Biochemical and Biophysical Research Communications **273,** 45–49 (2000)
doi:10.1006/bbrc.2000.2899, available online at http://www.idealibrary.com on IDEAL®

Bile Acids Reduce the Apoptosis-Inducing Effects of Sodium Butyrate on Human Colon Adenoma (AA/C1) Cells: Implications for Colon Carcinogenesis

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Received May 17, 2000

Butyrate is produced in the colon by fermentation of dietary fibre and induces apoptosis in colon adenoma and cancer cell lines, which may contribute to the protective effect of a high fibre diet against colorectal cancer (CRC). However, butyrate is present in the colon together with unconjugated bile acids, which are tumour promoters in the colon. We show here that bile acids deoxycholate (DCA) and chenodeoxycholate (CDCA), at levels present in the colon, gave a modest increase in cell proliferation and decreased spontaneous apoptosis in AA/C1 adenoma cells. Bile acids significantly inhibited the induction of apoptosis by butyrate in AA/C1 cells. However, the survival-inducing effects of bile acids on AA/C1 cells could be overcome by increasing the concentration of sodium butyrate. These results suggest that dysregulation of apoptosis in colonic epithelial cells by dietary factors is a key factor in the pathophysiology of CRC.

In colorectal cancer (CRC) genetic (1) and environmental factors contribute to the malignant transformation of colorectal epithelial cells. Epidemiological data implicate diet as the major environmental factor in colorectal carcinogenesis (2). A diet high in saturated fats and low in dietary fibre is associated with an increased incidence of colorectal cancer (3). The detrimental effect of a diet high in saturated fats has been attributed to increased levels of secondary bile acids in the colon (4). Bile acids are normal constituents of the colon and function as trophic factors for the gut epithelium. Secondary bile acids, deoxycholic acid (DCA), ursodeoxycholic acid (UDCA) and lithocholic acid, are produced in the colon by the actions of intestinal bacteria on primary bile acids. However, unconjugated bile acids, including chenodeoxycholic acid (CDCA) and DCA, have also been shown to be tumour promoting in animal studies (5, 6) and raised levels of secondary bile acids have been reported in patients with adenomatous polyps and colon cancer (7, 8). To date their mechanism of action is poorly understood. In contrast, a high intake of dietary fibre is associated with reduced risk of CRC (9) and this has been attributed to the fermentation of fibre in the gut to short chain fatty acids, including butyrate. Butyrate has been shown to increase apoptosis in both colon adenoma and cancer cell lines in a p53 independent way (10), an effect that is likely to contribute significantly to its protective effects.

The majority of studies in the literature concerning the effects of butyrate and bile acids on colonic epithelial cell proliferation and apoptosis, have considered these dietary factors separately. *In vivo* both are present in the colon and may influence each others actions directly or indirectly. For example, butyrate is known to lower colonic pH, inhibiting the bacterial transformation of primary to secondary bile acids (11). We reasoned that the ability of these two dietary factors to modulate each others function *in vivo* may extend further and include the regulation of apoptosis. Bile acids have been shown to activate the PKC signalling pathway (12), which is involved in regulating cell proliferation, differentiation and apoptosis (13). Moreover, pharmacological tumour promoters such as phorbol esters are potent inhibitors of apoptosis in many cell types (14). We have therefore investigated the effects of primary and secondary bile acids, CDCA and DCA respectively, on butyrate-induced apoptosis in adenoma (AA/C1) and cancer (HT29) cell lines. Bile acids inhibited spontaneous and butyrate induced apoptosis in AA/C1 adenoma cells, though butyrate could overcome this inhibition at higher concentrations.

MATERIALS AND METHODS

Cell culture. The colorectal carcinoma cell line HT29 (25) and an adenoma cell line AA/C1 (26) were cultured as described previously (27). Prior to treatment with bile acids or butyrate, cells were trypsinised and seeded at 3×10^5 cells per well in 6-well tissue culture plates in DMEM (Gibco-BRL), containing 20% FCS (Sera Laboratories), 2 mM glutamine, 0.2 U/ml insulin, 1 μg/ml hydrocor-

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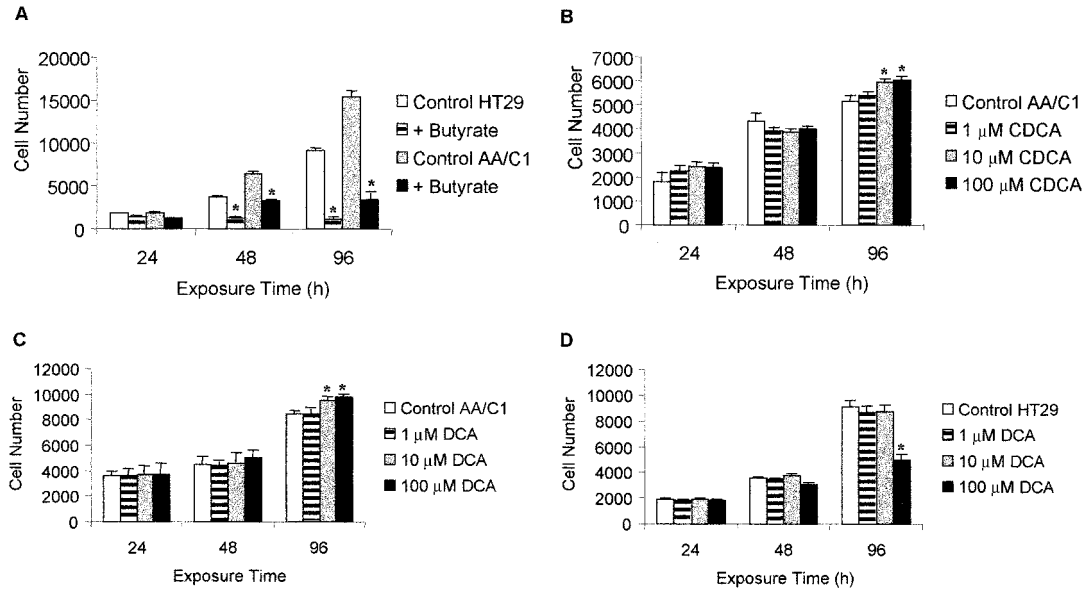

FIG. 1. Effect of sodium butyrate on HT29 and AA/C1 cell proliferation. HT29 or AA/C1 cells were incubated with (A) 4 mM sodium butyrate or (D) 1–100 μM DCA for up to 96 h. AA/C1 cells were also incubated with (B) 1–100 μM sodium chenodeoxycholate (CDCA) or (C) sodium deoxycholate (DCA) for up to 96 h. In each case cell proliferation was measured by enumeration of attached cells. Data are means ± SD of three separate experiments. *$P < 0.05$.

tisone sodium succinate (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were allowed to attach and after 3 days the medium was replaced with medium containing sodium butyrate (0.4–40 mM) or the bile acids sodium DCA or sodium CDCA (1–100 μM, Sigma), concentrations that are within the physiological range. Bile acids were added to medium from a 10 mM stock in DMSO and solvent controls were used throughout.

Measurement of cell proliferation. Proliferation of cells was assessed by two methods, by determining the increase in number of attached cells to give the attached cell yield (10) and by measuring incorporation of [^{3}H]thymidine in to DNA (27). Measurements of proliferation were made between 24 and 96 h.

Measurement of apoptosis. In studies to determine the effect of bile acids and butyrate on cell survival, these agents were added separately or in combination to the culture medium and apoptosis was measured after 48 or 72 h. Apoptosis was measured by counting the number of Annexin V positive cells detached from the culture plate and expressing this figure as a percentage of the number of cells (10). As apoptotic cells can progress to secondary necrosis, the floating cells were harvested every 24 h and a cumulative total for the treatment period determined. Annexin V staining was determined using a commercial kit and FACS analysis (Boehringer-Mannheim). That Annexin V positive and detached cells were apoptotic was also confirmed by analysis of cell morphology and TUNEL staining (data not shown).

RESULTS

Effect of Sodium Butyrate and Bile Acids on Proliferation of AA/C1 and HT29 Cells

As shown previously (10), sodium butyrate (4 mM) decreased attached cell yield in adenoma (AA/C1) and colon cancer (HT29) cell lines (Fig. 1A). We also show that incorporation of [^{3}H]thymidine into DNA was reduced in HT29 and AA/C1 cells treated with sodium butyrate for 48 h. Moreover the effects of butyrate were concentration dependent (Table 1). 0.4 mM butyrate had no significant effect, whereas 4 and 40 mM caused dramatic reductions in [^{3}H]thymidine incorporation. The effects of butyrate were rapid, with reduced DNA synthesis and attached cell yields first detected by 24 h and reaching significant levels by 48 h.

In contrast, AA/C1 cells treated with the primary bile acid CDCA showed a small but significant increase in cell yield (Fig. 1B) and tritiated thymidine incorporation in to DNA (Table 1). Similar effects were seen with the secondary bile acid DCA (Fig. 1C). The effects of bile acids were concentration dependent and took slightly longer to develop, not reaching significance until 96 h of treatment. The proliferative effects of bile acids were restricted to the adenoma cells as DCA and CDCA had no effect on cell number (Fig. 1D, DCA only) or tritiated thymidine incorporation (Table 1) in HT29 cells at concentrations up to 10 μM. Bile acids at 100 μM induced a dramatic decrease in HT29 cell numbers and DNA synthesis (Fig. 1D and Table 1), which may reflect increased sensitivity of HT29 cells to the detergent properties of higher concentrations of bile acids.

Effect of Sodium Butyrate and Bile Acids on Apoptosis of AA/C1 and HT29 Cells

The basal level of apoptosis in cultures of AA/C1 and HT29 cells assessed after 48h was similar at 3.8 ± 0.3% and 2.1 ± 0.2%, respectively. 4 mM butyrate induced a significant increase in apoptosis in both AA/C1 adenoma cells (Fig. 2A) and HT29 colon cancer

TABLE 1

Effect of Bile Acids and Butyrate on DNA Synthesis in HT29 and AA/C1

	[^{3}H]Thymidine incorporation (10^3 dpm per well)										
	DCA				CDCA			Butyrate			
	Control	1 μM	10 μM	100 μM	1 μM	10 μM	100 μM	Control	0.4 mM	4 mM	40 mM
HT29	153.7 ± 10.1	158.9 ± 9.8	155.6 ± 11.5	21.5* ± 2.9	161.2 ± 8.4	156.1 ± 5.3	70.4* ± 8.0	118.5 ± 5.6	115.4 ± 4.9	19.7* ± 2.1	10.3* ± 1.9
AA/C1	25.03 ± 0.59	25.25 ± 0.51	29.53* ± 0.57	28.61* ± 0.45	26.04 ± 0.8	26.94* ± 0.51	28.60* ± 0.61	20.4 ± 1.8	16.8 ± 3.0	9.3* ± 0.8	1.6* ± 0.1

Note. AA/C1 and HT29 cells were incubated with 1–100 μM sodium chenodeoxycholate (CDCA), sodium deoxycholate (DCA), or 0.4–40 mM sodium butyrate. Cell proliferation was determined by measuring the incorporation of [^{3}H]thymidine in to DNA, as described under Materials and Methods, after 96 h of treatment with bile acids and 48 h for butyrate treatment. Data are means ± SD of three separate experiments.
* Denotes $P < 0.05$.

cells (Fig. 2A). The effect of butyrate on apoptosis was also concentration dependent (data not shown), confirming previous publications (10). As we have shown recently that attached cells can also enter apoptosis prior to detachment from tissue culture plastic (15), we assessed apoptosis in attached HT29 cells treated with 4 mM butyrate using Annexin V staining. The data showed that a significant number of untreated, attached cells, 15.2 ± 0.5%, were apoptotic and this was increased to 20.4 ± 0.6% ($n = 3$, $P < 0.05$) by butyrate treatment.

The bile acids DCA and CDCA, reduced the level of spontaneous apoptosis in AA/C1 cultures after 96 h of treatment (Fig. 2B), but had no effect on HT29 cell apoptosis (Fig. 2B). Taken together these data show that the effects of butyrate and bile acids on cell numbers are mediated via modulation of cell proliferation and apoptosis.

Effect of Sodium Butyrate and Bile Acids in Combination on Apoptosis in AA/C1 and HT29 Cells

We next investigated whether bile acids could modify the potent apoptosis inducing effects of butyrate. Addition of either 10 μM DCA or CDCA significantly reduced the level of apoptosis in AA/C1 cells treated with 4 mM butyrate for 48 h (Fig. 3). Interestingly if the level of butyrate was increased to 10 mM, bile acids were no longer effective in preventing apoptosis (Fig. 3), even if levels of bile acid (DCA) were increased to 100 μM, the upper end of the physiological range (Fig. 3). As expected, bile acids did not modify butyrate-induced apoptosis in HT29 cells (data not shown).

DISCUSSION

In the colon, the basic functional unit is the colonic crypt. Colonic crypt stem cells are located at the base of

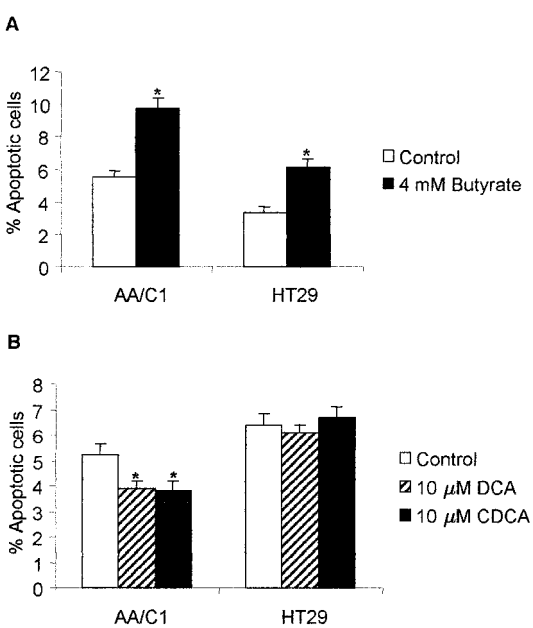

FIG. 2. Effect of butyrate and bile acids on apoptosis. AA/C1 colon adenoma cells or HT29 colon cancer cells were cultured in medium alone, or medium containing (A) 4 mM sodium butyrate for 48 h or (B) 10 μM DCA or 10 μM CDCA for 96 h. The number of Annexin V-positive apoptotic cells were enumerated and expressed as a fraction of the total cell population. Data are means ± SD of three separate experiments. *$P < 0.05$.

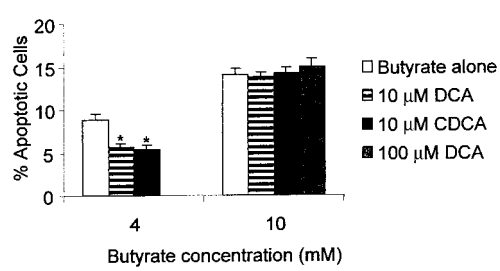

FIG. 3. Effect of bile acids and butyrate in combination on colon cell apoptosis. AA/C1 adenoma cells were cultured in medium for 48 h containing 4 or 10 mM sodium butyrate in the absence or presence of 10 μM DCA, 100 μM DCA or 10 μM CDCA. In each case the percentage of Annexin V positive apoptotic cells was determined and data are means ± SD of three separate experiments. *$P < 0.05$.

the crypt and as they differentiate they progress up the crypt, eventually dying by apoptosis and are sloughed off into the colon (16). Total crypt cell number and phenotype are strictly maintained by a balance between cell proliferation, differentiation and apoptosis. Dysregulation of this balance has significant consequences for colonic crypt homeostasis (17), leading to the development of adenomas and eventually cancer. The high incidence of CRC in Western society has been attributed to a diet that is low in dietary fibre and high in animal (saturated) fat. A high intake of saturated fat results in increased production of bile acids and in particular to raised levels of secondary bile acids, which are produced in the colon. Studies measuring faecal content of specific bile acids in normal volunteers and patients with adenomatous polyps and colorectal cancer, have shown that the ratio of secondary to primary bile acids was raised in patients with adenomas and cancer (8). Understanding their mode of action and particularly their interaction with short chain fatty acids, will improve understanding of the role of dietary factors in colon carcinogenesis. The higher concentrations of bile acid used here, i.e. 10 μM and 100 μM represent levels detected in patients with CRC (8) and the data reported here are therefore relevant to the *in vivo* situation.

Bile acids are not mutagens, but they do act as tumour promoters (18) and PKC has been identified as their molecular target (12, 19). As PKC is known to regulate a variety of cellular processes, including cell proliferation (13) and apoptosis (14), we would predict that bile acids would be able to regulate these processes. Our data show that bile acids, namely DCA and CDCA, can increase cell numbers in cultures of adenoma cells, by increasing proliferation and decreasing apoptosis. Whilst these effects were modest, the overall outcome would be significant *in vivo* in a tissue like the colon that turns over very rapidly. Interestingly, bile acids were ineffective on HT29 cancer cells, further suggesting that these dietary factors will play a crucial role in the progression of a tumour rather than its initiation. In fact, recent studies on biopsies of normal colon from patients with CRC and normal volunteers, showed that bile acids promoted apoptosis in normal epithelium from healthy volunteers. In contrast, histologically normal tissue from CRC patients was resistant to the effects of bile acids, suggesting that altered responsiveness to bile acids may occur very early in the carcinogenic process, possibly resulting from one of the many genetic mutations associated with tumour initiation (1).

We show here for the first time that bile acids are able to inhibit the apoptosis-inducing effects of the short chain fatty acid, butyrate. However, if levels of butyrate were increased, but remaining within the physiological range, the effects of bile acids were overcome. Thus butyrate was able to block the survival effects of bile acids on colon adenoma cells at concentrations found in the human intestine (20). Although not considered here, it is interesting that both butyrate and bile acids are known to modify the PKC signalling pathway (12, 21). PKC is a family of 11 isoenzymes with distinct roles in cellular regulation (22) and it is probable that butyrate and bile acids may target different PKC isoenzymes with pro- and anti-apoptotic functions respectively. Indeed we have in the past shown that secondary bile acids are potent activators of PKC-β *in vitro* (19). PKC-β appears to play a predominantly anti-apoptotic role. PKC-βII has been shown to be a mitotic lamin kinase (23) and is activated during the prevention of apoptosis mediated by the oncogene v-abl (24). Ongoing studies in this laboratory aim to identify which PKC isoenzymes are regulated by sodium butyrate in colon cancer and adenoma cells.

In conclusion, our data suggest that the beneficial effects of butyrate with regard to protection against colon cancer, relate not only to its ability to induce apoptosis in colon cells per se, but also to the inhibition of the survival effects provided by secondary bile acids. If these results are to be interpreted to inform dietary intervention protocols to reduce risk of CRC, data will also be required for the effects of these agents on normal human colonocytes.

ACKNOWLEDGMENTS

We are grateful to Professor Chris Paraskeva for providing the AA/C1 cells. This work was supported by a grant from the Ministry of Agriculture Fisheries and Food (ANO313). J.M.L. is a Royal Society University Research Fellow.

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