Abstract

Cyclooxygenases (COXs) are key enzymes that convert arachidonic acid to prostaglandins. Overexpression of one of the COX isozymes, COX2, has been shown to play an important role in colorectal cancer progression. Recently, however, low expression of COX2 has been reported in a subset of colorectal and gastric cancers. Aberrant CpG island methylation and associated transcriptional silencing are common in colorectal cancer, and we therefore investigated the potential role of methylation in the transcriptional silencing of COX2. We examined the methylation status of the COX2 5’ CpG island in a series of tumor cell lines. Among the 33 cell lines examined, dense methylation (>70%) of COX2 was detected in 5 cell lines, and partial methylation was detected in 10 cell lines. Detailed methylation mapping using bisulfite genomic sequencing revealed that loss of expression of COX2 mRNA was closely correlated with methylation of a region upstream of exon 1, and expression could be restored by demethylation using the DNA methyltransferase inhibitor 5-aza-deoxycytidine. Aberrant methylation of COX2 was also detected in 12 of 92 (13%) unselected sporadic primary colorectal cancers and 7 of 50 (14%) colorectal adenomas. COX2 methylation was strongly associated with the presence of the CpG island methylator phenotype (P < 0.01), inversely related to p53 gene mutation (P < 0.01), and unrelated to microsatellite instability status. We propose that COX2 expression in colorectal tumors is modulated by functional factors that favor high expression and by the CpG island methylator phenotype that favors silencing in a subset of cases. These results raise the possibility that tumors with COX2 methylation may be less sensitive to treatment using specific COX2 inhibitors.

Introduction

Various epidemiological studies indicate that the use of nonsteroidal anti-inflammatory drugs can reduce the risk of colorectal cancer (1–3). One of the important target genes of nonsteroidal anti-inflammatory drugs is thought to be COX, a rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandin H2. Two isoforms of COX, COX1 and COX2, have been identified and exhibit distinctive functional differences. COX1 is constitutively expressed in a wide variety of tissues. COX2 is a primary response gene whose expression is induced by cytokines, growth factors, and tumor promoters (4). Overexpression of COX2 is reported in various types of tumors, including colorectal, gastric, lung, prostate, and breast cancers (5–9). This overexpression appears to contribute to colorectal tumorigenesis, as evidenced by dramatic reductions in the number of colorectal tumors in mice deficient in the APC tumor suppressor gene when COX2 was deleted by knockout strategies (10). Moreover, constitutive expression of COX2 can inhibit apoptosis by inducing the proapoptotic gene BCL2 (11) and can also induce the metastatic phenotype in colorectal cancer cells (12).

COX2 overexpression is frequent but not universal in colorectal cancer. Initial studies indicated that about 85% of cancers had detectable expression of COX2 (5). More recently, it was reported that COX2 expression is reduced in a subset of sporadic colorectal cancers with MSI resulting from defective DNA mismatch repair and in colorectal cancer associated with hereditary nonpolyposis colorectal cancer (13, 14). Similar reduced expression of COX2 was reported in gastric cancers with MSI (15). In contrast, rectal cancers appear to express significantly higher levels of COX2 than cancers in other sites (16). These results indicate that expression of COX2 is not necessary for the development of a subset of colorectal tumors, which must then arise from a pathway independent of COX2. The molecular mechanism of COX2 activation, or lack thereof, in colorectal cancers remains unclear. It has been reported that COX2 expression is repressed by wild-type p53 (17), suggesting interactions between classical genetic changes and COX2 expression in these tumors. The lack of COX2 expression in subsets of cancers also raised the possibility of a specific silencing mechanism for the gene.

Cytosine methylation of CpG islands is an epigenetic mechanism of gene silencing through modulation of the chromatin structure (18). Aberrant hypermethylation of 5’ CpG islands has been implicated in the transcriptional silencing of various genes in aging and cancer (19, 20). Because subsets of sporadic colorectal cancers are often associated with hypermethylation of multiple loci (21, 22), and these subsets often include tumors with MSI because of the methylation and silencing of the hMLH1 mismatch repair gene promoter, we hypothesized that COX2 could be silenced by hypermethylation of its 5’ CpG island in some cases. In this study, we have examined the methylation status of COX2 in a series of cell lines and colorectal tumors. Our results indicate that COX2 is inactivated in association with aberrant methylation in a subset of colorectal tumors that are CIMP positive.

Materials and Methods

Samples and Cell Lines. Normal tissues examined included colon, placenta, bone marrow, normal fibroblast cell line IMR90, and normal breast epithelium cell line HMEC. Eight colorectal cancer cell lines (Caco2, RKO, SW48, HCT116, DLD-1, LOVO, SW837, and HT29), 4 lung cancer cell lines (OH3, H249, H157, and H209), 3 brain tumor cell lines (Daoy, D283, and SW15), 3 lymphoid cell lines (CEM, KG1A, and HL60), and one hepatoblastoma cell line (HepG2) were analyzed in this study. Primary colorectal tumors were obtained from the Johns Hopkins Hospital (Baltimore, MD) as described previously (22). In total, 142 colorectal tumors (92 colorectal cancers and 50 colorectal adenomas) were used for methylation analysis. Some of these tumors were typed previously for aberrant hypermethylation of multiple loci and the presence of the CIMP (22). Data on MSI, CIMP, COX2 expression, and death charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
dinucleotide tracts (21), and mutations of p53 (exons 2–11), determined by single-stranded conformational polymorphism followed by direct sequencing of shifted bands, were available on a subset of the cases (23). In addition, 18 colorectal tumors that had previously been studied for COX2 protein expression by IHC (14) were also studied for COX2 methylation. These 18 tumors were obtained from The University of Texas M. D. Anderson Cancer Center (Houston, TX). Genomic DNA was extracted using standard procedures. RNA was extracted using Trizol (Life Technologies, Inc.) according to the manufacturer’s protocol. For the demethylation experiments, the RKO and DuPro cell lines were treated with 1 mM 5-aza-deoxycytidine daily for 5 days.

COBRA and Bisulfite Sequencing. Bisulfite treatment of DNA and COBRA were performed as described previously (22–25). After treatment, 2 µl of aliquot were amplified in 50 µl of solution containing 1× buffer, 1.25 mM deoxynucleotide triphosphate mixtures, 2.5 pmol of each primer, and 1 unit of Taq DNA polymerase (Life Technologies, Inc.). Touchdown PCR was carried out as follows. After a hot start, the cycling parameters were: (a) 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s for 3 cycles; (b) 94°C for 30 s, 51°C for 30 s, and 72°C for 30 s for 4 cycles; (c) 94°C for 30 s, 49°C for 30 s, and 72°C for 30 s for 5 cycles; and (d) 94°C for 30 s, 47°C for 30 s, and 72°C for 30 s for 26 cycles. Primers were designed based on the nucleotide sequences submitted to GenBank (AF044206, HSU44805, and D28235). Primers used for COBRA were as follows: (a) COX2-AR, 5′-CTGATTTGTAGTGAGYGTTAGGAGT-3′; (b) COX2-BF, 5′-CGGGAGTGGTTTTGTTGTTGTG-3′; and (c) COX2-I, 5′-GTTYGYGTTTTGTTGTTGTG-3′. These primers were used to amplify the 1-kb region of the 5′ regulatory region of COX2.

Results

Hypermethylation of COX2 in Cancer Cell Lines. The 5′ region of COX2 has a typical CpG island spanning 350 bp (Fig. 1A). The CpG:GpC ratio is 0.8, and the GC content is 64%, satisfying the criteria for a CpG island (27). This CpG island is somewhat atypical in that it appears to start just after the transcription start site. First, we examined the methylation status of selected COX2 sites within this CpG island in several normal tissues including colon mucosa, placenta, and fibroblasts. Methylation was not detected (<5%) in the RsaI and Tail sites of the normal colon (Fig. 1). In contrast, all colorectal cell lines had highly methylated COX2 regions, with the exception of LNCAP. COBRA and bisulfite sequen-
sites in region 1 by the semi-quantitative methylation assay, COBRA (Fig. 1, B and C). In contrast, all normal tissues showed a slight amount of methylation in the Tail site in region 2, which is 40 bp downstream from the exon/intron boundary of exon 1. To investigate whether aberrant methylation is detected in this CpG island, a total of 33 cell lines from various tissue types were investigated for the methylation status of region 1 (Fig. 1C). Five of the 33 cell lines [RKO (colon), DUPRO (prostate), HL60 (acute myelogenous leukemia), MB-474 (breast), and CAMA1 (breast)] showed a methylation level of more than 70%, and 10 cell lines (D283, CEM, Raji, DU145, LNCAP, MB-435, MB-453, BT20, MB-468, and HepG2) showed partial methylation, ranging from 13–64%. Eighteen cell lines had no detectable methylation (<5%). Concordant results were obtained for the RsaI and Tail sites in region 1. Of note, the hypermethylated colon cancer cell line RKO is mismatch repair deficient due to promoter methylation of the hMLH1 gene. The other hypermethylated cell lines are not known to be mismatch repair deficient.

We used bisulphite sequencing to examine the methylation status of 24 CpG sites within this CpG island in more detail [see sequence fragment (Sequence) in Fig. 1A and the examples in Fig. 1D]. In normal colon mucosa from an 18-year-old patient without tumor, a slight amount of methylation was detected (in 10 alleles sequenced, methylation averaged 2.3 of 24 CpG sites or 10%). This methylation was predominantly in the 5’ region of the island in intron 1, which is consistent with our earlier COBRA data. No methylation was detected in the five CpG sites closest to the transcription start site. In a colorectal cancer cell line, LOVO, which was unmethylated by COBRA, very little methylation in the entire region was observed (2 of 312 CpG sites in 14 total alleles sequenced). In contrast, the colorectal cancer cell line RKO, which appeared highly methylated by COBRA, showed methylation of nearly all CpG sites examined in the region (232 of 240 CpGs in 10 alleles sequenced). The LNCAP cell line, which was partially methylated by COBRA, also showed a high degree of methylation by sequencing, but 1 of 10 alleles sequenced was completely unmethylated.

**Denise Methylation of COX2 Is Associated with Transcriptional Silencing.** To examine whether methylation of the 5’ region of COX2 is associated with loss of expression, we performed RT-PCR using cDNA from normal colon as well as six unmethylated cell lines, two heavily methylated cell lines, and one partially methylated cell line. COX2 was expressed at varying levels in normal colon, consistent with previous reports (Fig. 2A). RKO and DUPRO, which showed dense methylation, did not express COX2 (Fig. 2A). LNCAP, which showed heterogeneous methylation, expressed a slight amount of COX2. All of the unmethylated cell lines expressed COX2, albeit at various levels. These results suggest that dense methylation of the upstream region of COX2 is associated with transcriptional silencing of the gene.

To further confirm the involvement of methylation in silencing of COX2, two of the heavily methylated cell lines, RKO and DUPRO, were treated with a DNA methyltransferase inhibitor, 5-aza-deoxycytidine. In both cell lines, expression of COX2 was restored after treatment, together with a significant amount of demethylation in the 5’ region of COX2, as determined by bisulphite sequencing (Fig. 2B; data not shown). These results indicated that methylation in the 5’ region of COX2 plays a role in the loss of expression of this gene in normal cells.

**Aberrant Methylation of COX2 in Primary Colorectal Tumors.** To determine whether aberrant methylation of COX2 is also present in primary colorectal tumors, we investigated a series of colorectal adenomas and cancers by COBRA (see the examples in Fig. 3A). Aberrant methylation of COX2 (>5%) was detected in 12 of 92 (13%) colorectal cancers and was observed in 50 of 143 colorectal adenomas. The similar frequency of COX2 methylation in preneoplastic adenomas and carcinomas indicates that it is an early event in colorectal tumorigenesis. The results obtained using digestion with RsaI were identical to those obtained using digestion with Tail. In both adenomas and carcinomas, methylated alleles coexisted with unmethylated alleles. Because we used tumor samples without microdissection, these results could be due to infiltration of the tumor samples with nonneoplastic cells. Alternatively, they could be due to intratumor heterogeneity (some alleles are methylated, whereas others are not) or to partial heterogeneous methylation (all alleles are lightly methylated).

To determine the extent of COX2 methylation in more detail, bisulphite sequencing was performed in three methylated and three unmethylated tumors (Fig. 3B). The cases that showed aberrant methylation by COBRA had dense methylation of multiple alleles throughout the region examined. In each case, the densely methylated alleles coexisted with completely unmethylated alleles, which likely originated from nonneoplastic cells. In contrast, the three unmethylated tumors (by COBRA) had very little methylation throughout the CpG island. In one case (C13), 1 of 10 alleles appeared to have dense methylation, suggesting intratumor heterogeneity in methylation.

We next examined clinicopathological and molecular features of colorectal cancers with or without COX2 methylation. There was no association between COX2 methylation and the location of tumors, age, gender, or stage of the patients. The presence of CIMP was determined previously for 92 of these cases. There was a significant concordance between COX2 methylation and the presence of CIMP: methylation was present in 11 of 46 (24%) CIMP-positive tumors versus 2 of 46 (4%) CIMP-negative tumors (two-sided P = 0.01, Fisher’s exact test). Methylation of COX2 was inversely related to
mutations of p53. Overall, 1 of 13 (8%) cases with COX2 methylation has p53 mutations, whereas 40 of 79 (51%) cases without COX2 methylation had p53 mutations (two-sided \( P = 0.005 \), Fisher’s exact test). MSI had been determined previously for 46 cancers, and 19 cases were MSI positive. Of these, 3 of 19 (16%) MSI-positive cases showed COX2 methylation, compared with 7 of 27 (26%) MSI-negative cases \( (P = 0.61) \).

Finally, we examined whether hypermethylation of COX2 is associated with transcriptional silencing of the gene in primary tumors. We examined 18 cancers that had been studied previously for COX2 protein expression by IHC (14). Of the 18 tumors, 10 cases had no detectable methylation, and 8 of these expressed COX2 protein. Six cases had low levels of methylation (5–20%), and three of these cases were COX2 negative, whereas the other three expressed the protein (examples are shown in Fig. 3A). Two cases had high methylation levels (>20%), and both were negative for COX2 expression by IHC.

**Discussion**

In the present study, we showed that aberrant methylation of the 5’ CpG island of COX2 is present in colorectal cancer and other neoplastic cell lines and appears to be associated with transcriptional silencing of the gene. Detailed methylation mapping of 24 CpG sites revealed that methylation closest to the transcription start site is limited to neoplastic tissues and is associated with loss of expression of COX2. In contrast, methylation of downstream CpG sites was detected in normal tissues as well as neoplastic tissues. The impact of this downstream methylation on expression is unclear (28), but it is possible that this methylation serves as a trigger to recruit the methylation machinery to the region. In methylated tumors, there appears to be no critical region or hot spot of methylation in COX2, and the density of methylation likely contributes to loss of expression through recruiting methyl-binding proteins and histone deacetylases (18).

The mechanism of aberrant methylation of COX2 remains to be determined. We have shown previously that genes slightly methylated in normal colon mucosa tend to be methylated very frequently in colorectal cancers. These genes include ER (29), N33 (30), MYOD (30), and VERSICAN (26). On the other hand, methylation of some genes appears to be exclusive to a subset of cancers that are characterized by CIMP (22, 31). From the data presented here, methylation of the upstream part of the COX2 CpG island seems to be tumor specific because methylation was detected only in a subset of cancers and adenomas. Indeed, there was a significant correlation between COX2 methylation and the presence of the CIMP. However, the frequency of COX2 methylation is lower than that of p16 (22), THBS1 (21, 22), and CACNA1G (31), which is consistent with the fact that in some tumors, loss of COX2 might result in growth disadvantage. Thus, one can envision that the expression of COX2 in CIMP-positive colorectal tumors results from a balance between the pressure to silence the gene via methylation and the pressure to keep the gene active because of its positive effect on growth (through inhibited apoptosis). Those cases in which a separate pathway replaces COX2 activation can then become methylated as a result of CIMP. This model may then explain the relatively high degree of heterogeneity observed for COX2 methylation in primary tumors. This heterogeneity is well exemplified by case C13 (Fig. 3B), which is CIMP positive and has less than 5% COX2 methylation by COBRA but still has a densely

![Fig. 3. Aberrant methylation of COX2 in primary colorectal cancers and adenomas. A. Methylation analysis by COBRA. Methylated alleles are indicated by arrows. The percentage of methylated alleles is shown at the bottom of each lane. Note the presence of methylation in cases C1, C26, C23, C46, C118, P10, and P27. The results obtained with RsaI digestion (top) were identical to those obtained with Tail digestion (middle). The bottom gel shows methylation analysis of colon cancers previously shown to be positive or negative for COX2 expression by IHC. The PCR products were digested with RsaI. Note that, in general, tumors with significant methylation tended to be IHC+, but there is significant heterogeneity, with IHC+ tumors showing some methylation, and occasional IHC− tumors that lack methylation. B, bisulfite sequencing of the 5’ CpG island of COX2 in colorectal cancers with (C1, C29, and C118) or without (C2, C13, and C49) methylation detected by COBRA.](image-url)
methylated allele (among unmethylated alleles) detected by sequencing. It also could explain our finding that some cases with low levels of COX2 methylation (5–20%) have detectable COX2 protein by IHC.

One alternative explanation for COX2 methylation is that it follows loss of expression (or failure to activate the gene in some cases). Such a hypothesis has been proposed to explain methylation of E-cadherin in breast cancer (32). Interestingly, we found an inverse correlation between COX2 methylation and p53 mutations in primary tumors. Recently, COX2 expression was shown to be repressed by wild-type p53 (17). Thus, it is possible that tumors without p53 mutations fail to activate the gene efficiently and become predisposed to methylation that way. This possibility deserves further exploration, but the fact that the gene can be readily reactivated by demethylation in RKO (a cell line with wild-type p53) does not support this hypothesis. In addition, the HCT116 and MCF7 cell lines are also p53 wild type, but they do not hypermethylate COX2, indicating that factors other than p53 mutations must be important for this process to occur.

What are the functional consequences of aberrant methylation of COX2 in colorectal tumorigenesis? It has recently been reported that COX2 is not expressed in some colorectal cancers with MSI (13, 14). In these tumors, overproduction of arachidonic acids may not be necessary for tumor progression. Alternatively, colorectal cancers without COX2 expression might have altered regulation of the pros-taglandin synthesis pathway through other enzymes such as COX1. Although we found a strong association between COX2 methylation and CIMP, we did not find such an association with MSI. One possible explanation for this is posttranscriptional extinction of COX2 expression in some cases (14). In fact, the MSI-positive colorectal cancer cell line HCT116, which was reported to express COX2 mRNA (33) but not COX2 protein (14), is completely unmethylated at this CpG island. Furthermore, low levels of COX2 expression in MSI-positive cancers could also be related to the fact that most of these tumors lack p53 mutations. Regardless of the mechanism of COX2 down-regulation, the existence of tumors with little COX2 expression raises the possibility that these tumors may be relatively resistant to treatment with specific COX2 inhibitors (3).

References
Aberrant Methylation of the Cyclooxygenase 2 CpG Island in Colorectal Tumors

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