Effect of Growth and Sodium Butyrate on Brush Border Membrane-associated Hydrolases in Human Colorectal Cancer Cell Lines

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ABSTRACT

The activities of brush border membrane-associated hydrolases such as alkaline phosphatase (Alkpase), aminopeptidase, dipeptidyl aminopeptidase IV (DAP-IV), sucrase, lactase, and trehalase were studied in 14 different human colorectal cancer cell lines. The effect of sodium butyrate, a known differentiating agent, and cell growth on the activities of these enzymes was also examined. All 14 cell lines exhibited brush border membrane enzyme activities, and in general, the activity of Alkpase, aminopeptidase, and DAP-IV was much higher than the disaccharidases. However, the specific enzyme activities varied among different cell lines. The induction of Alkpase activity by sodium butyrate occurred in most of the 14 cell lines (2- to 123-fold), while induction of the other enzyme activities was observed in several (1.5- to 3.5-fold). In some instances, butyrate caused a decrease in enzyme activity. There was no statistically significant correlation between the induction of Alkpase activity and that of other enzyme activities by sodium butyrate. Levels of aminopeptidase and DAP-IV activity were found to be dependent on cell density and increased 3- to 4-fold by the tenth day in most of the cell lines.

Sodium butyrate altered the subcellular distribution pattern of the disaccharidases, causing a significant increase in activity associated with the soluble (cytoplasmic) fraction. Other enzymes such as Alkpase and DAP-IV continued to be predominantly associated with the membrane fraction in butyrate-treated cells. These data suggest that brush border membrane hydrolase activity and the effect of sodium butyrate may provide useful information regarding the differentiation of human colorectal cancer cells.

INTRODUCTION

The levels and characteristics of various enzyme activities in human tumors including colon cancer have been examined in an effort to begin to elucidate the mechanism of tumorigenesis (1-3). Recently, several cell surface membrane-associated hydrolase activities which are normally associated with small intestinal mucosal cells (4, 5) have been reported to be present in some human rectal and colorectal cancer cell lines in culture (6-9). Many of these reports deal with Alkpase activity, which is modulated by factors such as short chain fatty acid (6, 8-11), glucocorticoids (6, 12, 13), hyperosmolality (14, 15), nutritional status (16), and DNA synthesis (17, 18) in the various cell lines.

In previous studies from our laboratory, we have reported on the effect of 3 putative differentiating agents, sodium butyrate, dimethyl sulfoxide, and retinoic acid, on human colonic and rectal cancer cell lines. An increase in Alkpase and sucrase activities by sodium butyrate was found in human rectal cancer cell line HRT-18 (9), and human colon cancer cell lines SW-480 and SW-620 also showed an induction of aminopeptidase activity by sodium butyrate.

In the present study, we sought to determine if cell surface brush border membrane-associated hydrolases such as Alkpase, aminopeptidase, DAP-IV, sucrase, lactase, and trehalase are expressed in 14 human colonic and rectal cancer cell lines and to examine the effects of sodium butyrate and cell growth on the activities and subcellular localization of these enzymes.

MATERIALS AND METHODS

Cell Lines. The human rectal and colon cancer cell lines HRT-18 and HCT-48 were generously given to us by Dr. R. M. Schultz of the National Cancer Institute (Bethesda, MD) (19, 20). Human colon cancer cell lines CaCo-2 and HT-29 were kindly provided by Dr. J. Fogh of the Sloan-Kettering Institute for Cancer Research (Rye, NY) (21, 22). Human colon cancer cell lines SW-403, SW-480, and SW-1116 were developed at the Scott and White Clinic (Temple, TX) and were generously provided by Col. Albert Leibovitz (23). Human colon cancer cell lines HCT-116, HCT-116a, and HCT-116b were kindly provided by Dr. M. G. Brattain of the Baylor College of Medicine. Colon cancer cell lines DLD-2 and HCT-15 were generous gifts from Dr. D. L. Dexter of Brown University (24). LS174T colon cancer cell line was a gift from Dr. B. D. Kahan of the University of Texas (Houston, TX) (25). Colon cancer cell line LoVo was provided by the American Type Culture Collection.

Cell Culture and Treatment with Sodium Butyrate. All colon cancer cell lines were maintained as monolayers cultured in 75-cm² tissue culture flasks in 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 and 95% air. Treatment of cells with sodium butyrate was carried out by culturing them in the same medium supplemented with sodium butyrate (2 mM). In both control and sodium butyrate-treated groups, the culture medium was changed every other day after experimental samples had been taken. All cultures were free of Mycoplasma contamination when these experiments were performed.

Preparation of Cell Homogenate, Membrane, and Cytoplasmic Fractions. For cell growth studies, control and sodium butyrate-treated cells were harvested at various time intervals (2, 4, 6, 8, and 10 days). The cells were scraped from the culture flasks after washing 4 times with phosphate-buffered saline and suspended in isotonic phosphate-buffered saline at pH 7.4. The cell suspension was sonicated twice for 15 s on ice and designated the cell homogenate. In order to obtain the subcellular fractions, the cell homogenate was centrifuged at 100,000 × g for 1 h to obtain a membrane pellet and soluble (cytoplasmic) fraction.

Protein Assay. Protein content was estimated by the modified method of Lowry et al. (26) using bovine serum albumin as standard.

Enzyme Assay. All enzyme assays were carried out at 37°C under...
conditions where the reaction was linear with respect to enzyme concentration and time. AlkPase activity was measured using p-nitrophenyl phosphate as substrate (27). The reaction mixture consisted of 100 mM NaHCO₃ buffer (pH 10.0), 5 mM MgCl₂, 0.5 mM ZnSO₄, and 3 mM substrate. The reaction was terminated by the addition of 2.5 ml of 0.02 mM NaOH, and the extent of hydrolysis was determined spectrophotometrically at 410 nm. L-Leucyl-β-naphthylamide and Gly-L-Pro-β-naphthylamide were used as the substrates in the assay for aminopeptidase and DAP-IV, respectively. The released β-naphthylamide was assayed as described previously (28, 29). Assays of sucrase, lactase, and trehalase were carried out by the method of Dahlqvist (30) using the corresponding disaccharides as substrate. In all cases, the specific enzyme activities were expressed as units per mg of cellular protein, with 1 unit equal to 1 nmol of substrate hydrolyzed per min.

RESULTS

Enzyme Activities and the Effect of Sodium Butyrate. Mean values of brush border membrane-associated hydrolase activities of 14 human colorectal cancer cell lines harvested on the eighth day are summarized in Table 1. All subsequent experiments were performed using cells harvested at this time, because the cultures were confluent and provided adequate numbers of cells. The activities of AlkPase, aminopeptidase, and DAP-IV were much higher than those of sucrase, lactase, and trehalase in the control groups. The activities of AlkPase, aminopeptidase, and DAP-IV were compared to control groups. However, the extent of increase varied from 2- to 123-fold depending on the cell line. The DAP-IV activity of most cell lines was either slightly increased or unchanged, and butyrate caused a general reduction of aminopeptidase activity in all cell lines except for HCT-116a and SW480. Several cell lines (HCT-116, HRT-18, LoVo, DLD-2, HCT-116b, and CaCo-2) that had relatively high levels of amniopentidase in control groups had markedly decreased aminopeptidase following butyrate treatment. In 7 cell lines, sodium butyrate increased sucrase activity [LS174T (3.5-fold); HRT-18 (3-fold); HCT-116a (2.5-fold); and SW-403, LoVo, HCT-116, and SW1116 (1.5-fold)], while 7 other cell lines were insensitive to sodium butyrate treatment. Sodium butyrate also caused an increase in lactase and trehalase activities in several cell lines of 2- to 3-fold. It is noteworthy that the SW1116 cell line had the highest activities of most of the enzymes among all the cell lines examined, and the activities of these enzymes were further increased by treatment with butyrate.

When the correlation between induction by sodium butyrate of AlkPase activity and that of the 5 other enzyme activities was examined in the 14 cell lines, it was found that there was no statistically significant correlation between the induction of AlkPase and the other enzyme activities (Chart 2).

Cellular Levels of AlkPase Activity, Effects of Sodium Butyrate, and the Degree of Tumor Differentiation. AlkPase activity showed the most consistent response to sodium butyrate of any of the brush border membrane enzymes examined in the 14 colorectal cell lines. Table 2 compares the levels of activity before and after sodium butyrate treatment with the degree of differentiation of the tumor from which they were originally obtained. As can be seen, the highest levels of AlkPase were found in cell lines derived from tumors originally classified as moderate to well differentiated. Even though sodium butyrate increased AlkPase activity in 10 of the 14 cell lines, those from well-differentiated tumors also tended to have the greatest fold increase in activity. On the other hand, cell lines derived from poorly differentiated tumors in many cases not only had lower levels of AlkPase but did not respond as readily to sodium butyrate treatment.

The Effect of Cell Growth on Enzyme Activity. Fourteen cell lines were examined every other day up to 10 days for the effect of cell growth on enzyme activity (Chart 3). Cells were plated so that they reached confluency on Day 8. Aminopeptidase activity at the eighth and tenth day was increased 3-fold compared to that of the second day, while DAP-IV activity at the eighth and tenth day was increased 3.3-fold and 4-fold, respectively (Chart 3). This occurred in 10 and 11 of the 14 cell lines for aminopeptidase and DAP-IV, respectively. When averaged together, cell growth had no apparent effect on the activities of AlkPase, sucrase, lactase, and trehalase (Chart 3), although some increases were noted in a few individual cell lines (not shown).

Subcellular Distribution of Enzyme Activities. The subcellular distribution of enzyme activities in the control and sodium butyrate-treated groups was studied in 4 selected cell lines (HRT-18, CaCo-2, LS174T, and SW1116). These were chosen from the 14 cell lines studied due to the high induction of AlkPase activity by sodium butyrate and moderate induction of most of the other 5 enzyme activities. Since aminopeptidase activity was not increased by sodium butyrate in most of the cell lines, it was not studied. AlkPase activity was predominantly associated with the membrane fraction (74 to 77%) in control groups, and growth of cells in sodium butyrate-containing medium caused a slight increase in the percentage of activity associated with the membrane fraction (89 to 97%) (Chart 4f). Sodium butyrate did not alter the cellular distribution of DAP-IV activity, and in all 4 cell lines, 60 to 70% of the total DAP-IV activity was associated with the membrane fraction (Chart 4f). As shown in Chart 4, III to V, butyrate treatment caused considerable changes in the subcellular distribution profiles of the disaccharidases. Following treat-

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**Table 1**

<table>
<thead>
<tr>
<th>Membrane-associated enzyme activities in 14 cell lines</th>
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</thead>
<tbody>
<tr>
<td>AlkPase</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>Control group</td>
</tr>
<tr>
<td>Butyrate group</td>
</tr>
</tbody>
</table>

*Mean ± SE of 14 cell lines.*

*P < 0.05 as compared with control.

*Not statistically significant.*
MEMBRANE HYDROLASES AND COLORECTAL CANCER CELL LINES

Chart 1. The effect of sodium butyrate (2 mM) on the activities of membrane-associated enzymes. Cells were harvested at the eighth day. The enzyme activities represent the mean of duplicate measurements, which varied by less than 10%. Numbers in parentheses, enzyme activities higher than those indicated on the ordinate. C, control group; B, sodium butyrate group; 1, DLD-2; 2, CaCo-2; 3, HRT-18; 4, HCT-48; 5, LS174T; 6, HCT-116; 7, HCT-116b; 8, HCT-116; 9, SW1116; 10, SW-480; 11, SW-403; 12, LoVo; 13, HCT-15; 14, HT-29. AP, aminopeptidase.

ment with sodium butyrate, most of the sucrase activity was associated with the cytoplasmic fraction, whereas in control groups, it was in the membrane fraction. This was true even for the CaCo-2 cell line which had decreased total cellular sucrase activity following butyrate treatment. The same trend towards an increase in cytoplasmic lactase activity was observed in the 4 cell lines (Chart 4V). For trehalase activity, this phenomenon was observed only in the CaCo-2 and LS174T cell lines (Chart 4V).

DISCUSSION

Brush border membrane-associated hydrolases are integral membrane proteins (4) normally found in small intestinal mucosal
MEMBRANE HYDROLASES AND COLORECTAL CANCER CELL LINES

Table 2

<table>
<thead>
<tr>
<th>Degree of differentiation</th>
<th>Alkpase (units/mg protein)</th>
<th>Control</th>
<th>Butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original tumor</td>
<td>Cell Line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>DLD-2</td>
<td>3.5</td>
<td>6.9</td>
</tr>
<tr>
<td>M-W</td>
<td>CaCo-2</td>
<td>116.2</td>
<td>1106.9</td>
</tr>
<tr>
<td>M</td>
<td>HRT-18</td>
<td>6.9</td>
<td>106.4</td>
</tr>
<tr>
<td>P</td>
<td>HCT-48</td>
<td>12.9</td>
<td>39.7</td>
</tr>
<tr>
<td>W</td>
<td>LS174T</td>
<td>21.6</td>
<td>1075.5</td>
</tr>
<tr>
<td>P</td>
<td>HCT-116a</td>
<td>10.3</td>
<td>72.3</td>
</tr>
<tr>
<td>P-M</td>
<td>HCT-116b</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>P</td>
<td>HCT-116</td>
<td>3.1</td>
<td>4.0</td>
</tr>
<tr>
<td>W</td>
<td>SW1116</td>
<td>21.7</td>
<td>1547.5</td>
</tr>
<tr>
<td>W</td>
<td>SW-400</td>
<td>4.0</td>
<td>30.9</td>
</tr>
<tr>
<td>P-M</td>
<td>SW-403</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>P-M</td>
<td>LoVo</td>
<td>3.2</td>
<td>390.9</td>
</tr>
<tr>
<td>M</td>
<td>HCT-15</td>
<td>14.9</td>
<td>7.2</td>
</tr>
<tr>
<td>M</td>
<td>HT-29</td>
<td>6.3</td>
<td>78.9</td>
</tr>
</tbody>
</table>

* Ref. 47.

W, well; M, moderate; P, poor.
—, did not grow.

While the characterization and purification of these enzymes have been investigated in detail (31–35), comparable studies have not been carried out in the colon. However, several membrane-associated hydrolases have been reported to be present in various human colonic cancer cell lines (6, 7, 10, 11, 36–38). Among the most extensively studied of these membrane-associated hydrolases in tumor cells is Alkpase (6, 8, 10, 11, 36, 39). Other enzymes such as aminopeptidase, sucrase, γ-glutamyl transpeptidase, and K+-stimulated phosphatase have also been reported to be in some human colorectal cancer cell lines (7, 9, 36). However, the number of colon cancer cell lines which have been studied is limited, and the conditions and the methods used to assay enzyme activity have varied among the different laboratories. Therefore, a direct comparison between the different cell lines studied is not possible. In this paper, we have examined brush border membrane-associated hydrolytic enzyme activities in 14 different human colorectal cancer cell lines maintained in tissue culture. Our data indicate that all 14 cell lines examined expressed the 6 brush border membrane enzyme activities. However, the specific enzyme activity varied between different cell lines and different enzymes. In general, the activity of Alkpase, aminopeptidase, and DAP-IV was much higher than the disaccharidases. Among the disaccharidases, lactase was the highest activity present in the various cell lines. The SW1116 cell line had the highest activity of all the enzymes examined except for aminopeptidase. High sucrase activity has previously been reported in cultured cells and xenograft tumors from CaCo-2 (36, 38), and this cell line had the highest sucrase and lactase activity among the 14 cell lines in our study. These 2 human colon cancer cell lines (SW1116 and CaCo-2) are derived from well-differentiated tumors (22, 23), and these data suggest that there may be a correlation between the degree of differentiation and the levels of sucrase and lactase activity in these tumor cells. A similar type of correlation between enzyme levels and the extent of cellular differentiation has been studied with Alkpase in various cell lines (40–43).

Sodium butyrate is a natural metabolic product of the colon.
MEMBRANE HYDROLASES AND COLORECTAL CANCER CELL LINES

Chart 3. The effect of cell growth on enzyme activities. Cells were harvested every other day up to 10 days. AP, aminopeptidase. Points, mean of 14 cell lines; bars, SE. *, P < 0.001; **, P < 0.05 as compared with second day.

Chart 4. Subcellular distribution of enzyme activity and the effect of sodium butyrate in 4 cell lines. Cells were harvested on the eighth day. C, control group; B, sodium butyrate group. •, activity in membrane fraction; †, activity in cytosol fraction. Columns, mean of 4 dishes, which varied by less than 10%. I, Alkpase; II, DAP-IV; III, sucrase; IV, lactase; V, trehalase.
flora (44), which has been demonstrated to have a number of biological and biochemical effects on mammalian cells that mimic cell differentiation (10, 45). In addition, the induction of several enzyme activities by sodium butyrate has been reported (6, 7, 9, 11, 45). In this study, the activity of Alkpase was increased in 10 of 14 cell lines (2- to 123-fold) by treatment with sodium butyrate. However, DAP-IV and disaccharidase activities were increased only 1.5- to 3.5-fold in several cell lines, and there was no correlation between the increase of Alkpase activity and other enzymes by sodium butyrate. In half of the cell lines examined, butyrate caused a decrease in aminopeptidase activity. Thus, these data show that the effect of butyrate on many of the brush border membrane hydrolases varied considerably among the different cell lines.

Neoplasia is thought to be closely associated with cellular differentiation, which is a multistep process that has been broadly divided into early and late stages. Currently, however, there are very few stage-specific markers for colonic mucosal cells, although levels of carcinoembryonic antigen and Alkpase activity have been advanced as putative markers in some cell lines (46). The consistent pattern of induction of Alkpase activity in the majority of the colon cancer cell lines examined in this study indicates that this enzyme may indeed be of importance as a marker in monitoring the effect of differentiating agents. Although additional studies are required, our results also suggest that differentiating agents such as sodium butyrate may have their greatest effect on colorectal cancer cell lines derived from tumors that are in the “later” stages of differentiation. Those cell lines in the earlier stages of differentiation may be less susceptible to phenotypic alteration of Alkpase activity by sodium butyrate.

Although the precise biochemical effect of sodium butyrate on mammalian cells is not known, it has been shown to acetylate nuclear histones leading to an increase in DNA transcription (47-49). The butyrate-induced increase in Alkpase activity and carcinoembryonic antigen (8, 9) is probably due to an increase in transcription, since this induction process has been shown to require new RNA and protein synthesis (50, 51). Therefore, the butyrate-induced changes in brush border membrane hydrolase activity observed in this study may be due to similar alterations in gene expression, although the particular phenotype of expression is very different among the various cell lines.

In 10 of the 14 cell lines studied, cell growth had a dramatic effect on the levels of aminopeptidase and DAP-IV, so that after 10 days, they were increased 3- to 4-fold. In contrast, the other 4 enzyme activities did not respond in this manner except for one. In the CaCo-2 cell line, sucrase activity was increased 5-fold by the tenth day when compared to the fourth. Similarly, Nitowsky et al. (52) and Brahmacupta (39) have reported that Alkpase activity in Chang liver cells and HeLa cells could be correlated with cell growth. It is well known that the activities of small intestinal brush border membrane enzymes vary along the villus-crypt cell axis and that increases in the levels of these enzymes correspond to functional differentiation and cell growth. Therefore, it is likely that cell growth may affect cell differentiation and brush border enzyme levels in colon cancer cells maintained in tissue culture.

The brush border membrane hydrolases of the small intestine are associated with intracellular membranes during their biosynthesis, although soluble cytoplasmic precursors of some enzymes have been described by investigators (53, 54). Since comparable data are not available for the colon, the subcellular distribution of enzyme activity in 4 cell lines (HRT-18, CaCo-2, LS174T, and SW1116) was examined in the present study. These data show that Alkpase and DAP-IV activity was primarily associated with the membrane fraction which is similar to the situation in the small intestine. Furthermore, sodium butyrate did not alter the subcellular distribution of these 2 enzymes. In contrast, there was a significant change in the subcellular distribution profile of the disaccharidases. Following butyrate treatment, there was a general increase in the amount of enzyme associated with the cytoplasmic fraction, so that in some instances, the soluble enzyme constituted the majority of the total cellular disaccharidase activity.

At the present time, the nature of the soluble enzyme components is not known; however, other investigators have described soluble forms of Alkpase (55) and maltase (56) in the small intestinal enterocytes of suckling rat intestine. This component is apparently an isozyme of the membrane-bound enzyme with the ratio of the soluble and membrane-bound forms changing during intestinal development in the rat (56, 57). Thus, it is possible that sodium butyrate, a known differentiating agent, induces changes in the isozyme patterns of brush border membrane hydrolases in cultured colon cancer cells. This phenomenon may provide important information regarding the mechanisms of differentiation and tumorigenesis in colon cancer cells and merits further investigation. Studies in this regard are currently under way in our laboratory.

REFERENCES


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