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ORIGINAL CONTRIBUTION

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Mediation of differentiating effects of butyrate on the intestinal cell line Caco-2 by transforming growth factor-β1

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Summary *Background:* Beside their role as the main energy source in the colonic mucosa, short chain fatty acids were found to act as potent antiproliferative and differentiation agents in various cancer cell lines. It has recently been shown that butyrate also induces TGF-β1 mRNA in human keratinocytes, suggesting that TGF-β1 may play a role in butyrate induced cell differentiation.

Aim of the study: The objective of our study was to investigate the possible role of exogenous and endogenous TGF-β on butyrate induced differentiation of intestinal epithelium.

Methods: Studies were performed in Caco-2 cells, a cell line resembling functionally normal enterocytes. Cells, cultured in standard medium were studied over a 15-day period. Sodium butyrate (5 mM), TGF-β1 (2 ng/ml) or butyrate (5 mM) + anti-human TGF-β1 antibody (30 μg/ml) were added to the medium. At day 4, 8, 11 and 15 total protein content, alkaline phosphatase activity, lactate dehydrogenase activity and transepithelial resistance were measured.

Results: Under culture conditions both, butyrate and TGF-β1 inhibited growth accompanied by an induction of cell differentiation approved by increased alkaline phosphatase activitity and transepithelial resistance. The differentiating effect of butyrate was accompanied by an increased endogenous TGF-β1, but not TGF-β2 expression in Caco-2 cells. Co-incubation of butyrate with anti-human TGF-β1 antibody suppressed at least in part the differentiating effects of butyrate.

Conclusions: Our results directly implicate that the TGF-β isoform TGF-β1 is necessary for butyrate-induced Caco-2 cell differentiation, but other molecular mechanisms may also play a role in the differentiation of this cell line.

Key words Short chain fatty acids – transforming growth factor – colonic cancer

Abbreviations used in this paper
DMEM = Dulbecco's modified Eagle's medium; *FCS* = fetal calf serum; *SCFA* = short chain fatty acids; *TGF-β1/2* = transforming

Introduction

Colorectal cancer is one of the most common malignancies in Western Europe, and this high incidence is thought to be due largely to dietary factors. Burkitt proposed that a low-fiber diet leads to increased risk of colorectal cancer (4) and recent epidemiological studies have provided strong support for this association (11).

Short chain fatty acids (SCFA), mainly acetate, propionate, and butyrate, are the main end-products of anaerobic bacterial fermentation of carbohydrates in the human colon (6). The intraluminal concentration of SCFA has been estimated to be between 100 and 240 mM, most of which is butyrate (14). This four carbon compound has well-described potent effects on growth and differentiation of a wide variety of cell types (1, 10, 12, 16, 17). Several studies have examined the effects of butyrate on gastrointestinal epithelium. For example, in vivo experiments have suggested a role of butyrate in the maintenance of a normal colonic epithelium and have emphasized the potential for butyrate in the treatment of colonic disease (10, 16). In vitro studies have indicated that butyrate modulates cell growth and differentiation of colon adenocarcinoma cells in culture (1, 12, 17). The cellular and molecular mechanisms by which butyrate may affect cellular differentiation are not completely clear, particularly in gastrointestinal cells, where it may be of unique physiological relevance.

TGF-β1 acts as a negative regulator of growth for many epithelial cells, while their transformed derivatives are often resistant to its growth inhibitory effects (15). In addition, TGF-β1 induces a range of differentiation-like responses in colorectal carcinoma cells (5). It could be demonstrated that epithelial cells in the non-proliferating upper parts of the human colonic crypt show enhanced levels of TGF-β1 as compared with cells in the proliferative compartment (2). TGF-β1 may, therefore, be an important regulator of growth for colorectal epithelium.

Recent data in normal human keratinocytes demonstrated an important role of TGF-β1 in the butyrate induced differentiation of keratinocytes (18). Since butyrate and TGF-β1 have been strongly implicated in the control of colonic epithelium, yet possible interactions between these two growth regulating factors in intestinal cells have not been studied, we designed a study to elucidate the role of exogenous and endogenous TGF-β1 on differentiation of intestinal epithelial cells in response to butyrate. As a model we chose the colon tumor cell line Caco-2, which displays enterocytic differentiation in vitro, the differentiation process starting directly after confluence.

Materials and methods

Material

Dulbecco's modified Eagle's medium (DMEM) and FCS were obtained by Gibco (Karlsruhe, Germany). All other reagents were purchased from Sigma (Deisenhofen, Germany) and were of highest purity grade.

Cell culture

Caco-2 cells of passages 30-35 were kept in Dulbecco's modified eagle medium (DMEM), supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin under 5% CO_2 and 95% air. Cells were passaged using Dulbecco's PBS containing 0.25% trypsin and 1% EDTA and seeded on Millipore filters (0.45 µm, 30 mm in diameter) at a density of $4 \times 10^5/cm^2$. The medium was changed every second day.

Transepithelial resistance

Transepithelial resistance of Caco-2 cells was monitored with an EVOM™ volt-ohmmeter, which measures resistance by passing alternate current across the monolayer, using an ENDOHM™ 2.4 measurement chamber at day 4, 8, 11, and 15.

Growth rate

Growth rate was estimated by measuring the total protein content per dish. This parameter linearly correlates with the number of cells. Total protein content per dish was determined at the indicated time over a 15-day period of culture, using the method of Bradford (3). Bovine albumine was used as the standard.

Assay of enzymes

All enzymes were assayed at 37 °C. Alkaline phosphatase was measured according to Haase et al. (9) and lactate dehydrogenase was measured as recommended by the German Society of Clinical Chemistry (8).

Assay of TGF-β1 and TGF-β2

Antibody titers of TGF-β1 and TGF-β2 were estimated by standard enzyme-linked immunosorbent assay (ELISA) technique.

Calculations and statistics

All studies were carried out in triplicate from $n = 5 - 6$ separate membrane preparations. The values are expressed as means ± SE. Statistical analysis was done by the nonparametric U-Test (Mann-Whitney-Wilcoxon) for unpaired data. Differences were considered significant at $p < 0.05$.

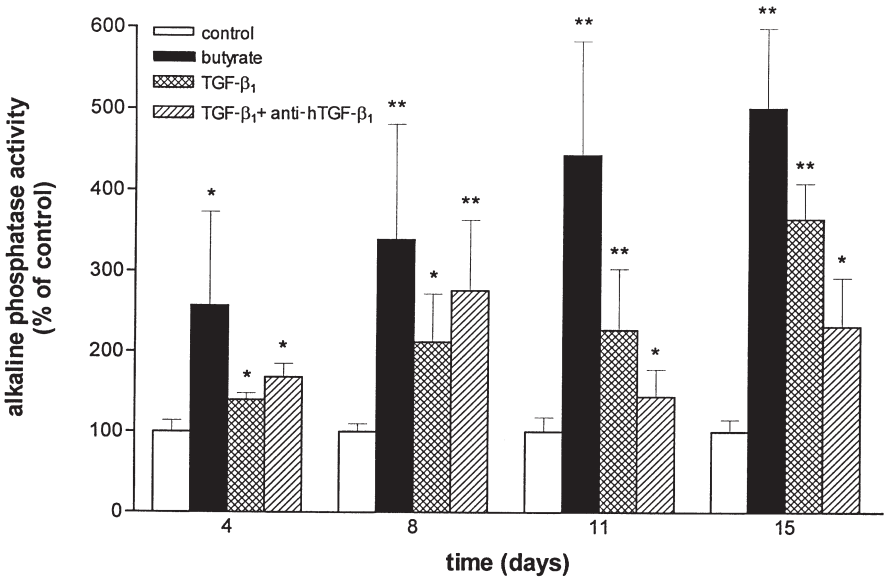

Fig. 1 Effects of butyrate (5 mM), TGF-β_1 (2 ng/ml), and butyrate (5 mM) + anti-hTGF-β1 (30 µg/ml) on alkaline phosphatase activity (% of control) in Caco-2 cells until day 15 post-confluence. (*:p<0.05; **:p<0.001, vs. control).

Results

Markers of cell differentiation

In order to test whether the antiproliferative effect of butyrate and TGF-β1 is correlated with an induction of cellular differentiation, the activity of alkaline phosphatase activity was measured. Fig. 1 shows the evolution of alkaline phosphatase activity during a 15-day period of culture treated with 1% FCS alone, or in combination with 5 mM butyrate, 2 ng/ml TGF-β1 or 5 mM butyrate + 30 µg/ml anti-human TGF-β1 antibody. Addition of butyrate to Caco-2 cells led to an increased alkaline phosphatase activity after 4 days followed by a sustained increase in alkaline phosphatase activity for the consecutive next 11 d. TGF-β1 also induced an increase in alkaline phosphatase activity during the whole period of incubation, although to a somewhat lower extent as compared with butyrate. When Caco-2 cells were co-incubated with butyrate and TGF-β1-antibody alkaline phosphatase activity was significantly reduced from day 11 to 15 of incubation when compared with butyrate and TGF-β1.

The influence of 5 mM butyrate on transepithelial electrical resistance (TEER) was examined to evaluate changes in the epithelial barrier integrity of the Caco-2 monolayer under conditions identical to those used for alkaline phosphatase activity (Fig. 2). Compared with the control group butyrate enhanced transmonolayer resistance significantly during the whole time period studied, indicating a strengthening of tight junctions between adjacent epithelial cells. In contrast, significant effects on TEER under treatment of Caco-2 cells with TGF-β1

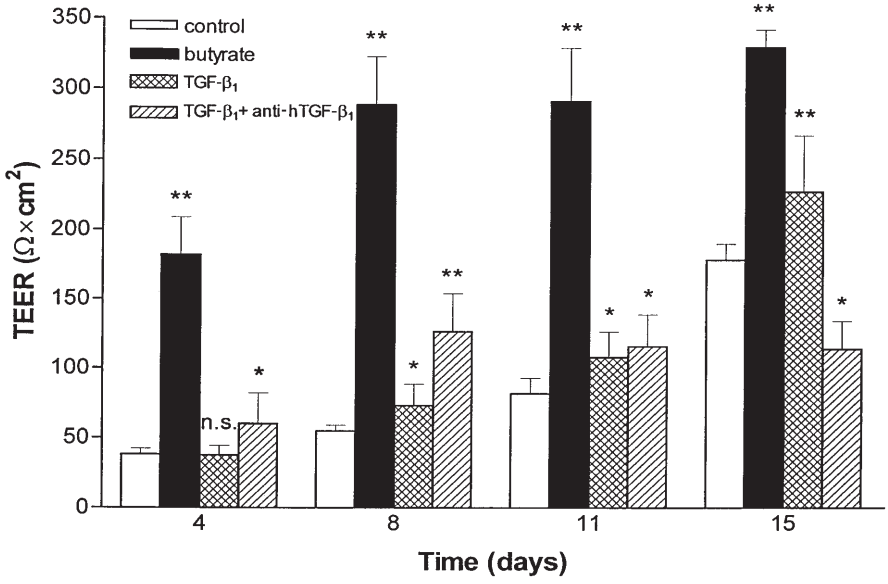

Fig. 2 Effects of butyrate (5 mM), TGF-β_1 (2 ng/ml), and butyrate (5 mM) + anti-hTGF-β1 (30 µg/ml) on transepithelial electrical resistance (TEER) (Ωxcm²) in Caco-2 cells until day 15 post-confluence. (*:p<0.05; **:p<0.001, vs. control).

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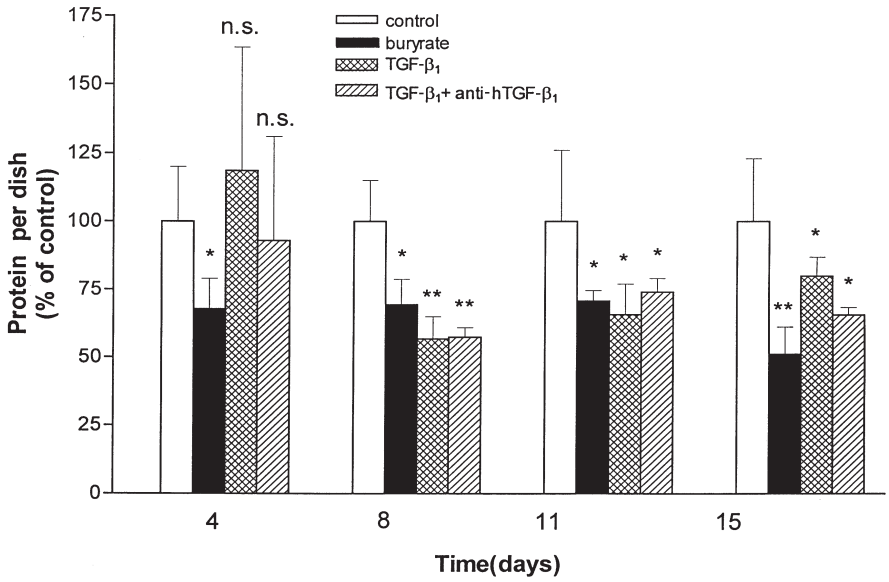

Fig. 3 Effects of butyrate (5 mM), TGF-β_1 (2 ng/ml), and butyrate (5 mM) + anti-hTGF-β1 (30 µg/ml) on protein content (% of control) in Caco-2 cells until day 15 post-confluence. (*:p<0.05; **:p<0.001, vs. control).

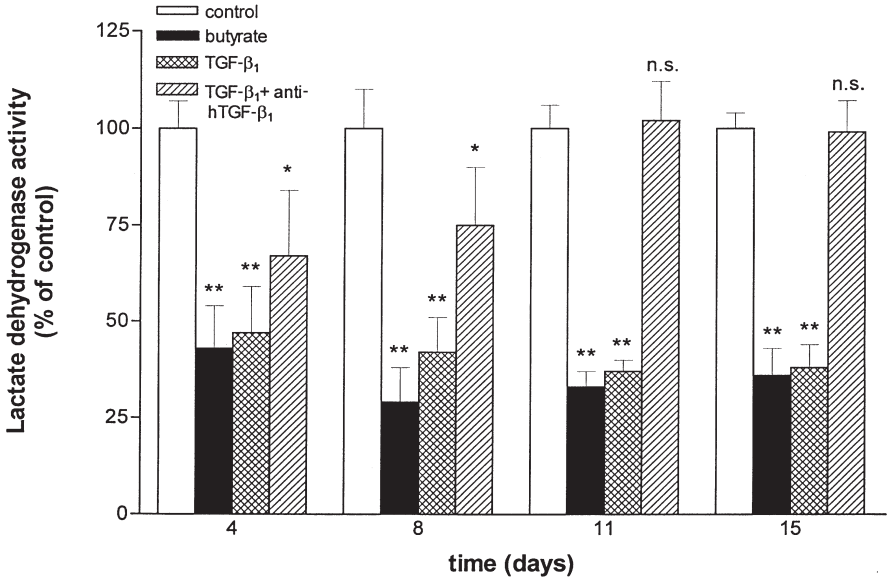

Fig. 4 Effects of butyrate (5 mM), TGF-β_1 (2 ng/ml), and butyrate (5 mM) + anti-hTGF-β1 (30 µg/ml) on lactase dehydrogenase activity (% of control) in Caco-2 cells until day 15 post-confluence. (*:p<0.05; **:p<0.001, vs. control).

could only be observed on day 15. Butyrate-induced increase of TEER was nearly totally abolished for the whole period of incubation, when the cells were co-incubated with anti-human antibody of TGF-β1.

Markers of cell proliferation

Cell proliferation was estimated by measuring total protein content per dish over a 15-day period of culture in the absence and presence of butyrate, TGF-β1 or butyrate + anti-human antibody of TGF-β1. The results of this long-term experiment (Fig. 3) confirm unambiguously the antiproliferative effect of butyrate (whole period studied) and TGF-β1 (after 4 days of incubation). Both agents led to a decrease in protein expression of 30-50% when compared with the control. Treatment of Caco-2 cells with butyrate and TGF-β1 antibody showed similar results as incubation with TGF-β1 alone, also demonstrating an antiproliferative effect at day 8, 11, and 15.

As shown in Fig. 4, the antiproliferative effect of butyrate and TGF-β1 is not due to any toxicity towards the cells, since lactate dehydrogenase activities were reduced in treated cells compared to control cells during the whole course of the experiment. Addition of 30 µg/ml anti-human TGF-β1 to butyrate treated Caco-2 cells completely neutralized the effect provoked by butyrate at day 11 and 15; at day 4 and 8 lactate dehydrogenase expression was slightly higher than in butyrate or TGF-β1 treated cells, but still significantly lower than in control cells.

Table 1A Effect of butyrate (5 mM), TGF-β1 (2 ng/ml) and butyrate (5 mM) + anti-human TGF-β1 antibody (30 μg/mL) on endogenous expression of TGF-β1 (in ng/ml) by Caco-2 cells

Day	Control	Butyrate	TGF-β1	butyrate + anti TGF-β1
4	1.18±0.33	2.17±0.25	1.21±0.09	0.63±0.21
8	2.51±0.47	2.90±0.79	1.71±0.26	1.23±0.18
11	2.37±0.19	4.18±0.74	1.91±0.13	1.99±0.15
15	4.49±0.74	6.13±1.44	2.93±0.73	2.97±0.56

Table 1B Effect of butyrate (5 mM), TGF-β1 (2 ng/ml) and butyrate (5 mM) + anti-human TGF-β1antibody (30 μg/mL) on endogenous expression of TGF-β2 (in ng/ml) by Caco-2 cells

Day	Control	Butyrate	TGF-β1	butyrate + anti TGF-β1
4	0.05±0.01	0.06±0.01	0.08±0.01	0.08±0.01
8	0.06±0.01	0.09±0.03	0.06±0.01	0.08±0.04
11	0.17±0.01	0.16±0.01	0.16±0.01	0.17±0.00
15	0.17±0.02	0.17±0.00	0.17±0.01	0.16±0.00

Effect of butyrate on TGF-β1 and TGF-β2 expression in Caco-2 monolayers

Further experiments were designed to test whether inhibition of cell growth and induction of cell differentiation by butyrate is correlated with TGF-β isoform(s) expression. For this purpose, Caco-2 cells were stimulated either with 5 mM butyrate or 1% FCS (control) and the expression of TGF-β1 and TGF-β2 in Caco-2 cells determined. As Table 1A demonstrates, 5 mM butyrate induced a TGF-β1 expression linear to the time of treatment. In contrast, addition of TGF-β1 or butyrate + antibody to TGF-β1 led to a reduced expression of endogenous TGF-β1.

When Caco-2 cells were examined for TGF-β2 expression only marginal endogenous expression of this isoform could be measured either in the control cells or in the treated cells (Table 1B).

Discussion

The protective effect of dietary fibers against colorectal cancer is widely accepted. Nevertheless, the underlying molecular mechanism by which such dietary compounds prevent carcinogenesis of the colon is yet poorly understood.

Among the fermentation products of the colonic microflora, butyrate appears to be of special interest in colorectal cancer, since patients suffering from adenomatous polyps or colon cancer have a significantly higher incidence of low butyrate fermentation (19). In support of this, a number of studies have demonstrated this SCFA to have a number of biological and biochemical effects on mammalian cells that mimic cell differentiation (1, 10, 12, 16, 17). Nevertheless, cellular and molecular mechanisms by which butyrate may affect cellular differentiation are not yet established.

TGF-β regulates a wide range of biological activities including cell growth, differentiation, gene expression, wound healing, and tissue morphogenesis. In addition, alteration of normal TGF-β function has been causally associated with the pathogenesis of several diseases, including cancer. Recent studies suggested that endogenous TGF-β may play an important role in promoting repair after epithelial injury (8). In the present study, we investigated the effects of butyrate on differentiation of the colonic adenocarcinoma cell line Caco-2 and explored its influence on TGF-β1 and TGF-β2 expression of this human colon adenocarcinoma cell line.

Our results clearly show that butyrate can exert an antiproliferative effect. Addition of butyrate in a concentration of 5 mM resulted in about 40–60% inhibition of protein concentration indicating that the observed effect may be of physiologic relevance.

By estimating lactate dehydrogenase activities over a 15-day period of culture in the presence of butyrate, we evaluated a possible toxicity as a cause for its antiproliferative effect. Lactate dehydrogenase activities were reduced up to 75% compared to control cells indicating that the antiproliferative effect is not due any toxicity toward the Caco-2 cells.

The inhibition of growth observed in butyrate-treated cells is associated with an induction of the alkaline phosphatase activity. In the (small) intestine, this enzyme is localized in the brush-border membrane of epithelial cells and its activity is considerably higher in differentiated cells from the villus than in proliferative cells from the crypt. Malignant transformation of a variety of cells provokes a decrease in the activity of alkaline phosphatase. The activity of the enzyme is low during the exponential phase of growth but can be enhanced by several differentiating agents. Therefore, the increase in activity in butyrate-treated cells compared with untreated cells may reflect a more differentiated phenotype. The observed increase in transepithelial resistance after confluence indicates an extended expression of the intercellular tight-junctions also consistent with a differentiating effect of butyrate.

It has been hypothesized that TGF-β is a physiological regulator of normal intestinal epithelial growth and that loss of such regulation plays a role in malignant transformation of colonic epithelium. The TGF-β family consists of at least five genes encoding distinct proteins in vertebrates referred to as TGF-β1-5. Most of the available evidence concerning the biological properties and function of the TGF-βs has been obtained from studies of TGF-β1, which is the protein initially isolated and characterized by Roberts et al. (13). In our experiments, comparable results with regard to cell differentiation and proliferation over time were obtained when Caco-2 cells were treated with TGF-β1 in a concentration of 2 ng/ml. It could also be demonstrated that the differentiating effects of butyrate were associated with increased levels of TGF-β1 in the Caco-2 cell line, whereas TGF-β2 was not expressed by the Caco-2 cells either with or without butyrate treatment. Moreover, neutralization of TGF-β1 activity with anti-human TGF-β1 antibody significantly inhibited butyrate-induced cell differentiation except for protein concentration. Thus, it is tempting to speculate that TGF-β1 plays an important role in the normal cascade of events which culminate in the butyrate-induced differentiation of Caco-2 cells. TGF-β2, another isoform of the TGF-β family, which is often present in mammalian cells, is not expressed by butyrate treated Caco-2 cells and, therefore, cannot be responsible for the induction of differentiation and growth inhibition in this cell line.

In conclusion, our results directly implicate that the TGF-β isoform TGF-β1 is necessary for butyrate-induced Caco-2 cell differentiation, but other molecular mechanisms may also play a role in the differentiation of this cell line. Whether the modulating influence of TGF-β1 is restricted to our model, the Caco-2 cell line, or can be transferred to other intestinal in vitro models or even in vivo needs further studies.

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