Transcriptional and Posttranscriptional Modulation of Calcitonin Gene Expression by Sodium \( n \)-Butyrate in Cultured Human Medullary Thyroid Carcinoma

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ABSTRACT

The TT cell line of human medullary thyroid carcinoma produces large quantities of calcitonin (CT) and calcitonin gene-related peptide (CGRP) mRNAs by alternative splicing of a primary CT gene transcript. We have previously shown that the relative levels of these mRNAs depend on the growth stages of the TT cells in culture and that these mRNAs can be increased acutely at the transcripational level by phorbol esters (12-O-tetradecanoylphorbol-13-acetate) and the cyclic nucleotide, cyclic AMP.

We show here that the naturally occurring fatty acid butyrate, unlike 12-O-tetradecanoylphorbol-13-acetate or cyclic AMP, has a delayed stimulatory effect on CT gene transcription, and also can modulate the posttranscriptional processing of RNA from this gene. Treatment of the TT cells with butyrate leads to a 5-fold increase in CT gene transcription after a lag period of 48 h and to a sustained increase in the calcitonin gene-related peptide to CT mRNA ratio throughout the growth curve of these cells. In addition to its effects on CT gene expression, butyrate also decreases cellular proliferation and CT mRNA expression in the TT cells.

These changes suggest that butyrate induces cultured human medullary thyroid carcinoma cells to acquire in vitro properties more consistent with the differentiated phenotype of the mature thyroid C-cell which is characterized by a low calcitonin gene-related peptide to CT ratio.

INTRODUCTION

The TT cell line of MTC represents one of the few established cultures of a polypeptide hormone secreting human epithelial cell (1). Our previous studies have revealed these cells to be an excellent model system to study the regulation of expression of the CT gene, including the alternative processing of its primary RNA transcript to separate mRNA species for the hormones CT and CGRP (2-6). Recently, we have shown that the production of CT is dynamically linked to the growth stages of the TT cells in culture, such that it is highest during stationary growth phase and lowest during exponential growth phase (4, 5). These changes in culture may relate closely to the behavior of MTC in patients, since individuals who die from rapidly growing, widely metastatic disease have a marked decrease in CT content of their tumors (7-9).

Based on the above dynamics in culture and in patients, it is important to understand further the regulatory factors responsible for the transcriptional and posttranscriptional control of CT gene expression. In our previous studies, we identified agents, 12-O-tetradecanoylphorbol-13-acetate and cyclic AMP, which induced apparent differentiation of the TT cells and increased transcription of the CT gene within 2 to 4 hours (2, 3). These agents, however, did not alter the CGRP to CT mRNA ratio over the time period studied. By contrast, we now show that butyrate, a naturally occurring fatty acid and an agent also known to cause differentiation of several types of tumor cells (10, 11), increases transcription of the CT gene after a lag period of 48 h and also increases CT mRNA levels relative to CGRP mRNA levels throughout the growth curve of the TT cells in culture. These findings suggest that butyrate modulates the regulation of CT gene RNA processing, and enhances the use of TT cells as a model system for studying factors which control posttranscriptional processing of this gene.

MATERIALS AND METHODS

Cell Culture. The human medullary thyroid carcinoma cell line TT has been previously described (1). The cells were grown in 75-cm\(^2\) plastic flasks (Corning Glass Works, Corning, NY) as monolayers in RPMI 1640 (B&B/Scott, Fiskeville, RI) supplemented with 16% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 \( \mu \)g/ml streptomycin.

Growth Study. Sodium \( n \)-butyrate (Sigma Chemical Co.) was dissolved in distilled water to a concentration of 200 mM, stored at -20°C, and diluted with the culture medium just before use.

TT cells were subcultured in 9.6-cm\(^2\) well multiwells for protein measurements, CT secretion, and thymidine incorporation studies and in 75-cm\(^2\) plastic flasks for cytoplasmic RNA extraction and nuclear runoff experiments (1 \( \times \) 10\(^5\) cells/cm\(^2\)). At twenty-four h after seeding (Day 0), the medium was changed and either control medium or medium containing butyrate at concentrations of 0.4, 0.6, 0.8, and 1.0 mM was added. Every fourth day for the next 11 days, the medium was changed. Calcitonin secretion in the medium and cell protein content were measured at 24-h intervals during these 11 days. In all experiments, the cell number was determined with a hemocytometer and results were correlated with the cell protein content as measured by the method of Bradford (12). In most studies, the protein content was used as an index to quantitate total cellular growth.

Thymidine Incorporation. Rates of DNA synthesis were evaluated by measuring the incorporation of [\( ^{3}H \)]thymidine into acid-insoluble material as previously described (2, 11).

Secretion of Calcitonin. Medium from treated or untreated cells was centrifuged (1500 \( \times \) g for 3 min), and the concentration of calcitonin in the culture medium was determined by a previously described radioimmunoassay (13) and normalized to cell protein content. Control medium, which was not exposed to the cells, had no detectable levels of calcitonin.

cDNA Probes. The CT (pTT1062) and CGRP-containing (pTT42) cDNA probes have been previously described (6). pTT1062 contains only CT-specific sequences and no 5' common region or CGRP sequences (6). pTT83 contains a subclone PvuII-PstI restriction fragment from pTT42 and contains only CGRP-specific sequences including 17 base pairs of exon 5 and all of exon 6 of the CT gene (6).

Recombinant plasmids containing human actin and \( \beta \)-myc sequences (14) were provided by Dr. Don Cleveland, Johns Hopkins University School of Medicine and the American Type Culture Collection, respectively. These probes were labeled, as in our previous studies (2, 3, 5, 6), with [\( ^{32}P \)]dCTP to a specific activity of up to 1 \( \times \) 10\(^{6}\) cpm/\( \mu \)g by random priming with Klenow DNA polymerase (15).

Quantitation of CT, CGRP, and \( \beta \)-Actin mRNAs. Cytoplasmic RNA was prepared as previously detailed (2, 16), subjected to Northern blotting, and hybridized to the \( ^{32}P \)-labeled cDNA probes for CGRP (pTT83), \( \beta \)-actin, and CT (pTT1062) in succession, removing the
previous probe each time as described (2, 5). After autoradiography, quantitation of the hybridization signal for CT and CGRP by densitometry (Model TBX; Tobias Associates, Inc., Ivyland, PA) was expressed relative to the β-actin signal as done previously (2, 3, 5).

Quantitation of c-myc mRNA. Since c-myc expression is much lower than that of the CT gene in TT cells, polyadenylated RNA isolation was required for analysis, and done by passage through an oligodeoxynucleotidylic acid cellulose column. Electrophoresis and hybridization were done as described previously (2).

Nuclear Runoff Transcription. Isolation of nuclei, in vitro nuclear transcription, and isolation of 32P-labeled RNA were performed as previously described (2). Equal amounts of radioactive RNA (5–7 × 10⁶ cpm) from nuclei from treated and control cells were hybridized to plasmids containing calcitonin, CGRP, and β-actin specific cDNA sequences immobilized on a nitrocellulose filter. Hybridization conditions were as described previously (2).

RESULTS

Biological Effects of Butyrate on TT Cells. We first showed that butyrate markedly slows but does not stop the growth of TT cells. As measured by protein content of culture wells (Fig. 1), 1.0 mM butyrate treatment decreased the growth rate of the TT cells to 20% of that for control cells by day 6 of treatment. These changes were also documented by rates of DNA synthesis as determined by [3H]thymidine uptake and by cell numbers (17 and 20% of control cells, respectively, by day 6 of treatment with 1.0 mM butyrate). The growth suppression of TT cells by butyrate was readily reversible by removing the compound (Fig. 1), and there was no loss of cell viability as analyzed by trypan blue exclusion.

Butyrate treatment initially (first 2 days of treatment, data not shown) did not alter steady state mRNA levels for the growth associated c-myc oncogene but later led to a 43% decrease in the expression of this gene by day 6 (Fig. 2). Such a decrease was seen previously with phorbol esters but the effects of phorbol esters were much more acute, causing an 85% fall in c-myc RNA within 24 h (2).

We then studied the effects of butyrate on CT secretion, a parameter of the differentiated function of the TT cells. Unlike cAMP and phorbol esters (2, 3), butyrate does not induce an immediate (over the first 24 h) change in CT secretion. However, after 48 h of treatment with 1.0 mM butyrate, CT secretion begins to increase (Fig. 3). This increased secretion is sustained during continued butyrate administration and reaches 456% of control cell levels by day 6 (P < 0.005, Fig. 3). These changes are readily reversible by withdrawing butyrate from the culture media (data not shown).

Effects of Butyrate on CT and CGRP mRNA in TT Cells. Having found the above effects of butyrate on cell growth and secretion of CT, we next studied whether the compound might modulate CT gene expression. Initially, we studied a 6-day treatment of cells with various doses of butyrate. We found that butyrate caused increased levels of both CT and CGRP cytoplasmic transcripts, as measured by Northern hybridization (Fig. 4; 4.4- and 3.4-fold for CT and CGRP mRNA, respectively). Interestingly, levels of CT mRNA increased dose-dependently by butyrate treatment from 0.6–1.0 mM, while increase of CGRP mRNA was maximum by 0.6 mM butyrate and

![Fig. 1. Cell protein content in TT cells grown in the absence (C) and presence (O) of 1.0 mM butyrate. Cells were subcultured in 6-well plates and 24 h later (day 0) the medium was changed and butyrate was added to the final concentration of 1.0 mM.], time point at which butyrate treated cells were washed twice with fresh medium and replaced with butyrate-free medium. Cells were harvested by 0.08% sodium dodecyl sulfate to determine protein content. Points, mean of duplicate wells from three separate experiments; SDs (bars) were less than 10% of the mean for each time point.

![Fig. 2. Polyadenylated RNA (3 μg/lane) extracted from control cells (left lanes) and from cells treated with 1.0 mM butyrate (right lanes) for 6 days and hybridized to the 32P-labeled human c-myc probe (14). Polyadenylated RNA was used in this study to detect the c-myc mRNA which is much less abundant than that for CT and CGRP. After autoradiography, the filter was rehybridized with 32P-labeled β-actin cDNA probe. Quantitation of the hybridization signal for c-myc by densitometry is expressed relative to the β-actin signal as done previously (2) and revealed that

![Fig. 3. Effect of butyrate on calcitonin release into the medium. Cells were subcultured and 24 h later (day 0), the medium was changed, and butyrate was added to give final concentration of 1.0 mM. The medium was replaced by fresh medium with or without butyrate 24 h prior to cell harvest. The means and SEs (bars) of four wells are shown. Calcitonin secretion by butyrate-treated cells has been statistically compared to control cells for each day shown and is statistically increased on days 2, 3, 4, 5, and 6. *, P < 0.01; **, P < 0.005.

![Fig. 4. Polyadenylated RNA (3 μg/lane) extracted from control cells (left lanes) and from cells treated with 1.0 mM butyrate (right lanes) for 6 days and hybridized to the 32P-labeled human c-myc probe (14). Polyadenylated RNA was used in this study to detect the c-myc mRNA which is much less abundant than that for CT and CGRP. After autoradiography, the filter was rehybridized with 32P-labeled β-actin cDNA probe. Quantitation of the hybridization signal for c-myc by densitometry is expressed relative to the β-actin signal as done previously (2) and revealed that

![Fig. 5. Effect of butyrate on calcitonin release into the medium. Cells were subcultured and 24 h later (day 0), the medium was changed, and butyrate was added to give final concentration of 1.0 mM. The medium was replaced by fresh medium with or without butyrate 24 h prior to cell harvest. The means and SEs (bars) of four wells are shown. Calcitonin secretion by butyrate-treated cells has been statistically compared to control cells for each day shown and is statistically increased on days 2, 3, 4, 5, and 6. *, P < 0.01; **, P < 0.005.
was actually lower thereafter (Fig. 4). This decreased ratio of CGRP to CT mRNA induced by 1.0 mM butyrate was suggestive of a possible role for this agent in the posttranscriptional regulation of the CT gene. We therefore used this concentration of butyrate throughout our studies. Levels of the 2.2-kilobase 0-actin transcript remained unchanged by the butyrate treatment over this time course. 0-actin was thus used as a control for a subsequent series of experiments, as was done in our previous studies (2, 3).

We then studied the effects of butyrate on CT gene expression at multiple time points during the growth curve of the TT cells. As shown in Fig. 5A, CGRP-specific mRNA changes differently from CT mRNA during the growth curve of untreated TT cells. Thus, while levels of CT mRNA increase gradually throughout the growth period achieving a stable plateau in confluent cells, CGRP mRNA levels rise more quickly to a peak at day 6, and then fall precipitously thereafter, returning by day 9 to near the levels seen in freshly seeded cells.

In the 1.0 mM butyrate-treated cells, the levels of both CT and CGRP mRNAs were markedly increased from days 2 through 6, reaching levels which were 4.0-fold over control cells for CT mRNA at day 6 and 3.5-fold over control cells for CGRP mRNA at day 5. After day 6, in both control and treated cells, there was a dramatic fall in levels of CGRP mRNA while CT mRNA levels remained stable (Fig. 5A). Thus, while the steady-state levels of CT and CGRP mRNA are both increased by butyrate treatment, the changing patterns for these mRNAs during the growth curve of the TT cells are maintained in the treated cells.

An important point of this study is that although the absolute levels for both CT and CGRP mRNAs are increased in the butyrate-treated cells, the calculated ratio of CGRP to CT mRNA is decreased by butyrate on days 1 through 5 (Fig. 5B).

Nuclear Runoff Transcription. In order to study whether the increased CT and CGRP mRNAs were due to the stimulation of CT gene transcription by butyrate, we looked at rates of runoff transcription in nuclei isolated from control cells and cells treated with butyrate for various periods of time.

No effect on CT gene transcription was observed at 24 h. However, after 2 days of treatment of TT cells with 1.0 mM butyrate, there was a marked increase in both CT and CGRP-specific nascent RNA. This increase in CT gene transcription in butyrate as compared to control cells appears to be approximately 5-fold relative to that of control cells (Table 1).

This suggests that the increased CT and CGRP mRNA levels, at least during the early part of the growth curve, are due to the increased CT gene transcription induced by butyrate. However, the lower ratio of CGRP to CT mRNA induced by butyrate (Fig. 5B) suggests an additional effect of this compound on the posttranscriptional processing of the CT gene in favor of CT mRNA. This effect is suggested even more strongly at day 6, where levels of CGRP mRNA fall dramatically while levels of CT mRNA remain elevated. At this time point, the butyrate-
treated TT cells do not exhibit any difference in the rate of transcription of the CT gene from that for control cells (Table 1).

DISCUSSION

We show here that butyrate treatment alters the expression of the CT gene in the TT cell line of human MTC on at least two levels: (a) after a 48-h lag period, butyrate increases the steady state levels of CT and CGRP specific mRNAs by stimulating CT gene transcription; (b) butyrate appears to alter posttranscriptional RNA processing of the CT gene, because the CGRP/CT mRNA ratio is significantly decreased by butyrate. Since both the CT and CGRP mRNAs are derived from a common primary RNA transcript, the dissociation between the levels of these two species must be due to differential posttranscriptional processing of this gene (17–20). It is important to note that the endogenous factors which modulate control of CT gene expression during growth of the TT cells are not abolished by butyrate, since similar to growth related changes in the ratios of CT to CGRP in control TT cells, a dramatic decrease in these ratios is still seen in butyrate-treated cells during days 6–9 of growth (Fig. 5B). In addition to the effects noted above, butyrate appears to differentially affect the phenotype of TT cells at different concentrations; for example, lower concentrations (0.6 mm) do not appear to decrease the ratio of CGRP to CT mRNA, but higher concentrations (1.0 mm) decrease the ratio of CGRP to CT mRNA (Fig. 4).

The effects of butyrate on the TT cells are complex and appear to be different from the pure transcriptional stimulation of the CT gene which we observed previously with TPA and cyclic AMP (2, 3). Butyrate has been noted to affect DNA structure and nuclear histone composition through acetylation (21). Numerous correlations have been noted between increased acetylation of the histones and increased RNA synthesis (22). More recently, butyrate has been demonstrated to activate transcription for the β-gonadotropin gene in HeLa cells and for the metallothionein-I gene in hepatoma cells (23, 24). However, to our knowledge, there has been no report that butyrate has effects on the posttranscriptional processing (stability or splicing) steps for expression of genes.

The data presented here suggest that butyrate indeed induces differentiation of human medullary thyroid carcinoma cells in culture. It not only increases calcitonin gene expression but also inhibits growth and decreases the levels of mRNA for the c-myC oncogene. Thus, in the TT cell line, butyrate appears to selectively modulate gene expression such that a program of differentiation is induced, while cell growth is decreased. These findings may be of potential clinical relevance. In most patients, medullary thyroid carcinoma is relatively indolent and is characterized by high levels of CT, the major secretory product of the thyroid C-cell from which medullary thyroid carcinoma arises. However, in some patients, the disease is aggressive and tumor tissues from such patients are characterized by a marked decrease in content of CT (6–8, 25). These features suggest that in vivo as well as in our cell culture model the progression of medullary thyroid carcinoma cells to a less differentiated phenotype may be characteristic of a more rapid growth state. Butyrate slows cell growth and alters posttranscriptional processing of the CT gene in a direction more characteristic of the mature normal thyroid C-cell, which produces mostly calcitonin. The marked phenotypic changes induced by butyrate then suggest that this compound causes the TT cells to acquire in vitro properties more consistent with a well-differentiated, more indolent type of medullary thyroid carcinoma. In addition, recently, while our work was in progress, another compound, dexamethasone, was also shown to favor production of CT over CGRP in the TT cells (26). The effects of such compounds on alternative RNA processing events as well as their therapeutic value in medullary thyroid carcinoma merit further exploration.

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REFERENCES


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