Transcriptional upregulation of the insulin-like growth factor binding protein IGFBP-3 by sodium butyrate increases IGF-independent apoptosis in human colonic adenoma-derived epithelial cells

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Sodium butyrate (NaBt) and the pro-apoptotic IGFBP-3 protein, expressed at the top of the normal colonic crypt, have both been implicated in the regulation of apoptosis in colonic epithelial cells. Recent studies in human breast and hepatic cell lines have shown that NaBt can transcriptionally upregulate IGFBP-3 expression. However, the role of butyrate in the regulation of IGFBP-3 expression in the colon is less clear, with reports of both up- and downregulation of the IGFBP-3 protein in colorectal cancer cell lines. In this study we have shown that the level of IGFBP-3 protein expression in colonic epithelial cells correlates with the p53 status of the cells; wildtype p53 cells secrete higher levels of IGFBP3 protein than mutant p53 cell lines. Data presented shows that, when treated with a dose of NaBt that induced significant apoptosis (4 mM for 48 h), there was an upregulation of IGFBP-3 protein in both wildtype and mutant p53 expressing cell lines. The NaBt-induced increase in secreted IGFBP-3 protein was associated with transcriptional upregulation of the IGFBP-3 gene. Using a transfected derivative of the S/RG/C2 adenoma-derived cell line, which stably expressed exogenous IGFBP-3 protein at levels equivalent to that secreted by the 4 mM NaBt-treated parental line (1–3 ng/10^6 cells), we have shown a >2-fold increase in the sensitivity of the cells to NaBt-induced apoptosis when compared with the vector control and parental cell lines. Furthermore, inhibition of the secreted IGFBP-3 protein, by addition of neutralizing antibodies, resulted in a significant decrease in NaBt-induced apoptosis. These data suggest that IGFBP-3 may act as a positive regulator of NaBt-induced apoptosis in colonic epithelial cells, and represents a potentially important mechanism whereby the sensitivity of colonic epithelial cells to NaBt-induced apoptosis can be increased.

Introduction

Sodium butyrate (NaBt), a naturally occurring short chain fatty acid resulting from carbohydrate metabolism in the gut, has been implicated in the induction of cell cycle arrest, differentiation and apoptosis in colonic epithelial cells (1–3). The concentration of NaBt needed to initiate these effects in vitro is less than the levels of NaBt found in vivo, as NaBt is present in 10–20 mM concentrations in the gut lumen (4). Furthermore, as NaBt is used as a preferred energy source, it is readily metabolized by normal colonic epithelial cells (5,6). However, the mechanisms by which NaBt promotes differentiation and apoptosis in colonic epithelial cells are poorly understood. For example, NaBt is a known inhibitor of histone deacetylase (HDAC) activity (7,8), therefore potentially affecting the overall level of transcription, whereas NaBt responsive elements have also been identified in the promoters of a number of genes (refs 9 and 10 for example) implicating a more specific mechanism of gene regulation as well.

Recent work from our laboratory has shown that an insulin-like growth factor binding protein IGFBP-3 is differentially expressed along the colonic crypt (11), with IGFBP-3 being highly expressed at the luminal surface coincident with exposure to higher concentrations of NaBt. IGFBP-3 is a member of a family of high affinity binding proteins known to regulate the function of insulin-like growth factors (IGF-I and IGF-II) through modulating their interactions with the signalling receptor IGF-I receptor (IGF-IR). IGFBP-3 is the most predominant IGF binding protein in adult serum, secretion of which varies greatly with cell type and species of origin (reviewed in ref. 12). Although IGFBP-3 is known to inhibit cell growth by sequestering IGFs, there is growing evidence that IGFBP-3 can have direct IGF-independent effects on cell growth (reviewed in ref. 13). A number of reports have described growth inhibition by IGFBP-3 that appears to be IGF-independent (in breast cancer cells [13,14], in colonic epithelial cells [16], and in fibroblasts [17,18] for example). Supporting its role as a primary growth inhibitor, the antiproliferative effects of retinoic acid (19,20), TNFα (21), wildtype p53 (22), vitamin D (23), anti-estrogens (24), and TGFβ (25,26) have all been shown to be mediated, in part, through IGFBP-3. There is now accumulating evidence to suggest that, in addition to the anti-proliferative effects of IGFBP-3, it is involved in the induction of apoptosis. Using an IGF-IR negative prostate cell line (PC-3), Rajah et al. (27) reported IGF-independent induction of apoptosis following addition of recombinant IGFBP-3, and showed that IGFBP-3 is a mediator of TGFβ-induced apoptosis in PC-3 cells (27). We have also recently demonstrated the IGF-independent, pro-apoptotic effects of IGFBP-3 in human breast cancer cells (15,28).

There have also been a number of studies that show that IGFBP-3 can potentiate the apoptotic effects of various agents. IGFBP-3 has been shown to increase ceramide-induced apoptosis (a physiological mediator of apoptosis induced by radiation and cytokines such as TNFα and IL-1β) in a breast cancer cell line, although there was no induction of apoptosis by the addition of IGFBP-3 alone (29). Furthermore, IGFBP-3 enhanced p53-dependent apoptosis in response to γ-irradiation of colon epithelial cells (11) and UV irradiation of an oesophageal carcinoma cell line (30), again despite the fact that IGFBP-3 alone had no effect on cell growth or cell survival. Stable transfection of IGFBP-3 cDNA in breast cancer cells results in both increased apoptosis and a potentiation of radiation-induced apoptosis (28). These results suggest that
IGFBP-3 may interact with mediators of apoptosis leading to an enhanced level of cell death.

Recently, the transcriptional upregulation of IGFBP-3 by NaBt has been described in breast cancer cells but not in normal breast epithelial cells (31,32). In the colon however, it has been reported that NaBt both decreased secretion of IGFBP-3 in the colon carcinoma-derived Caco-2 cells (33) and induced IGFBP-3 expression in LIM 2405 human colon cancer cells (34). As NaBt is implicated in the regulation of tissue homeostasis in the colon (1) the aim of this study was to establish whether NaBt regulates expression and secretion of the IGFBP-3 protein in adenoma as well as carcinoma-derived colonic epithelial cells. In addition, as IGFBP-3 has previously been shown to potentiate induced apoptosis in colon adenoma-derived cells (11) we wished to establish whether IGFBP-3 can regulate NaBt-induced apoptosis. As IGFBP-3 has been found to be expressed in the normal colonic crypt (11), it is possible that IGFBP-3 expression may play a role in regulating the sensitivity of colonic epithelial cells to NaBt-induced apoptosis.

Materials and methods

Cell lines and culture conditions

S/RG/C2 is a clonogenic, non-tumorigenic human colonic adenoma-derived cell line hemizygous for the p53 gene, with the remaining allele having a 282 (Arg→Trp) mutation. S/AN/C1 and PC/AA/C1 are clonogenic, non-tumorigenic human adenoma-derived cell lines, which expresses wildtype p53. PC/AA/SB10 is a tumorigenic derivative of the PC/AA/C1 cell line shown to express increased levels of wildtype p53 protein compared with the parental cell line (11,35). These cell lines were maintained on conditioned medium (refer to ref. 35). The human colonic carcinoma-derived cell line HT29 with a 273 (Arg→His) p53 mutation and the human carcinoma-derived PC/JW cell line that is null for p53 protein expression were routinely cultured in DMEM (Invitrogen) supplemented with 100 units/ml penicillin, 100 mg/ml
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Fig. 3. (A) Induction of apoptosis in the A(i) S/RG/C2 adenoma-derived cell line and A(ii) HT29 carcinoma-derived cell line after 24 and 48 h treatment with 4 mM NaBt. The results shown are the mean of three separate experiments ±SEM, statistical difference determined by two-way ANOVA with replication; *P < 0.05, **P < 0.01, ***P < 0.001. (B) Western blot showing IGFBP-3 protein in 5× concentrated conditioned serum-free medium collected from 5 × 10⁶ B(i) S/RG/C2 and B(ii) HT29 cells 24 and 48 h after treatment with or without 4 mM NaBt. (C) α-Tubulin used as a loading control for attached cell number from which conditioned medium was harvested is included. 1. 24 h SFM control. 2. 48 h SFM control. 3. 24 h SFM from 4 mM NaBt-treated cells. 4. 48 h SFM from 4 mM NaBt-treated cells. 5. 80 ng non-glycosylated IGFBP-3 protein control. 6. 80 ng glycosylated IGFBP-3 protein control. The bands on these gels represent post-transcriptionally modified protein. In addition to glycosylation variants, there are a number of common proteolytic and phosphorylation variants of IGFBP-3. Combinations of these different post-translational modifications result in the well-established microheterogeneity of the IGFBP-3 protein. Whilst IGFBP-3 generally resolves as a number of broad bands on electrophoresis, the protein appears to have resolved as a series of more discrete bands in these cell lines. (D) Western blot showing PARP cleavage in 1 × 10⁵ attached and floating cells of D(i) S/RG/C2 and D(ii) HT29 cells 24 and 48 h after treatment with or without 4 mM NaBt. α-Tubulin used as a loading control for cell number. Note all the PARP is cleaved in the floating (apoptotic) cells (lanes 5–8). 1. 24 h untreated attached cells. 2. 48 h untreated attached cells. 3. 24 h 4 mM NaBt-treated attached cells. 4. 48 h 4 mM NaBt-treated attached cells. 5. 24 h untreated floating cell population. 6. 48 h untreated floating cell population. 7. 24 h 4 mM NaBt-treated floating cell population. 8. 48 h 4 mM NaBt-treated floating cell population.

Streptomyces, 2 mM glutamine and 10% foetal bovine serum (referred to as 10% FBS DMEM medium). S/RG/C2 cells transfected with, and stably expressing a vector containing IGFBP-3 (from Rob Baxter, Kolling Institute for Medical Research, Sydney) RG/BP3, and a vector control, RG/OP13, were grown in conditioned medium supplemented with conditioned medium and 200 µg/ml G418 as previously described (36).

Stable transfection
S/RG/C2 cells (passage 30) were transfected with a 0.9-kilobase pair IGFBP-3 cDNA fragment in the expression vector pOP13 (Invitrogen, Carlsbad, CA) as described previously (28). Cells were stably transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After 48 h, the transfected cells were selected for using G418 (400 µg/ml), cell lines grown up from single colonies and maintained in conditioned medium supplemented with 200 µg/ml G418.

Treatment with 4 mM NaBt
Cells were seeded in duplicate flasks and grown under standard growth conditions until ~70% confluent. Preliminary investigations showed that all the cell lines studied were able to tolerate serum-free growth conditions (SFM) for up to 72–96 h (SFM = standard non-conditioned growth medium without addition of FBS). Cells were grown for 24 h in SFM to remove IGFBP-3 present in the serum and then grown for up to 48 h in SFM supplemented with or without 4 mM NaBt (Sigma), previously reported to induce apoptosis.
Effect of 4 mM NaBt treatment on IGFBP-3 mRNA expression in the PC/AA/C1 colon adenoma cell lines. (A) Expression of IGFBP-3 mRNA in 24 and 48 h NaBt-treated. (B) 18S, used to control for RNA loading. Results shown are representative of those obtained from four separate experiments. 1. 24 h untreated cells. 2. 24 h 4 mM NaBt-treated cells. 3. 48 h untreated cells. 4. 48 h 4 mM NaBt-treated cells.

(1). The treatment with NaBt was not extended further than 48 h to prevent prolonged incubation in SFM. Attached and floating (apoptotic, refer below) cell yields were determined from parallel flasks treated for 24 and 48 h.

Assessment of apoptosis

The level of apoptosis was assessed by measuring the proportion of the total cell population that detached from the monolayer and were floating in the medium and by determining the fraction of these floating cells that were apoptotic, as described in ref. 11. The attached and floating cell populations were stained with 5 mg/ml acidine orange in PBS, and analysed by fluorescent microscopy for morphological features of apoptosis (most obviously the characteristic condensed chromatin, data not shown, as previously described in refs 1 and 37). As the fraction of floating cells that were apoptotic did not vary between treated and control untreated cell populations, the number of floating cells could be used as a measure of the induction of apoptosis. Apoptosis was also confirmed by PARP cleavage as previously reported for NaBt-treated cells (38).

Assessment of IGFBP-3, IGF-I and IGF-II secreted into the medium

Sub-confluent monolayers were grown for 24 h in SFM. The cells were then grown for a further 24 or 48 h in SFM, with or without NaBt, the media harvested, centrifuged to remove floating cells and stored at –70°C. Attached cell numbers were determined using a haemocytometer. Proteins from conditioned medium were concentrated using 'Microsep' 10K centrifuge columns from Gelman Laboratory. The levels of IGFBP-3 were assessed by SDS–PAGE immunoblotting, the IGFBP-3 protein detected by anti-IGFBP-3 antisera (polyclonal from Diagnostic System Laboratory). Levels of IGFBP-3, IGF-I and IGF-II in conditioned medium were also measured using a radioimmunoassay (RIA) previously described (39,40), proteins from conditioned medium were concentrated (×5) using Millipore Ultrafree-MC filter units and the final concentration of IGFs and IGFBP-3 corrected for cell number.

Treatment with NaBt ± αr3 antibody

Cells were incubated in SFM for 24 h, followed by SFM plus the anti IGF-IR antibody αr3 (Santa Cruz) at concentrations of 1 and 5 μg/ml (41) for 48 h ± 4 mM NaBt. The cell yield and floating cell population in the presence of the αr3 antibody were counted using a haemocytometer.

Blocking secreted IGFBP-3 protein function by treatment with neutralizing antibody

In order to block secreted IGFBP-3 function, cells were incubated in SFM for 24 h, followed by SFM ± 4 mM NaBt plus the anti-IGFBP-3 antibody (anti-IGFBP-3 polyclonal from Diagnostic System Laboratory) at concentrations of 10 μg/ml. After 48 h incubation in the presence of the IGFBP-3 neutralizing antibody ± 4 mM NaBt the cell yield and floating cell population were determined.

Northern blotting

1 × 10^7 – 3 × 10^7 cells were washed in PBS and total RNA was extracted using the Qiagen (Chatsworth, CA) RNeasy mini kit according to the instructions of the manufacturer. 15 μg of total RNA was separated on a 0.9% agarose gel, containing 3% formamide, and transferred to a nylon membrane (Genescreen Plus, NEN Life Sciences, MA). α-32P-dATP labelled probes for IGFBP-3 (template cDNA from Rob Baxter, Kolling Institute of Medical Research, University of Sydney, Australia) and 18S loading control (template from Maria Davies, Department of Dental Medicine, University of Bristol) were prepared using a random-primer DNA-labelling kit (Stratagene, CA) and hybridized overnight at 68°C. Filters were washed to a final stringency of 0.2 × SSC/1% SDS (10 min, 68°C) and bands visualized by autoradiography.

Statistical analysis

The data represent the mean of three separate experiments. (Each experiment was carried out in duplicate parallel flasks. The experiment was repeated three times, and results presented as the mean of the three separate experiments.) Statistical analysis was carried out using SPSS for Windows statistical software (release 10.0.5, SPSS, Chicago, IL). Two-way analysis of variance (ANOVA) was used to determine differences amongst means. Pairwise comparisons were made using Tukey’s post hoc test for multiple comparisons.

Results

IGFBP-3 is secreted by both colonic adenoma and carcinoma-derived cell lines

IGFBP-3 secreted protein was measured in 24 h serum-free medium from both adenoma and carcinoma-derived cell lines as shown in Figure 1. Interestingly, the level of secreted glycosylated IGFBP-3 protein was found to correlate with the p53 status of the cell line. Adenoma and carcinoma cell lines that have lost wildtype p53 function (lanes 1, 3, 4) secrete very low levels of the IGFBP-3 protein (the carcinoma HT29 and adenoma S/RG/C2 cell lines have a 273(Arg→His) and 282 (Arg→Trp) mutation respectively, the carcinoma-derived PC/JW/2 is null for the p53 protein). In each of these cell lines the amount of secreted IGFBP-3 protein was undetectable by radioimmunoassay (<1 ng/10^6 cells). In contrast, the adenoma-derived cell lines that are wildtype for p53 secreted between 10 and 26 ng/10^6 cells (PC/AA/C1 lane 5) and 4.0 to 29 ng/10^6 cells (S/AN/C1, lane 2) in 24 h. In the PC/AA/C1 tumorigenic derivative cell line (AA/C1/SB10, lane 6) with stabilized wildtype p53 function (42) there was an additional 3.5-fold increase in secreted protein compared with the parental cell line (67.3 ± 19.8 ng/10^6 cells in 24 h, Figure 1). (All represent pooled results from ten independent experiments.)

Induction of apoptosis by NaBt results in an increase in secreted IGFBP-3 protein in colorectal epithelial cells

PC/AA/C1 cells (which secrete quantifiable amounts of IGFBP-3 by RIA) were grown in SFM ± 4 mM NaBt and attached and floating cell yields determined. Results are summarized in Figures 2 and 3. The results show that there was a >2-fold increase in apoptosis on treatment with 4 mM NaBt for 48 h (Figure 2A), in concurrence with previous reports (1,38). NaBt treatment resulted in an increase in secreted IGFBP-3 protein coincident with the induction of apoptosis (Figure 2B). There was a 50% increase in the level of IGFBP-3 secreted by the NaBt-treated cells compared with untreated controls (Figure 2B).

Furthermore, NaBt-induced apoptosis was also shown to be associated with an increase in IGFBP-3 secretion in mutant p53 expressing cells. There was a 2-fold increase in apoptosis in the adenoma-derived S/RG/C2 cells and 4-fold increase in the carcinoma-derived HT/29 cell line with NaBt treatment (Figure 3A). NaBt treatment over 48 h resulted in an increase in secreted IGFBP-3 protein in both the adenoma- and carcinoma-derived cell lines (Figure 3B). These results suggest that NaBt-induced apoptosis is associated with an increase in secreted IGFBP-3 protein levels, in both wildtype and mutant p53 expressing cell lines.

IGFBP-3 is transcriptionally upregulated by NaBt treatment

Total RNA was prepared from the NaBt-treated PC/AA/C1 adenoma-derived cell line and IGFBP-3 mRNA expression...
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Fig. 5. (A) Western blot shows representative results of IGFBP-3 protein in 5× concentrated 24 h conditioned serum-free medium from 5×10⁶ attached cells. (B) α-Tubulin used as a loading control for attached cell number from which conditioned medium was harvested. 1. S/RG/C2 parental cell line. 2. RG/C2/OP13 vector control cell line. 3. RG/C2/BP-3 IGFBP-3 expressing cell line. IGFBP-3 resolves as a number of bands on electrophoresis, representing post-transcriptionally modified protein. Induction of apoptosis in (C) vector control RG/OP13 and (D) IGFBP-3 expressing RG/BP-3 cells after 24 and 48 h treatment with or without 4 mM NaBt. Induction of apoptosis is shown by floating cell number expressed as a percentage of the total cell population (refer to Materials and methods). Results shown are the mean of three separate experiments ± SEM, statistical difference determined by two way ANOVA with replication; *P < 0.05, **P < 0.01, ***P < 0.001.

determined using northern blotting analysis (Figure 4). These findings show that there is a notable upregulation in IGFBP-3 mRNA in 24 h NaBt-treated cultures, and to a lesser extent after 48 h NaBt treatment.

Overexpression of IGFBP-3 in the S/RG/C2 adenoma-derived cell line increases the sensitivity of the cells to NaBt-induced apoptosis

IGFBP-3 was stably expressed in the S/RG/C2 adenoma-derived cell line. This cell line was selected as it secretes low levels of IGFBP-3 protein, and hence stable expression of the IGFBP-3 cDNA resulted in a significant increase in secreted IGFBP-3 as confirmed by western blot (1–3 ng/10⁶ cells, equivalent to levels secreted by 48 h NaBt-treated cultures). Results are summarized in Figure 5. Increased expression of IGFBP-3 in RG/C2/BP-3 cells resulted in an increase in the level of spontaneous apoptosis as well as an increase in the level of NaBt-induced apoptosis. Contrary to the findings of Butt et al. (28), this increase in apoptosis in the IGFBP-3 expressing RG/C2/BP3 cell line was not associated with an increase in Bax protein expression (data not shown).

To determine whether IGFBP-3 increases NaBt-induced apoptosis through sequestering IGFs, it was important to determine the levels of IGF-I and IGF-II secreted by the S/RG/C2 epithelial cell lines (as all experiments were carried out in SFM, and therefore only endogenous protein would be present). S/RG/C2 and transfected cell lines did not express detectable levels of IGF-I and only expressed low levels of IGF-II protein; 5.7 ±0.53 ng/10⁶cells, hence the concentration
NaBt-induced apoptosis is increased via an IGF-independent mechanism.

NaBt-induced apoptosis is inhibited when the function of the secreted IGFBP-3 protein is blocked

S/RG/C2 adenoma-derived cells were treated with 4 mM NaBt in the presence or absence of IGFBP-3 neutralizing antibodies. Results are summarized in Figure 7. NaBt-induced apoptosis was inhibited by the addition of IGFBP-3 neutralizing antibodies; the antibody alone had no effect on cell yield and apoptosis (Figure 7A and C). In addition, inhibition of NaBt-induced apoptosis by IGFBP-3 neutralizing antibodies could also be detected in the HT29 carcinoma-derived cell line (Figure 7B and D). These results suggest that IGFBP-3 has to be secreted before potentiating NaBt-induced apoptosis (either through binding to a receptor or through a receptor independent mechanism).

Discussion

In the current study we have investigated the effect of NaBt on the secretion of IGFBP-3, previously shown to be a potent enhancer of p53-dependent radiation-induced apoptosis in colorectal epithelial cells (11). NaBt is known to induce p53 independent apoptosis in colonic cell lines (1,38), and proposed to be important in regulating differentiation and apoptosis in the normal colonic crypt. As IGFBP-3 has been reported to be pro-apoptotic, the aim of this investigation was to determine whether expression and secretion of the IGFBP-3 protein is regulated by NaBt and whether IGFBP-3 regulates NaBt-induced apoptosis in human colonic epithelial cell lines.

It was initially demonstrated that the levels of IGFBP-3 in conditioned medium from both adenoma and carcinoma-derived cell lines correlated with the p53 status of the cells, consistent with the finding that IGFBP-3 is a p53 regulated gene (22). In addition, it has been shown that IGFBP-3 is transcriptionally upregulated in adenoma-derived colonic epithelial cells by NaBt treatment. Recently Leng et al. (34) have shown that NaBt-induced IGFBP-3 expression in LIM 2405 human colon cancer cells. They reported a dose-dependent increase in IGFBP-3 protein in conditioned medium and showed that TSA (a more specific histone deacetylase [HDAC] inhibitor) was able to upregulate IGFBP-3 protein expression. Furthermore, TSA has been shown to upregulate IGFBP-3 mRNA in HEP3B hepatic cell line (44). Walker et al. (32) reported upregulation of the IGFBP-3 promoter activity by both NaBt and TSA in breast cancer cells. They identified a 45 bp butyrate responsive element in the IGFBP-3 promoter and suggest that NaBt increases IGFBP-3 expression by activating the promoter via an Sp1/Sp3 multiprotein complex.

The importance of the findings of this study is that NaBt has been shown to transcriptionally upregulate IGFBP-3 in colonic adenoma-derived cells which express wildtype p53 function. Although transcriptional upregulation is likely to be mediated via inhibition of HDAC activity as reported by Walker et al. (32) it should be noted that Bax was not upregulated in the same cells, and hence may imply some target specificity in the regulation of IGFBP-3 expression (potentially through the SP1/SP3 complex [32]). These findings demonstrate that the levels of IGFBP-3 expressed by epithelial cells in the colonic crypt could be regulated by the concentration of NaBt in the colonic lumen.

Using a transfected adenoma-derived cell line we were able to show that increased IGFBP-3 protein expression (detectable of IGF-II in the flask was < 40 ng/ml. 48 h treatment with between 50 ng/ml and 50 μg/ml IGF-II (from Growpep, Adelaide, Australia) had no effect on S/RG/C2 cell survival (data not shown). Furthermore, when the S/RG/C2 cells were treated with the IGF-I analogue (10–100 ng/ml) which has a markedly decreased binding affinity for IGF binding proteins (Long R3 IGF-I from Gropep, Adelaide, Australia) for 48 h in SFM, there was no effect on cell yield or induction of apoptosis (data not shown). These results indicate that S/RG/C2 cells are insensitive to the anti-apoptotic effects of IGF-I and IGF-II.

In addition, cells were treated with 4 mM NaBt in the presence of the IGF-IR neutralizing antibody (αIR3, previously used to inhibit IGF activity [11]). Results are shown in Figure 6. Addition of the αIR3 antibody had no effect on cell yield or apoptosis in the NaBt-treated cells. Taken together, the data indicate that endogenous IGF-II in the SFM does not affect the growth of the cells, IGFBP-3 does not act through sequestering endogenous IGFs, and therefore the cellular sensitivity to
in conditioned medium from the cells) increased levels of both spontaneous and NaBt-induced apoptosis. The data presented suggest that IGFBP-3 is able to act through an IGF-independent mechanism as the only IGF produced is IGF-II. The adenoma-derived cell line used in this study was shown to be relatively resistant to IGF-II, as reported for other colon cancer cell lines, such as LIM1215 (34). The concentration of IGF-II required to enhance S/RG/C2 cell survival of this cell line was 10³-fold more than found to be produced by the cells (43). In addition, blocking the actions of IGF through the addition of neutralizing antibodies to the IGF-IR was not sufficient to enhance NaBt-induced apoptosis, whereas inhibition of the function of the secreted IGFBP-3 did suppress NaBt-induced apoptosis. This is in agreement with findings from a number of studies in different cell systems where IGFBP-3 has been shown to enhance induced apoptosis through IGF-independent pathways (reviewed in ref. 13).

Previous findings from our laboratory have shown that addition of exogenous IGFBP-3 alone is not sufficient to induce apoptosis in colorectal epithelial cells (11). This suggests that, although NaBt-induced apoptosis can be inhibited by the addition of IGFBP-3 neutralizing antibodies to the medium, IGFBP-3 is not a downstream component in the NaBt-induced apoptotic signalling pathway, and does not directly mediate NaBt-induced apoptosis. Rather that IGFBP-3 interacts with and potentiates the NaBt-induced apoptotic signalling pathway. As levels of IGFBP-3 secreted by transfected cells were equivalent to those produced by NaBt-treated cultures and...
sufficient to increase the apoptotic response of the cells, this suggests the existence of a mechanism for increasing apoptosis in vivo. When cells are exposed to NaBt, there is not only induction of apoptosis, but also the transcriptional upregulation of IGFBP-3, resulting in an increase in secreted IGFBP-3 protein leading to a potentiation of apoptosis. This is of particular importance for the regulation of tissue homeostasis in the colon, as NaBt may be physiologically important in the regulation of apoptotic cell death in this tissue (1), and IGFBP-3 has been shown to be expressed towards the top of the normal colonic crypt (11). The possible therapeutic implications of this work are that by increasing the levels of extracellular IGFBP-3 in the colon, it may be possible to enhance the sensitivity of tumour cells to p53-independent apoptosis and hence act as an adjuvant to existing therapeutic regimes for colorectal carcinogenesis.

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