose-dependent manner. No such change was observed in HuH7 cells. The experiments were repeated three times and essentially similar data were obtained. Representative results were shown.

On the other hand, as was expected, all the examined CpG sites remained unmethylated in HuH7 cells after 5-Aza-CdR treatment (Figure 3b). At a concentration of 20 μ M, the agent was cytotoxic enough to detach both Hep3B and HuH7 cells from the plate (data not shown).

As shown in Figure 4a, 5-Aza-CdR treatment for 96 h stimulated COX-2 protein synthesis in Hep3B cells in a dose-dependent manner, whereas it had no effect on the protein expression in HuH7 cells (Figure 4b). RT-PCR confirmed this effect; mRNA levels were upregulated by the demethylator in Hep3B cells, but not in HuH7 cells. In other words, the demethylating agent dose-dependently reactivated the *Cox-2* transcription that had been silenced by promoter hypermethylation.

To examine the phenotypic change by COX-2 induction, cyclin D1, a hallmark of cell turnover, was examined by Western blotting (Figure 4). The

Figure 4 COX-2 restoration and cyclin D1 down-regulation by 5-Aza-CdR. (A) Hep3B cells, and (B) HuH7 cells. Treatment with 5-Aza-CdR induced COX-2 protein synthesis (a) (Western blotting) as well as transcriptional activation of the *Cox-2* gene (b) (RT-PCR) dose-dependently in Hep3B cells, but not in HuH7 cells. To confirm that the equal amount of the template was used for RT-PCR, the *GAPDH* gene was amplified (c). The Western blot used for COX-2 (a) was reprobed with cyclin D1 antibody. Cyclin D1 was downregulated along with the COX-2 restoration in Hep3B cells. No such change was observed in HuH7 cells (d). Western blotting for GAPDH confirmed that the equal amount of protein was loaded (e). The experiments were repeated three times and provided similar results.

blot used to examine COX-2 expression was reprobed with cyclin D1 antibody. Blots for GAPDH confirmed that the equal amount of protein was loaded. Of interest, 5-Aza-CdR treatment, in association with COX-2 induction, inhibited the protein synthesis of cyclin D1 in a dose-dependent manner, which implicated decelerated cell proliferation by demethylation. Therefore, the possibility was suggested that COX-2 restoration might delay cell cycle and COX-2 inhibition, in turn, might stimulate the growth of HCC cells.

5-Aza-CdR Treatment Increased PGE₂ Production via Enhanced COX-2 Activities

Figure 5a and b demonstrate PGE₂ synthesis in Hep3B and HuH7 cells after 5-Aza-CdR treatment. In parallel with the enhanced COX-2 expression, PGE₂ synthesis was stimulated by 5-Aza-CdR in a dose-dependent manner in Hep3B cells, and PGE₂ levels were significantly higher by the treatment at 5 and 10 μ M than in the control ($P < 0.05$ and $P < 0.01$, respectively). On the other hand, 5-Aza-CdR had no effect on the synthesis of PGE₂ and PGE₂ levels remained relatively low in HuH7 cells.

To confirm that the increased PGE₂ production by 5-Aza-CdR treatment in Hep3B cells was attributable to the enhanced COX-2 protein expression, the cells were treated with a COX-2-specific inhibitor celecoxib in combination with 5-Aza-CdR, and PGE₂ production was measured (Figure 5c).

Figure 5 PGE₂ production by 5-Aza-CdR and celecoxib. Treatment with 5-Aza-CdR for 96 h significantly stimulated PGE₂ production dose dependently in Hep3B cells (a), while no change was observed in HuH7 cells (b). The increased PGE₂ production in Hep3B cells was abrogated by 1 μ M of celecoxib treatment (c). * and ** denote $P < 0.05$ and $P < 0.01$ vs the control, respectively. PGE₂ production was measured in quadruplicate in three independent experiments.

In the controls (without 5-Aza-CdR treatment), PGE₂ production in Hep3B cells was comparable between with and without celecoxib treatment (Figure 5a and c), indicating that COX-2 inhibition had little effect on PGE₂ production in the controls. This result suggested that a majority of PGE₂ production was catalyzed by COX-1 instead of COX-2 in Hep3B cells under the regular culture condition. In Hep3B cells treated with celecoxib, PGE₂ levels were similar and unaffected by 5-Aza-CdR (Figure 5c). Collectively, this implied that the restored COX-2 activity was essentially abrogated by 1 μ M of celecoxib. Conversely, as shown in Figure 5a, an increase in PGE₂ production by 5-Aza-CdR treatment was mainly attributable to COX-2 induction.

5-Aza-CdR Treatment Suppressed Cell Growth

To explore the outcome of the COX-2 restoration by 5-Aza-CdR, changes in numbers of Hep3B cells were examined by WST-8 assay. As shown in Figure 6a (closed bars), 96 h of treatment with 5-Aza-CdR significantly inhibited the growth of Hep3B cells in a dose-dependent manner ($P < 0.01$ vs the control).

Next, to determine whether the enhanced COX-2 expression by 5-Aza-CdR inhibited cell growth, Hep3B cells were treated with celecoxib to suppress COX-2 activity and the cell growth was examined.

Celecoxib, which suppressed COX-2 activity and PGE₂ production almost completely at 1 μ M in the same cells (Figure 5c), reversed the growth inhibitory effect of 5-Aza-CdR (Figure 6a, open bars). In the presence of celecoxib, there was no significant change in the cell number at any concentrations of 5-Aza-CdR, compared with the control without 5-Aza-CdR treatment (0 μ M). This result implicated that COX-2 reactivation indeed suppressed the growth of Hep3B cells. Interestingly, without the addition of 5-Aza-CdR (at 0 μ M), celecoxib suppressed the growth of Hep3B cells. As shown in Figure 5a and c, celecoxib hardly affected the production of PGE₂ in the absence of 5-Aza-CdR (0 μ M), indicating that this growth suppression was mediated by the specific effect of celecoxib, rather than COX-2 inhibition or suppression of PGE₂ production.

The growth inhibition of Hep3B cells by the demethylator treatment was predicted in view of the previous result on cyclin D1. In agreement with cyclin D1 downregulation (Figure 4), restored COX-2 activities might delay cell growth, and the possibility was indicated that COX-2 suppression might accelerate the development of HCC.

On the other hand, 5-Aza-CdR treatment up to 10 μ M had little effect on the growth of HuH7 cells (Figure 6b, closed bars), which was in good contrast with the growth inhibition observed in Hep3B cells (Figure 6a). This result was expected because neither COX-2 expression nor PGE₂ production was unaltered by the demethylator in HuH7 cells (Figures 4b and 5b). As was found in Hep3B cells, celecoxib suppressed the growth of HuH7 cells in the absence of 5-Aza-CdR (0 μ M) (Figure 6b, open bars). Moreover, celecoxib, to a similar extent, suppressed the growth of HuH7 cells at all the examined concentrations of 5-Aza-CdR (Figure 6b, open bars). Considering that PGE₂ was synthesized at a low level and 5-Aza-CdR hardly affected COX-2 expression or PGE₂ production in HuH7 cells (Figures 4b and 5b), the result that the growth of HuH7 cells was inhibited also implicated the specific growth-inhibitory effect of celecoxib, rather than the outcome of COX-2 inhibition.

Taken together, despite the specific growth-inhibitory effect of celecoxib, at least found in these two cell lines, COX-2 reactivation by the demethylation of the *Cox-2* promoter delayed the growth of Hep3B cells.

Cell Viability by 5-Aza-CdR

To exclude the possibility that the growth of Hep3B cells was suppressed due to the cytotoxicity of 5-Aza-CdR, LDH release into the supernatant was measured. Up to 10 μ M of 5-Aza-CdR treatment, there was no increase in LDH values in either Hep3B or HuH7 cells, indicating that these cells were hardly killed by 5-Aza-CdR at the concentrations

Figure 6 Cell growth by 5-Aza-CdR and celecoxib. Cell viability was determined by the WST-8 assay. (a) 5-Aza-CdR inhibited growth of Hep3B cells dose dependently (closed bars), and the growth inhibition was abolished to some extent by 1 μ M of celecoxib treatment (open bars). (b) Dose-dependent growth inhibition by 5-Aza-CdR was not found in HuH7 cells (closed bars), and celecoxib treatment showed similar growth suppression irrespective of 5-Aza-CdR concentrations (open bars), indicating the specific growth-inhibitory effect of celecoxib. ** denotes $P < 0.01$ vs the control. The WST-8 assay was carried out in quadruplicate in three independent experiments.

Figure 7 No remarkable cytotoxicity by 5-Aza-CdR. Cytotoxicity and cell viability by 5-Aza-CdR treatment were examined by the LDH release into the supernatant. No remarkable cytotoxicity or impaired viability was observed in Hep3B (closed bars) and HuH7 cells (open bars).

examined (Figure 7). This result also supports the idea that the growth of Hep3B cells was inhibited not by the cytotoxicity of 5-Aza-CdR but by COX-2 reactivation.

Discussion

A number of reports have demonstrated that COX-2 upregulation is involved in carcinogenesis.^{14–29} Among them, it is particularly intriguing that COX-2 expression is highly upregulated in early HCC, but relatively downregulated over progression.^{23–29} However, the underlying mechanism to regulate COX-2 expression, especially in HCC, is poorly understood. This prompted us to hypothesize that COX-2 expression could be regulated by promoter hypermethylation in HCC cells.

In the present study, we provided compelling evidence that CpG sites in the *Cox-2* promoter in some HCC cell lines were hypermethylated and 5-Aza-CdR treatment could demethylate the promoter in Hep3B cells that originally had hypermethylated *Cox-2* promoter, resulting in the transcriptional activation of the *Cox-2* gene. In addition, we demonstrated that COX-2 reactivation by treatment with 5-Aza-CdR suppressed the growth of Hep3B cells.

In an attempt to clarify the methylation status of the *Cox-2* promoter, three CpG sites were examined: –431 nt (A), –372 nt (B), and –138 nt (C) from the transcription starting site. It is generally accepted that COX-2 expression is regulated predominantly by the activation of three transcription factors: NF- κ B, NF-IL6, and CRE. The consensus sequences for NF- κ B (–452/–433, –229/–209), NF-IL6 (–132/–124), and CRE (–59/–53) binding sites are known (Figure 1, GenBank: AF276953).^{44,53–55} The CpG sites (A) and (B) were located not within but close to the NF- κ B site, while the CpG site (C) was also not within but near the NF-IL6 site. Both NF- κ B and NF-IL6 sites were greatly involved in the transcription of *Cox-2*.⁵⁵ Hence, there is a substantial possibility that the methylation of CpG sites at these sites repressed *Cox-2* transcription in Hep3B cells under the regular culture condition, and demethylation by 5-Aza-CdR at these sites, probably through creating newly accessible NF- κ B and NF-IL6 binding sites for each transcription factor, reactivated *Cox-2* transcription.

DNA methylation has been shown to reduce the binding affinity of sequence-specific transcription factors.^{56,57} Furthermore, methylation-dependent, sequence-specific DNA-binding proteins, such as MDBP, may act as transcriptional repressors.⁵⁸ However, it has also been reported that the transcriptional repression by DNA methylation can be mediated through a sequence-independent process involving changes in chromatin structure and histone acetylation levels.^{59,60} In general, CpG sites in the promoter are not uniformly methylated,^{44,61}

and there is a mixed population of methylated and unmethylated clones at any CpG site. Indeed, as shown in Figure 2, heterogeneity was observed in the methylation status of CpG sites. As to the *Cox-2* gene, there are 51 CpG sites in the promoter region from –590 to +186 nt.⁴⁴ Collectively, it needs further investigation to conclude which CpG site is pivotal in regulating *Cox-2* transcription and thereby causing a change in chromatin structure and accessibility of transcription factors to the binding sites. However, we could at least demonstrate that the methylation status of the CpG sites examined in this study was associated with the COX-2 expression and these sites seemed to play potentially a critical role in the transcriptional repression and reactivation.

Recently a high level of cancer-specific methylation in human cancer has been referred to as the CpG island methylator phenotype (CIMP).⁶¹ Tumors exhibiting this phenotype are characterized by a high degree of concurrent CpG island methylation in a variety of genes, such as *p16*, *hMLH1*, and *THBS1*. In HCC cells with hypermethylated *Cox-2* promoter including Hep3B cells, promoters of other genes can also be hypermethylated; in fact, promoters in the estrogen receptor, *p16*, and T-type calcium channel gene are hypermethylated.^{46,62}

In Hep3B cells, promoter hypermethylation was found in the Ras association domain family 1A (*RASSF1A*) gene,⁶³ pi-class glutathione S-transferase (*GSTP1*) gene,^{64–66} but not in *p16*.⁶³ Interestingly, promoter hypermethylation of *RASSF1A* was found in 85% of HCC tissues examined while in 0% in the adjacent nontumorous liver tissues.^{63,67} These previous reports indicate that the methylation status in human HCC tissue may be, to some extent, retained in some HCC cell lines as well.

Treatment with 5-Aza-CdR may demethylate and reactivate other genes involved in cell growth, such as the *p16* gene, and result in growth suppression. Therefore, the involvement of such genes cannot be completely excluded in interpreting the results of the experiments using a demethylator. However, considering that 5-Aza-CdR, in the presence of celecoxib, did not affect the growth of HuH7 cells in a dose-dependent manner, the abrogation of cell growth suppression by celecoxib found in Hep3B cells implicated that reactivated COX-2 by promoter demethylation, rather than reactivation of other formerly methylated genes, predominantly suppressed or delayed the cell growth.

Intestinal epithelial cells overexpressing COX-2 have the increased duration of the G₁ phase of cell cycle, lower levels of cyclin D1 protein, and a marked decrease in retinoblastoma kinase activity associated with cyclin-dependent kinase 4.³⁰ In addition, overexpression of COX-2 arrested cell cycle in other cell types such as fibroblasts and endothelial cells.³¹ Furthermore, there have been a number of reports that PGE₂ and its derivatives suppressed growth of a variety of cells,^{32–37} whereas

they could also stimulate colony formation and transactivate EGF receptor, which presumably led to cell proliferation.^{38,39}

Not surprisingly, consistent with these reports showing that upregulated COX-2 and PGE₂ delayed cell growth, our results provided evidence that growth of HCC cells was suppressed or retarded, at least in part, by demethylation of the *Cox-2* promoter, that is, COX-2 reactivation, and it was related with cyclin D1 downregulation. We could also demonstrate that treatment with a COX-2 inhibitor abrogated the cell growth suppression by 5-Aza-CdR. Therefore, in spite of the possible involvement of other genes whose promoters are originally methylated and can be demethylated by a demethylator, COX-2 reactivation seems to play a major causative role in growth inhibition in HCC. In this study, the effect of PGs on cell cycle was not directly investigated in HCC cells. We are currently investigating the effect of PGs, especially PGE₂, on cell cycle in HCC cells.

The underlying mechanism how the *Cox-2* promoter becomes hypermethylated is poorly understood. DNA methyltransferases are presumed to play a significant role in DNA methylation. Compared with the corresponding noncancerous liver tissue, significant overexpression of DNMT3b, which had *de novo* DNA methylation activity,⁶⁸ was observed in HCC.⁶² Nevertheless, DNMT3b mRNA levels did not change significantly over dedifferentiation,⁶² and whether this mechanism may be involved in the promoter hypermethylation in HCC cells remains unclear.

Methyl-CpG binding domain (MBD) proteins, at least in part, mediate CpG island hypermethylation and repress gene transcription. Bakker *et al*⁶⁴ demonstrated that MBD2, among MBD family proteins, repressed *GSTP1* transcription via hypermethylation of the promoter in Hep3B cells. Therefore, although the exact mechanism how the *Cox-2* promoter can be hypermethylated remains unclear and needs further investigation, MBD2 is a potential candidate that mediated the hypermethylation process.

Given that the IC₅₀ of celecoxib is about 0.04 μ M, the concentration of celecoxib used in this study (1 μ M) was considered reasonably enough to suppress the COX-2 activity. Indeed, judging from the reduced PGE₂ production, COX-2 inhibitor celecoxib almost completely abolished the COX-2 activity restored by 5-Aza-CdR. In contrast, COX-2-specific inhibitors are known to have proapoptotic and antiproliferative potentials *in vitro* at markedly high concentrations ($\sim 100 \mu$ M).^{26,69} Should COX-2 inhibitors be used at such high concentrations, they may exhibit chemopreventive effect on HCC. This possibility must be investigated *in vivo* in the future.

In conclusion, our present study indicates that promoter hypermethylation silences the *Cox-2* gene and the reactivation of *Cox-2* may lead to growth retardation in some HCC cell lines. Although we did

not investigate on human HCC tissue in this study, it would be intriguing to examine the distribution patterns of the cells with hypermethylated *Cox-2* promoter within tumors.

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