Butyrate Rapidly Induces Growth Inhibition and Differentiation in HT-29 Cells

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Abstract
Selected G1 events associated with butyrate-induced differentiation were examined in HT-29 colon adenocarcinoma cells. [3H]Thymidine incorporation by HT-29 cells was decreased to 40% of control levels by treatment with 5 mM butyrate for 24 h, and cell numbers decreased to 21% of control levels after 48 h of treatment. Cells released by butyrate arrest entered S phase approximately 24 h after release, and serum-deprived HT-29 cells escaped growth inhibition if butyrate was added 8 h or more after serum restimulation. Northern analysis of RNA isolated from rapidly growing HT-29 cells showed a marked induction of alkaline phosphatase mRNA expression within 12 h of treatment with 5 mM butyrate. The appearance of alkaline phosphatase mRNA was temporally associated with a 5-fold increase in expression of transforming growth factor β1 (TGF-β1) mRNA. Expression of the nuclear protooncogene c-myc began to decrease 30 min after treatment with butyrate and was decreased 4.5-fold 4 h after treatment; however, expression of other immediate-early genes (c Jun/475 and zif268) was not significantly affected. Histochemical staining of HT-29 monolayers showed that no cells were positive for alkaline phosphatase protein prior to treatment, and 90% were positive 48 h after treatment. TGF-β1 and TGF-β2 had no effect on HT-29 cell growth. TGF-β3 did not induce alkaline phosphatase mRNA or histochemical positivity. These data indicate that butyrate arrests HT-29 cell growth early in G1. Sodium butyrate-mediated inhibition of HT-29 cell growth is associated with rapid emergence of a marker of differentiation, down-regulation of c-myc, and up-regulation of TGF-β1 mRNA expression, although TGF-β3 does not appear to be a primary differentiating agent for these cells.

Introduction
Cellular differentiation is a complex phenomenon associated with exit from the cell cycle and entry into an alternate pathway in which a more sophisticated phenotype and function are assumed. In the small intestine and colon, epithelial cells arise from stem cells located in crypts at the base of villi (small intestine) or near the base of the colonic glands. As intestinal epithelial cells migrate away from this proliferative zone, an increasingly differentiated phenotype is assumed. For example, absorptive epithelial cells acquire brush border membrane-associated transporter proteins and an assortment of membrane-anchored hydrolases and other digestive enzymes that characterize the differentiated phenotype (1). It has been suggested that the differentiation status of a cell is governed by a dynamic stoichiometry of positive and negative "regulators" (2), but "regulators" pertinent to differentiation of the intestinal epithelial cell have not been identified. Studies by Gordon and coworkers (reviewed in Ref. 3) have begun to identify cis-acting sequences in the rat liver fatty acid-binding protein gene that modulate the distribution of this differentiation marker along the "horizontal" (proximal to distal) dimension of the intestine. In addition to cis-acting elements, it is likely that both positive and negative trans-acting factors also contribute to expression of differentiation markers along both the vertical (crypt to villus) and horizontal dimension in the intestine. These trans-acting factors are regulated, in part, by the complex intraluminal and serosal compartments to which polarized intestinal epithelial cells are exposed.

Investigation of molecules that regulate intestinal epithelial cell growth and differentiation have focused largely on polypeptide growth factors and less on nutrients and other solutes in the serosal and intraluminal compartments. The most abundant solutes in the intraluminal compartment of the colon are short chain fatty acids, compounds generated by the metabolism of complex carbohydrates by the colonic bacterial flora (4). The intraluminal concentration of short chain fatty acids in the human colon has been estimated to be between 100 and 240 mM, most of which is butyrate (5). This four-carbon compound has well-described potent effects on the growth and differentiation of a wide variety of cell types. Several studies have examined the effects of butyrate on the gastrointestinal epithelium. For example, in vivo experiments have suggested a role for butyrate in maintenance of a normal colonic epithelium and have emphasized the potential for butyrate in the treatment of colonic disease (6, 7). In vitro studies have indicated that butyrate modulates growth and differentiation of colon adenocarcinoma cells in culture (8-19). The cellular and molecular mechanisms by which butyrate may affect cellular differentiation are not completely clear, particularly in gastrointestinal cells, where there may be a unique physiological relevance. In the present study, we have explored the effects of butyrate on selected growth-related cell cycle events in a clone [HT-29(E)] of the human colon cancer cell line HT-29.

Results
Effect of Butyrate on HT-29(E) Cell Growth. The effect
Butyrate Induces Growth Inhibition

Fig. 1. A, thymidine incorporation by HT-29(E) cells treated with butyrate. Cells were seeded at a density of 25,000 cells/well and allowed to attach for at least 24 h in serum-containing medium. Preliminary [3H]thymidine experiments indicated that serum deprivation of HT-29(E) cells for 24 h resulted in growth arrest. Each well was washed twice with solution A (dextrose 0.18 g/liter, KCl 0.022 g/liter, NaCl 0.76 g/liter, Na2HPO4·H2O 0.07 g/liter, phenol red 0.00012 g/liter, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-Tris 0.715 g/liter, pH 7.4) and replaced with serum-free DMEM for 24 h. The indicated concentrations of butyrate or TGF-β1, in DMEM with 10% serum or serum-free medium (S.F.) were added at the 24th h. Twenty-one h later, the cells were pulse labeled with 4 μCi/ml [3H]thymidine for 3 h. Radioactivity was measured as described in "Materials and Methods." Results are expressed as mean ± SE (bars) of triplicate determinations. B, cell counts after butyrate treatment. Cells were plated at a density of 20,000 cells/well in 6-well plates and allowed to attach for at least 24 h. Each well was washed twice with solution A and replaced with serum-free DMEM for 24 h. The indicated concentrations of butyrate and TGF-β1, and TGF-β2, were added in 10% DMEM for 48 h, and cells were then counted. Results are expressed as mean ± SE for triplicate determinations.

Fig. 2. Duration of butyrate exposure required for growth inhibition. Cells were seeded as described in Fig. 1A. After 24 h, growth in serum-containing medium, butyrate (NaBu) treatment was initiated (in the continued presence of serum). At the indicated time, monolayers were washed twice with butyrate-free medium and supplied with serum-containing, butyrate-free medium for the remainder of the experiment. [3H]Thymidine uptake was determined as described in legend to Fig. 1A, and results are expressed as mean ± SE (bars) for triplicate determinations. S.F., serum-free medium.

of sodium butyrate on HT-29(E) DNA synthesis was examined by assay for [3H]thymidine incorporation and cell counts. Butyrate caused a dose-dependent decrease in [3H]thymidine incorporation by HT-29(E) cells. Half-maximal inhibition of DNA synthesis occurred at a butyrate concentration between 1 and 5 mM (Fig. 1A). These findings were corroborated by cell counting experiments (Fig. 1B). Additional experiments (data not shown) indicated that the growth-inhibitory effect of butyrate was fully reversible, suggesting that growth inhibition was not due to a cytotoxic effect of butyrate. Fig. 1 also shows that 10 ng/ml TGF-β1 and/or TGF-β2 had no effect on HT-29(E) growth. The duration of butyrate exposure required for growth inhibition was determined by removing butyrate-containing medium from HT-29(E) cells after various intervals of treatment and replacing the medium with fresh, butyrate-free medium (Fig. 2). Exposure to 5 mM butyrate for 4–8 h was sufficient to fully effect growth inhibition.

Cell Proliferation Kinetics in Butyrate-treated Cells. Previous studies have suggested that butyrate arrests cells in the G1 phase of the cell cycle (20, 21). We designed several experiments to determine more precisely the interval within G1 in which butyrate causes growth inhibition of HT-29(E) cells. Initially, it was necessary to develop a definition of G1 traverse in HT-29(E) cells. Rapidly growing, subconfluent cells were treated with nocodazole, an agent that arrests cells in metaphase (22). Release from nocodazole arrest resulted in an increase of [3H]thymidine uptake (S phase entry) after a G1 traverse of approximately 20 h (Fig. 3, top panel). The kinetics of G1 traverse and entry into S phase after release from 24 h of butyrate treatment were similar to those for nocodazole, suggesting that butyrate-induced inhibition of HT-29(E) cells must occur quite early in G1 (Fig. 3, middle panel). Next, we postulated that if there exists a specific target for butyrate-mediated growth inhibition, it is likely an early G1 event, and cells that have progressed beyond this event (into mid- or late G1) should be insensitive to the addition of butyrate. Fig. 3 (bottom panel) shows that addition of butyrate after approximately 8 h of G1 traverse resulted in no inhibition of DNA synthesis, whereas addition at any time earlier in G1 resulted in growth inhibition to a magnitude equal to

The abbreviations used are: TGF-β, transforming growth factor β; FACS, fluorescence-activated cell sorter; HMBA, N,N'-hexamethylene bisacetamide; DMEM, Dulbecco’s modified Eagle’s medium.
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expression after treatment of HT-29(E) cells with 5 mm

sodium butyrate. A dose response for induction of alkaline

phosphatase is also shown. Expression of c-myc, an

immediate early gene with helix-loop-helix and leucine

zipper domains, is characteristically inversely correlated

with differentiation (23). We observed a decrease in c-

muc expression that began as early as 30 min after butyrate

treatment and continued until the 12th hour, when

expression began to increase (Fig. 6). Repeated experi-

ments showed that butyrate exposure for 2 h consistently

serum-free DMEM for 48 h. The cells were then refed with 10% DMEM to

effect a synchronous entry into G1, and 5 mm butyrate was added at

the time points shown. Twenty-one h after restimulation, 4 µCi/well

[3H]thymidine were added. Incorporation was terminated at the 24th h

and measured as described in "Materials and Methods."
decreased c-myc expression 4-8-fold below untreated cells. nup/475 and zif/268 are immediate-early genes that are rapidly induced by serum treatment of mouse fibroblasts and are regulated in the intestine by starvation and refeeding (24). nup/475 encodes a nuclear protein with a novel zinc-binding domain (25). zif/268 contains three consensus zinc finger repeat units, suggesting that it is a transcription factor (26). Expression of nup/475 and zif/268 remained relatively constant when examined relative to aldolase expression. These observations suggest that the down-regulation of c-myc by butyrate is not a general phenomenon involving immediate-early genes.

Previous studies have indicated that butyrate induces alkaline phosphatase protein activity in a variety of colon carcinoma cell lines (8, 9, 12), and a single study has shown that alkaline phosphatase mRNA expression in LS174T cells is induced by butyrate 3 days after treatment (8). Figs. 5 and 6 show that alkaline phosphatase expression was first detectable 12 h after butyrate treatment of HT-29(E) cells and further increased 24 h after treatment. Additional experiments showed that expression was detectable as early as 6 h after treatment and continued to increase between 24 and 48 h after treatment (data not shown). It was also of interest to examine TGF-β expression following butyrate treatment, since previous studies have shown that TGF-β mRNA and protein levels are positively correlated with the extent of intestinal epithelial cell differentiation (27, 28). In addition, differentiating agents such as HMBA and retinoic acid up-regulate TGF-β expression in epithelial cell lines (29, 30), and Nathan et al. (13) found that selected clones of the colon cancer line HCT-116 showed a 2-fold increase in TGF-β expression 96 h after butyrate treatment. In the present study, HT-29(E) cells treated with butyrate exhibited 1.8- and 5-fold increases in TGF-β mRNA expression, respectively, 12 and 24 h after treatment with butyrate (Figs. 5 and 6). Although it has been proposed that differentiating agents like HMBA and retinoic acid may influence butyrate expression by up-regulation of TGF-β expression, the kinetics of alkaline phosphatase and TGF-β induction by butyrate observed herein are not supportive of this view. Indeed, treatment of HT-29(E) cells with TGF-β did not induce alkaline phosphatase mRNA or protein expression (data not shown).

**Development of Histochemical Positivity for Alkaline Phosphatase.** The development of alkaline phosphatase activity in HT-29(E) cells was examined using several concentrations of butyrate (Fig. 7). Histochemical positivity was not seen in untreated cells, a finding consistent with the previously reported absence of alkaline phosphatase activity in the HT-29 line (18). Occasional staining was seen in cells treated with 1 mM butyrate for 48 h, and increased levels of staining were seen with 5 and 10 mM treatment. Heterogeneity in the intensity of staining was apparent, as all cells were not histochemically positive after 48 h of treatment.

**Discussion**

Eukaryotic cells must prepare for DNA synthesis by progression through an orderly sequence of complex cellular events during G1 (31). Recent work has begun to define how polypeptide growth factors facilitate or hinder G1 traverse and thus stimulate or inhibit growth, by modulating specific G1 events. For example, TGF-β, regulates activity of the retinoblastoma gene product, and this may be an essential step in TGF-β-mediated suppression of c-myc expression and growth inhibition (32, 33). Cellular differentiation is also initiated in G1, but the precise sequence of events and the various factors that regulate these events in differentiating eukaryotic cells are less well defined.

The gastrointestinal epithelial cell is an attractive model for the study of differentiation. Anchored stem cells located at the base of the intestinal villus provide a lifelong supply of daughter cells that migrate toward the villus tip and rapidly acquire a more differentiated phenotype. Terminally differentiated cells are then extruded from the villus tip within 72 h of leaving the crypt. Study of this dynamic process is potentially quite complicated...
because of the functional polarity of the intestinal epithelial cell and the complexity of the two separate and distinct compartments (intraluminal and serosal) to which the cell is exposed. Recent studies have begun to dissect out roles for several putative autocrine growth factors in regulating intestinal epithelial cell proliferation and differentiation (27, 34), but the potential role for intraluminal solutes and nutrients has received less attention.

In the present study, the effect of the four-carbon free fatty acid, butyrate, on intestinal epithelial cell growth and differentiation was examined. Although the potent effects of butyrate on cellular growth and differentiation are well known and have proved useful in the analysis of molecular mechanisms of cellular differentiation, these effects assume a more definitive physiological significance when cells of gastrointestinal origin are studied because of the high concentrations of intraluminal free fatty acids (including butyrate) to which enterocytes are continuously exposed in vivo (5).

Our data demonstrate that HT-29(E) cells are reversibly growth inhibited and induced to differentiate by butyrate at concentrations as low as 0.1–1 mm. Early studies indicated that butyrate inhibits cell growth in G1 (20, 21). A more precise definition of the point at which butyrate arrests growth in G1 could potentially enable development of a hypothesis regarding the molecular mechanism by which butyrate arrests growth. Both [3H]thymidine uptake data (Fig. 3, top and middle panels) as well as FACS analysis (Fig. 4) indicated a prolonged interval (nearly 24 h) of G1 traverse before initiation of DNA synthesis in cells released from butyrate arrest. In fact, the kinetics of S phase entry after release from butyrate arrest were quite similar to those observed after release from nocodazole, an agent that arrests cells in metaphase, suggesting that butyrate arrests cells at an early point in G1. In addition, butyrate is effective in inducing growth arrest only when added early in the G1 phase of the cell cycle (Fig. 3, bottom panel). Exposure for 4–8 h was all that was required to achieve the level of inhibition seen after continuous treatment for 24 h. Taken together, these results suggest that an early G1–G1 event, such as expression of an “immediate-early” gene (31), may be a target for butyrate action. Fig. 4 shows that expression of c-myc was markedly decreased within 30 min of butyrate treatment. This effect appears to be specific, since expression of nup/475 and zil/268 is not similarly affected. Elucidation of the precise mechanisms by which c-Myc regulates gene expression and cell growth continues to evolve, but as a general principle, its expression is generally associated with cell growth and inversely associated with differentiation (23). Constitutive overexpression of c-Myc has been shown to prevent differentiation in murine erythroleukemia cells (35), and antisense c-myc oligomers inhibit HL-60 promyelocytic cell growth and differentiation (36). Specific regions within the molecule appear to confer the capacity for inhibition of differentiation (37). Thus, it is plausible that the downregulation of c-myc expression by butyrate observed in the present study may be an obligatory step in the induction of enterocyte growth inhibition and differentiation, but additional studies are required. An alternate interpretation, that decreased c-myc expression is only associated with the process of differentiation, must also be addressed.

Several indirect lines of evidence suggest that TGF-β, may be important in mediating growth inhibition and differentiation of the intestinal epithelial cell. Kurokowa et al. (38) reported that TGF-β1 induced sucrase activity in the IEC-6 cell line, although we have been unable to confirm this observation (27). There does, however, appear to be an association between TGF-β, mRNA levels (27), as well as TGF-β, immunoreactivity* (28), and the differentiated phenotype in the small intestinal and colonic epithelium. Similar observations have been made in other epithelia (28, 29, 30); for example, TGF-β, expression in normal human suprabasal keratinocytes is induced 4-fold by sodium butyrate, but no induction is seen in the basal keratinocyte population (41). In addition, differentiating agents such as HMBA and retinoic acid induce TGF-β expression in vivo and in vitro (29, 30). We observed that the differentiating effects of butyrate were associated with increased levels of TGF-β, mRNA in HT-29(E) cells; however, addition of TGF-β,- to this cell line did not inhibit cell growth or induce differentiation. Thus, the preponderance of evidence suggests that TGF-β, is probably not the single primary differentiating agent for intestinal epithelial cells; notwithstanding, TGF-β expression in a number of systems is consistently associated with the differentiated phenotype.

Early studies suggested that butyrate modulates gene expression by a general mechanism involving regulation of DNA methylation (42) and histone acetylation (43). Several recent studies have begun to analyze the mechanism of butyrate-induced growth inhibition by examination of the expression of protooncogenes and other growth-associated genes. For example, induction of c-myc and c-fos expression in serum-stimulated Swiss 3T3 fibroblasts is unaffected by concurrent treatment with butyrate, whereas expression of p53 and thymidine kinase is decreased (44). Butyrate treatment of F-98 rat glioma cells results in induction of c-fos and c-sis, whereas expression of Ki-ras was unaffected (45). Foss and coworkers (16) found that butyrate-induced differentiation of several human colon carcinoma cell lines was associ-
ated with decreased levels of two members of the src-related protein kinase family, pp60^src^ and pp56^x^. Increased binding of ^1^H^-epidermal growth factor to its receptor has been described in HCT-116 colon lines (13). Two additional studies indicate that butyrate treatment of colon carcinoma cell lines results in down-regulation of c-myc or c-myc protein (46, 47). Of particular interest are observations by Herold and Rothberg (46) which indirectly suggest that down-regulation of c-myc by butyrate is associated with induction of a labile “activity” that has a negative effect on c-myc abundance.

Further supporting the specificity of butyrate in modulating differentiation are studies in differentiating erythroid cells in which specific 5’-flanking sequences in the embryonic globin gene appear to be required for butyrate responsiveness (48). Induction of differentiation in this system occurred in the absence of histone deacetylation. Deng and coworkers (49) have identified a broad region (between nucleotides ~363 and ~170) in the placental-like alkaline phosphatase gene that appears to modulate butyrate sensitivity in L5174T colon cancer cells. It has also been observed that butyrate inhibits differentiation in myoblasts by interfering with the transcriptional activating function of the helix-loop-helix proteins Myo-D and myogenin (50). The helix-loop-helix domain of Myo-D was essential for butyrate inhibition of differentiation. All of these studies, as well as the present report, suggest that butyrate affects cell growth and differentiation by selectively and specifically altering the expression of growth-related genes. Butyrate treatment of intestinal cell lines such as the HT-29 line may be an important model for the study of early molecular events associated with intestinal epithelial differentiation.

Materials and Methods

Cell Line. The parental HT-29 cell line was obtained from the American Type Culture Collection. Cells were grown in DMEM (glucose, 4.5 g/liter) supplemented with 10% fetal bovine serum. The clone designated HT-29(E) was isolated by limiting dilution of parental cells into 96-well flat-bottomed tissue culture plates. HT-29(E) cells were passed as soon as confluence was reached and were supplied with fresh medium every 48–72 h.

Isolation of Polyadenylated RNA and Northern Blot Analysis. Total cellular RNA was extracted by the method described by Schub et al. (51). Oligo(dT)-selected RNA was separated by electrophoresis in a 1.2% agarose-formaldehyde gel, and Northern blotting was performed as previously reported (52, 53). Isolated complementary DNA probes were radiolabeled by random primer extension using [32P]dCTP and the Megaprime DNA labeling system from Amersham (Arlington Heights, IL). The alkaline phosphatase probe, a kind gift from Dr. David Alpers, is an EcoRI clone encoding 301 base pairs of the 3’ untranslated region of the rat placental alkaline phosphatase gene (54). The TGF-β1 probe is a 974-base pair Smal clone of the mouse TGF-β gene (55). The c-myc, nup475, and zif268 probes have been described previously (25, 26, 56). Hybridizations and posthybridization washes were performed under conditions described previously (27).

[^H]Thymidine and Growth Assays. [^H]Thymidine incorporation assays were carried out using 24-well tissue culture plates. Typically, cells were seeded at a density of 25,000 cells/well and allowed to attach for at least 24 h. Subsequent manipulations and treatments were performed as described in the figure legends. Cells were labeled with 4 μCi/ml [^H]thymidine (50–80 Ci/mmol; New England Nuclear, Boston MA). Radioactivity incorporated into trichloroacetic acid-insoluble material was determined by scintillation counting, and results are presented as the mean ± SE for triplicate or quadruplicate determinations. Each experiment was repeated at least once. The effect of butyrate on cell growth was also determined by cell counts. Cells were seeded into 6-well plates at a density of 20,000 cells/well. Butyrate was added 24 h later. Forty-eight h after treatment, cells were trypsinized and counted using a hemocytometer. Results are expressed as mean ± SE of triplicate determinations, and each experiment was repeated at least once.

Histochemical Staining for Alkaline Phosphatase. HT-29(E) cells were seeded into 8-well Lab-Tek slides (Nunc, Naperville, IL) and treated with butyrate 24 h later. After 48 h treatment, histochemical staining for alkaline phosphatase was performed using Sigma kit 86R (Sigma Diagnostics, St. Louis, MO), which detects alkaline phosphatase activity colorimetrically by formation of stable diazonium salts. Cells were counterstained with hematoxylin, and histochemically positive cells were counted and expressed relative to total number of cells.

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References


