Induction of apoptosis by the 16-kDa amino-terminal fragment of the insulin-like growth factor binding protein 3 in human colonic carcinoma cells

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Abstract. The insulin-like growth factor binding protein 3 (IGFBP-3) is the major circulating IGF binding protein, its function regulated by proteolytic cleavage. The fragments generated have recently been suggested to have IGF-independent biological activity. We have previously established that IGFBP-3 can potentiate apoptosis in colorectal epithelial cells, although its use as a therapeutic reagent may be limited by the fact that it is cleaved in the circulation. Therefore the aim of these experiments was to determine whether the 16-kDa proteolytic fragment ($^{1\text{-}95}$IGFBP-3) would have IGF-independent pro-apoptotic activity in human colonic carcinoma derived cells. We report that the enforced expression of $^{1\text{-}95}$IGFBP-3 increased the induction of apoptosis by the naturally occurring short chain fatty acid sodium butyrate (NaBt) in the IGF non-responsive HT29 human colorectal carcinoma cell line. Furthermore, the addition of condition medium containing the secreted $^{1\text{-}95}$IGFBP-3 was as effective as the intact IGFBP-3 protein at potentiating apoptosis. Although not associated with changes in Bcl-2, Bcl-X_L, Bax, Bad or Bak expression levels, we report that the expression of the pro-apoptotic $^{1\text{-}95}$IGFBP-3 fragment is associated with the inhibition of TNFα-induced NF-κB activity, similar to that reported for the full length IGFBP-3 protein. These results suggest that the 16-kDa $^{1\text{-}95}$IGFBP-3 fragment is as effective as an intact recombinant protein when used in combination with apoptosis inducing agents, and due to its relative stability in the circulation, it may be important for use as an adjuvant in the treatment of colorectal cancer.

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Introduction

It has been reported that colorectal cancer risk is positively related to the insulin-like growth factor 1 (IGF-1) and inversely related to insulin-like growth factor binding protein 3 (IGFBP-3) (1,2). The IGFBP-3 protein is a multifunctional protein with a complex physiological role. Evidence suggests that IGFBP-3-induced growth inhibition and/or apoptosis may occur by sequestering and inhibiting IGF binding to its cognate receptor (3). However, IGFBP-3 has also been shown to promote apoptosis through an IGF-independent mechanism (4-7). Previous studies from our group have shown that the addition of human recombinant IGFBP-3 protein can potentiate p53-dependent γ-radiation-induced apoptosis in colorectal tumour cells (8) and enhances the sensitivity of colonic adenoma cells to p53-independent apoptosis (9).

Proteolytic modification of IGFBP-3 is thought to be a principal mechanism for regulating not only IGF-1 availability but also the IGF-independent functions of IGFBP-3 (10). Increased IGFBP-3 proteolysis in serum has been reported in cancer (11); in a study of patients with colon cancer, a group with increased IGFBP-3 proteolytic activity suffered fewer metastases than did another with less IGFBP-3 proteolytic activity (12). Three naturally occurring fragments of IGFBP-3 have been detected in human hemofiltrates with molecular mass of 34, 16 and 11-kDa (13). The 16-kDa N-terminal fragment has been shown to have virtually no affinity for IGFs (14), and yet is reported to induce potent growth inhibition and/or apoptosis in both human breast cancer cells (15,16) and prostate PC3 cancer cell lines (17).

We have previously established that both the addition of recombinant IGFBP-3 and the enforced expression of IGFBP-3 can potentiate apoptosis in colorectal epithelial cells (8,9), leading to the possibility that IGFBP-3 may be of use as an adjuvant for existing therapies in colorectal cancer. However, one possible difficulty with this approach is that it would be difficult to maintain the therapeutic levels of intact IGFBP-3 protein in the patient, as its levels are tightly regulated through proteolysis. The report that increased IGFBP-3 proteolytic activity correlates with improved prognosis in colon cancer patients (12), suggests that it is

possible that the IGFBP-3 proteolytic fragments may themselves have pro-apoptotic activity in colorectal cancer cells. Given the potential importance of the IGF system in colorectal cancer, and the fact that there have been no previous studies looking at the affect of IGFBP-3 proteolytic fragments in colorectal cancer cells, the aim of these experiments was to determine whether the $^{1\text{-}95}$IGFBP-3 16-kDa fragment is biologically active in the IGF non-responsive HT29 human colonic carcinoma cell line. In addition, experiments were carried to out to determine the mechanism(s) through which $^{1\text{-}95}$IGFBP-3 may potentiate apoptosis; whether the induction of apoptosis is associated with the regulation of pro-apoptotic (Bax and Bad) to anti-apoptotic (Bcl-2 and Bcl-X_L) proteins (6) and/or inhibition of NF-κB survival signalling (18). The purpose is to establish whether the IGFBP-3 fragment (which unlike the intact glycosylated protein is not cleaved in the circulation), could enhance the induction of apoptosis in colorectal tumour cells. This would raise the possibility that the 16-kDa fragment may have potential as a possible therapeutic adjuvant in colorectal cancer.

Materials and methods

Cell lines, culture media and cell treatment. The human colonic carcinoma derived cell line HT29 was studied. This cell line has a p53 mutation (273 Arg-His), is IGF-1 non-responsive and has undetectable expression levels of IGFBP-3 (19). For standard growth condition, the HT29 cell line was grown in DMEM (Invitrogen, UK) supplemented with 10% fetal bovine serum (batch selected), glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml).

Construction of N-terminal 16-kDa fragment of IGFBP-3. Human IGFBP-3 cDNA in pCP2A was cut with *Eco*RI restriction enzyme and cloned into the predigested expression vector pCDNA3.1 (Invitrogen). The cDNA of the IGFBP-3 fragment encoding the amino-terminal was generated by PCR amplification from the human IGFBP-3 cDNA using forward primer 5' CGC GGA TCC GGC GTC ATG CAG CGG GCG; and reverse primer 5' CGA ATT CCT AGC GGC TGA CGG CAC TAG (16). *Bam*HI and *Eco*RI restriction sites were incorporated into the 5' and 3' ends of the coding sequence to permit subsequent cloning of the PCR fragment into the expression vector, pcDNA3.1

Transfection of HT29 cells. Sub confluent HT29 cells were transfected with either the full IGFBP-3 cDNA (2.4 kb, a gift from Professor Jeff Holly, Division of Surgery, Bristol Royal Infirmary, Bristol, UK) or the N-terminal 16-kDa IGFBP-3 cDNA (376 bp) in the pCDNA3.1 expression vector. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, and selected using 200 μg/ml G418 (Sigma). Single colonies were expanded, cell lines established and examined for gene expression at the mRNA and protein levels.

RNA preparation, cDNA synthesis and RT-PCR. Total cellular RNA was isolated using the RNeasy RNA mini kit (Qiagen) according to the manufacturer's instructions. RNA (1 μg) was used for cDNA synthesis using the cDNA Synthesis kit (Promega). RT-PCR screening was performed on the HT29 parental cell line, HT29 cells transfected with the empty expression vector and cells transfected with IGFBP-3 or the 16-kDa $^{1\text{-}95}$IGFBP-3 fragment. The primer pairs used in this study for PCR screening were the cloning primers at positions 126-502 of IGFBP-3 cDNA.

Northern blot. Northern analysis of total RNA was carried out using 'Northern Max' kit (Ambion Inc.). The probe was an *Eco*RI/*Bam*HI fragment of IGFBP-3 which was radiolabelled with [α-^{32}P]-deoxycytidine triphosphate (Amersham Bioscience) using rediprime II labelling system (Amersham Bioscience). Blots were exposed to Biomax MS film (Kodak) and membranes were then re-probed with a PCR generated fragment of GAPDH, which acted as a loading control for the RNA samples.

Treatment with sodium butyrate (NaBt). Cells were seeded in triplicate flasks and grown under standard growth conditions until ~70% confluent. Previous investigations showed that HT29 cells were able to tolerate serum-free growth conditions (SFM) for up to 96 h (SFM = standard non-conditioned growth medium without the addition of FBS) (9). 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 (9). Attached and floating (apoptotic, refer below) cell yields were determined from parallel flasks treated for 48 h.

Medium exchange experiments. Sub-confluent monolayers of HT29 cells either expressing IGFBP-3, 16-kDa $^{1\text{-}95}$IGFBP-3 fragment or the empty expression vector were grown for 24 h in SFM. The cells were then grown for 48 h in SFM, the media harvested, centrifuged to remove floating cells and stored at -70˚C. Attached cell numbers were determined using a Neubauer counting chamber (VWR, UK). The secretion of IGFBP-3 or the 16-kDa N-terminal fragment was verified by SDS PAGE immunoblotting, and the proteins detected by anti-IGFBP-3 antiserum (polyclonal from Diagnostic Systems Laboratory). Proteins from the conditioned medium were concentrated (approximately x5) using Millipore Ultrafree-MC filter units and volumes of media loaded onto the gel were adjusted for equivalent cell numbers ($5x10^6$ cells). Parental HT29 cells were seeded in triplicate flasks and grown under standard growth conditions until 70% confluent. Cells were grown for 24 h in SFM and then for up to 48 h in the conditioned medium from IGFBP-3 or 16-kDa $^{1\text{-}95}$IGFBP-3 expressing HT29 cells ±4 mM NaBt (Sigma). The conditioned medium from the HT29 cells transfected with the empty vector was used as a control for this study. Attached and floating (apoptotic, refer below) cell yields were determined.

Apoptosis assays. The level of apoptosis was assessed by measuring the proportion of the total cell population that detached from the monolayer and was floating in the medium and by determining the fraction of these floating cells that were apoptotic, as described (8). The attached and floating

cell populations were stained with 5 μg/ml acridine orange in PBS, and analysed by fluorescent microscopy for morphological features of apoptosis (most obviously the characteristically condensed chromatin, as previously described) (20,21). As the fraction of floating cells that were apoptotic did not vary between the treated and the 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 (22).

Bcl-2, Bcl-X_L, Bad, Bak and Bax protein expression. The levels of Bcl-2, Bcl-X_L, Bad, Bak and Bax were assessed by SDS PAGE immunoblotting, detected by the monoclonal antibodies Bcl-2 (100) Santa Cruz, USA; Bcl-$X_{L/S}$ (L-10) polyclonal, Santa Cruz; Bad Cell Signaling Technology Inc., USA; Bak Pharmingen (BD) Europe; Bax (n-20) Santa Cruz, respectively) using an ECL detection system (KPL, USA).

NF-κB reporter assays. Cells were transiently transfected with either the NF-κB reporter plasmid pNF-κB-TA-luc or with the control reporter plasmid pTA-luc (Clontech, BD, Europe). pNF-κB-TA-luc contains four copies of a consensus NF-κB binding sequence (GGGAATTTCC) in addition to a minimal promoter (P_{TA}, the TATA box from the herpes simplex virus thymidine kinase promoter) located upstream of the firefly luciferase (*luc*) gene. The consensus NF-κB binding sequences are absent from the control vector pTA-luc. All transfections also included the renilla luciferase vector pRL-SV40 (Promega, UK) as an internal control for transfection efficiency.

For transient transfection, all cells were grown to 70% confluence in T12.5 flasks. Triplicate flasks were co-transfected with one of the two reporter constructs (pTA-luc or pNF-κB-TA-luc) and with the renilla construct (pRL-SV40) in a ratio of 50:1. Each flask was incubated for 6 h with 2 μg of plasmid DNA and 5 μl of Lipofectamine 2000 (Invitrogen) diluted in Opti-MEM serum-free medium according to manufacturer's instructions. Following transfection, cells were allowed to recover overnight prior to 24 h treatment with TNFα (100 ng/ml for 24 h) in SFM.

Luciferase reporter assay. Twenty-four hours after treatment, cells were washed in PBS and lysates prepared in 1X PLB (Promega, UK) according to manufacturer's instructions. Reporter activity was measured using the Dual-Luciferase reporter assay system (Promega) and a Jade Luminometer (Labtech, UK) set for a 10 sec read. Sample readings were corrected for background autoluminescence using untransfected cells as a control.

Statistical analysis. The data represents the mean of 3 separate experiments (each experiment was carried out in triplicate parallel flasks). Statistical analysis was carried out using SPSS for Windows statistical software (release 10.0.5, SPSS Inc. Chicago, IL, USA). Two-way analysis of variance (ANOVA) was used to determine the differences amongst the means. Pairwise comparisons were made using Tukey's *post hoc* test for multiple comparisons.

Results

Expression of IGFBP-3 or the 16-kDa $^{1\text{-}95}$IGFBP-3 fragment in the HT29 carcinoma cell line. HT29 cells were stably transfected with either intact IGFBP-3 or the $^{1\text{-}95}$IGFBP-3 fragment, and expression was determined by RT-PCR, Northern hybridization and Western blotting. Results are summarized in Fig. 1. The PCR products (Fig. 1A) were generated using the same 5' to 3' oligonucleotide primers as designed for the N-terminus of IGFBP-3 cDNA; hence a 376 bp product (confirmed by sequence analysis, data not shown) was generated in the cells expressing either the whole gene or the $^{1\text{-}95}$IGFBP-3 fragment. No amplification of IGFBP-3 sequences was found in parental or in cells transfected with the empty expression vector (vector control cells). To further confirm expression, the intact IGFBP-3 and $^{1\text{-}95}$IGFBP-3 transcripts were detected by Northern analysis using the PCR generated fragment as the probe (Fig. 1B). Once again there was no detectable mRNA of endogenous IGFBP-3 in the parental and vector control cells; these findings are consistent with the published data showing non-expression or very low expression of IGFBP-3 in the HT29 cell line (9,19). In addition, secreted IGFBP-3 derived proteins from transfected cells could be detected by Western analysis [the 16-kDa $^{1\text{-}95}$IGFBP-3 fragment in Fig. 1C(i) and 42-kDa IGFBP-3 protein in Fig. 1C(ii)].

IGFBP-3 or the 16-kDa $^{1\text{-}95}$IGFBP-3 fragment increases sodium butyrate-induced apoptosis in HT29 cells. Sodium butyrate (NaBt) is a naturally occurring short chain fatty acid which has been shown to induce apoptosis in colorectal epithelial cells (20). It has been proposed that NaBt may have potential as a chemopreventive/therapeutic agent for colorectal cancer, and that IGFBP-3 may act as a positive regulator of NaBt-induced apoptosis in colonic epithelial cells (9). The aim was to investigate whether HT29 cells expressing either the whole IGFBP-3 protein or the 16-kDa N-terminal fragment were more sensitive to NaBt-induced apoptosis than vector control cells. The results are summarized in Fig. 2. Enforced expression of the $^{1\text{-}95}$IGFBP-3 fragment significantly enhanced NaBt-induced apoptosis as compared to the vector control cells. Similar results were obtained when compared to the parental cell line (data not shown). Importantly, the expression of the $^{1\text{-}95}$IGFBP-3 fragment was as potent as the whole IGFBP-3 protein at increasing NaBt-induced apoptosis in HT29 cancer cells.

We then assessed whether protein secreted from the HT29 transfected cells (either IGFBP-3 or the 16-kDa $^{1\text{-}95}$IGFBP-3 fragment) could increase NaBt-induced apoptosis in the parental non-transfected cell line. HT29 cells were grown to 70% confluence and treated with 4 mM NaBt in conditioned medium from the IGFBP-3 or 16-kDa $^{1\text{-}95}$IGFBP-3 expressing cells. Conditioned medium from the vector control cells was added to the parental HT29 cell cultures as a control for the experimental procedure. The results are summarized in Fig. 3. Both the IGFBP-3 and the $^{1\text{-}95}$IGFBP-3 secreted proteins in the CM significantly increased NaBt-induced apoptosis ($P<0.01$). Once again, the fragment was as potent as the whole IGFBP-3 protein at increasing apoptosis.

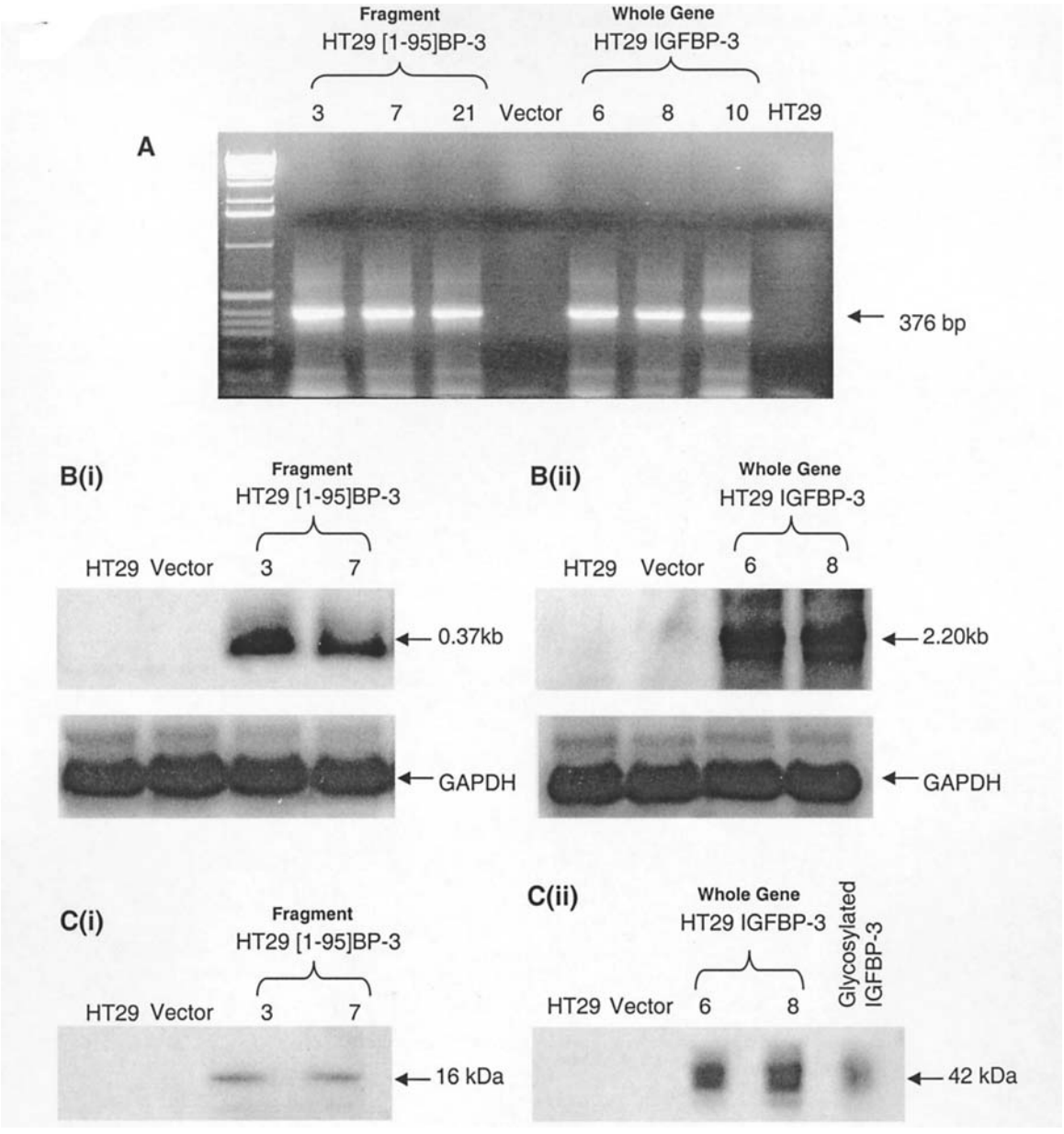

Figure 1. Expression of the16-kDa $^{1\text{-}95}$IGFBP-3 fragment or IGFBP-3 in HT29 cells. (A) Expression of the16-kDa $^{1\text{-}95}$IGFBP-3 fragment or IGFBP-3 in HT29 cells as shown by RT-PCR. Using primers which amplify N-terminal sequences, the expression of the whole gene or 16-kDa fragment was detected in independent clones isolated after transfection with either the 16-kDa $^{1\text{-}95}$IGFBP-3 fragment (results for 3 independent clones) or IGFBP-3 cDNA (results for 3 independent clones). No expression was detected either in the HT29 cells transfected with the empty expression vector or the parental HT29 cell line. Molecular weight markers are shown in lane 1. Numbers above lanes denote individual clones analysed. (B) Northern analysis to show $^{1\text{-}95}$IGFBP-3 or IGFBP-3 mRNA expression in HT29 transfected cells. (i) Expression of the 16-kDa $^{1\text{-}95}$IGFBP-3 fragment [(1-95)BP-3]. (ii) Expression of the exogenous whole IGFBP-3. Results are presented for 2 independent clones. GAPDH was used to control for RNA loading. (C) Western blot showing the 16-kDa $^{1\text{-}95}$IGFBP-3 fragment or intact IGFBP-3 protein secreted by transfected HT29 cells. Aliquots of 5x concentrated conditioned serum free medium from $5x10^6$ transfected cells were analysed by SDS-PAGE, the protein detected by anti-IGFBP-3 antiserum. (i) Expression of the N-terminal $^{1\text{-}95}$IGFBP-3 protein fragment (16-kDa). (ii) Expression of the IGFBP-3 protein (42-kDa). No protein was detected in conditioned medium from the parental or vector control cell lines. (Glycosylated IGFBP-3 (100 ng) (Gropep, Aus) is shown as a control. IGFBP-3 resolves as a number of bands on electrophoresis, representing post-trancriptionally modified protein (9). Numbers above lanes denote individual clones analysed.

Taken together these results demonstrate that the 16-kDa N-terminal fragment ($^{1\text{-}95}$IGFBP-3) is a pro-apoptotic regulator in human carcinoma derived colorectal epithelial cells. In addition, the $^{1\text{-}95}$IGFBP-3 fragment is as potent as the whole IGFBP-3 protein at increasing both spontaneous and NaBt-induced apoptosis in the HT29 cell line.

IGFBP-3 but not the 16-kDa $^{1\text{-}95}$IGFBP-3 fragment inhibits Bcl-2 expression in HT29 cells. IGFBP-3 has previously been suggested to favour apoptosis by increasing the ratio of pro-apoptotic (Bax and Bad) to anti-apoptotic (Bcl-2 and Bcl-X_L) proteins in breast cancer cells (6). To elucidate the mechanism through which the $^{1\text{-}95}$IGFBP-3 fragment increases apoptosis, the levels of Bcl-2, Bcl-X_L, Bax, Bak or Bad were determined and the results summarized in Fig. 4A. Neither enforced expression of IGFBP-3 nor the 16-kDa NBP-3 fragment had any effect on the level of Bcl-X_L, Bax, Bak or Bad protein expression. However, expression of the whole IGFBP-3 did

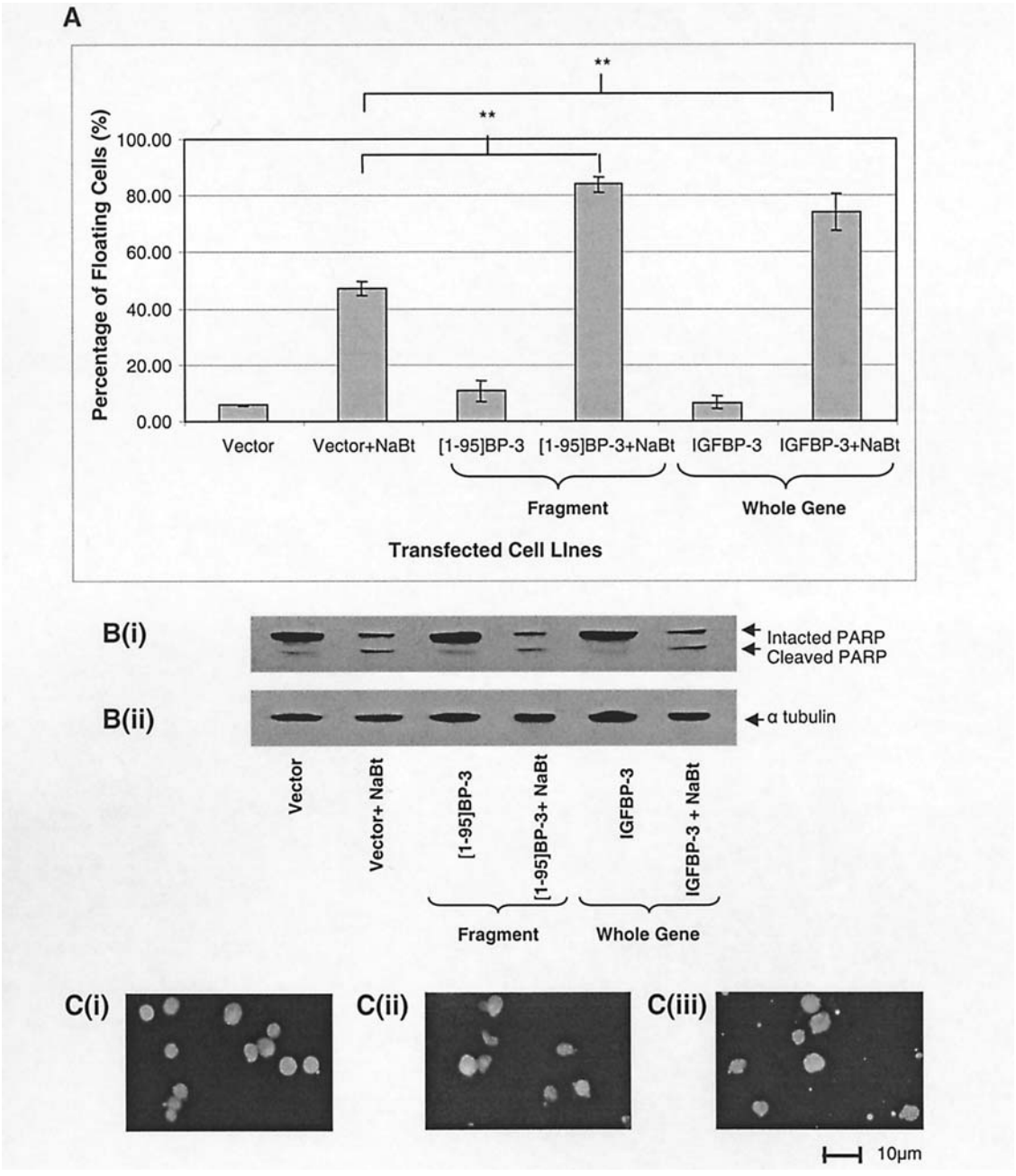

Figure 2. IGFBP-3 and the 16-kDa $^{1\text{-}95}$IGFBP-3 fragment increase sodium butyrate-induced apoptosis in HT29 cells. (A) Induction of apoptosis in the HT29 carcinoma derived cell line stably expressing either the 16-kDa $^{1\text{-}95}$IGFBP-3 fragment [(1-95)BP-3] or the whole protein (IGFBP-3) after 48 h treatment with 4 mM NaBt in SFM. The results shown are the mean of 3 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 (i) PARP expression and (ii) α-tubulin (loading control), in mixed attached and floating HT29 cell populations 48 h after treatment±NaBt (4 mM). Cleavage of PARP in the NaBt treated cells indicates induction of apoptosis above spontaneous levels. (C) Acridine orange staining of mixed attached and floating HT29 cells from the NaBt treated (i) vector control, (ii) $^{1\text{-}95}$IGFBP-3-expressing [(1-95)BP-3], and (iii) IGFBP-3 expressing cell populations, showing the morphological characteristics of apoptosis.

significantly suppress Bcl-2 protein expression in HT29 cells, although it should be stressed that this was not seen in other human colorectal adenoma (S/RG/C2) or carcinoma (SW480) derived cell lines tested, where expression of Bcl-2, Bcl-X_L, Bax, Bak or Bad did not change (data not shown). In contrast, the expression of the 16-kDa $^{1\text{-}95}$IGFBP-3 fragment had no effect on Bcl-2 protein expression (Fig. 4A).

The 16-kDa $^{1\text{-}95}$IGFBP-3 fragment inhibits NFκB activity in TNFα-treated HT29 cells. We have recently reported that IGFBP-3 can potentiate apoptosis through inhibition of the NF-κB survival pathway (18). The aim of this investigation was to determine whether the expression of the $^{1\text{-}95}$IGFBP-3 fragment also inhibited NF-κB activation. As the death receptor mediated apoptotic signalling results in concurrent activation of the NF-κB survival pathway (23), we used a reporter assay to determine NF-κB activation in the 16-kDa $^{1\text{-}95}$IGFBP-3-expressing HT29 cells treated with TNFα (100 ng/ml for 24 h). The results are summarized in Fig. 4B. Expression of the $^{1\text{-}95}$IGFBP-3 fragment suppresses NF-κB

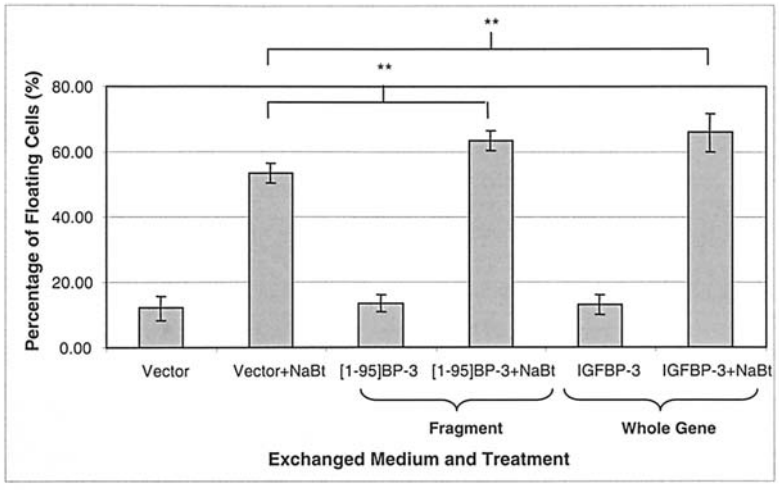

Figure 3. Addition of IGFBP-3 protein or the 16-kDa $^{1\text{-}95}$IGFBP-3 fragment increases NaBt-induced apoptosis in the parental non-transfected HT29 cell line. Induction of apoptosis in the parental HT29 carcinoma derived cell line after 48 h treatment with conditioned medium containing either the 16-kDa $^{1\text{-}95}$IGFBP-3 fragment [(1-95)BP-3 CM] or the whole IGFBP-3 protein (IGFBP-3 CM)±4 mM NaBt. The results shown are the mean of 3 separate experiments±SEM, statistical difference determined by two way ANOVA with replication, *P<0.05, **P<0.01, ***P<0.001. The conditioned medium from the HT29 cells transfected with the empty vector was used as a control for this study. Cells were confirmed as apoptotic by changes in morphology as detected by acridine orange staining and cleavage of PARP as described in detail previously (9,18) (data not shown).

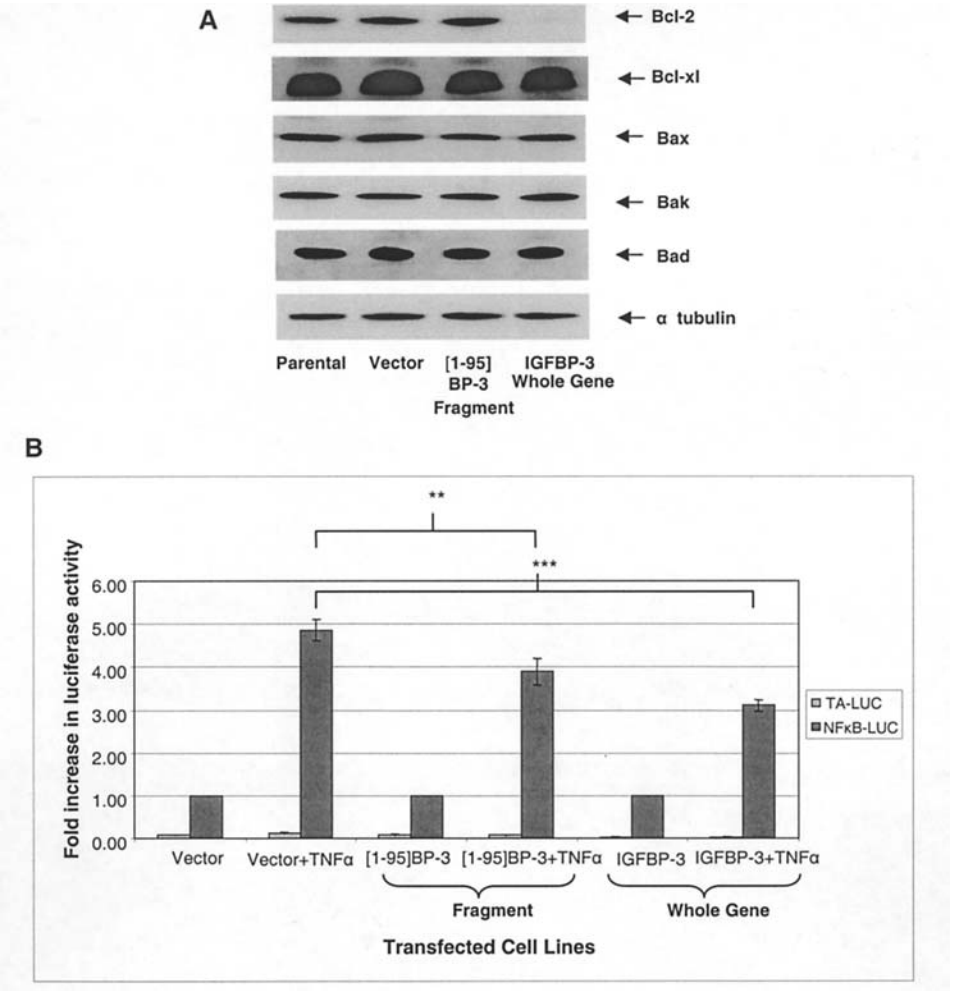

Figure 4. (A) IGFBP-3 but not the 16-kDa $^{1\text{-}95}$IGFBP-3 fragment inhibits Bcl-2 expression in HT29 cells. Western blot showing Bcl-2, Bcl-xl, Bax, Bak, Bad and α-tubulin (loading control) protein expression in HT29 cells expressing the 16-kDa $^{1\text{-}95}$IGFBP-3 fragment [(1-95)BP-3] or the whole IGFBP-3 protein (IGFBP-3). Expression levels are compared to vector control and non-transfected parental HT29 cells. (B) IGFBP-3 and the 16-kDa $^{1\text{-}95}$IGFBP-3 fragment inhibit NF-κB activity in TNFα-treated HT29 cells. The effect of enforced expression of the 16-kDa $^{1\text{-}95}$IGFBP-3 fragment [(1-95)BP-3] or the whole IGFBP-3 protein (IGFBP-3) on NF-κB activation in TNFα-treated (100 ng/ml for 24 h) HT29 cells. pNF-κB-TA-luc:firefly renilla ratios are expressed as a percentage of vector only untreated cells, results shown are representative of 4 independent experiments ±SEM, statistical difference determined by two way ANOVA with replication, *P<0.05, **P<0.01, ***P<0.001.

activation, suggesting that the pro-apoptotic activity of the 16-kDa $^{1-95}$IGFBP-3 fragment may also be mediated through the inhibition of the NF-κB survival pathway.

Discussion

In the current study we show that the enforced expression of the $^{1-95}$IGFBP-3 fragment is able to increase the sensitivity of HT29 cells to the induction of apoptosis by NaBt treatment. As HT29 cells have mutant p53 protein expression (24) and are IGF non-responsive (19), this induction of apoptosis is both p53 and IGF-1-independent. Furthermore, as cell-association sites have not been reported in the amino terminus of IGFBP-3, the inhibitory effects of $^{1-95}$IGFBP-3 may involve the internalization of the protein and subsequent intracellular actions (15). Significantly, we have demonstrated that the $^{1-95}$IGFBP-3 amino fragment has a pro-apoptotic function equivalent to that of the whole protein in colorectal cancer cells.

The mechanism through which the 16-kDa $^{1-95}$IGFBP-3 fragment of IGFBP-3 increases apoptosis remains unclear. Previous reports have suggested that IGFBP-3 may favour apoptosis by increasing the ratio of pro-apoptotic (Bax and Bad) to anti-apoptotic (Bcl-2 and Bcl-X_L) proteins (6). However, in the current study neither the enforced expression of IGFBP-3 nor the 16-kDa $^{1-95}$IGFBP-3 fragment had any effect on the level of Bax, Bad, Bak or Bcl-X_L protein expression. Expression of the whole IGFBP-3 protein did significantly suppress Bcl-2 protein expression in HT29 cells (although not in other human colorectal cell lines). In contrast, the expression of the 16-kDa $^{1-95}$IGFBP-3 fragment had no effect on Bcl-2 protein expression. The significance of this finding remains unknown; whereas increased apoptotic susceptibility mediated by IGFBP-3 protein(s) may be attributed in part to the regulation of the Bcl-2 protein family in a subset of tumours, the molecular mechanism underlying the increased susceptibility in the $^{1-95}$IGFBP-3 expressing cells was not due to suppression of Bcl-2 expression. However, expression of the 16-kDa $^{1-95}$IGFBP-3 fragment was shown to suppress NF-κB activation (as does the whole protein) (18) and therefore we propose that the pro-apoptotic activity of the 16-kDa $^{1-95}$IGFBP-3 fragment may also be mediated through the suppression of the NF-κB survival pathway. As NaBt has previously been reported to induce apoptosis at least in part through the inhibition of constitutive NF-κB activity in cancer cells (25,26), data presented here would suggest that both IGFBP-3 and the 16-kDa $^{1-95}$IGFBP-3 fragment could potentiate NaBt-induced cell death through acting synergistically with NaBt, to suppress NF-κB activity.

IGFBP-3 has previously been proposed as a possible adjuvant to existing cancer therapy, although one potential difficulty with the therapeutic use of IGFBP-3 is proteolysis of the whole protein in the circulation and cell environment (10). Results from this study suggest that the addition of the proteolytic N-terminal fragment of IGFBP-3 may be as effective as the whole protein in enhancing the induction of apoptosis, with the advantage that it has low affinity and hence would not disrupt IGF signalling in normal tissues. Another advantage in administering the $^{1-95}$IGFBP-3 fragment rather than the whole protein is that, although proteolysis of IGFBP-3 would increase the levels of the 16-kDa fragment, it also yields a number of other protein fragments, the largest of which (22-25 kDa) may have opposing action to the 16-kDa $^{1-95}$IGFBP-3 fragment, as they have been reported to induce cell growth in prostate cancer cells (17). In summary, we have shown for the first time that the N-terminal 16-kDa fragment of IGFBP-3 ($^{1-95}$IGFBP-3) enhances the effect of apoptosis-inducing agents in colon carcinoma cells *in vitro* and suggest that $^{1-95}$IGFBP-3 could play a role in increasing the actions of anticancer drugs *in vivo*.

Acknowledgements

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