Effect of Sodium Butyrate on Alkaline Phosphatase in HRT-18, a Human Rectal Cancer Cell Line

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ABSTRACT

Treatment of the human rectal cancer cell line HRT-18 with sodium butyrate caused a reversible elevation of alkaline phosphatase activity which was inhibited by cycloheximide and actinomycin D. The alkaline phosphatase in untreated cells was heat stable at neutral pH and inhibited by phenylalanine but not by homoarginine, and 80% of the enzyme activity was precipitated by antibody against human term-placental enzyme. Following butyrate treatment, the enzyme became more heat stable at neutral pH and inhibited by phenylalanine and amino acid and peptide inhibitors. These results were analyzed and compared to the patterns obtained with alkaline phosphatase from human placenta, human normal colon mucosa, and human colon cancer tissues. The alkaline phosphatase from four human colon cancer tissues was of the early placental form, while the enzyme from human normal mucosa was of the intestinal type.

INTRODUCTION

Butyrate, a 4-carbon fatty acid, is a natural product of colonic flora and has been shown to be a potent "differentiating agent" in some cancer cells such as teratocarcinoma, erythroblasts, and HeLa cells. It has also been reported that, in several cancer cell lines, butyrate increases the activities of a number of enzymes including alkaline phosphatase (23, 24).

In our previous papers (16, 17, 28), we have reported that sodium butyrate causes an increase in the activity of alkaline phosphatase in colon and rectal cancer cell lines concomitant with changes in the profile of cell surface proteins and a decrease in in vitro tumorigenicity. Although comparable data are not available for the colon, alkaline phosphatase in the small intestine is localized on the brush border membranes, and its activity is considerably higher in more differentiated villus cells as compared to the less differentiated crypt cells (7, 22). Histochemical studies from several laboratories have demonstrated that alkaline phosphatase activity in normal human colonic mucosa is low and that activity in colon or rectal cancer tissues is absent or minimal (2, 6, 7, 10, 20), although there is one report showing that alkaline phosphatase activity is higher in colon and rectal cancer tissue than in surrounding normal mucosa (9). It has been reported that, after in vitro malignant transformation of a variety of cells including those of human placental origin, the activity of alkaline phosphatase decreases (1, 3, 25). A reduction in enzyme activity has also been found after experimental carcinogenesis (18). Therefore, the increase of alkaline phosphatase activity in butyrate-treated cancer cells may reflect a more differentiated phenotype than the untreated control cancer cells.

In this paper, the induction of alkaline phosphatase by sodium butyrate in a human rectal adenocarcinoma cell line (HRT-18) was studied in detail. Alkaline phosphatase from control and sodium butyrate-treated HRT-18 cells was characterized by its thermostability and inhibition patterns using various amino acid and peptide inhibitors. These results were analyzed and compared to the patterns obtained with alkaline phosphatase from human placenta, human normal colonic mucosa, and human colon cancer tissues.

MATERIALS AND METHODS

Cell Line. Human rectal cancer cell line HRT-18 derived from a patient with primary adenocarcinoma of the rectum was a generous gift from Dr. N. A. F. Tompkins (27). HRT-18 cells exhibited human type B glucose-6-phosphate dehydrogenase isozyme and had Y chromosome, indicating that this cell line has no HeLa cell contamination.

Cell Culture. The cells were routinely cultured as a monolayer in plastic T-75 flasks or 35-mm petri dishes using Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO2-95% air. The cells were subcultured weekly using 0.05% trypsin-0.02% EDTA in Hanks’ balanced salt solution without Ca2+ and Mg2+. The culture medium was changed every 4 days. The cultures were examined periodically for the presence of Mycoplasma and found to be free of contamination.

To study the effect of sodium butyrate, the culture medium was changed to that containing the appropriate amount of sodium butyrate 48 hr after cell transfer. To harvest the control and experimental cultures, the cells were washed 4 times with 10 mM Tris-HCl buffer, pH 7.5, and scraped with a rubber policeman. The cells were then suspended in 10 mM Tris-HCl buffer, pH 7.5, and homogenized by sonication. Cell homogenates were kept at −20°C and used within a week. When a measure of the cell number was required, aliquots of 1×10⁸ cells were seeded in 35-mm Petri dishes. Triplicate cultures were harvested using a mixture of 0.05% trypsin and 0.02% EDTA, and the number of cells was determined using a Coulter Counter.

Tissue Preparation. Normal human colon and colon cancer tissues were obtained at surgery. Normal mucosa were scraped with a glass slide and homogenized in 10 mM Tris-HCl buffer, pH 7.5, by sonication. Cancer tissues were homogenized in a similar manner after the tissues were minced with a razor blade. Homogenates were centrifuged at 100,000 × g for 1 hr, and the total membrane pellets were utilized after suspending in 10 mM Tris-HCl buffer, pH 7.5.
Alkaline Phosphatase and Protein Assay. Alkaline phosphatase activity was assayed at 37° using p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) as substrate. The standard assay mixture consisted of 100 mM bicarbonate buffer, pH 10.0, 5 mM MgCl₂, and 10 mM substrate in a final volume of 500 µl including 50 µl of enzyme.

The reaction was terminated by adding 1 ml of 0.1 N NaOH, and the extent of hydrolysis was measured spectrophotometrically at 410 nm.

Enzyme activity was expressed in units, 1 unit being equal to the number of µmol of substrate hydrolyzed per min, and the specific enzyme activity was defined as units per mg of cellular protein. The ratio of the specific enzyme activity of the experimental to the control cultures referred to as the "induction index." Protein was assayed by the method of Lowry et al. (19) using crystallized bovine serum albumin (Sigma) as standard.

Effect of Protein Synthesis Inhibitors. The effects of 2 protein synthesis inhibitors, cycloheximide and actinomycin D (Sigma), were examined with regard to the induction of alkaline phosphatase by sodium butyrate. They were included in the culture medium at concentrations of 2 and 0.2 µg/ml, respectively. Control and sodium butyrate-treated cells were cultured in 35-mm plastic dishes for a total of 4 days, and the culture medium was changed after the first 2 days. The experiments were divided into a series of 4 groups. The number of dishes per experimental group was 4. In Group 1, inhibitor was included throughout the entire experimental period. In Group 2, inhibitor was present for only the first 2 days and removed from the culture medium for the last 2 days. In Group 3, cells were cultured in the absence of inhibitor for the first 2 days, and inhibitor was included for the last 2 days. In Group 4, no inhibitor was added. All cultures were harvested on the fourth day. Cell homogenates were assayed for alkaline phosphatase, and protein and induction index were calculated for each group.

Inhibition Study. In order to characterize alkaline phosphatase from control and sodium butyrate-treated cells, the following inhibitors were used: L-phenylalanine; L-homoarginine; L-leucine; L-Leu-Gly-Gly; and L-Phe-Gly-Gly. Each inhibitor was added to the assay mixture at a final concentration of 5 mM except for homoarginine which was used at 10 mM. The effects of inhibitors, where available, were used as controls. The thermal stability of alkaline phosphatase was examined by heating samples in 10 mM Tris-HCl buffer, pH 7.5, or 750 mM 2-amino-2-methyl-1,3-propanediol-HCl buffer, pH 10.0, at 65° for 5 min and comparing the resulting enzyme activity to a control kept at 4°. In the case of experiments carried out at pH 10.0, enzyme assays were done using 2-amino-2-methyl-1,3-propanediol-HCl buffer, pH 10.0, instead of bicarbonate buffer.

Polyacrylamide Gel Electrophoresis. A vertical slab gel (7.0% acrylamide-0.67% bisacrylamide) buffered with 125 mM Tris-HCl buffer, pH 8.5, was used. The gel and electrode buffer of 66 mM Tris-80 mM glycine, pH 8.9, contained 0.5% Triton X-100. No stacking gel was used. Samples of n-butyl alcohol-extracted enzymes or purified human placental alkaline phosphatase were brought to 0.5% Triton X-100 and 10% glycerol and subjected to electrophoresis at 20 mA/gel for 2 hr. Histochemical enzyme staining of gels was carried out by the method of Fishman (11).

RESULTS

Effect of Sodium Butyrate at Various Concentrations. As shown in Chart 1, increasing concentrations of sodium butyrate caused an increase in alkaline phosphatase activity in HRT-18 cells. The cells were cultured in 35-mm plastic dishes and harvested after 4 days of exposure to various concentrations of sodium butyrate. The increase in specific enzyme activity was maximum at 3 mM. However, treatment of cells with sodium butyrate at concentrations above 2 mM caused cellular uptake of trypan blue. Therefore, treatment of the cells with sodium butyrate was performed at a concentration of 2 mM in subsequent experiments.

Time Course Study. When sodium butyrate was included in the culture medium at a concentration of 2 mM, a marked reduction occurred in the growth rate of HRT-18 cells as shown in Chart 2, although this was a reversible phenomenon. Chart 2 also shows the effect of 2 mM sodium butyrate on the activity of alkaline phosphatase. In contrast to the reduction in the cellular growth rate, specific activity per unit of cellular protein reached a maximum value on Day 4 (72.5-fold compared to control) and subsequently decreased with longer cell culture. When sodium butyrate was removed from the culture medium, enzyme activity decreased significantly, indicating that the effect of sodium butyrate on alkaline phosphatase was reversible in HRT-18 cells.

Effect of Protein Synthesis Inhibitors. The enhancement of alkaline phosphatase activity by sodium butyrate treatment in HRT-18 cells was inhibited by the addition of cycloheximide (2 µg/ml) or actinomycin D (0.2 µg/ml) when these agents were
present in the culture medium throughout the 4 days of the experimental protocol. This indicates that both RNA and protein synthesis are necessary for the enhancement of alkaline phosphatase activity by sodium butyrate in HRT-18 cells. As can be seen in Chart 3, however, some differences in the mode of action were noted between these 2 agents. When cycloheximide was removed from the culture after the first 2 days, there was an enhancement of enzyme activity at the end of 4 days, suggesting that the effect was reversible. This was not observed with actinomycin D. Furthermore, when cycloheximide was included in the culture medium after 2 days of sodium butyrate treatment, induction of alkaline phosphatase was partially inhibited, while under the same conditions, actinomycin D showed no effect.

Characterization of Alkaline Phosphatase. In order to characterize the alkaline phosphatase of HRT-18 cells before and after sodium butyrate treatment, its thermostability characteristics and inhibition patterns with various amino acids and peptides were examined. Purified human placental alkaline phosphatase and the total membrane fractions isolated from human normal colonic mucosa and colon cancer tissues obtained at surgery were included for comparison.

The stability of the enzymes at 65°C for 5 min at neutral and alkaline pH is shown in Chart 4. At pH 7.5, the alkaline phosphatase of control and sodium butyrate-treated HRT-18 cells was heat stable, and their patterns were comparable to that of human placental enzyme. At alkaline pH, the placental enzyme was heat stable, but the enzymes from HRT-18 cells were not stable, indicating that they were not identical with placental alkaline phosphatase. The alkaline phosphatase from normal human colonic mucosa was heat labile at pH 10.0. After sodium butyrate treatment, the alkaline phosphatase from HRT-18 cells was somewhat more heat stable at neutral pH (p < 0.001), and its thermostability at alkaline pH remained unchanged.

The results of inhibition studies using various amino acids and peptides are summarized in Chart 5. Alkaline phosphatase activity in both control and sodium butyrate-treated HRT-18 cells was phenylalanine sensitive and homoaarginine insensitive, indicating that they are different from a liver, bone, kidney type alkaline phosphatase. Inhibition of alkaline phosphatase by peptides such as Leu-Gly-Gly and Phe-Gly-Gly has been reported to be a useful measure to distinguish the placental and intestinal enzyme (8). The normal human colon enzyme was relatively insensitive to these peptides, but human placental enzyme was very sensitive as can be seen in Chart 5. However, the enzymes from HRT-18 cells showed increased inhibition by phenylalanine (p < 0.001), homoaarginine (p < 0.001), and leucine (p < 0.001), while inhibition by Leu-Gly-Gly and Phe-
Gly-Gly remained unchanged. When a higher concentration (10 mM) of these peptides was tested, the enzyme from sodium butyrate-treated cells showed increased inhibition by these peptides as follows: Leu-Gly-Gly: control, 49.4 ± 2.2%, and butyrate treated, 43.4 ± 2.3% (p < 0.001); Phe-Gly-Gly: control, 32.1 ± 2.4%, and butyrate treated, 22.3 ± 1.2% (p < 0.001). These data indicate that sodium butyrate treatment of HRT-18 cells increases the sensitivity of their alkaline phosphatase to inhibition by amino acids and peptides.

Since the original tumor tissue from which the HRT-18 cell line was derived is not available, 4 other colon cancer tissues were obtained at surgery, and their alkaline phosphatases were characterized for comparison. Total membrane fractions were isolated and subjected to the same experiments as described above. The alkaline phosphatase from all 4 cancer tissues was heat labile and sensitive to homologanine but insensitive to phenylalanine (Table 1). Thus, it appears that these enzymes are of the liver, bone, kidney type and clearly different from the enzyme found in normal colon mucosa. Enzyme from normal colon mucosa is of the intestinal type.

**Kinetic Analysis.** A kinetic analysis of alkaline phosphatase showed that sodium butyrate treatment affected both K<sub>m</sub> and V<sub>max</sub>. The K<sub>m</sub> was lowered slightly from 3.1 ± 0.4 to 1.8 ± 0.1 mM (p < 0.001, n = 4), while the V<sub>max</sub> was markedly enhanced from 4.9 ± 0.6 to 112.1 ± 3.6 units/mg protein (p < 0.001) after sodium butyrate treatment for 8 days. These data suggest that sodium butyrate exerts its effect on the catalytic efficiency of the enzyme and/or the content of enzyme protein in HRT-18 cells rather than on the affinity of the enzyme for the substrate.

**Immunoprecipitation of Alkaline Phosphatase.** The immunological properties of alkaline phosphatase in homogenates from control and sodium butyrate-treated HRT-18 cells were examined in Nonidet P-40-solubilized supernatants which were subjected to immunoprecipitation with anti-human placental alkaline phosphatase antibody. Fixed S. aureus containing protein A was utilized to precipitate the antigen-antibody complex. An excess of antibody was assured by preliminary experiments. The results are shown in Table 2. The enzyme from butyrate-treated cells was completely precipitated by anti-human placental alkaline phosphatase antibody, while approximately 20% of the enzyme activity from control HRT-18 cells was not precipitated.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>% of activity remaining in human colon cancer tissues</th>
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<tbody>
<tr>
<td></td>
<td>Group 1</td>
</tr>
<tr>
<td>Phenylalanine (5 mM)</td>
<td>72.2</td>
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<tr>
<td>Hoomoarginine (10 mM)</td>
<td>23.9</td>
</tr>
<tr>
<td>Leucine (5 mM)</td>
<td>62.8</td>
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<tr>
<td>Leu-Gly-Gly (5 mM)</td>
<td>71.8</td>
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<tr>
<td>Phe-Gly-Gly (5 mM)</td>
<td>76.9</td>
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<tr>
<td>Heat (65°, 5 min)</td>
<td></td>
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<tr>
<td>pH 7.5</td>
<td>9.8</td>
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<tr>
<td>pH 10.0</td>
<td>4.4</td>
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**DISCUSSION**

It has been shown that treatment of cultured human cancer cells with glucocorticoids (5), hypertonic medium (21), DNA synthesis inhibitors (26), nucleoside analogs (13), and sodium butyrate (4, 14–17, 28, 29) induces an increased level of...
alkaline phosphatase. The induction of alkaline phosphatase by sodium butyrate has been observed in HeLa cells (14, 29), choriocarcinoma cells (4), and colon cancer cells (15-17, 28). When HeLa S3 and JEG-3 human choriocarcinoma cells were exposed to sodium butyrate, changes in thermostability of the enzyme were observed (4, 29). In the case of HT-29 human colon cancer cells, however, alkaline phosphatase induced by sodium butyrate showed the same properties as did the enzyme from the control cells which was of the intestinal form (15).

The present study shows that sodium butyrate causes a reversible elevation of alkaline phosphatase activity in HRT-18 human rectal cancer cells. Alkaline phosphatase from control HRT-18 cells exhibited the following characteristics. (a) The enzyme is heat stable at neutral pH, sensitive to phenylalanine, but insensitive to homoaarginine, indicating that the enzyme is mainly of the placental and/or intestinal type. (b) The degree of inhibition by peptides and heat at alkaline pH was intermediate to the values for placental and colon enzyme, indicating that the enzyme is not a typical placental or intestinal enzyme. (c) Approximately 20% of the enzyme activity remained un-reacted with anti-human placental alkaline phosphatase antibody. These observations suggest that alkaline phosphatase from HRT-18 cells is heterogeneous and may consist of more than one type of isozyme or that, regarding this enzyme, the HRT-18 cell line may not be homogeneous and consists of cells which express different forms of alkaline phosphatase.

After sodium butyrate treatment, alkaline phosphatase from HRT-18 cells was more sensitive to all the amino acid and peptide inhibitors tested and became more heat stable at neutral pH, although an obvious shift of isozyme class was not observed. Butyrate also caused changes in the kinetic parameters of the enzyme. The K_m was slightly decreased, and there was a large increase in the V_max. In addition, the enzyme from sodium butyrate-treated cells was found to be almost exclusively precipitable by antibody against human placental enzyme. These results indicate that sodium butyrate may not induce all the alkaline phosphatase isozymes present in control HRT-18 cells. This was clearly demonstrable on polyacrylamide gel electrophoresis. The mobility of the major bands of enzyme from butyrate-treated cells was different from that of control cells, and the faster migrating heat-labile enzyme band was found only in the control cells. The present study also indicates that the induction of alkaline phosphatase in HRT-18 cells by sodium butyrate requires new RNA and protein synthesis, since cycloheximide and actinomycin D inhibit the effect of sodium butyrate. It is probable that sodium butyrate acts at the transcriptional rather than translational level for the following reasons. (a) Actinomycin D did not block induction of alkaline phosphatase if included in the culture medium at the rapid phase of increase of enzyme activity, suggesting that translation of preformed mRNA is unaffected. (b) Removal of cycloheximide from the culture medium after 2 days caused a 15-fold increase in enzyme activity, while only a 1.5-fold increase was observed with actinomycin D. These data indicate that sodium butyrate-treated cells translate RNA which has already been synthesized into new alkaline phosphatase following removal of cycloheximide, while time is required for new RNA synthesis in the case of actinomycin D.

Since original tumor tissue from which HRT-18 cells derived was not available, we obtained colon cancer tissues at surgery and compared their alkaline phosphatase with the enzyme from HRT-18 cells. Although the number of tissues we studied was limited, all 4 colon cancer tissues had enzymes which were heat labile, sensitive to homoaarginine, but insensitive to phenylalanine. Clearly, these enzymes are similar to the liver, bone, kidney alkaline phosphatase and are different from the enzyme found in normal colonic mucosa. Among alkaline phosphatase isozymes found in human cancers, these enzymes are likely to be the "non-Regan" or "early placental" isozyme (12). Interestingly, these enzymes exhibited similar electrophoretic mobility to the fast migrating heat-labile enzyme from control HRT-18 cells which was insensitive to the treatment with sodium butyrate.

Although it is not clear if the early placental enzyme actually occurs in the original tumor tissue from which HRT-18 cells were derived, these data suggest that HRT-18 cells may contain an alkaline phosphatase which is similar to the enzyme found in the colon cancer tissues and that this enzyme is not induced by sodium butyrate. It would be premature to conclude that the induction of alkaline phosphatase by sodium butyrate is due to the suppression of the malignant state. However, it seems probable that sodium butyrate might be a useful tool in the elucidation of molecular mechanisms involved in the differentiation of human colon cells.

REFERENCES


Fig. 2. Polyacrylamide gel electrophoresis of alkaline phosphatase from human colon cancer tissues and control and sodium butyrate-treated HRT-18 cells. Lane 1, sodium butyrate-treated HRT-18 cells, heat treated (65°C, 5 min); Lane 2, sodium butyrate-treated HRT-18 cells, no treatment; Lane 3, control HRT-18 cells, heat treated (65°C, 5 min); Lane 4, control HRT-18 cells, no treatment; Lanes 5 and 7, 3 human colon cancer tissues. Arrow, a heat-labile minor component.
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