Abnormal Methylation of the Calcitonin Gene in Human Colon Neoplasms


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ABSTRACT

Earlier studies of the methylation status of total genomic DNA and of specific genes have demonstrated, predominantly, hypomethylation in human neoplasms. However, we have recently documented the presence of new sites of methylation in the calcitonin gene in human lymphomas (100%), small cell lung carcinomas (92%), and acute myeloid leukemias (95%). We now report that these same novel calcitonin gene methylation sites are also a feature of DNA from human colon adenomas (13 out of 14 studied), colon carcinomas (4/13), and established colon carcinoma cell lines (18/19), despite the presence of overall genomic DNA hypomethylation in these neoplasms. The data provide further evidence that regional increases in DNA methylation, like gene hypomethylation, occur in benign colon neoplasms prior to malignant transformation. The fact that abnormalities of calcitonin gene methylation are less frequent in DNA from human colon carcinomas than from adenomas and colon carcinoma cell culture lines is of special interest. This finding suggests that a more heterogeneous population of cells is present in the carcinomas and that the calcitonin gene hypermethylation may be inherent to cells which are initially selected for growth in culture or are capable of prolonged survival under culture conditions.

INTRODUCTION

Methylation of cytosine at the 5' position is the only known nucleotide modification to occur in vertebrate DNA and provides an important mechanism for modulating gene expression (1–3). Of the total cytosine residues present in the genome, approximately 2–7% are methylated depending on the host (1–3). In order to ensure equal strand modification, the modification is present almost exclusively in the sequence CpG and is catalyzed by a methyltransferase enzyme which is specific for the dinucleotide sequence (1–3).

Alterations of DNA methylation have been reported in human cancers (4–10) and such changes can potentially alter patterns of gene expression which are important to normal cell growth and differentiation. Previous reports have most frequently demonstrated total genomic hypomethylation as well as hypomethylation of specific genes in carcinomas (4–7) although total genomic hypermethylation has been reported in neoplastic cells in culture (11) in addition to a report of hypermethylation in a few genes from malignant cells in culture (12). In the specific case of colon neoplasms, hypomethylation can be traced to premalignant lesions as well as carcinomas (7). Recently, we determined that several genes on the short arm of chromosome 11 including the calcitonin gene, are extensively hypermethylated in human tumors (8–10). The presence of increased gene methylation may be of particular significance, since, in experimental studies, new sites of 5' region methylation act as a dominant event to decrease gene expression (13–15). In contrast, hypomethylation may or may not increase gene expression depending upon multiple other cellular factors (133). Most of the CG sites in the calcitonin gene are located in the 5' regulatory portion of the gene. Although calcitonin is not expressed in most tumors we have examined, the hypermethylation present in the 5' region of somatic tumor cells correlates with a conformation which is not favorable for gene expression (10). The increased methylation for the calcitonin gene occurs in over 85% of lymphoid tumors (8, 9), acute myeloid leukemias (9), and small cell lung carcinoma (8), but the incidence for such hypermethylation was much lower (<40%) for other solid tumors examined including a very preliminary study of human colon carcinoma (8).

The current study was directed at three issues concerning hypermethylation of the calcitonin gene in human colon neoplasms. First, we sought to establish whether regional DNA hypermethylation, such as that on chromosome 11, occurs in the setting of the widespread gene hypomethylation previously described in colon neoplasms (5–7). Second, established culture lines of colon carcinomas were extensively studied to determine whether, in this setting, hypermethylation changes could be detected with a higher frequency than in fresh carcinomas. This concept was based on our findings (9) that within single clones of leukemia cells, a variable number of cells demonstrate CT gene methylation. In solid tumors, cell culture might select for such an important population. Finally, we sought to determine at which stage of colon neoplasia altered CT gene methylation occurs. Our previous studies have documented that this abnormality appears relatively early in human cells infected with the Epstein-Barr, human T-cell leukemia, and SV40 viruses (10). The methylation change could, thus, play a role in tumor progression prior to final malignant changes.

Colon cancer represents an important model in which to study methylation because premalignant lesions which can have the potential to progress to carcinoma can be examined. Also a familial syndrome with an autosomal dominant transmission of colon cancer can be studied from early adenomatous changes to cancer. There is also evidence that abnormalities of cell division (by thymidine labeling and biochemical markers) exist in the normal mucosa of patients predisposed to colon cancer (16–18).

In the present study we show that the novel methylation patterns of the CT gene which occur in the presence of widespread genomic hypomethylation, are virtually a constant property of cultured colon carcinoma cells, and appear in colon neoplasms prior to the time of malignant transformation.

MATERIALS AND METHODS

Calcitonin Gene DNA. The probe used in these studies have been previously described and consists of a 1.7-kilobase genomic fragment of the 5' region of the CT gene (8–9). Fig. 1 contains an abbreviated map of the gene as well as the position of the probe.

Tissues and Cells. Colonic tissue specimens were obtained under protocols approved by the Committee for Clinical Investigation for The Johns Hopkins Hospital. DNA was prepared from cryostat sections of...
fresh colonic mucosa of neoplasms and surrounding normal tissue to remove as much nonepithelial tissue as possible. This technique has been previously described (19). Sixteen of the human colorectal cancer cell lines investigated were established at the National Cancer Institute (JP and AFG-20) and three colon cancer cell lines, lines COLO 320, SW48, and Widr, were obtained from American Type Culture Collection (Rockville, MD). Control colon tissue was also obtained from patients without underlying neoplasms and was from specimens obtained for medical and surgical indications and included tissue from patients with inflammatory bowel disease, diverticulosis, and diverticulitis.

DNA Hybridizations. High molecular weight DNA was extracted as previously described (21) and was digested with either Mspl (6–8 units/µg DNA) or HpaII (3–9 units/µg DNA) for 12–16 h at 37°C. Several of the hypermethylated samples were digested with a 50-fold excess of HpaII to ensure complete DNA restriction. Approximately 5 µg of DNA was applied to each gel lane for all of the studies. The digested samples were electrophoresed on 1% agarose gels, transferred to nylon filters (Dupont), and hybridized for 48 h, at 60°C, with the 5′ CT gene probe, which was nick translated (reagents from Bethesda Research Labs) in the vector to a specific activity of 0.5–1.0 × 10^6 cpm/fg DNA. 5.0–7.5 × 10^6 cpm of CT probe were used for each hybridization. The hybridization solution has been previously described (22), and the final filter wash was with 0.1 × SSC (0.015 M NaCl-0.03 M Na citrate), 0.1% SDS at 65°C for 30–60 min. Filters were exposed to Kodak XAR films at −70°C for 24–96 h.

RESULTS

CT Gene Methylation in Normal Intestinal Mucosa. Methylation of CCGG sites in the CT gene was studied by digesting control and tumor DNA with the isoschizomers Mspl and HpaII. Mspl recognizes the sequence CCGG whether or not the internal cytosine is methylated and HpaII recognizes the sequence only when the internal cytosine is unmethylated. As previously described (8, 9), there are seven Mspl restriction sites in the human CT gene (shown in Fig. 1). The 1.7-kilobase probe detects bands of 0.5 (from site M2 to M3 - Fig. 1), 0.6 (site M1 to M2), and 1.0 kilobase (M2 to M3) in DNA from all normal and neoplastic tissues digested with Mspl (8, 9). In all normal human tissue previously studied (8, 9), an additional 2.0-kilobase band (M4 to M5), with reduction of the 1.0-kilobase band, was detected in HpaII digests. The 2.0-kilobase band appears because of methylation of site M5 (see Fig. 1 and Refs. 8, 9). Normal intestinal mucosa contains a minor difference in that, in some normal specimens and to a variable degree in different patient samples, an additional 1.4-kilobase band appears (Fig. 2) which has not been precisely mapped. This band is usually of light intensity in DNA from normal intestinal mucosa, suggesting that 50% or less of the cells have cytosine methylation at this site.

Studies of Colonic Neoplasms. For analysis of DNA from colon neoplasms, 14 adenomas and 13 colon carcinomas were studied and compared to adjacent normal tissue from the same patient. Prior to hybridization of the genomic DNA with the CT gene probe, we estimated the degree of overall DNA methylation in normal versus neoplastic tissue by examining the ethidium bromide staining patterns of the electrophoresed samples. As previously reported (5–7), the colon adenomas and carcinomas had evidence of overall DNA hypomethylation as compared to adjacent normal tissue (note in Fig. 3 that HpaII cuts adenoma DNA more easily than it cuts normal mucosa DNA as evidenced by increased migration of the restricted DNA on agarose gels).

Of the 14 adenomas studied, and despite the overall DNA hypomethylation seen in Fig. 3, 13 had, for the CT gene, novel patterns of methylation of CCGG sites. Eleven of these benign tumors showed one or two new sites of CT gene methylation
compared to the normal DNA from the same patient (most subtle changes shown in Fig. 4A and more striking ones in Fig. 4B) and two adenomas had many cells methylated at essentially all 5' CT gene CCGG sites (Fig. 4C). It is important to note that the degree to which an abnormality is detected relates to the fact that only a variable number of cells in the samples have methylation changes since the new bands, which are discussed below, coexist with background normal patterns (Fig. 4, A–C) in which the 2.0-, 1.0-, 0.6-, and the 0.5-kilobase bands are still seen. We have previously shown that this heterogeneity for cellular CT gene methylation exists in leukemias proven to be of clonal origin by molecular biology techniques (9).

The methylation patterns of the HpaII digests of the colonic adenomas include the following. In those adenomas showing one or two new sites of methylation in the CT gene (Fig. 4, A and B), the most frequent abnormal band seen is a 1.2-kilobase band which cannot be detected, or only to a questionably trace amount, the DNA from normal colonic mucosa (see Fig. 4A, Lanes 1, 3, 4, 5, and 6, and especially in Fig. 4B, Lanes 2–4). This is best exemplified in Lane 2 in Fig. 4B where many cells have the alteration, and the normal 0.6- and 0.5-kilobase are diminished in the digest with appearance of the 1.2-kilobase band. A second abnormality involves appearance of a 0.7-kilobase band (Fig. 4A, Lanes 3–6). This alteration has been previously described in small cell lung carcinoma, leukemias and virally transformed human cells (8–10). The 0.7-kilobase band arises as the result of methylation of site M1 (Fig. 1) which eliminates or decreases the normal 0.5-kilobase band (Fig. 4A, Lanes 3–6) which spans the MspI sites M2 to M5.

In two adenomas (Fig. 4C, Lanes 1 and 3), some cells show methylation of virtually all the mapped MspI sites. The abnormal 2.6- and 3.1-kilobase bands are similar to those we have previously identified in other tumors (8, 9) and arise from methylation of sites M1 through M6 or M3 through M6 (Fig. 1). The heterogeneity of cell distribution is evident in these two samples in that one adenoma has virtually all cells with the abnormal pattern (Fig. 4C, Lane 1). Note the virtual loss of the normal 0.5-, 0.6-, and 1.0-kilobase bands in this DNA. Less cells are involved in the second adenoma (Fig. 4C, Lane 3) as evidenced by the persistence of the normal bands despite the presence of the abnormal 2.6- and 3.1-kilobase bands.

To ensure that the above results in adenomas were not secondary to incomplete genomic digestion, two techniques which were employed. First, DNA from two of the neoplasms which had extensive CT gene methylation were digested with a 50-fold excess of HpaII and no change was detected in the restriction pattern (Fig. 4A, Lane 5 compared to Lane 6). Second, as in our previous studies in which we demonstrated either hypomethylation or no changes in methylation in specific genes not from chromosome 11 (transferrin receptor, C-fos, etc.) (9–10), probes for other genes (c-myc and c-Harvey-ras) were hybridized to the same filters and showed evidence of either normal or decreased methylation indicating complete genomic digestion (data not shown).

The colon cancer DNA samples yielded a different set of results from the adenomas, and only four of 13 tumors had novel sites of CT gene methylation, including two with extensive involvement of the CCGG sites (Fig. 5C). Four carcinomas had no significant changes in methylation of the CT gene compared to the normal mucosa from the same patients (Fig. 5A), while five tumors actually had relative hypomethylation of the CT gene (Fig. 5B, note in Lanes 1, 3, and 5 the increased density of the 1.0-kilobase band relative to the 2.0-kilobase band as compared to controls in Lanes 2, 4, and 6). There was
no correlation between the methylation alterations of the calcitonin gene and the cellular histology of the colon cancers.

In three patients we studied both an adenoma and a carcinoma arising from that adenoma (Fig. 6). Of these, one adenoma (S7) revealed new methylation sites (1.2-kilobase band) while no changes were seen in the cancer DNA as compared to surrounding normal tissue. In one patient (S105), the same new methylation sites (1.2-kilobase band) were found in the adenoma and the carcinoma. In the last patient (S122) there was unusual prominence of the 1.4-kilobase band in the adenoma DNA while relative CT gene hypomethylation was found in the carcinoma as evidenced by the markedly abnormal increase in ratio of the 1.0- to the 2.0-kilobase band.

Taken together, the above results might indicate that new sites of CCGG methylation in the CT gene is a virtually constant feature of colon adenomas, but may be lost or is less frequent in carcinomas. However, as discussed above, individual tumors can be highly heterogeneous in terms of cell populations, with respect to the methylation abnormalities we are studying (10), and for other properties. A small but important population of cells with various characteristics (for example, propensity to metastasize, etc.) can be missed when examining whole tumors (for review see Ref. 23). To examine this possibility with respect to CT gene methylation, we studied established cell lines of human colon carcinomas.

Of 19 colon cancer cell lines examined, 18 had either extensive methylation of CCGG sites in the CT gene or involvement of one or more novel sites as compared to normal tissues. DNA from four cancers had extensive methylation of the CT gene, containing only abnormal bands of 1.6-kilobase or greater indicating that most of the cells were involved (examples shown, Fig. 7, Lanes 1–4; note virtual loss of all bands below 1.6-kilobase and/or novel high molecular weight bands above 2 kilobases). Nine additional cell lines had evidence for one or more new sites of CT gene methylation in most cells (examples, Fig. 7, Lanes 5–13; note the absence or marked decrease in the normal 0.6- and/or 0.5-kilobase bands in Lanes 5–13 resulting from methylation of sites M2 or M3 or both sites simultaneously. Also, note abnormal bands of >2 kilobases in Lanes 5 and 6, and abnormal bands of 1.6-kilobase in Lanes 8 and 10, of 1.2 kilobase in Lanes 6, 8, 10–13, and 0.7 kilobase in Lane 13). Finally, some cell lines had a loss or decrease in the normal CT gene methylation at site M5, either as the sole change seen (Fig. 7, Lane 16; note marked increase in ratio of 1.0- to 2.0-kilobase bands) or simultaneously with methylation of other sites (Fig. 7, Lanes 14 and 15).

**DISCUSSION**

We now report increased methylation of a chromosome 11 gene in a spectrum of colon neoplasms in which the present
and previous data (5-7) have documented generalized hypomethylation of DNA and of specific nonexpressed genes. The significance of localized DNA hypermethylation in colonic neoplasms, as well as other neoplasms in which it is found, is not yet clear. However, there is a growing body of experimental evidence, including our own recent data (10), to suggest that such changes could be important markers for and/or mechanistically involved in DNA events resulting in gene inactivation. First, there is recent experimental data to indicate that extensive methylation of genes in vitro causes loss of expression when the gene is inserted into cells (13). Second, it is known that de novo methylation of viruses can inactivate viral gene expression and/or infectivity (14). Also, methylation of host murine DNA within 1 kilobase of the insertion site of a Moloney murine leukemia virus results in concomitant inactivation of the host gene within the methylated region (15). Third, increased methylation is associated with normal inactivation of X chromosome genes in mammals (24, 25). It is not proven whether this methylation of the inactive X chromosome is a primary event or serves to mark other cellular events. Recent data from Lock, et al., exploring the timing of methylation in the inactivation of the X chromosome, suggests that methylation may occur soon after inactivation, but may be important in “locking in” the inactive state of the gene (25). Finally, our recent studies show that an inactive conformational state of the CT gene correlates with the presence of hypermethylation in human tumors (10).

It is now clear that the type of chromosomal inactivation discussed above and the associated loss of gene expression may play a major role in tumor initiation and/or progression for some human cancers [for example, retinoblastoma and Wilm’s tumor (26, 27)]. Interestingly, at least one of these nonexpressed tumor suppressor genes may be located on the short arm of chromosome 11 (27, 28) where we have now demonstrated not only increased methylation of the CT gene (8, 9) but also of other genes in this region in human cancers (10). The potential importance of such aberrant methylation is also emphasized by data for tumors induced by adenovirus types 12 and 5 in newborn hamsters and rats where explantation and culture of the tumors reveals an association between host DNA methylation and maintenance of the tumorigenic phenotype (29, 30).

Considering the above chromosomal events which could be associated with regional hypermethylation, it is important to consider the behavior of cell populations bearing the abnor-

malities we have observed. One intriguing aspect of our data, in this regard, concerns the marked differences in frequency in which CT gene hypermethylation occurs in colon adenomas, carcinomas, and cultured tumor cells. The fact that the aberrant methylation is seen more commonly in adenomas than carcinomas, and that abnormal methylation patterns differ even in carcinomas arising from the same adenoma, suggests that there may be heterogeneous populations of cells which are constantly changing as growth evolves. At all stages of transformation, the cells with abnormal methylation could constitute an important population with regards to tumor growth. Our present data with colon cancer cell culture lines suggests that the cells which are propagated exhibit, more often, hypermethylation of the CT gene than cells in fresh tumors. Our recent studies suggest that, for the CT gene, such changes are not the result of cell culture alone, since continued passage of normal human fibroblasts and lymphocytes causes either no changes, or actual loss of the normal M5 site methylation in the CT gene (10). It is important, however, to note that others have observed distinct changes in DNA methylation patterns arising in cultured nonneoplastic human cells (31, 32). At present, our results at least suggest the possibility that, in culture, colon cancer cells with the CT gene hypermethylation initially survive better or have a selective growth advantage with time. It will be important to try to determine whether this population of cells could possess important phenotypic characteristics with respect to the clinical behavior of colon cancer.

Our studies further emphasize the need to understand the role of DNA methylation in abnormal cell growth and transformation. The spectrum of abnormalities present in patients at risk for development of colon cancer offers a unique opportunity for exploring such events. Investigations of the DNA methylation status of chromosome 11 genes in DNA from “normal” appearing mucosa of patients with familial polyposis or from patients at direct risk for inheriting this gene are warranted. Also, cell cultures of colon carcinoma represent an important model to determine further how chromosome 11 hypermethylation might be associated with the phenotypic characteristics of cells that may play an essential role in the clinical behavior of colon cancer.

REFERENCES

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