Butyrate- but not TGF β_1 -induced apoptosis of colorectal adenoma cells is associated with increased expression of the differentiation markers E-cadherin and alkaline phosphatase

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Abstract

Sodium butyrate and transforming growth factor β (TGF β_1) are growth inhibitory to colonic adenoma cell lines. Butyrate induces apoptosis, whereas in some adenoma cell lines, TGF β_1 can be growth inhibitory without apoptosis. In this report, we show that the adenoma cell line PC/BH/C1 undergoes apoptosis in response to TGF β_1 . Butyrate induced cell death is preceded by the induction of two markers of colonic differentiation—alkaline phosphatase (ALP) activity and E-cadherin protein expression. However, TGF β_1 -induced apoptosis was not accompanied by induction of these differentiation markers. It is possible that the apoptosis induced by TGF β_1 in the adenoma cell line PC/BH/C1 is due to conflicting signals, as downregulation of c-myc protein in response to TGF β_1 occurs only slowly in this cell line. Development of resistance to TGF β_1 in colonic tumours may involve two separate stages—resistance to growth inhibition and resistance to TGF β_1 -induced apoptosis. Our results indicate that sodium butyrate induces apoptosis via differentiation, but TGF β_1 induces apoptosis by a differentiation-independent mechanism. As for butyrate, the induction of E-cadherin expression is a potentially important chemopreventative action, since increased E-cadherin expression has been correlated with decreased metastatic potential. This is the first report of induction of E-cadherin by a naturally occurring factor in the diet. Butyrate may reduce tumour growth and invasion, not only as a result of the induction of apoptosis, but also through increased expression of E-cadherin.

Keywords: TGF β_1 ; butyrate; colon; apoptosis; differentiation; E-cadherin; chemoprevention

Abbreviations: TGF β , transforming growth factor β ; ALP, alkaline phosphatase; ECM, extracellular matrix

Introduction

In the human colonic crypt, tissue homeostasis is maintained by a precise balance between an increase in cell number due to mitosis at the base of the crypt, and a decrease in cell number by cell death at the luminal surface. The regulation of colonic cell number may be influenced by growth factors endogenous to the colon, for example, transforming growth factor- β_1 (TGF β_1). This autocrine growth inhibitor has been demonstrated to negatively regulate intestinal epithelial cell growth *in vitro* (Barnard *et al*, 1989; Hoosein *et al*, 1989; Manning *et al*, 1991; Geiser *et al*, 1992; Wu *et al*, 1992). In addition, immunostaining for TGF β_1 protein expression in the normal human colonic mucosa has shown it to be differentially expressed within epithelial cells of the crypt, with enhanced immunoreactivity in the non-proliferating more differentiated cells in the upper parts of the crypt and an absence of expression in the lower proliferating cell compartment (Avery *et al*, 1993).

In addition to endogenous growth regulators like TGF β_1 , there is evidence that dietary factors may also play an important role in growth control in the colorectum. Epidemiological studies have suggested that a diet low in fat and high in fibre could be protective against colorectal cancer (Burkitt, 1971; Howe *et al*, 1992). Conversely, studies by Sandler *et al* (1993) have demonstrated that diets high in fat and low in fibre typical of those found in industrialised countries, increase the risk of the development of colon cancer. In the colonic lumen, dietary fibre is fermented by symbiotic bacteria producing the short-chain fatty acids butyrate, propionate and acetate as major by-products in millimolar amounts (Cummings, 1981). A putative role for sodium butyrate in the negative regulation of growth in the colon has been suggested by several studies that have demonstrated its antiproliferative effects on colon carcinoma cells and the induction of differentiation in these cells *in vitro* (Kim *et al*, 1982; Whitehead *et al*, 1986; Barnard and Warwick, 1993). The pleiotrophic effects of butyrate have been suggested to result from the inhibition of histone deacetylase by butyrate leading to altered gene expression (Kruh *et al*, 1982).

We have previously shown that sodium butyrate and TGF β_1 have growth inhibitory effects on colonic adenoma-derived cell lines *in vitro* (Manning *et al*, 1991, 1993). However, in two of the adenoma cell lines studied (S/RG/C2 and PC/AA/C1) it has been shown that butyrate treatment induced apoptosis whereas TGF β_1 did not, suggesting that these factors may act through different growth-inhibitory pathways (Hague *et al*, 1993a). In this paper, we demonstrate that TGF β_1 -mediated growth

inhibition can lead to an induction of apoptosis in another adenoma-derived cell line, PC/BH/C1. This finding is consistent with a recent report by Wang *et al* (1995) who showed that TGF β_1 can induce apoptosis in the human colon adenoma cell line VACO-330. Therefore, TGF β_1 is growth inhibitory to colonic epithelial cells and, depending on the cell line, TGF β_1 induces apoptosis in some colorectal tumour cell lines but not others.

In vivo studies have suggested that apoptosis occurs at the luminal surface of the colonic crypt as the final step in a terminal differentiation pathway (Gavrieli *et al*, 1992; Hall *et al*, 1994). Further evidence to support this has come from *in vitro* studies which have shown that the differentiation agent butyrate is able to induce apoptosis in colorectal tumour cell lines (Hague *et al*, 1993a; Heerdt *et al*, 1994) and that the apoptotic cells retain a marker of colonic differentiation that is expressed by the non-apoptotic population (Heerdt *et al*, 1994). However, despite its clear importance, relatively little is known about the relationship between growth inhibition, differentiation and apoptosis in the colon, the factors which regulate these processes or the possible pathways to apoptosis. Although there have been a number of studies to determine the mechanism(s) of TGR β_1 induced G1 arrest little is known regarding the mechanisms by which TGF β_1 induces apoptosis (Markowitz and Roberts, 1996). This is an important question since there is significant evidence that TGF β_1 is a negative growth regulator in the colon.

In this study, we have examined the relationship between butyrate- and TGF β_1 -mediated growth inhibition and the induction of differentiation and apoptosis. In particular, we addressed the question as to whether the TGF β_1 -induced apoptosis seen in the adenoma cell line PC/BH/C1 is *via* a terminal differentiation pathway or potentially by a differentiation-independent pathway, for example, due to conflicting growth signals (Askew *et al*, 1991; Evan *et al*, 1992; Shi *et al*, 1992). We examined alkaline phosphatase activity, a well-documented parameter of the degree of colonic epithelial differentiation (Yamamoto and Yasuda, 1973; Herz *et al*, 1981; Chung *et al*, 1985; Henthorn *et al*, 1988). Furthermore, we have used E-cadherin protein expression as an additional marker of colonic cell differentiation. Cell-cell and cell-matrix interactions play an essential role in the induction and maintenance of a differentiated epithelial cell phenotype (Nigam *et al*, 1993) and the expression of the cell-cell adhesion molecule, E-cadherin, has been shown to play an important role in the induction and maintenance of a differentiated colonic epithelial cell phenotype (Nigam *et al*, 1993; Birchmeier and Behrens, 1994). Loss of E-cadherin expression both *in vitro* and *in vivo* has been shown to correlate with a loss of cellular differentiation (Liu *et al*, 1993; Nigam *et al*, 1993; Pignatelli *et al*, 1993; Hermiston and Gordon, 1995). In addition, E-cadherin has been demonstrated to prevent invasiveness of malignant cells (Chen and Obrink, 1991; Frixen *et al*, 1991; Vleminkx *et al*, 1991), suggesting it may have an important role as a tumour (invasion) suppressor (Becker *et al*, 1994; Birchmeier, 1995).

Thus, in this paper we examined whether apoptosis induced by two potentially important regulators of colonic

epithelial cell growth, TGF β_1 and butyrate, is *via* a differentiation pathway and/or whether they alter the expression of the tumour (invasion) suppressor E-cadherin. These studies could have important implications for our understanding of the pathways to differentiation and apoptosis in the colon and also the potential role of these factors in chemoprevention.

Results

Sodium butyrate, but not TGF β_1 , induces expression of markers of colonic differentiation in adherent colonic adenoma- and carcinoma-derived cells

To determine the relationship between growth inhibition and the induction of a more differentiated phenotype, we analyzed markers of colonic differentiation in the adenoma-derived cell lines PC/AA/C1 and S/RG/C2 and the transformed adenoma cell line PC/AA/C1/SB/10 following treatment with concentrations of sodium butyrate (1–3 mM) previously shown to be growth inhibitory and to induce apoptosis (Hague *et al*, 1993a). In addition, the carcinoma-derived cell line PC/JW/FI was analyzed following butyrate treatment as this cell line has previously been shown to be relatively resistant to butyrate-induced growth inhibition (Berry and Paraskeva, 1988). Results show that in all cell lines examined, 48 h treatment with sodium butyrate resulted in a significant increase in ALP activity in adherent cells compared to controls (Figure 1). TGF β_1 -treatment (0.5–10 ng/ml) has also been shown to be growth inhibitory to PC/AA/C1 and S/RG/C2 cells (Manning *et al*, 1991). However, treatment of the above cell lines with TGF β_1 did not significantly alter the levels of ALP activity, after either 48 h (Figure 1) or 96 h (results not shown).

We extended these initial studies by examining an additional marker of colonic differentiation to provide a more comprehensive picture of the effects of butyrate and TGF β_1 on the induction of differentiation. Thus, adherent cells were examined for expression of the cell-cell adhesion protein E-cadherin following SDS–PAGE and Western blotting with the HECD-1 antibody. Figure 2 shows a dose-dependent increase in E-cadherin expression in PC/AA/C1 following treatment with concentrations of sodium butyrate (1–3 mM). Analogous results were obtained with the adenoma cell line S/RG/C2, the transformed adenoma cell line PC/AA/C1/SB10 and the carcinoma cell line PC/JW/FI (data not shown).

TGF β_1 treatment for 96 h did not result in any detectable change in the expression of E-cadherin protein in the two adenoma-derived cell lines PC/AA/C1 and S/RG/C2 that have previously been demonstrated to be sensitive to its antiproliferative effects (Manning *et al*, 1991), but are not induced to apoptose following treatment (Hague *et al*, 1993a). The results obtained for S/RG/C2 are shown in Figure 3. PC/AA/C1 gave similar results (data not shown).

The results of the E-cadherin protein expression studies correlate with the ALP activity assays, and suggest that growth inhibition induced by sodium butyrate leads to differentiation in these adherent cells. However, PC/AA/C1 and S/RG/C2 could be growth inhibited by TGF β_1 without

the induction of two markers of colonic differentiation. These two cell lines did not undergo apoptosis in response to $TGF\beta_1$.

Both spontaneously-derived and butyrate-induced apoptosis occurs via a pathway of terminal differentiation

In vivo, apoptosis is seen amongst terminally differentiated cells at the luminal surface of the colonic crypt (Gavrieli *et al*, 1992; Hall *et al*, 1994). In our *in vitro* cell culture system,

cells which spontaneously detach from the tissue culture flask during routine cell culture and float in the medium have previously been shown to be apoptotic (Hague *et al*, 1993a; Bracey *et al*, 1995). This apoptotic population has previously been demonstrated to increase in the adenoma-derived cell lines PC/AA/C1 and S/RG/C2 and PC/AA/C1/SB10 following treatment with sodium butyrate, but not with $TGF\beta_1$ (Hague *et al*, 1993a, 1995). To determine whether spontaneous and butyrate-induced floating, apoptotic cells were the end point of a differentiation pathway (i.e. were terminally differentiated), adherent and floating

Figure 1 Effect of 48 h treatment with sodium butyrate and $TGF\beta_1$ on alkaline phosphatase activity in adherent adenoma- and -carcinoma-derived cell lines. (A) PC/AA/C1 (passages 79–81), (B) PC/AA/C1/SB10 (passages 106–112), (C) S/RG/C2 (passages 47–50), (D) PC/JW/F1 (passages 49–51). A: Control samples, B: 2 mM sodium butyrate, C: 10 ng/ml $TGF\beta_1$. The results shown are the means $\pm$ s.e.m. of three experiments for each of the cell lines

Figure 2 Effect of 48h treatment with sodium butyrate on E-cadherin expression in the adenoma cell line PC/AA/C1 (passage 80). The HECD-1 antibody recognised the E-cadherin protein (120 kD). Lane 1: Control sample, Lane 2: 1 mM butyrate, Lane 3: 2 mM butyrate, Lane 4: 3 mM butyrate

Figure 3 Effect of 96 h treatment with $TGF\beta_1$ on E-cadherin expression in the adenoma-derived cell line S/RG/C2 (passage 50). Lane 1: Control sample, Lane 2: 0.5 ng/ml $TGF\beta_1$, Lane 3: 2 ng/ml $TGF\beta_1$, Lane 4: 10 ng/ml $TGF\beta_1$

cells in control and treated cultures were analyzed for ALP activity.

After treatment with butyrate, ALP activity was increased in the adherent cells, suggesting the induction of differentiation by butyrate (Figure 1). Levels of ALP activity in the spontaneously-derived apoptotic cell population were generally higher than levels observed in adherent cells of the four cell lines examined, in agreement with previous observations by Heerdt *et al* (1994). For example, at the 72 h time point the ALP activity in the floating cells was 3.4-fold higher than the controls for PC/AA/C1, 6.7-fold higher for PC/AA/C1/SB/10, 1.7-fold higher for S/RG/C2 and 6.0-fold higher for PC/JW/FI. These results suggest that cells may spontaneously apoptose during routine culture as the end point of a pathway of terminal differentiation. Furthermore, in butyrate-treated cultures, levels of ALP activity were similarly increased in floating apoptotic cells compared to adherent cells (data not shown). The apoptotic population in these conditions is comprised of both spontaneously-derived and butyrate-induced apoptotic cells. A level of ALP activity in the apoptotic population that is similar or higher than that observed in adherent, non-apoptotic cells, suggests that these apoptotic cells are derived from the differentiated population of adherent cells.

TGF β_1 induces apoptosis in responsive cells via a differentiation-independent pathway

Although the two adenoma cell lines examined previously (PC/AA/C1 and S/RG/C2) did not apoptose following treatment with TGF β_1 (Hague *et al*, 1993a), our recent studies have demonstrated that a further adenoma-derived cell line, PC/BH/C1, is responsive to both TGF β_1 -induced growth inhibition (approximately 50% growth inhibition compared to controls at 72 h) and apoptosis (Figure 4). Note that the PC/BH/C1 cell line exhibits high levels of spontaneous apoptosis (Figure 4) and undergoes apoptosis in response to 1–4 mM butyrate (this dose range gives a 2–4-fold increase in the percentage of cells floating compared to the controls after a 96 h treatment). As for the other adenoma cell lines, both spontaneous and butyrate-induced floating apoptotic cells had high levels of ALP activity and the attached cells of butyrate treated cultures had higher activity than the controls. Therefore, PC/BH/C1 is responsive to butyrate-induced differentiation and apoptosis, as shown previously in several other colonic tumour cell lines (Hague *et al*, 1993, 1995; Heerdt *et al*, 1994). However, in response to TGF β_1 analysis of markers of colonic differentiation in these cells revealed no detectable differences in either E-cadherin expression (Figure 5) or ALP activity (Figure 6) compared to controls. Thus, these results suggest that, unlike butyrate, TGF β_1 may induce apoptosis in these cells via a pathway separate from the induction of markers of colonic differentiation.

Discussion

The maintenance of normal tissue homeostasis in the colonic crypt is dependent upon a precise balance between proliferation, differentiation and cell death. A breakdown in

Figure 4 Effect of TGF β_1 (10 ng/ml) on apoptosis in the adenoma-derived cell line PC/BH/C1 (passages 86–96). After 48 and 72 h of treatment, apoptosis was measured as the percentage of cells floating in the medium, with confirmation that the floating cells were apoptotic by morphological analysis of acridine orange stained cells. Results shown are means $\pm$ s.e.m. of six experiments. The percentage of cells floating was measured by counting the number of cells floating in the medium on day of harvest i.e. the number shed into the medium during the treatment time. The attached cell yield on the day of harvest was also counted. The percentage of cells floating represents the floating cell yield as a percentage of the total number of cells in the flask

Figure 5 E-cadherin expression after 48 and 72 h treatment of the adenoma-derived cell line PC/BH/C1 (passage 92) with TGF β_1 (10 ng/ml). Lane 1: Control 48 h, Lane 2: TGF β_1 48 h, Lane 3: Control 72 h, Lane 4: TGF β_1 72 h

this balance is believed, at least in part, to be important in the uncontrolled growth characteristic of colonic carcinogenesis. This deregulation of growth can occur through a reduced response to growth inhibition by negative regulators of growth such as TGF β_1 (Manning *et al*, 1991; Markowitz *et al*, 1995) and/or due to reduced levels of apoptosis (Hague *et al*, 1993b; Bedi *et al*, 1995).

Despite evidence that the end point of the colonic differentiation pathway *in vivo* is apoptosis at the luminal surface (Gavrieli *et al*, 1992; Hall *et al*, 1994), relatively little is known about the relationship between growth inhibition, differentiation and apoptosis in the colon, the factors which regulate these processes, or possible different pathways to apoptosis. To examine the relationship between differentia-

Figure 6 Effect of 72h treatment with TGFβ₁ on alkaline phosphatase activity in adherent cells of the adenoma-derived cell line PC/BH/C1 (passages 87–89). 1. Control samples, 2. 10 ng/ml TGFβ₁. The results shown are means ± s.e.m. of three experiments

tion and apoptosis in colonic cells, we have examined whether TGFβ₁- and butyrate-induced growth inhibition and/or apoptosis are via differentiation dependent or independent pathways. We have used ALP activity and E-cadherin protein expression as markers of colonic differentiation. Furthermore, we have addressed these questions using adenoma cell lines as well as carcinoma cell lines, since the former retain certain features of normal cells, such as being non-tumorigenic and retaining sensitivity to TGFβ₁-induced growth inhibition (Manning *et al*, 1991; Wang *et al*, 1995).

In this paper, we have demonstrated that sodium butyrate inhibits growth and induces apoptosis in colonic adenoma- and carcinoma-derived cell lines and this is accompanied by the induction of a more differentiated phenotype. The ability of the naturally occurring dietary factor, butyrate, to increase the expression of E-cadherin protein is of interest since increased E-cadherin expression correlates with decreased tumour invasiveness (Frixen *et al*, 1991). Indeed, a recent study by Barshishat *et al* (1996) has demonstrated that butyrate treatment abolished the liver colonising ability of human colon cancer cell lines previously shown to have high liver metastasising activity. Thus, butyrate, in addition to promoting differentiation and apoptosis, may have a role in protecting against tumour progression by reducing the invasiveness of a tumour through increased expression of E-cadherin.

In contrast to the effects of butyrate, there is no evidence from these studies that TGFβ₁ induces a more differentiated phenotype in colonic adenoma cells, whether it induces apoptosis or not. Although both butyrate and TGFβ₁ are growth inhibitory to adenoma cells, these results suggests that they may act through different growth regulatory pathways. Previous studies have suggested that TGFβ₁ may inhibit intestinal epithelial cell growth by induction of a terminal differentiation pathway (Kurokawa *et al*, 1987; Hott and Baird, 1986). However, subsequent studies by Barnard *et al* (1989) did not reveal any evidence of a direct role for TGFβ₁ in enterocyte differentiation in the

IEC-6 intestinal cell line, suggesting that TGFβ₁ may function together with other stimuli *in vivo* to induce a differentiation programme. Our results may reflect the *in vivo* role of TGFβ₁ in the colon, by inducing a growth arrest in responsive cells which then require additional signals from other growth and/or dietary factors, e.g. sodium butyrate, to proceed through a differentiation programme. Indeed, the presence of TGFβ₁ protein expression in the non-proliferating region of the human colonic crypt (Avery *et al*, 1993), may suggest a role for the growth factor in mediating a stable growth arrest which is a prerequisite for differentiation. In addition, differentiating agents such as HMBA, retinoid acid and sodium butyrate can induce TGFβ₁ expression (Glick *et al*, 1989; Schroy *et al*, 1990; Barnard and Warwick, 1993) suggesting some interaction between these factors in the maintenance of a differentiated phenotype. Thus, it seems plausible that the role of TGFβ₁ in the human colonic crypt may be in the maintenance of a non-proliferative state, to allow subsequent induction and stabilisation of a differentiated phenotype by additional growth and/or dietary factors and through interactions with the extracellular matrix (ECM).

Although it is unclear why TGFβ₁ induces apoptosis in some adenoma cell lines and not others, the response to TGFβ₁ may depend on the degree of sensitivity to TGFβ₁-induced growth inhibition which may be determined, in part, by the particular genes mutated in the adenoma cells (Wang *et al*, 1995). For example, it is plausible that TGFβ₁ may induce an apoptotic response in PC/BH/C1 by eliciting conflicting growth signals in these cells, for example by an inability to downregulate Myc expression, as has been suggested by studies in fibroblasts (Evan *et al*, 1992), myeloid (Askew *et al*, 1991) and T cells (Shi *et al*, 1991). Indeed, Western blotting analysis of Myc expression in PC/BH/C1 cells following treatment with TGFβ₁ showed a slower downregulation of expression in treated cells (after 48 h, data not shown) compared to the rapid downregulation observed in PC/AA/C1 and S/RG/C2 (after 4 h, data not shown). These results may, therefore, suggest some deregulation of Myc in these cells and that the apoptotic response to TGFβ₁ observed in PC/BH/C1 may be along a pathway that is distinct from a terminal differentiation pathway, possibly due to conflicting growth signals. Whatever the mechanism by which TGFβ₁ induces apoptosis in colonic epithelial cells, the development of resistance is important in colorectal tumorigenesis (Manning *et al*, 1991; Markowitz *et al*, 1995). There may be two stages to the development of TGFβ₁ resistance – resistance to growth inhibition and resistance to TGFβ₁-induced apoptosis.

In summary, we have demonstrated that butyrate-mediated apoptosis occurs as the end point of a terminal differentiation pathway, thereby potentially mimicking *in vivo* processes in which differentiated cells at the top of the colonic crypt undergo apoptosis (Gavrieli *et al*, 1992; Hall *et al*, 1994). In addition, we have demonstrated that sodium butyrate may play an important role in growth regulation in the colon, not only in inducing apoptosis, but also by protecting against tumour invasion by inducing expression of the tumour invasion suppressor E-cadherin. This is the

first report of an endogenous dietary factor increasing the expression of E-cadherin which, together with the growth inhibitory and apoptosis inducing effects of butyrate and TGF β_1 , could have important implications for cancer chemoprevention. In contrast, TGF β_1 -treatment does not induce differentiation in adenoma cells that are responsive to its antiproliferative and, in some cases, apoptosis-inducing effects. Thus, although both sodium butyrate and TGF β_1 are clearly growth inhibitory to colonic epithelial cells, they may have divergent and/or complementing roles in the regulation of growth in the colonic crypt *in vivo*.

Materials and Methods

Cell culture

The human adenoma cell lines used in this study, S/RG/C2, PC/BH/C1 and PC/AA/C1, were clonogenic variants of the sporadically derived S/RG cell line (Paraskeva *et al*, 1989a), and the PC/BH and PC/AA derived cell lines from patients with the inherited condition familial adenomatous polyposis (Paraskeva *et al*, 1984, 1989b) respectively. Two adenocarcinoma cell lines were also investigated. PC/JW/FI is a 3T3 feeder independent variant of the carcinoma cell line PC/JW, derived from a FAP patient and previously described (Paraskeva *et al*, 1984). PC/AA/C1/SB/10 is a tumorigenic *in vitro* transformed derivative of the PC/AA/C1 cell line (Williams *et al*, 1990). The PC/AA/C1 and PC/BH/C1 adenoma cell lines are both wild type for p53 whereas the S/RG adenoma cell line has a mutant p53 (Baker *et al*, 1989; Williams *et al*, 1993). All the adenoma cell lines are TGF β_1 sensitive and non tumorigenic in athymic nude mice whereas PC/JW and PC/AA/C1/SB/10 are TGF β_1 resistant and tumorigenic (Paraskeva *et al*, 1984; Williams *et al*, 1990; Manning *et al*, 1991).

Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% foetal bovine serum (bath tested), hydrocortisone sodium succinate (1 μ g/ml), insulin (0.2 units/ml), glutamine (2 mM), penicillin (100 units/ml) streptomycin (100 mg/ml). Cells were grown at 37°C in a 5% CO₂ air incubator.

Measurement of apoptosis

We and others (Hague *et al*, 1993; Heerdt *et al*, 1994; Tsujii and DuBois, 1995) have previously reported that during routine cell culture, epithelial cells spontaneously detach from the tissue culture flask and float in the medium. These detached cells have been demonstrated to be apoptotic (Hague *et al*, 1993a) both morphologically using acridine orange staining (Gregory *et al*, 1991) and biochemically by internucleosomal fragmentation of the DNA to produce a DNA ladder on an electrophoretic gel (Wyllie, 1980). The level of apoptosis has been demonstrated to increase following treatment with sodium butyrate, but not with TGF β_1 , in the two original adenoma cell lines analyzed, PC/AA/C1 and S/RG/C2 (Hague *et al*, 1993a). However, our recent studies presented here have shown that the adenoma cell line PC/BH/C1 is sensitive to TGF β_1 -induced apoptosis.

Treatment with sodium butyrate

The two adenoma-derived cell lines, PC/AA/C1 and S/RG/C1, the tumorigenic transformed adenoma cell line PC/AA/C1/SB10 and the carcinoma-derived cell line PC/JW/FI were used in experiments to determine the effects of sodium butyrate on expression of markers of

colonic differentiation. PC/JW/FI and PC/AA/C1/SB10 have previously been demonstrated to be relatively insensitive to butyrate-induced growth inhibition (Berry and Paraskeva, 1988; Williams *et al*, 1990) and apoptosis (Hague *et al*, 1995). Cells were trypsinised, and seeded in triplicate at 2×10^6 cells per T25 flask in standard culture medium. After 72 h when the cells were in exponential growth phase, the medium was replaced with fresh medium containing concentrations of sodium butyrate (1–3 mM) previously demonstrated to produce growth inhibition (Hague *et al*, 1993a) and arrest in G1 phase of the cell cycle (data not shown). Cells were treated for 48 h for analysis of E-cadherin protein expression and 48, 72 and 96 h for the alkaline phosphatase activity study.

Treatment with TGF β_1

The adenoma-derived cell lines PC/AA/C1, S/RG/C2 were used in experiments to investigate the effect of TGF β_1 on E-cadherin expression and alkaline phosphatase activity. These cell lines have previously been demonstrated to be sensitive to the antiproliferative effects of TGF β_1 (Manning *et al*, 1991) and are arrested in the G1 phase of the cell cycle following treatment (data not shown), but are not induced to apoptose following treatment (Hague *et al*, 1993a). More recent studies have shown that the adenoma-derived cell line PC/BH/C1 is sensitive to both TGF β_1 -mediated growth inhibition and apoptosis (Figure 5).

Cells were trypsinised and seeded at a density of 2×10^6 cells per T25 flask in standard culture medium and treated with a range of concentrations of TGF β_1 (0.5–10 ng/ml) in fresh medium after 72 h when cells were in exponential growth phase. Initially, 48 h of treatment resulted in no change in E-cadherin protein expression in PC/AA/C1 and S/RG/C2 (data not shown), so a longer treatment time of 96 h was used in subsequent experiments with these cell lines. E-cadherin expression in PC/BH/C1 was analyzed after 72 h of treatment, since this was the time point at which the greatest induction of apoptosis occurred. All the cell lines examined were treated for 48, 72 and 96 h for analysis of alkaline phosphatase activity.

Immunoblotting

E-cadherin is a transmembrane protein with a large extracellular domain involved in mediating homotypic cell-cell binding. In order to maintain the structure of the full-length E-cadherin protein for detection by immunoblotting, cells were scraped from the tissue culture flask rather than trypsinised. Cell number was estimated from trypsinising and counting duplicate flasks. Cells to be used for sample preparation were rinsed twice with PBS and 100 μ l lysis buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 0.2% glycerol, 2 mM EDTA, 1.43 M β -mercaptoethanol) per 1×10^6 cells was added. The cells were removed by gentle scraping and kept on ice. A further 50 μ l of lysis buffer per 1×10^6 cells was added to the flask to remove any remaining cells. Hence, the final concentration was 1×10^6 cells per 150 μ l of lysis buffer. Samples were boiled for 10 min with periodic vortexing then placed on ice for 5 min. The supernatant was removed and microfuged at 13 000 r.p.m. for 3 min. The samples were aliquoted at 150 μ l and stored at –70°C. Before SDS–PAGE analysis, 15 μ l of lysis buffer with bromophenol blue (10 mg) was added and the samples were boiled for 5 min.

Samples were subjected to SDS–PAGE (7.5% acrylamide for E-cadherin) following the method of Laemmli (1970) and transferred to a polyvinylidene difluoride membrane (PVDF; Millipore) by electroblotting for a minimum of 5 h (using transfer buffer of 192 mM glycine, 25 mM Tris

with 20% methanol). The membrane was blocked for at least 1 h with milk block (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 5% non fat dried milk) and probed with the E-cadherin monoclonal antibody (HECD-1, gift from Sir Walter Bodmer, ICRF, London) diluted 1:100 with milk block, overnight at 4°C. Following the primary incubation, the membrane was washed twice in milk block buffer for 10 min, then twice in Tween buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.2% Tween) for 10 min, followed by a final 10 min wash in milk block buffer. The membrane was probed with goat anti-mouse IgG horseradish peroxidase conjugate (Sigma) at a concentration of 1:1000 in milk block buffer for 1 h at 4°C. Following one 20 min wash in milk block buffer and one 10 min wash in Tween buffer, the membrane was rinsed in distilled water and peroxidase activity was visualised using enhanced chemiluminescence (ECL: Amersham).

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was used as an additional marker of the degree of colonic differentiation induced by either sodium butyrate or TGF β_1 in the cell lines studied. Attached and floating cells were washed and lysed with 0.25% sodium deoxycholate essentially as described by Herz *et al* (1981). ALP activity was determined using the Sigma Diagnostics ALP reagent (No. 245). Total cellular protein content of the samples was determined in a microassay procedure as described by Bradford (1976) using the Coomassie Protein Assay Reagent Kit (Pierce). ALP activity was calculated as units of activity per μ g of protein.

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