Correlation of DNA Hypomethylation with Expression of Carcinoembryonic Antigen in Human Colon Carcinoma Cells

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ABSTRACT

Using an assay based on the binding of a carcinoembryonic antigen (CEA)-specific monoclonal antibody, we have examined the expression of carcinoembryonic antigen genes in human colon tumor and normal fibroblast cell lines. CEA expression was not detectable in the normal fibroblast cell lines, whereas varying levels of high CEA expression were found in the colon tumor cell lines LS-174T, GEO, and WIDR. We have used a 550-base pair CEA probe derived from cloned complementary DNA to carry out Southern analysis of the DNA isolated from the normal and colon tumor cell lines. At high stringency, the CEA probe detected seven BamHI fragments in all DNAs analyzed. At low stringency, however, 14 BamHI fragments ranging from 1.5 to 23 kilobases were detected. Results of the Southern analysis demonstrate no amplification or rearrangement of the CEA genes in tumor cells. We used methylation-sensitive restriction endonucleases, HpaII and HhaI, to compare the degree of methylation of CEA family of genes in normal and colon tumor cell lines. Our results demonstrate that the CEA family of genes exists in a state of hypermethylation in the normal cell lines. In contrast, the CEA gene(s) are relatively hypomethylated in the tumor cell lines, suggesting a correlation between the state of methylation and degree of expression of the CEA gene(s). A comparison of the state of methylation of the CEA gene(s) in cells before and after treatment with the γ-interferon (which up-regulates CEA steady-state mRNA levels) showed no detectable difference in the degree of DNA methylation. The segments of CEA genes that are hypermethylated in normal cells, but are hypomethylated in tumor cells, were also identified. Thus, these studies may help identify the sites of methylation that are crucial for the control of CEA gene regulation.

INTRODUCTION

CEA, a Mr 180,000 glycoprotein, belongs to a family of immunologically related oncodevelopmental antigens. CEA expression is associated with human embryogenesis and also with the development of a variety of human tumors of epithelial origin (1, 2). Because CEA is present on a variety of human tumor cells and in sera of cancer patients, it has been widely used as a human tumor marker (3-5). However, the tumor specificity of the antigen is clouded due to the presence of several CEA-related molecules in normal cells (6-10). Previous studies (11, 12) have shown that the level of antigen expression varies among carcinomas from different patients. The molecular basis for this differential expression in different tumors and tumor cell lines remains obscure.

It has been suggested that mutations, gene rearrangement, gene amplification, deletion, or alteration in DNA methylation pattern may perturb gene expression, which in turn may have a bearing on neoplastic transformation of cells. Transcriptional activity of several eukaryotic and viral genes has been found to be inversely correlated to the degree of methylation of cytosine residues at CpG dinucleotides located in the DNA sequence of the gene (reviewed in Ref. 13). Absence of DNA methylation is an essential prerequisite for gene expression, though not all hypomethylated genes are necessarily transcriptionally active. In addition, genomic DNA from a large number of human and animal tumor cells is found to be hypomethylated (14). For example, it has been shown that the ras gene family is hypomethylated in primary colon and lung carcinomas (15), and a specific site of the c-myc gene undergoes hypomethylation in human tumor cell lines (16).

The present study was undertaken to begin to elucidate the molecular basis of the regulation of CEA gene expression in colon tumor cell lines. The data presented here rule out gene amplification and rearrangement as the basis for enhanced expression of the CEA gene. Our results suggest that, in several human tumor cell lines, the elevated level of CEA expression is correlated with hypomethylation of the CEA gene.

MATERIALS AND METHODS

Cell Culture. The human epithelial colon carcinoma cell lines WIDR and GEO were gifts from Dr. P. Noguchi (NIH, Bethesda, MD) and Dr. M. Brattain (Baylor University School of Medicine, Houston, TX), respectively. The human colon carcinoma cell line LS-174T (ATCC CCL 188) and the human fibroblast cell lines, WI-38 (ATCC CCL 75) and MRC-5 (ATCC CCL 171), were purchased from the American Type Culture Collection (Rockville, MD). All cell lines were grown as monolayer cultures in growth medium as recommended by their respective sources. The growth medium was supplemented with 10% heat-inactivated fetal calf serum and 50 μg/ml gentamicin. Human lymphocytes were obtained from blood samples derived from two normal individuals and were also obtained as cell pellets from Creighton University (Lincoln, NE).

MAb. The details of the production, isolation, and characterization of the IgG2a MAb COL-4 have been reported elsewhere (12).

Interferon Treatment. Recombinant Hu-IFN-γ was obtained from Hoffmann-La Roche Inc. (Nutley, NJ) at a specific activity of 1 × 10^7 antiviral units/ml. It was diluted to a concentration of 2.5 × 10^6 antiviral units/ml in RPMI 1640 containing 1% (v/v) BSA and 25 mm N-2-hydroxymethylpiperezine-N'-2-ethanesulfonic acid. The stock solution was stored at 4°C. All interferon concentrations are expressed in international reference units as determined by appropriate comparison with the International Reference Reagent Gxgol-901-535 supplied by the Research Resource Branch, National Institute of Allergy and Infectious Diseases, NIH. Treatment of monolayer cultures with Hu-IFN-γ was performed when cell cultures were 75% confluent. Culture medium was replaced with fresh medium supplemented with Hu-IFN-γ at a final concentration of 2000 antiviral units/ml. Cultures were incubated at 37°C for 72 h.
Solid Phase RIA. Cell extracts of the colon carcinoma and fibroblast cell lines were prepared, and the binding of MAb to the cell extracts was determined in a RIA as described by Horan Hand et al. (11). Briefly, 5 μg of cell extract were added to the wells of a microtiter plate and dried at 37°C. The wells were treated with 5% BSA (w/v) in 1× phosphate-buffered saline for 1 h to minimize nonspecific binding. Cells were then incubated with MAb COL-4 at 37°C for 1 h. After one washing with 1× phosphate-buffered saline containing 1% BSA, 125I-labeled goat anti-mouse IgG antibodies were added, and the plates were incubated for 1 h at 37°C. The plates were washed, and the radioactivity bound to each well was determined with a LKB RiaGamma counter (Model 1274; Piscataway, NJ).

DNA Isolation and Restriction Endonuclease Cleavage. High-molecular-weight DNA was isolated from cultured cells using a procedure described by Krieg et al. (17). Restriction endonuclease digestion of DNA was carried out as recommended by the suppliers of the enzymes (Bethesda Research Laboratories, Gaithersburg, MD). Digestions using two enzymes with the same salt requirement were performed simultaneously. For two enzymes with different salt requirements, cleavage was carried out sequentially with an ethanol precipitation step in between.

Southern Blot Analysis. Approximately 10 μg of each cleaved DNA were fractionated by horizontal electrophoresis through 0.8% agarose gel. HindIII fragments of wild type λ DNA and HaeIII fragments of the replicative form of φX174 DNA were used as size markers. DNA was transferred to Bio-Rad nylon membranes (ICN, Irvine, CA) by the Southern procedure (18). Filters were air dried and baked in a vacuum oven for 2 h at 80°C. They were prehybridized overnight at 37°C in 3 M NaHPO₄ containing 50% formamide, 10% dextran sulfate, 0.1% BSA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 1.0% SDS, and 100 μg/ml denatured salmon sperm DNA. Hybridization was carried out for 18 h at 37°C by replacing the prehybridization mixture with a fresh aliquot supplemented with 5.0 × 10⁶ cpm/ml of the CEA probe. A 32P-labeled CEA probe was synthesized by nick translating a 550-base pair PstI fragment of a CEA cDNA clone, which represents the repeating domain of the CEA gene (19). Following hybridization, the filters were washed twice for 15 min in a solution containing 0.5% SDS in 6 × SSC at room temperature and then subsequently washed twice for 30 min in a solution containing either 0.5% SDS in 1 × SSC (relaxed) or 0.1% SDS in 0.1 × SSC (stringent) at 65°C. The blots were exposed to Kodak XAR-2 film with intensifying screens at −80°C.

RESULTS

Constitutive Levels of CEA Glycoprotein Expression in Human Colon Carcinoma and Normal Fibroblast Cell Lines. The constitutive levels of CEA expression in the human colon carcinoma cell lines LS-174T, GEO, and WIDR and in the normal fibroblast cell lines WI-38 and MRC-5 were determined by a solid phase RIA based on the binding of COL-4, a CEA-specific MAb, to the cell extracts. Table 1 shows the reactivity of total cell extracts of these cell lines with decreasing dilutions of MAb COL-4. Some of these results have been reported elsewhere (20) but are shown in Table 1 to put the rest of the data reported in this study in proper perspective. The three colon tumor cell lines showed different levels of CEA expression, as evidenced by differential binding of COL-4. The highly differentiated epithelial colon tumor cell lines LS-174T and GEO showed high levels of CEA expression, whereas WIDR expressed the tumor antigen at a level that was 4- and 10-fold lower than that found in LS-174T and GEO cells, respectively. No expression of CEA could be detected in normal fibroblast cell lines included in this experiment. In contrast, the three human colorectal tumor lines and the two normal fibroblast cell lines constitutively expressed the class I HLA-ABC antigen determinants. Utilizing MAb W6/32, the range of reactivity for these five cell lines was 2000–5000 cpm when analyzed in a solid phase RIA (21, 22).

Analysis of CEA and CEA-related Genes. We have used a 550-base pair cloned cDNA probe (19) representing the repeating domains of the CEA gene to detect CEA-related genes in the total human cellular DNA (Fig. 1). BamHI-digested DNA from the LS-174T, GEO, and WIDR colon tumor cell lines (Fig. 1, Lanes 1–3, respectively), normal fibroblasts (Lanes 4 and 5), and normal lymphocytes (Lanes 6 and 7) was electrophoresed. Following hybridizations, the Southern blots were washed under relaxed (Fig. 1A) and highly stringent (Fig. 1B) conditions. At higher stringency, the CEA probe hybridized with only seven of the BamHI fragments, which are estimated to be approximately 21, 9, 5.8, 3.6, 3.1, 2, and 1.6 kilobases long. Under similar conditions of stringency but using a CEA cDNA probe of 900 base pairs, Kamark et al. (23) have also reported seven CEA-specific fragments when BamHI-digested DNA of LS-174T cells was hybridized. At low stringency, however, at least 14 BamHI fragments ranging in size between 23 and 1.5 kilobases were hybridized to the CEA probe. At low stringency, Thompson et al. (19) detected 9 to 11 hybridization bands when SstI or EcoRI fragments of human normal leucocytes, colon tumors, and cell line LS-174T were hybridized to two different probes derived from a cDNA clone of the CEA gene family. However, only one band of hybridization remained for each probe when the same filters were washed at a higher stringency. The hybridization of the probe to a large number of restriction endonuclease cellular DNA fragments following relaxed conditions of blot washing, as reported here and by Thompson et al. (19), supports the theory that CEA belongs to a family of multiple genes. Hereafter, we conducted the washing of the Southern blots under highly stringent conditions to further ensure the specificity of hybridization.

An examination of Fig. 1 (A and B) reveals no significant difference in either pattern or intensity of hybridization of DNAs isolated from the tumor (Lanes 1–3) and normal (Lanes 4–7) cells. Similar results were obtained by Southern analysis of HindIII fragments (Fig. 1C) and DNA fragments generated by several other restriction endonucleases (data not shown). The lower intensity of hybridization of the largest band in Lanes 1–4 (Fig. 1C) is a technical artifact as revealed by later experiments. These observations rule out the possibility of amplification or rearrangement of the CEA genes in tumor cells. Thus, our findings confirm and extend earlier reports (19, 23) showing no rearrangement of the CEA-related genes in the colon tumor cell lines LoVo and LS-174T.

Analysis of the Methylation Patterns of the CEA Gene(s) in Normal and Colon Tumor Cell Lines. To investigate a possible correlation between CEA gene expression and its state of methylation, we studied the methylation pattern of the CEA family.

<table>
<thead>
<tr>
<th>Anti-CEA MAb COL-4 (ng/well)</th>
<th>Colon carcinoma cell lines</th>
<th>Normal cell lines</th>
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<tbody>
<tr>
<td></td>
<td>LS-174T</td>
<td>GEO</td>
</tr>
<tr>
<td>20</td>
<td>7,540</td>
<td>18,220</td>
</tr>
<tr>
<td>0.8</td>
<td>2,630</td>
<td>10,760</td>
</tr>
<tr>
<td>0.032</td>
<td>1,080</td>
<td>3,230</td>
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* Some of the above results have been reported previously (20) but are shown here to put the data which follow in proper perspective. NEC, negative.

1 cpM/well containing 5 μg of protein.

2 J. Kantor, R. Tran, J. Greiner, S. Pestka, P. Fisher, J. E. Shively, and J. Schlam. Regulation of carcinoembryonic antigen mRNA levels in human colon carcinoma cells by recombinant human γ-interferon, submitted for publication.
ANALYSIS OF CEA-RELATED GENES IN HUMAN CARCINOMA CELLS

Fig. 1. Hybridization pattern of DNAs from human colon carcinoma cell lines, fibroblast cell lines, and normal lymphocytes to a CEA cDNA probe. DNAs from colon carcinoma cell lines LS-174T (Lane 1), GEO (Lane 2), and WIDR (Lane 3); normal fibroblast cell lines WI-38 (Lane 4) and MRC-5 (Lane 5); and normal lymphocytes (Lanes 6 and 7) were digested with BamHI (A and B) or HindIII (C). Southern blots were hybridized to a 32P-labeled CEA cDNA probe and washed at 65°C either with 1 x SSC containing 0.5% SDS (A) or 0.1 x SSC containing 0.1% SDS (B and C). The exposure time of the autoradiograms was 3 days. Molecular weight markers (HindIII-digested bacteriophage λ DNA and Haellll-digested replicative form of φX174 DNA) are indicated in kilobase pairs (Kb).

Fig. 2. Methylation of the CEA genes in human colon carcinoma and normal fibroblast cell lines. DNAs isolated from the colon carcinoma and normal fibroblast cell lines were digested with eitherMspI (A), HpaII (B), or HhaI (C). Southern blots were hybridized to the CEA cDNA probe, and the blots were washed at 65°C with 0.1 x SSC containing 0.1% SDS. The order of the samples on the gel is: colon carcinoma cell lines LS-174T (Lane 1), GEO (Lane 2), and WIDR (Lane 3) and normal fibroblast cell lines WI-38 (Lane 4) and MRC-5 (Lane 5). Kb, kilobases.

Results of the Southern analysis of MspI-, HpaII-, and HhaI-treated DNAs from colon tumor and normal fibroblast cell lines are presented in Fig. 2. MspI digestion of DNAs (Fig. 2A) invariably generated a number of small fragments ranging in size between 1 and 2 kilobases. The patterns of CEA-specific fragments generated by MspI treatment of the colon tumor and normal fibroblast cell line DNAs were remarkably similar. In contrast, digestion of the DNAs with methylation-sensitive enzyme HpaII (Fig. 2B) produced CEA-specific fragments of a very large size. HpaII cleavage of the DNAs from two normal

of genes in normal fibroblast and colon tumor cell lines using methylation-sensitive restriction endonucleases. One such enzyme, HhaI, which recognizes the sequence 5'-GCGC-3', does not cleave DNA when the internal cytosine of the recognition sequence is methylated. Especially useful is the isoschizomeric pair of restriction endonucleases, MspI and HpaII, which recognize the sequence 5'-CCGG-3'. Of these isoschizomers, MspI cleaves the DNA regardless of the methylation state of the internal cytosine, whereas HpaII cuts the DNA only when this internal cytosine is not methylated (24).
fibroblast cell lines (Lanes 4 and 5) produced several CEA-specific fragments longer than 23 kilobases, which overlapped each other because of the poor resolving power of the gel for this size range. In addition, HpaII digestion of these DNAs generated fragments of approximately 20, 13, 9, and 5 kilobases. The HpaII digestion patterns of colon tumor cell DNA (Lanes 1–3) were conspicuously different from those of normal fibroblast DNAs. Furthermore, DNA from each tumor cell line yielded a unique pattern of hybridization. None of the tumor cell DNA yielded the CEA-specific fragments of 23 kilobases or larger that are so prominent among the HpaII fragments of the normal fibroblast cell DNA. HpaII digestion of the high CEA-expressing LS-174T and GEO cells produced an array of smaller fragments not produced by HpaII-digested normal fibroblast cell DNA. However, CEA-specific fragments generated by HpaII digestion of WIDR cell DNA were relatively larger.

Similarly, HhaI digestion patterns of normal fibroblast and colon tumor cell DNA are remarkably different from each other (Fig. 2C). DNAs isolated from tumor cell lines LS-174T and GEO (Lanes 1 and 2) are most extensively fragmented by HhaI digestion, whereas WIDR cell DNA (Lane 3) yields relatively larger HhaI fragments, although it is significantly more susceptible to HhaI digestion than DNA isolated from the normal fibroblast cells (Lanes 4 and 5).

These results clearly establish that the CEA genes exist in a state of hypermethylation in the normal fibroblast cell lines, WI-38 and MRC-5, which do not express detectable levels of CEA antigen. In the colon carcinoma cell line WIDR, a low expressor of CEA, the CEA-specific sequences show a low degree of hypomethylation; whereas in the tumor cell lines LS-174T and GEO, high expressors of CEA, the degree of hypomethylation is significantly higher. These observations suggest a correlation between the degree of DNA methylation and CEA gene expression.

Effect of Hu-IFN-γ on CEA at the DNA Level. In a previous study (21), we reported that Hu-IFN-γ, a biological modifier, can modulate the level of CEA gene expression in certain human colon tumor cell lines. Specifically, treatment of the WIDR cell line with Hu-IFN-γ resulted in a 6-fold increase in the level of CEA glycopeptide expression. This increase in protein level is concomitant with an increase in CEA mRNA transcripts in the cells. However, treatment of LS-174T and GEO cells with γ-interferon showed little, if any, effect on CEA expression (21). In an attempt to study the molecular mechanism of modulation of CEA gene expression by Hu-IFN-γ, we have examined the effect of interferon treatment at the DNA level. The colon tumor cell lines LS-174T, GEO, and WIDR and the normal fibroblast cell line WI-38 were treated with γ-interferon at 2000 antiviral units/ml for 72 h. Our previous study (20) demonstrated that the concentration and the duration of treatment of the interferon used here were optimal for enhancing CEA expression. High-molecular-weight cellular DNA was isolated from cells treated with Hu-IFN-γ and untreated, and aliquots of cell extracts were assayed for CEA. The DNA was cleaved with the appropriate restriction endonucleases, electrophoresed, and subjected to Southern blot analysis. The interferon-treated cells showed no evidence of amplification or rearrangement of the CEA genes (data not shown). We then analyzed the methylation patterns of the DNA using the methylation-sensitive enzymes HpaII (Fig. 3A) and HhaI (Fig. 3B). The results showed no difference in the state of methylation of the CEA genes in colon tumor cells before or after treatment with Hu-IFN-γ. Thus, interferon does not modulate the CEA gene expression through DNA methylation.

Identification of Hypomethylated Segments of CEA Genes in Colon Tumor Cells. We have also attempted to identify those hypermethylated segments of the CEA genes in normal fibroblast cell lines that exist in the hypomethylated state in tumor cell lines. To that end, we have examined the state of methylation of the CEA-specific BamHI fragments of normal fibroblast and colon tumor cell line DNA. The choice of BamHI was based on the results of our earlier experiments showing that these fragments, ranging in size from 21 to 1.5 kilobases, are well resolved on agarose gel (Fig. 4, Lane 6). BamHI-treated

Fig. 3. Methylation status of the CEA genes in human colon carcinoma cell lines treated with recombinant human interferon (Hu-IFN-γ). DNA from cell lines LS-174T (Lane 1), GEO (Lane 2), WIDR (Lane 3), and WI-38 (Lane 4) was isolated before (−) and after (+) treatment with Hu-IFN-γ. Interferon treatment was carried out as described in “Materials and Methods.” (A) HpaII- and (B) HhaI-cleaved DNAs were electrophoresed and subjected to Southern blot analysis as described in Fig. 2. Kb, kilobase pairs.
colon carcinoma cell lines. High-molecular-weight cellular DNAs were cleaved by the methylation-sensitive enzyme HpaII and thus are hypermethylated. LS-174T cell DNA was subsequently digested with HpaII and was isolated from LS-174T cells.

DISCUSSION

In this paper, we have attempted to begin to elucidate the mechanism(s) of the regulation of CEA gene expression at the DNA level. Specifically, we have looked for gross genomic abnormalities such as gene rearrangement (25-28), gene amplification (29-30), and alteration in DNA methylation (14-16, 31, 32), all of which have been reported to occur in neoplastic tissue. Under stringent conditions of Southern hybridization and blot washings, our probe, representing the repeating domains of CEA, hybridized with seven major BamHI fragments of genomic DNA. This conforms to an earlier report by Kamarck et al. (23). Parenthetically, we have shown that more than 14 BamHI fragments of the CEA gene are detected when conditions of blot washing are relaxed. These findings are yet another indication that CEA gene belongs to a family of related genes (19, 23, 33).

Results of the Southern analysis of DNA cleaved with BamHI, HindIII, and several other restriction endonucleases did not reveal any evidence of gross rearrangement or amplification of the CEA gene. However, our results cannot rule out the possibility of any point mutations or small deletions suffered by the CEA genes in tumor cell lines.

Southern analysis of the DNAs treated with methylation-sensitive restriction endonucleases, HpaII and HhaI, clearly demonstrate that the CEA genes, which are hypermethylated in the normal fibroblast cells, exist in a state of hypomethylation in tumor cell lines. Moreover, the degree of hypomethylation is higher in the colon tumor cell lines LS-174T and GEO, which express high levels of CEA.

We have reported earlier that γ-interferon can modulate the level of CEA gene expression in certain human colon tumor cell lines (22). Treatment of the WIDR cell line, a low expressor of CEA, with Hu-IFN-γ results in an increase in CEA-specific mRNA and a 6-fold increase in the level of CEA glycopeptide expression. However, interferon treatment does not alter the level of CEA gene expression in those cell lines that constitutively express high levels of the antigen (21). The mechanism of interferon action remains unclear. We have not detected any gross demethylation of the CEA gene after treatment of tumor cells with interferon. It is to be noted, however, that a subtle change in DNA methylation critical for the regulation of the gene might remain undetected by our technique. In fact, only a small percentage of the CpG methylations are detectable by the available methylation-sensitive restriction endonucleases. A more rigorous analysis of specific sites of DNA methylation may be needed to establish whether or not interferon modulates CEA gene expression through DNA methylation. It is expected that an antigen associated with development and malignancy is regulated by several complex mechanisms operating at different levels of transcription and protein synthesis. It is, therefore, likely that interferon modulates CEA gene expression by a mechanism that does not involve DNA methylation.

Our data suggest a correlation between the state of methyla-
tion and expression of the CEA gene. An inverse correlation between expression and the degree of methylation has been demonstrated for several eukaryotic genes (13), and alteration in DNA methylation has been implicated in human and animal malignancies. Furthermore, it has been shown that specific genes (14, 31, 32), including cellular oncogenes (15, 16), exist in a state of hypomethylation in tumor cell lines. It has not escaped our attention that methylation of only the regulatory elements of a particular gene determines its transcriptional activity. It is now important to identify the specific sites of DNA methylation that control CEA gene expression. In this study, we have identified those DNA fragments of the CEA gene(s) that are hypermethylated in normal cells but exist in a hypomethylated state in the colon tumor cell lines. An extension of this approach may help to identify the sites of methylation that are crucial for CEA gene regulation.

REFERENCES


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