Induction of carcinoembryonic-antigen-gene expression in human colorectal carcinoma by sodium butyrate

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The effect of sodium butyrate on the expression of the carcinoembryonic-antigen (CEA) gene was studied in two poorly differentiated colorectal-carcinoma cell lines (Clone-A and MIP-101) and in one well-differentiated cell line (LS-174T; A.T.C.C. no. CCL 188). Northern-blot and dot-blot analyses indicated a steady increase in CEA mRNA from day 4 to a maximal level by day 14 after these cells were exposed to 2 mM-sodium butyrate. Studies using nuclear run-off assays followed by dot-blot hybridization to a partial CEA cDNA clone demonstrated that specific increases in gene transcription rates (3-fold in MIP-101, 4-fold in LS-174T and 6-fold in Clone-A) are not sufficient to account for the observed increases in CEA mRNA abundance. Further studies showed that CEA-specific transcripts have a half-life of about 60–80 min, and treatment with sodium butyrate increased the stability of CEA-specific transcripts to about 340 min in LS-174T cells and to about 500 min in Clone-A cells. We conclude that the induction of the CEA-gene expression by sodium butyrate in colorectal-cancer cells is mediated by both transcriptional and post-transcriptional mechanisms, with CEA mRNA stability as one of the major check-points.

INTRODUCTION

Carcinoembryonic antigen (CEA), a high-molecular-mass glycoprotein (180 kDa) containing 50–60 % carbohydrate, is one of the most widely used tumour markers for colorectal cancer. It is expressed at the cell surface and secreted by many human carcinomas, especially those of the colon, lung and breast. More is known about its structure and metabolism than any other cancer marker [1]. Sensitive immunoassays are now available that can detect CEA at levels below 0.5 ng/ml in serum, and CEA levels are used to indicate prognosis and to monitor response to therapy [2]. CEA was one of the first tumour markers to be used for the immunolocalization of primary cancers and their metastases [3]. CEA is one member of a family of molecules that includes non-specific cross-reacting antigen and biliary glycoprotein. These glycoproteins have structural similarity to members of the immunoglobulin superfamily, such as neural-cell adhesion molecule [4], Thy-1 antigen [5] and myelin-associated glycoprotein [6]. Molecular cloning and sequencing of the cDNAs for CEA-gene family members [7–11] has helped to clarify the relationship between the CEA-gene family and that of the immunoglobulins and has suggested possible functions. Recently it has been suggested that CEA can function as an adhesion molecule that may be important in homotypic cell interactions, particularly in the fetal colon [12]. It has also been suggested that CEA production offers a selective advantage to colorectal carcinoma cells to form hepatic metastases [13].

Sodium butyrate [14] and human γ -interferon [15] have been shown to induce differentiation and increase CEA expression in human colon-cancer-cell lines. The reasons for this increased CEA mRNA content are not known, although increased transcription rates have been suggested to be mainly responsible [14]. Sodium butyrate has also been implicated in the complex processes of tumour promotion and progression in the epithelial cells of the colon [16].

The present study demonstrates that the treatment of Clone-A and MIP-101 (two poorly differentiated human colorectal-carcinoma-cell lines) and LS-174T cells with sodium butyrate is

responsible for the appearance of CEA-specific transcripts. The level of CEA expression is regulated by both transcriptional and post-transcriptional mechanisms, with CEA mRNA stability as one of the major regulatory check-points.

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MATERIALS AND METHODS

Cell culture

Clone-A [17], MIP-101 [18] and LS 174T [A.T.C.C. (American Type Culture Collection, Bethesda, MD, U.S.A.) CCL 188] cells were grown and maintained at 37 °C in a humidified atmosphere containing 5 % CO₂ in RPMI-1640 medium supplemented with 10 % (v/v) fetal-calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) with medium changed every other day. Monolayers (80–85 % confluent) of cells in 100 (or 150) mm × 20 mm plastic dishes were harvested after incubation in the above medium with or without 2 mM-sodium butyrate and, at the indicated times, CEA assay and nuclei/or RNA isolations were performed.

A decrease in the rate of growth of both MIP-101 and Clone-A cells occurs after butyrate treatment. Untreated cells have a doubling time of approx. 30 h, whereas the treated cells, after an initial phase of no growth, have a doubling time of 60 h [18].

CEA assay

Cellular and secreted forms of the CEA were assayed in the cells and medium respectively, with a CEA double-monoclonal-antibody enzyme-immunoassay kit (Roche CEA-EIA; Hoffman-LaRoche, Nutley, NJ, U.S.A.).

RNA preparation and Northern analyses

Total RNA was isolated as described by Chirgwin *et al.* [19]. After aspirating the medium, the cells were washed twice with

Abbreviations used: CEA, carcinoembryonic antigen; PBS, phosphate-buffered saline.

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phosphate-buffered saline (PBS; 0.1 M-sodium phosphate/0.15 M-NaCl, pH 7.4) and homogenized in guanidinium isothiocyanate buffer [4.0 M-guanidinium isothiocyanate/25 mM-sodium citrate (pH 7.0)/0.5 % Sarcosyl/100 mM- β -mercaptoethanol]. Total RNA was isolated by centrifugation through CsCl and repeated ethanol precipitation [19]. Total RNA (20–25 μ g) was electrophoresed on 1.0 % agarose/0.7 M-formaldehyde gels and transferred to Gene Screen Plus nylon membranes (New England Nuclear, Boston, MA, U.S.A.) as per the manufacturer's recommendations.

Hybridization was done for 22 h at 43 °C in 50 % (deionized) formamide/5 $\times$ Denhardt (1 $\times$ Denhardt is 0.02 % BSA/0.02 % Ficoll/0.02 % polyvinylpyrrolidone) 5 $\times$ SSPE (1 $\times$ SSPE is 0.15 M-NaCl/0.01 M-NaH₂PO₄/1.0 mM-EDTA, pH 7.4)/0.1 % SDS/denatured salmon sperm DNA (250 μ g/ml)/10 % dextran sulphate/randomly primed ³²P-labelled [20] CEA cDNA (pcLV7; an 860-nucleotide sequence which encompasses the second and third loop domains of CEA; this was generously provided by Dr. Tom Barnett, Molecular Diagnostics, West Haven, CT, U.S.A.) [(2–3) $\times$ 10⁶ c.p.m./ml]. Blots were also probed with β -actin cDNA to check for differences in RNA loading (results not shown). The filters were washed to a final stringency of 0.1 $\times$ SSPE and 0.1 % SDS at 65 °C and exposed to X-ray film (Kodak XRP-5) for 1–3 days.

Nuclear run-off transcription assays

Isolation of nuclei and nuclear run-off transcription *in vitro* were carried out essentially as described by Greenberg & Ziff [21]. Incubation of (5–10) $\times$ 10⁶ nuclei for 30 min at 30 °C in the presence of 100 Ci of [α -³²P]UTP (New England Nuclear) resulted in the incorporation of between 3 $\times$ 10⁶ and 4 $\times$ 10⁶ c.p.m. into total RNA.

DNA-excess filter hybridization

Control (p Bluescript KS⁺; Stratagene), β -actin probe and CEA cDNA (pcLV7) (5 μ g/filter) were immobilized on 7 mm-diameter nitrocellulose filters (grade BA-83; Schleicher and Schuell, Keene, NH, U.S.A. [22]) and hybridized with (1–4) $\times$ 10⁶ c.p.m. of ³²P-labelled newly transcribed RNA at 65 °C for 48 h [21,23]. After hybridization, washing of filters and release of RNA from the filters was accomplished as described by McKnight & Palmiter [24]. The results are expressed as parts of CEA RNA transcribed per million parts of total RNA transcribed (p.p.m.).

DNA-excess filter hybridization was also used to quantify CEA mRNA abundance in sodium butyrate-treated and untreated MIP-101 and Clone-A cells (results not shown).

CEA mRNA half-life measurements

The uridine pulse-decay method [25] was employed to determine the half-life of CEA-specific transcripts. This technique has been routinely employed by us [26] and others [27] to study the kinetics of mRNA decay. Untreated and sodium butyrate-treated Clone-A and LS-174T cells were exposed to [5,6-³H]uridine (100 μ Ci/dish; 50 Ci/mmol) for 4 h to radiolabel newly synthesized RNA. To terminate radioactive labelling, the cells were incubated for 10 min with medium (sodium butyrate-treated and untreated) containing 10 mM-glucosamine and 5 mM each of cytidine and uridine. The radioactive label was 'chased' out with the respective medium containing 5 mM each of cytidine and uridine. At 0, 1, 2 and 4 h the cells were washed twice with PBS, and RNA was extracted [19]. The quantification of CEA mRNA is described above under 'DNA-excess filter hybridization'. CEA-specific transcripts accounted for > 100 p.p.m. in untreated Clone-A cells and > 2000 p.p.m. in the other treatment groups. The half-life of CEA mRNA was determined from the rate of dis-

appearance of ³H-labelled CEA-specific transcripts. The radioactivity bound to the control (p Bluescript KS⁺) filters was less than 10 % of that hybridized to pcLV7. Binding to the β -actin cDNA caused < 5 % variability among the untreated and sodium butyrate-treated Clone-A and LS-174T cells.

RESULTS AND DISCUSSION

Mechanisms involved in the stimulation of CEA synthesis by sodium butyrate were investigated by measuring levels and rates of synthesis of CEA mRNA in two poorly differentiated and one well-differentiated colorectal-cancer-cell line. Neither of the two poorly differentiated cell lines secrete detectable amounts of CEA into the medium when grown in culture. These tumours when grown subcutaneously as xenografts in nude mice also do not secrete detectable amounts of CEA into the blood. However, previous studies from our laboratory [18] have shown induction of CEA production by these colon-cancer-cell lines in culture after they are exposed to butyrate. Butyric acid, a C₄ volatile fatty acid, is a natural fermentation product of the gut flora and has been shown to induce differentiation in a variety of tumour-cell lines [16–28]. Recently butyrate has also been implicated in the regulation of human pregnancy-specific β_1 -glycoprotein (another member of the immunoglobulin supergene family, and one that shows structural similarity to CEA at both the nucleotide and amino acid levels) gene expression in placental fibroblasts mainly by increasing the steady-state levels of its three mRNAs [29]. However, the molecular mechanisms of the action of butyrate on CEA gene expression in tumour cells is unknown.

The effect of different concentrations of sodium butyrate on CEA production and cytotoxicity showed 2 mM to be the optimal [16], and this concentration has been used throughout this study. Time-course studies on CEA production and CEA mRNA appearance rate showed the appearance of transcripts (detectable by Northern blotting) by day 4 and a progressive increase up to 14 days (Table 1, Fig. 1a; and results not shown), which is consistent with a previous study where immunofluorescence was used to measure cell-surface CEA and an e.l.i.s.a. procedure for secreted CEA [18]. In the present study using Clone-A we found that the secreted and cellular forms of CEA were elevated more than 50-fold between 8 and 14 days of sodium butyrate treatment (Table 1).

Nuclear transcription assays (Table 2) showed that sodium butyrate increased transcription of the CEA gene in Clone-A (6-fold), MIP-101 (3-fold) and LS 174T (4-fold) cells. This induction of transcription, however, did not account entirely for the increase in CEA mRNA abundance caused by sodium butyrate. It appears that butyrate exerts a positive effect on CEA mRNA at post-transcriptional step(s). Using the pulse-chase technique [25] we now show that sodium butyrate also stabilizes CEA mRNA, increasing its half-life from 60–80 min to about 340 min in LS 174T cells. In butyrate-treated Clone-A cells the half-life is about

Table 1. Effect of sodium butyrate on the cellular and secreted forms of CEA in Clone-A cells

Butyrate exposure (days)	CEA levels (ng/ml)	
	Cellular form	Secreted form
0	1.6	< 0.5
8	21.3	1.5
14	52.7	27.0

Cells were grown and maintained, and CEA assays were carried out, as described in the Materials and methods section. Results are means for two independent experiments.

Fig. 1. Northern-blot analysis

(a) Analysis of CEA mRNA from MIP-101 and Clone-A cell lines exposed to sodium butyrate. A portion (20–25 μ g) of total RNA was electrophoresed, transferred, hybridized and autoradiographed as described in the Materials and methods section. Lane 1, untreated MIP-101; lane 2, MIP-101 exposed to sodium butyrate for 14 days; lane 3, untreated Clone-A; lane 4, Clone-A exposed to sodium butyrate for 14 days. (b) Ethidium bromide fluorescence of the gel before transfer. Lanes 1–4 are same as in (a).

500 min. In untreated Clone-A cells, however, CEA transcripts remain below the detection limit. Nevertheless, extrapolating from the increase seen with LS 174T cells, it seems likely that butyrate has a major effect on CEA mRNA stability. Sodium butyrate-mediated effects on CEA-gene expression appear to involve a co-ordinate response at both transcriptional and post-transcriptional levels. Our results further indicate that butyrate exerts a selective effect on the synthesis and turnover of CEA mRNA rather than a more general phenomenon affecting all RNA-synthesis and turnover. Apart from the known effects of butyrate on gene transcription, this is, to our knowledge, the first report where it has been shown to stabilize preferentially a given mRNA. Extra- and intra-cellular modulators have been shown to regulate expression for a number of genes (reviewed in [30]). The modulation of CEA mRNA and CEA production by sodium butyrate provides a useful model to examine the molecular mechanisms underlying the regulation of the CEA-gene expression in colon carcinomas.

A recent report [14] has also shown increases in CEA mRNA abundance in other butyrate-treated colon-cancer-cell lines. Shively and his colleagues [14,31] have postulated that increases in CEA-gene-transcript levels must be primarily responsible for increased expression of CEA mRNA. They further concluded that butyrate-induced increases in CEA-gene-transcript levels is independent of gene amplification, rearrangement or DNA methylation.

Sealy & Chalkley [32] have proposed a model for the effects of sodium butyrate on histone-DNA interactions. They suggested that hyperacetylation of histones was due to the inhibition of histone deacetylase by sodium butyrate. This allows DNA to stay uncoiled and single-stranded instead of reverting to the supercoiled form. This increases the surface area for RNA polymerase to recognize specific nucleotide segments and start RNA synthesis. Alternatively, butyrate may act by simply reducing the growth rate of the cells by switching off genes that are not involved in the growth process. Many workers have shown that reduction of cell growth rate will increase CEA production in a large number of cancer-cell lines, but only where there is a measurable CEA level to begin with. In the case of Clone-A and MIP-101, other agents are effective in slowing growth (e.g. dimethyl formamide, retinoic acid or γ -interferon) without inducing CEA production [18]. It is therefore likely that, in the cell lines discussed here, sodium butyrate helps promote transcriptional processes in the region of active genes. The induction of CEA mRNA in the sodium butyrate-treated cells may be due to a direct effect of the butyrate on the state of DNA in the nucleus. It will be of considerable interest to know whether sodium butyrate increases transcription of the CEA-gene-family members by a mechanism similar to the one reported for histones. The activation of pre-existing transcription factor(s) or the synthesis of a new protein are other possibilities requiring further experimentation.

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Table 2. Effect of sodium butyrate on CEA-gene transcription in Clone-A, MIP-101 and LS 174T cell lines

Cell culture, isolated of nuclei and nuclear run-off transcription assays were as described in the Materials and methods section. Results are means for two independent experiments. The fold increase in CEA-gene transcription rate, relative to the control, is given in parentheses.

Treatment	$10^{-6} \times$ Input of [32 P]RNA in each hybridization (c.p.m.)	[32 P]RNA (c.p.m.) hybridized to:			32 P-labelled CEA mRNA hybridized (c.p.m.)	CEA-gene- transcription rate (p.p.m.)
		pcLV7	pBluescript KS ⁺	β -Actin		
Clone-A	1.19	104 $\pm$ 2	19 $\pm$ 2	43 $\pm$ 4	85 $\pm$ 3	71
Clone-A + butyrate	1.17	553 $\pm$ 5	36 $\pm$ 3	47 $\pm$ 3	517 $\pm$ 5	442 (6.2)
MIP-101	1.06	57 $\pm$ 1	20 $\pm$ 1	44 $\pm$ 2	37 $\pm$ 2	35
MIP-101 + butyrate	1.08	131 $\pm$ 4	25 $\pm$ 2	45 $\pm$ 5	106 $\pm$ 3	98 (2.8)
LS 174T	4.03	274 $\pm$ 5	24 $\pm$ 4	48 $\pm$ 5	250 $\pm$ 6	62
LS 174T + butyrate	3.66	934 $\pm$ 9	28 $\pm$ 3	52 $\pm$ 6	906 $\pm$ 8	248 (4.0)

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