Effects of Sodium Butyrate on the Synthesis and Methylation of DNA in Normal Cells and Their Transformed Counterparts

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ABSTRACT

The effects of various concentrations of sodium butyrate were examined on a normal embryonic lung fibroblast cell line (WI-38) and its two transformed counterparts, a simian virus 40-transformed line (SVWI-38) and a cell line transformed by γ-irradiation (CT-1). The rate of thymidine incorporation into DNA was inhibited by 60–80% in the WI-38 cells, even at butyrate concentrations as low as 5 mm. The two transformed cell lines showed no inhibition of DNA synthesis, even at concentrations of 75 mm butyrate. Analysis of RNA and protein synthesis revealed that the former was inhibited by ±20% at 5-10 mm butyrate in the normal WI-38 cell line, while protein synthesis was not inhibited at these concentrations. The inhibition of RNA synthesis was not dose dependent up to butyrate concentrations of 20 mm, and protein synthesis was inhibited less than 15% at this concentration. None of these inhibitory effects was observed in the case of the SVWI-38 or CT-1 cell lines. Analysis of the 5-methylcytosine content of DNA that was labeled either prior to or during treatment with butyrate revealed an increased content of methylcytosine when compared with control cells. Both preexisting and newly synthesized DNAs were subject to hypermethylation. Although all three cell lines showed a dose-dependent hypermethylation of DNA, the extent of this methylation differed in the normal and transformed lines, as preexisting DNA was more methylated in WI-38 cells compared with SVWI-38 and CT-1 cells, while methylation of newly synthesized DNA occurred to a greater extent in the SVWI-38 cells. These studies show that sodium butyrate affects major macromolecular synthetic processes as well as DNA methylation quite differently in normal and transformed cells.

INTRODUCTION

Ever since it was shown that sodium butyrate is capable of inducing globin gene expression in Friend erythroleukemia cells (1), attention has been focused on this short-chain 4-carbon, polar fatty acid as a potential antitumor agent (2). Treatment of human promyelocytic leukemia cells with butyrate caused their terminal differentiation into phagocytic myeloid cells (3). Butyrate has already been used clinically in a patient with acute myelogenous leukemia, where it was administered i.v. for 10 days, resulting in the elimination of peripheral myeloblasts, with an increase in the number of mature myeloid cells (4). Butyrate is to have further clinical applications, knowledge of its precise mode of action in cells is vital.

To date, the most notable effect of butyrate that has been observed on cultured cells has been its ability to induce hyperacetylation of the histone proteins, with histones H3 and H4 being most affected (5–7). This effect is reversible and is a direct consequence of the inhibition of the deacetylase enzyme(s) (8–10). Butyrate has also been shown to prevent phosphorylation of histones H1 and H2A (11, 12), apparently mediated through the inhibition of certain kinases. Hyperphosphorylation of the nuclear proteins HMG 14 and 17 caused by butyrate treatment has also been reported (13). Methylation of certain cellular proteins is inhibited by this fatty acid, probably due to the inhibition of some methylase enzymes and not due to a reduction in the pool of S-adenosyl-L-methionine (11).

Most of the effects of butyrate have been investigated in the case of transformed cells. Sodium butyrate has been shown to enhance α-fetoprotein, albumin, and transferrin production in a rat hepatoma cell line (14) and to cause neurite-like process formation in a mouse neuroblastoma cell line (15). Although butyrate appears to be specific in its actions on transformed cells, its effects on normal cells have not yet been examined. We have accordingly studied a number of effects of butyrate addition on cultured human lung fibroblasts. These effects have been compared with those obtained in two transformed counterparts of the same cell line. This comparison is important since butyrate will interact with normal as well as transformed cells when used clinically.

MATERIALS AND METHODS

Cell Culture. WI-38, a normal human embryonic lung fibroblast cell line, was obtained from the American Type Culture Collection (Rockville, MD). The two transformed counterparts were: (a) a SV-40-transformed cell line (SVWI-38)3 and (b) a γ-irradiation-transformed cell line (CT-1), a gift from Dr. M. Namba, Tokyo, Japan (29).

Cells were plated at a density of 5 × 104 cells per dish in 30-mm plastic Petri dishes in Eagle’s basal medium containing 10% fetal calf serum, supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). Treatment with sodium butyrate was for 14 h at 37°C in a humidified 95% air-5% CO2 incubator. Sodium butyrate was filtered sterilized (0.45-μm filter) and stored at 4°C as a 1 M stock solution in Eagle’s basal medium. Cell counts were obtained after trypsinization, using a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL).

Trypan blue exclusion tests (0.04% trypan blue in phosphate-buffered saline, for 2 min) showed that the cells still attached to dishes were viable under the above conditions.

DNA, RNA, and Protein Synthesis. Five μCi of [3H]thymidine (40 Ci/ mmol) were added per 30-mm dish to all three cell lines for periods of 14 h. The cells were then trypsinized and counted. An aliquot was precipitated with 10% TCA, washed thoroughly with 5% TCA on Whatman GFC filters. The amount of DNA synthesized was expressed as [3H]thymidine incorporated per 106 cells.

For RNA synthesis, cells were labeled with [5,6-3H]uridine (43 Ci/ mmol) and 50 μg of [3H]uridine (50 Ci/mmol) were added per 30-mm dish to all three cell lines for periods of 14 h. The cells were then trypsinized and counted. An aliquot was precipitated with 10% TCA, washed thoroughly with 5% TCA on Whatman GFC filters. The amount of DNA synthesized was expressed as [3H]uridine incorporated per 106 cells.

The abbreviations used are: SVWI-38, simian virus 40-transformed WI-38 fibroblasts; CT-1, γ-irradiation-transformed WI-38 fibroblasts; TCA, trichloroacetic acid.

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mmol) together with 10 μM cold deoxycytidine and 10 μM cold thymidine, in order to reduce conversion of uridine to dCTP and dTTP and its subsequent incorporation into DNA. Cells were counted and TCA precipitated as above. In order to determine protein synthetic activity, cells were labeled with [3H]leucine (52 Ci/mmol) for 14 h and then processed as above.

DNA Methylation. DNA methylation was monitored by the incorporation of [6-3H]uridine into DNA (15 Ci/mmol) after conversion into deoxyctydine and measuring the ratio of methylcytosine to cytosine in the hydrolysate of the isolated DNA (16). Label was added at a final concentration of 1 μCi/ml either 3 days prior to treatment with sodium butyrate or overnight (14 h) in the presence of sodium butyrate. DNA from the three cell lines was isolated as described by Wilson and Jones (16). After removal of the medium, the cells were lysed in 0.5% sodium dodecyl sulfate-0.3 M NaOH and incubated overnight at 37°C. After alkaline hydrolysis of the RNA, the NaOH was neutralized with HCl to pH 7.0. Protein removal was achieved by the addition of proteinase K (50 μg/ml). The DNA was precipitated with TCA, pelleted by centrifugation, rinsed in 70% ethanol, dried, and hydrolyzed in 68% formic acid in sealed capillary tubes at 100°C for 25 min. The formic acid was evaporated off, and the bases were resuspended in 0.1 N HCl and analyzed by high-pressure liquid chromatography using a Beckman Ultracil™-Cx column.

The percentage of methylation was calculated as follows.

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\frac{\text{[5-methylcytosine]}}{\text{[5-methylcytosine]} + \text{[cytosine]}} \times 100
\]

RESULTS

DNA, RNA, and Protein Synthesis. Although most studies on the biochemical effects of sodium butyrate have been conducted within the 1–20 mM range, it was decided in this study to determine the threshold concentration of sodium butyrate for cytotoxicity. Cells were treated with increasing concentrations of sodium butyrate until they showed altered morphology when examined under the light microscope after 14 h of treatment. The normal cells and their transformed counterparts showed no gross morphological alterations after treatment with butyrate at concentrations as high as 100 mM. At such high doses of sodium butyrate, however, cell viability was affected, probably by hypertonicity.

Sodium butyrate inhibited DNA synthesis (assessed by the incorporation of radiolabeled thymidine into DNA) by 60–80% at a concentration of 5 mM in WI-38 fibroblasts (Figs. 1A and 2), but cells treated with the same concentration of NaCl were unaffected (Fig. 1B). In fact, DNA synthesis in normal cells was not affected at all up to 25 mM sodium chloride, while a 10–15% inhibition occurred between 25 and 75 mM NaCl. The SVWI-38 and CT-1 cells, however, were not affected by butyrate at concentrations below 75 mM and NaCl below 125 mM. Above these concentrations, the hypertonicity of the medium caused decreased DNA synthesis. Trypan blue exclusion tests showed that, in the concentration range where DNA synthesis was not affected, the cells were viable and attached to the surface of the culture dishes.

The effects of butyrate treatment on DNA, RNA, and protein synthesis in the case of the WI-38, SVWI-38, and CT-1 cell lines are shown in Fig. 2. DNA synthesis was again inhibited maximally in the normal cell line, contrasting strongly with both transformed lines where no inhibition occurred up to 20 mM butyrate. RNA synthesis was not affected in either of the transformed lines, while the WI-38 cell line showed about 20% inhibition of RNA synthesis. This inhibition became more apparent at higher concentrations of butyrate, reaching a maximum of 80% at 100 mM. Protein synthesis was not inhibited at low butyrate concentrations in the transformed cell lines. A slight inhibition (10–15%) occurred at 20 mM butyrate and above in the case of the WI-38 cell line.

These data show that low concentrations of butyrate affected DNA synthesis only in the normal cell line. Both transformed cell lines were inhibited only at much higher concentrations of butyrate; the CT-1 cell line showed the least inhibition of DNA synthesis, reaching a maximum of 40% inhibition at 175 mM, while the SVWI-38 transformed line showed maximum inhibition of 80%
at 150 mM. Treatment with sodium chloride also showed approximately 40% DNA synthesis inhibition in CT-1 cells at 175 mM, while only 20% inhibition in SVWI-38 cells was noted at 150 mM (See Fig. 1B).

DNA Methylation. Sodium butyrate induced hypermethylation of DNA in all three cell lines. This hypermethylation occurred in DNA which had replicated prior to butyrate treatment (i.e., in the preexisting DNA), as well as in DNA which had undergone replication during butyrate treatment (i.e., in the newly synthesized DNA).

Fig. 3 shows the similar effects of sodium butyrate addition on DNA prelabeled for 3 days before addition of the indicated concentrations of butyrate in the case of all three cell lines. At lower sodium butyrate concentrations, there was an overall increase in the percentage of methylation. This increase fell back to that of the control value at higher butyrate concentrations, giving a bell-shaped dosage curve. Butyrate induced the largest increase in methylation in the normal cell line, where an increase of almost 100% was observed at 20 mM. The two transformed cell lines showed a similar qualitative response, with maximum methylation occurring at 10–20 mM.

Fig. 4 shows the extent of DNA methylation in the presence of sodium butyrate: hypermethylation of DNA occurred in all three cell lines, with the SVWI-38 cells giving a maximum methylation of around 8% at 20 mM, WI-38 cells a maximum methylation of 4% between 10 and 20 mM, while the CT-1 cell line gave a maximum methylation of 4.5% between 5 and 10 mM, decreasing to normal levels at 20 mM.

**DISCUSSION**

Most studies on the biochemical and morphological effects of butyrate have focused on specific tumor cell lines [e.g., human pancreatic tumor cells (17), uterine cervical cancer cells (SK-111a) (18), Hela cells (19–22), human breast cancer cells (MCF) (23), and many others (24, 25)]. In most cases, the loss of malignant properties caused by butyrate treatment has been accompanied by the concomitant appearance of more differentiated states [e.g., in mouse neuroblastoma cells (15)]. The lack of a suitable normal cell line for comparison has been a problem in many studies. A tumor may not have originated in the tissue in which it is found, or normal cells may comprise only a small percentage of the cells present in a tissue, e.g., in pancreatic cancer, where the tumor is thought to originate in the ductal cells of the organ, which make up only 4% of the pancreas (17). The use of a normal cell line, as well as its transformed counterparts, allowed us to compare directly the effects of butyrate on normal and transformed cells in tissue culture systems.

This study has clearly shown different responses to sodium butyrate in normal and transformed cells. In particular, DNA synthesis was maximally inhibited in normal cells at butyrate concentrations of 5–10 mM, while the transformed counterparts showed no inhibition at these concentrations. Of great interest is the fact that, although normal cells showed maximal DNA synthesis inhibition at low butyrate concentrations (i.e., 5–20 mM), cell viability was not affected. By contrast, DNA synthesis was not affected by butyrate in the transformed cells, although they showed decreased cell viability. This was probably due to prolongation of the mitotic phase and/or G1 phase of the cell cycle or to mitotic arrest. This would explain the large number of mitotic figures observed after butyrate treatment. Transformed cells appeared thus to have greater cytotoxicity when butyrate was present in the incubation medium.

It is clear that care should be taken if sodium butyrate is to be administered clinically as an antileukemic drug, for although normal cells appear not to be affected by butyrate on the basis of cellular morphology and survival, DNA synthesis was strongly inhibited by this agent in the untransformed cells studied. This could explain the results obtained by Novogrodsky et al. (4) when sodium butyrate was administered i.v. to a patient with acute myelogenous leukemia. The elimination of myeloblasts from the peripheral blood, with the appearance of mature myeloid cells, together with a reduction of [3H]thymidine uptake by the periph-
eral blood cells, was taken to indicate partial reversion to the normal state. Although butyrate appeared to reduce the number of leukemic cells, the observed decrease in [\(^{3}H\)]thymidine uptake may have been due to effects of sodium butyrate on the DNA-synthesizing ability of the mature myeloid cells. RNA synthesis was also slightly inhibited in the normal cell line (±20%) tested in this study, but this did not occur in the transformed lines.

Sodium butyrate did not appear to affect total protein synthesis in any of the three cell types, and only WI-38 showed a slight inhibition of protein synthesis at the higher butyrate concentrations of 20–50 mM. This indicates that butyrate inhibited DNA synthesis in the normal cells through a specific action on the DNA-synthesizing machinery of normal cells, possibly by acting on the DNA polymerase itself.

Sodium butyrate has been reported to exert diverse effects on many biochemical processes in cells. By inducing new gene expression in Friend erythroleukemia cells (26), sialyl transferase synthesis in the normal cells through a specific action on the DNA to the enzyme; (d) inhibition of demethylases; and (e) a hypermethylation of DNA when cells are treated with butyrate. In all cases, this DNA hypermethylation was concentration dependent and occurred maximally at 5–10 mM butyrate. This hypermethylation of daughter DNA could have resulted from any of the following: (a) an increase in the number of maintenance methylase molecules; (b) activation of maintenance methylase; (c) exposure of more methylatable sites in DNA to the enzyme; (d) inhibition of demethylases; and (e) a combination of the above.

Sodium butyrate may affect gene expression through the alteration of DNA methylation patterns. Our results clearly show hypermethylation of DNA when cells are treated with butyrate. This is an apparent contradiction of a previous report (26) indicating that Friend erythroleukemia cells become hypomethylated after sodium butyrate treatment. However, it is more likely, as can be seen from this study, that sodium butyrate affects different genes and different cell types in a tissue-specific manner. Because butyrate has been shown to affect different cell lines in different ways, this discrepancy possibly arises from differences in the cell types investigated. It has been reported that sodium butyrate inhibits gene expression in a number of systems for example, the synthesis of \(M_{3}, 53,000\), the transformation-related protein in 3T6 mouse fibroblasts (28); it was also noted that SV-40-transformed 3T6 cells showed resistance to this butyrate-related inhibition of \(M_{3}, 53,000\) protein. This agrees with our finding that butyrate affects normal and transformed cells differently. Since methylation changes are generally coupled with altered gene expression, sodium butyrate may be capable of both inducing and/or repressing expression of genes in different cell types.

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