Effect of Sodium Butyrate on Carcinoembryonic Antigen Production by Human Colonic Adenocarcinoma Cells in Culture

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ABSTRACT

The effects of sodium butyrate, a known differentiation-inducing agent, on carcinoembryonic antigen (CEA) production by human colonic adenocarcinoma cells in culture were studied using seven different colorectal tumor cell lines. Sodium butyrate (2 mM) treatment resulted in a marked induction of CEA content in two cell lines, HRT18 and HCT48; a moderate induction of CEA content in LS174T and SKCO-1; and no increase of CEA content in SW480, SW620, and SW1116. Of those showing an induction of CEA production by butyrate, a dose-related increase up to 5 mM butyrate concentration could be demonstrated. This enhanced phenotypic expression of CEA in cells treated with butyrate could be blocked by the protein synthesis inhibitor cycloheximide and by actinomycin D, an inhibitor of RNA transcription. Neither the subcellular distribution of CEA nor the rate of release of CEA into culture medium by the cells was changed by the treatment. Cell surface CEA was labeled with galactose oxidase, followed by reaction with sodium borotritide, and was subsequently immunoprecipitated specifically with anti-CEA antiserum. Examination of labeled cell surface CEA from control and butyrate-treated HCT48 and LS174T cells by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a major glycoprotein with a molecular weight of 200,000, indicating that no new form of CEA was induced with sodium butyrate treatment. A similar result was observed when the cell membrane proteins, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were transferred onto nitrocellulose paper and reacted with rabbit anti-CEA antiserum and 125I-Protein A. Lectin affinity chromatography of cellular CEA revealed a higher binding of CEA from butyrate-treated HCT48 cells to Ricinus communis agglutinin-Sepharose 4B than that from untreated cells, while the binding of CEA to wheat germ agglutinin-Sepharose 4B and concanavalin A agglutinin-Sepharose 4B was similar in butyrate-treated and untreated cells.

It is concluded that butyrate may enhance expression of the differentiated function of some human colorectal tumor cells, as indicated by an elevation of CEA production. This elevated CEA content in butyrate-treated cells appears to be caused by increased synthesis of the CEA molecule.

INTRODUCTION

Sodium butyrate has been shown to be an effective differentiation-inducing agent in a murine erythroleukemia system (12, 18). We have examined the effect of butyrate on 2 human colonic tumor cell lines, SW620 and SW480 (10), and one human rectal tumor cell line, HRT18 (23). A reduced in vitro tumorigenicity was found in butyrate-treated tumor cells together with elevated alkaline phosphatase activity and changes in the cell surface protein-labeling pattern.

One of the characteristics of human colorectal tumor cells is their production of CEA,3 an oncofetal protein produced only in trace amounts by the normal adult colorectal cells (3). Studies of 9 different colorectal tumor cell lines indicated that all of them contained CEA, although the relative amount of CEA varied considerably among cell lines.4 The tissue and serum levels of CEA in humans and their relationship to the degree of differentiation of colorectal tumors have been studied. In general, a higher tissue content and serum level appear to occur in the case of well-differentiated tumors, while a low or barely detectable level of CEA is associated with poorly differentiated or anaplastic colon cancer tissues (13). Thus, it would appear that the degree of expression of CEA is related to the degree of differentiation of the cancer cells.

In the present study, we sought to determine if a putative differentiating agent like sodium butyrate could selectively induce an increase in the CEA content of human colorectal tumor cells.

MATERIALS AND METHODS

Materials

The CEA-Roche assay kit was purchased from Hoffman-La Roche Inc. (Nutley, N. J.). Purified CEA from liver metastases of a carcinoma of the colon was kindly provided by Dr. H. J. Hansen, Hoffman-La Roche Inc. Triticated sodium borohydride (15.0 Ci/mmol) was a product of Amersham/Searle Corp. (Arlington Heights, Ill.). Con A-Sepharose, Sepharose 4B, and Sephadex G-200 were obtained from Pharmacia (Uppsala, Sweden). NP40 was from Particle Data Laboratories (Elmhurst, Ill.). Staphylococcus aureus Protein A and zymosin (fixed and killed) S. aureus were obtained from Zymed Laboratories (Burlingame, Calif.). Atofluor was from National Diagnostics (Somerville, N. J.). Lactose, 2-methyl-o-mannoside, N-acetylgalactosamine, galactose oxidase, neuraminidase, cycloheximide, actinomycin D, and sodium butyrate were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Methods

Treatment of Cell Cultures with Sodium Butyrate. The human colorectal cancer cell lines HCT48 and HRT18 were generously given

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3 The abbreviations used are: CEA, carcinoembryonic antigen; Con A, concanavalin A agglutinin; NP40, Nonident P-40; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; RCA, Ricinus communis agglutinin; WGA, wheat germ agglutinin.

to us by Dr. Richard M. Schultz, National Cancer Institute (Bethesda, Md.). The human colon cancer cell line SKCO-1 was kindly provided by Dr. Jorgen Fogh, Sloan-Kettering Institute for Cancer Research (Rye, N. Y.). The human colon cancer cell lines SW480, SW620, and SW1116 were developed at the Scott C. White Clinic (Temple, Texas) and were generously provided by Col. Albert Lebovitz. LS174T colon cancer cell line was a gift from Dr. Barry D. Kahan of the University of Texas (Houston, Texas). All of these cell lines were cultured as monolayers in 75-cm² tissue culture flasks or 60-mm Petri culture dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 ìg/ml). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

The treatment of cells with sodium butyrate was carried out by culturing them in culture medium supplemented with sodium butyrate at the concentrations indicated in the text, and the growth medium was changed every fourth day unless otherwise noted.

Preparation of Cell Homogenate, Membrane, and Cytoplasmic Fraction. For the determination of the subcellular distribution of CEA, cells cultured in 75-cm² tissue culture flask were used. The cells were scraped after 4 washings and suspended in isotonic PBS, pH 7.4. The cell suspension was sonicated (twice) for 15 sec on ice and designated cell homogenate. The homogenate was centrifuged at 100,000 × g for 1 hr to obtain the membrane pellet and the cytoplasmic fraction. Both the cytoplasmic and membrane fractions were solubilized in 1% NP40 with sonication and used for the CEA assay. Duplicate flasks were used for the determination of cell numbers.

CEA Assay. CEA levels were determined by the radioimmunoassay method as suggested by Hoffman-La Roche Inc.

Protein Determination. Protein content was estimated by the method of Lowry et al. (13) using crystalline bovine serum albumin as the standard.

Release of CEA into Medium. Human colon cancer cells chosen for this study were seeded in an aliquot of 3 × 10⁶ cells into 60-mm Petri culture dishes containing 8 ml of medium. After 24 hr, the medium was replaced with fresh medium with or without sodium butyrate. The medium was changed every 4 days of incubation thereafter. Dishes were harvested every 24 hr up to 10 days, collecting the medium and cells separately. The CEA contents of both media and cells were determined by direct CEA radioimmunoassay method. Protein content of the cell homogenate was determined by the method of Lowry et al. Net CEA released by cells per day was calculated by subtracting total CEA in the medium of the previous day and dividing by the total cell number measured in duplicate dishes that day.

Effect of Protein and RNA Synthesis Inhibitors. The protein synthesis inhibitor cycloheximide and actinomycin D, an inhibitor of RNA transcription, were used to examine the induction of CEA in HCT48 by sodium butyrate. The inhibitors were added at concentrations of 20 and 1 ìg/ml respectively, to the culture media of cells grown in the presence and absence of 2 mM sodium butyrate. The cells were cultured in 35-mm plastic Petri dishes for 4 days and then assayed for CEA and protein.

Surface Labeling of Cells. Labeling the surface glycoproteins of cells with galactose oxidase and tritiated sodium borohydride was performed as described previously (22). Cells cultured in 75-cm² tissue culture flasks were labeled in situ after 4 washings with isotonic PBS, pH 7.4. Washed cells were treated with 25 units (45 ìg) of galactose oxidase in 4 ml of Hanks' balanced salt solution, pH 7.0, at 37°C for 60 min. The cells were then washed once and incubated with 0.1 ml (2.0 µCi) of tritiated sodium borohydride (15 Ci/mmol) for 10 min at 25°C in 2 ml of Hanks' balanced salt solution, pH 8.0. Solid tritiated sodium borohydride was dissolved in 0.01 N NaOH and kept in liquid nitrogen until use. Labeled cells were washed 4 times with PBS and harvested with a rubber policeman. After centrifugation of the sonicated cell homogenates at 100,000 × g for 60 min, the pellet was considered the membrane fraction.

Treatment of Cells with Neuraminidase. To a flask of cells were added 4 ml of Hanks' balanced salt solution, pH 6.0, and 0.1 unit of Vibrio cholerae neuraminidase. The cells were incubated at 37°C for 30 min. The cells were then washed 4 times with PBS prior to use.

SDS-Polyacrylamide Gel Electrophoresis. SDS-polyacrylamide slab gel electrophoresis was run in a discontinuous buffer system and was carried out according to the method of Laemmli (11) with a vertical slab gel apparatus. Samples were dissolved in 0.021 M Tris buffer, pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol, 0.001% bromophenol blue, and 10% glycerol. Just prior to application to the gel, samples were heated at 100°C for 3 min. Gel thickness was 1.5 mm, and the current applied was 50 ma/gel. An average run finished within 2 hr. Gels were stained with Coomassie Brilliant Blue for proteins according to the method of Fairbanks et al. (6).

Preparation of Monospecific Anti-CEA Antiserum. Anti-CEA antiserum was prepared in rabbit using CEA purified from liver metastases of a colon adenocarcinoma. The antiserum was absorbed with glutaraldehyde-insolubilized normal human plasma proteins and human erythrocytes.

Immunoprecipitation of Labeled Cellular CEA. Detergent-solubilized, galactose oxidase-borotritide-labeled cell membrane containing 50 ìg of protein was incubated with 10 µl of rabbit anti-CEA antiserum overnight at 4°C. The mixture was then incubated with 50 µl of 10% Protein A-S. aureus for 30 min at room temperature and centrifuged at 2500 × g for 10 min in a Beckman tabletop centrifuge. The bacterial pellet was washed twice with PBS containing 1% deoxycholate and 0.1% NP40 and then twice with PBS. The washed pellet was resuspended in 0.5 ml 1 N NaOH and counted in 5 ml of scintillation fluid using a Beckman LS1800 scintillation counter.

 Autoradiography. Gels containing ßH-labeled proteins were fixed and stained with Coomassie Brilliant Blue. After a rinsing with deionized water, gels were incubated in Autofluor, dried, and exposed to X-ray film (Kodak X-omat R film).

Radioiodination of Staphylococcus Protein A. Radioiodination was performed by reacting 10 µg of S. aureus protein A with 20 µg of chloramine-T and 0.5 mCi of Na¹²⁵I in 200 µl of 0.5 % phosphate buffer, pH 7.4. The reaction was carried out in an ice bath for 5 min and stopped with 20 µl of sodium metabisulfite (2 mg/ml). A 5-ml Sephadex G-25 column was used to separate radioiodinated protein A from free ¹²⁵I.

Electrophoretic Transfer and Identification of Membrane-associated CEA. Electrophoretic transfer of proteins from SDS-polyacrylamide slab gels to nitrocellulose paper was performed by the method described by Towbin et al. (20). The transfer was carried out overnight at 80 V and 100 ma with a Trans-Blot cell (Bio-Rad Laboratories, Richmond, Calif.). The buffer used in the transfer was composed of 25 mM Tris-HCl, pH 8.3, containing 192 mM glycine and 20% methanol.

Binding of antibody and ¹²⁵I-Protein A to the nitrocellulose-immobilized proteins was performed according to the method of Burnette (22). Immediately following transfer, the nitrocellulose was incubated in a solution of 0.9% NaCl:10 mM Tris-HCl, pH 7.4, containing 2% bovine serum albumin for 30 min at 45°C. The paper was transferred to the same solution containing diluted CEA antiserum and incubated for 120 min at room temperature. Washing was carried out for 10-min intervals with isotonic PBS, pH 7.4, PBS containing 0.05% NP40 (twice), and PBS again. The nitrocellulose was then incubated in the bovine serum albumin solution containing 2 × 10⁶ to 5 × 10⁷ cpm of ¹²⁵I-Protein A per ml for 30 min at room temperature and washed as described above. The paper was blotted dry, wrapped in Handi-wrap, and exposed to Kodak XR-2 film utilizing a Dupont intensifying screen.

Lectin Affinity Chromatography. RCA was purified from castor beans according to a modification of a method of Nicholson and Blau-}


dstein (17), but no further separation of the lectin with a molecular weight of 120,000 (RCA1) from that with a molecular weight of 60,000 (RCA2) was attempted. WGA was purified according to the method of Bassett (1). Both purified lectins were coupled to CBN-activated Sepharose 4B as described by March et al. (14). Detergent-solubilized cell membrane preparations from cancer cells with or without treatment
by sodium butyrate were applied to a lectin column (volume, 0.5 ml) equilibrated with PBS-0.1% NP40. The column was washed with same buffer, and the bound CEA was eluted with the appropriate sugar (0.5 M α-methyl-β-mannoside for Con A-Sepharose, 0.2 M lactose for RCA-Sepharose, and 0.2 M N-acetylglucosamine for WGA-Sepharose) dissolved in PBS containing 0.1% NP40.

RESULTS

The effect of different concentrations of butyrate on human colorectal adenocarcinoma cells was examined. Tumor cell lines HRT18 and SW1116, which contained relatively low levels of CEA; HCT48 cells, which contained a medium level of CEA; and LS174T cells, which contained a high level of CEA, were used for the study. Induction of CEA production by butyrate was found in all cells studied except for SW1116 cells, which are relatively insensitive to butyrate treatment (Chart 1). This experiment was repeated with the same results. Cells for which CEA was inducible showed a dose-related increase of CEA activity with sodium butyrate concentration up to 5 mM. Sodium butyrate at a concentration of 5 mM or higher was cytotoxic. Therefore, we have used 2 mM sodium butyrate throughout our studies.

A time course study of cells in the presence of 2 mM sodium butyrate showed an increase in accumulation by the cells with longer incubation time (compared with untreated cells) (Chart 2). This experiment also was repeated, and the results were the same. However, at Day 10, both LS174T and HCT48 had a lower CEA content compared to that at Day 8. All of the subsequent experiments were performed with 8 days of exposure to butyrate.

The effect of sodium butyrate on 7 colonic tumor cell lines is summarized in Table 1. SW480, SW620, and SW1116, which contain low levels of CEA, showed no change of CEA activity after sodium butyrate treatment. These cells have previously been shown to have inducible alkaline phosphatase activity and cell surface membrane protein changes by butyrate. Although it also contains a low level of CEA activity, HRT18 showed a remarkable induction of its CEA content (60-fold) by butyrate. The CEA activity in HCT48 was increased 6-fold, while that of SKCO-1 and LS174T increased 1.6- and 2.4-fold, respectively. When its subcellular distribution was studied,
CEA was found to be mainly associated with the membrane fraction (80 to 90%), which is the same as we have reported previously (22). Treatment of cells with butyrate did not change this distribution. The amount of CEA activity found in the culture medium increased in cells treated with butyrate. However, the ratio of CEA released into the medium to total cell-bound CEA did not show any significant difference between treated and untreated cells.

Cycloheximide (20 μg/ml) and actinomycin D (1 μg/ml) were tested for their ability to inhibit the increased content of CEA caused by treatment with butyrate (Table 2). Cycloheximide inhibited 75% of cellular protein synthesis in HCT48. Both inhibitors completely blocked the increased CEA production caused by butyrate treatment. Cell surface-labeling methods utilizing galactose oxidase and sodium borotritide have been used previously to probe externally oriented glycoproteins of mammalian cell membranes (8). Cell surface glycoproteins of intact HCT48 and LS174T cells were labeled by this technique and solubilized with the non-ionic detergent NP40. Labeled cell surface CEA was specifically immunoprecipitated with rabbit anti-CEA antiserum and Protein A-bearing S. aureus and subsequently examined by SDS-polyacrylamide gel electrophoresis. The fluorograph of labeled CEA showed a major glycoprotein with a molecular weight of 200,000 in both cells (Fig. 1). Cell surface CEA showed an increase in labeling in cells treated with neuraminidase prior to galactose oxidase treatment. An even more dramatic increase in labeling of cell surface CEA was found in butyrate-treated HCT48 and LS174T cells.

For the characterization of cell membrane-associated CEA, human colonic tumor cell membrane fractions obtained by the ultracentrifugation of sonicated cells were solubilized in SDS, and the membrane proteins were separated by SDS-polyacrylamide gel electrophoresis. After the electrophoresis was completed, the separated membrane proteins were transferred electrophoretically to nitrocellulose paper. CEA was then identified by reacting the nitrocellulose paper with rabbit anti-CEA antiserum followed by 125I-Protein A.

Both HCT48 and LS174T showed a major CEA band at M, 200,000 (Fig. 2). This high-molecular-weight CEA was markedly increased in cells treated with butyrate, as indicated by an elevated radioactivity associated with CEA band. A minor low-molecular-weight CEA at M, 150,000 and M, 100,000 could be detected in HCT48 and LS174T, respectively, after butyrate treatment. However, no difference in the CEA pattern could be demonstrated when comparison of butyrate-treated and untreated cells was conducted at an identical CEA activity.

To further characterize the nature of the cell membrane CEA, membrane fractions containing CEA activity from untreated or butyrate-treated HCT48 cells were solubilized in NP40 and run on 3 different lectin columns in 0.1% NP40. The columns were washed, and bound CEA was eluted with specific sugars. In these experiments, membrane glycoproteins at amounts below the binding capacity of the lectin columns were applied to ensure sufficient binding of membrane CEA to the columns. The fractions separated by lectin affinity chromatography were monitored for their CEA activities. Relative binding of CEA from butyrate-treated HCT48 to RCA-Sepharose 4B column was increased 20% as compared to that from untreated cells. Binding of membrane CEA to WGA-Sepharose 4B and Con A-Sepharose 4B columns was similar in butyrate-treated and untreated cells, as shown in Chart 3.

**DISCUSSION**

CEA, a tumor-associated antigen, has been shown to correlate with the state of colonic tumor differentiation (15). High CEA activity was found in moderately to well-differentiated colonic tumors, while little was found in poorly differentiated colonic tumors. Our studies of the effect of sodium butyrate, a known differentiation-inducing agent, on 7 human colonic cell lines indicated that induction of CEA production is not a general phenomenon among cells treated with butyrate. Basically, the responses of cellular CEA contents to butyrate treatment can be divided into 3 groups. Colorectal tumor cells SW480,
LS174T, which produced large amounts of CEA, showed moderate induction of CEA by butyrate.

We have shown previously that the effect of butyrate on 2 human colonic cell lines SW620 and SW480 (10) and one human rectal tumor cell line HRT18 (23) was to reduce in vitro tumorigenicity together with elevating alkaline phosphatase levels and causing changes in cell surface-labeling pattern. Our present study has shown that CEA activity of SW620 and SW480 is not induced, while that of HRT18 is induced 60-fold. These results indicate that the effects of butyrate on gene expression may not be the result of a general phenomenon.

The subcellular distribution of CEA was first examined in SKCO-1 cells (22) using a radioimmunoassay and later extended to 8 other human colorectal cell lines. It was found that most (>80%) of the cellular CEA was associated with cell membrane fractions. The treatment of tumor cells with sodium butyrate did not change this distribution. The proportion of CEA found in the medium relative to the total cell-associated CEA was also not changed by the treatment. This indicates that the CEA produced after induction by sodium butyrate may still be metabolized in the same way.

The increased CEA in butyrate-treated cells is not due to a decreased release of cellular CEA, since the relative release rate of CEA into medium from cells was not altered by butyrate. It is also unlikely that the increase in CEA is due to a decreased CEA degradation. Although the data are not shown, we have found that cell membrane CEA is very resistant to protease. However, we cannot rule out the possibility that a specific cellular protease which is responsible for CEA degradation is altered by butyrate. Nevertheless, the requirement of RNA transcription and protein synthesis for the induction of CEA production is clearly demonstrated by the inhibition study with actinomycin D and cycloheximide. Sodium butyrate has been reported by Tallman et al. (19) and Deutsch et al. (4) to change the relative synthetic rate of specific proteins in HeLa cells and in mouse neuroblastoma cells. This is also true for CEA in some human colorectal tumor cells.

Cell surface glycoproteins of intact HCT48 and LS174T cells were labeled with tritiated sodium borohydride after oxidation of terminal galactosyl and N-acetylgalactosaminy1 residues with galactose oxidase. Immunoprecipitation of cell surface-labeled CEA from detergent-solubilized labeled membrane proteins with anti-CEA antiserum produced a major labeling glycoprotein with a molecular weight of 200,000 in both cells. The cell surface CEA showed an increase in labeling if the cells were treated with neuraminidase prior to galactose oxidase treatment. This indicates blocking of galactosyl and N-acetylgalactosaminy1 residues by sialic acid in the CEA molecule. Similar results were also found in SKCO-1 cells, as reported earlier by this laboratory (22). The cell surface CEA of butyrate-treated HCT48 and LS174T cells showed an even more dramatic increase in labeling. This coincides with our finding that membrane-associated CEA activity markedly increased after sodium butyrate treatment, as determined by radioimmunoassay. No additional labeled glycoproteins other than the M, 200,000 CEA band could be detected in cells treated with sodium butyrate.

A more sensitive method for detecting any membrane proteins which cross-react with CEA was utilized by electrophoretically transferring membrane proteins after SDS-polyacrylamide gel electrophoresis to a nitrocellulose sheet and subse...
quently reacting the nitrocellulose-bound membrane proteins with rabbit anti-CEA antisera. The membrane CEA was then localized by 125I-labeled protein A. Both HCT48 and LS174T showed a single CEA band at a molecular weight of 200,000. The intensity of labeling of this CEA was markedly increased in cells treated with butyrate. In addition, a minor band at M, 150,000 and M, 100,000 could be detected in HCT48 and LS174T, respectively. This low-molecular-weight CEA is not unique to butyrate-treated cells. Membrane proteins from untreated cells, when overloaded on SDS-polyacrylamide gel electrophoresis, also showed the same minor low-molecular-weight CEA in HCT48 and LS174T. Moreover, when membrane proteins of butyrate-treated and untreated cells were subjected to SDS-polyacrylamide gel electrophoresis at the same CEA concentration, an identical CEA pattern was found. Small amounts of CEA-like antigens with molecular weights of 100,-000 to 150,000 in human colonic tumor tissues have been reported by different laboratories (16, 21, 24). These include colonic CEA 2, colon carcinoma antigen III, and TEX. A similar heterogeneity of CEA is reflected in human colonic tumor cells in culture.

Properties of CEA in human colonic tumor cells were further studied by lectin affinity chromatography of solubilized cell membrane CEA. Lectins are sugar-binding proteins, and their interaction with glycoproteins reflects certain specific orientations of the carbohydrate structure of glycoproteins (9). Carbohydrate has been suggested to play an important role in cell differentiation (5, 7, 25). For instance, the lectin agglutinability and fluorescein-labeled lectin-staining patterns of well-differentiated small intestinal villus cells were reported to be considerably different from those of poorly differentiated small intestinal crypt cells. The binding affinity of cell membrane CEA of HCT48 cells to both Con A- and WGA-Sepharose 4B was found to be similar to that of butyrate-treated HCT48 cells. However, the binding of cell membrane CEA to RCA-Sepharose 4B was found to be higher in case of butyrate-treated HCT48. This result suggests that CEA in butyrate-treated HCT48 cells may contain more exposed terminal galactose sugar residues.

Further studies are necessary to determine the nature of possible qualitative differences in carbohydrate side chains of CEA molecules induced by sodium butyrate.

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