

Differential expression of IGF-binding protein-3 in normal and malignant colon and its influence on apoptosis

P J Jenkins, S Khalaf, W Ogunkolade, K McCarthy, T David, R E Hands¹, D Davies² and S A Bustin¹

Department of Endocrinology, William Harvey Research Institute, Barts and the London Queen Mary's School of Dentistry, John Vane Building, Charterhouse Square, London EC1M 6BQ, UK

¹Cancer Research UK, 61 Lincoln's Inn Fields, London WC2A 3PX, UK

²Department of Academic Surgery, Barts and the London Queen Mary's School of Dentistry, University of London, Turner Street, London E1 2AD, UK

(Requests for offprints should be addressed to P J Jenkins at the Department of Endocrinology, St Bartholomew's Hospital, West Smithfield, London EC1A 7BE, UK; Email: P.J.Jenkins@qmul.ac.uk)

Abstract

IGF-binding protein-3 (IGFBP-3) has been reported to exert a protective influence on the pathogenesis of colorectal cancer. This may reflect its modulation of IGF-I bioactivity as well as IGF-I-independent effects on cell proliferation and apoptosis. Although local expression of IGF-I in the colon is increasingly recognised as having important regulatory consequences, the role of locally expressed IGFBP-3 remains unknown. The aims of the present study were: (i) to quantify and localise the expression of IGFBP-3 in human normal and malignant colon; (ii) to relate this expression to that of other components of the IGF-I axis; and (iii) to investigate the effects of IGFBP-3 on colonic epithelial cell proliferation and apoptosis. RNA was extracted from 46 paired samples of normal and malignant colonic tissue. IGFBP-3, IGF-I, IGF-I receptor and GH receptor mRNA levels were quantified using real-time RT-PCR. Laser-capture microdissection of the same samples was used to isolate mRNA from epithelium and stromal components and localise mRNA expression. Expression was confirmed at a protein level by immunohistochemistry. Human colorectal cancer HT-29 and CaCo-2 cells were cultured with IGFBP-3 (200 ng/ml), +/- IGF-I (20 ng/ml), +/- sodium butyrate (5 mM). Cell number was assessed by an MTS assay (a modification of the MTT assay), and apoptosis assessed by cell morphology and FACS analysis using both annexin and propidium iodide staining. UO146, a MAP kinase inhibitor, and wortmannin, an inhibitor of the phosphatidylinositol 3-kinase (PI-3K) pathway, were used to determine the contribution of these signalling pathways on the effects of IGFBP-3. IGFBP-3 mRNA was detected in all samples (mean copy number/ μ g total RNA in normal colon, 2.6×10^6 compared with 1.3×10^7 in the cancers; $P < 0.0001$). Immunohistochemistry confirmed the expression and showed it to be equally distributed between epithelial and stromal components in normal tissue, but to be mainly restricted to the stromal component of malignant tissue. This differential expression was confirmed by RT-PCR of RNA from laser-capture microdissected samples. IGF-I mRNA was detected in 31 samples of normal colon; mean IGFBP-3 copy number was higher in the IGF-I-positive samples compared with IGF-I-negative samples. IGFBP-3 on its own induced apoptosis in HT-29 cells ($P < 0.001$). Co-incubation of 200 ng/ml IGFBP-3 with butyrate (5 mM) resulted in the potentiation of its apoptosis ($P < 0.0001$), which was not rescued by co-incubation with IGF-I ($P < 0.0001$). The addition of UO126 caused a decrease in cell number and increased the effects of IGFBP-3. IGFBP-3 is differentially expressed between stromal and epithelial components of normal and malignant colon, which may reflect its pro-apoptotic, IGF-I-independent effect on colonic epithelial cells. These effects are mediated in part by the PI-3K pathway in contrast to the MAP kinase pathway used by IGF-I.

Endocrine-Related Cancer (2005) 12 891–901

Introduction

Circulating insulin-like growth factor binding protein 3 (IGFBP-3) and insulin-like growth factor-I (IGF-I) are primarily hepatic-derived peptides that modulate cellular growth, differentiation and apoptotic responses. While serum levels of IGF-I, which are regulated by growth hormone (GH), are generally considered to be a positive risk factor for the development of colorectal cancer, the role of IGFBP-3 appears less clear. Some studies suggest it exerts a protective effect (Ma *et al.* 1999, Giovannucci *et al.* 2000) while others do not support such a role (Renehan *et al.* 2004). In addition to modulating the bioavailability of IGF-I, IGFBP-3 is also an important mediator of growth inhibitory agents, such as retinoic acid, vitamin D, transforming growth factor- β , anti-oestrogens, tumour necrosis factor- α and p53, independently of the IGF signalling system (Gill *et al.* 1997, Oh 1997, Baxter *et al.* 2000). Although IGF-I-independent growth inhibition by IGFBP-3 has been reported in colonic cell lines, a regulatory role in the colon remains uncertain. Positive immunostaining for IGFBP-3 protein has previously been reported in colonic cancers and it appears to be differentially expressed along the colonic crypt (Michell *et al.* 1997, Williams *et al.* 2000, Miraki-Moud *et al.* 2001). However, immunohistochemical staining cannot distinguish circulating IGFBP-3 protein from local paracrine/autocrine expression. This is particularly relevant given recent work emphasising the importance of autocrine and paracrine actions of IGF-I in normal growth and development (Le Roith *et al.* 2001), effects which we have also demonstrated in colorectal tumourigenesis and tumour neovascularisation (Bustin *et al.* 2002). Furthermore, the functional relevance of any expression of IGFBP-3 in the colon is also unknown as is its relationship to components of the IGF-I axis.

Dietary factors appear to play an important role in both the development and prevention of colorectal cancer. One such factor, sodium butyrate, has potent *in vitro* anti-tumourigenic effects on many colon cancer cell lines, including inhibition of growth and promotion of apoptosis. A recent report has indicated a significant increase in apoptosis accompanying the induction of IGFBP-3 in a colorectal adenoma cell line by butyrate (Collard *et al.* 2003). IGFBP-3 by itself has no effect on the viability of these cells but initiates an increase in p53-dependent apoptosis to previously gamma-irradiated cells (Williams *et al.* 2000). Interestingly, in mammary epithelial cells these pro-apoptotic effects of IGFBP-3 are limited to cancerous cells, which may point to an important mechanism for

growth inhibition by IGFBP-3 in cancer (McCaig *et al.* 2002). Altogether these observations suggest that IGFBP-3 may be a major downstream effector of growth inhibitory and apoptosis-inducing agents in cancers. As there have been no studies comparing the simultaneous expression of IGFBP-3 in normal and malignant colonic tissue, the aims of this study were first to quantify and localise expression of IGFBP-3 in normal and malignant human colon tissue, secondly to relate this expression to that of IGF-I and its receptor (IGF-IR) as well as the GH receptor (GH-R), thirdly to determine any association between this expression and the clinical phenotype of colon cancer, and fourthly to determine the IGF-I-independent effects of IGFBP-3 on colon epithelial cell proliferation and apoptosis.

Materials and methods

Patient samples

This study was approved by the Local Research Ethics Committee of the East London and City Health Authority and all patients gave informed consent. Paired samples of normal and malignant colon were taken from 46 consecutive patients undergoing surgery for colorectal carcinoma. Sixteen cancers were located in the proximal colon, and 30 distal to the splenic flexure.

RT-PCR

RNA was extracted from the samples as previously described (Bustin *et al.* 1999) except that the samples were homogenised in 2 ml cryotubes using a bead mill (Glen Creston Ltd, Stanmore, Middx, UK), with three steel beads per 0.5 ml lysis buffer. RNA quality and quantity was assessed, in triplicate, using RNA LabChips together with an Agilent 2100 Bioanalyser (Agilent Technologies, Waldbronn, Germany). Primers and probes were newly designed using Primer Express software (ABI, Warrington, Cheshire, UK) (Table 1). Real-time RT-PCR assays were performed according to the criteria previously described (Bustin 2000). Reactions were carried out and results recorded and analysed using the ABI 7700 Prism Sequence detection system. mRNA levels were quantified relative to amplicon-specific standard curves (Bustin 2000). Copy numbers were normalised relative to total RNA concentration and expressed as copy numbers/ μ g RNA. All serial dilutions were carried out in duplicate. The reactions to generate standard curves were repeated twice, each time in triplicate. All clinical samples were tested in triplicate and the average

Table 1 Primers and probes for real time RT-PCR mRNA quantification of IGFBP-3, IGF-I, IGF-IR and GH-R genes

Target gene	Primers and probes (5'-3')	Amplicon size (bp)
IGFBP-3	F: AGCACAGATACCCAGAACTTCTCC R: TCCATTCTCTACGGCAGGG P: CATATTCTGTCTCCCGCTTGGACTCGG	76
IGF-I	F: CTTCAGTTCGTGTGTGGAGACAG R: CGCCCTCCGACTGCTG P: TTTTATTTCAACAAGCCCACAGGGTATGGC	75
IGF-IR	F: CTCCTGTTTCTCTCCGCCG R: ATAGTCGTTGCGGATGTGCAT P: TGGCCCGCAGATTTCTCCACTCGT	84
GH-R	F: TTTGGAATATTTGGGCTAACAGTG R: TCACCTCCTCTAATTTTCCTTCCTT P: AAGGATTAATAATGCTGATTCTGCCCCAGT	84

F, forward, R, reverse primers; P, Taqman probe.

value of the triplicates was used for quantification. Two no-template controls were included with every amplification run; one was prepared before opening all the tubes and dispensing the various reagents, the other at the end of the experiment.

Laser-capture microdissection and RNA preparation

An empty, labelled cryomould was placed on dry ice for 1 min. The bottom of the cryomould was then lightly covered with OCT medium (Cryo-M-Bed; Bright Instruments, Huntingdon, Cambridgeshire, UK) and the tissue was placed against the bottom of the cryomould. Additional OCT medium was added to the cryomould until the tissue was completely covered and left to harden on dry ice. Blocks were placed into individual Petri dishes and stored at -80°C until required. Fresh frozen blocks were left to pre-cool for 15–20 min in the cryostat (-20°C) to avoid shattering of the sections. The cryostat was cleaned thoroughly (RNase-free) before sectioning and blades were changed for each block to avoid cross contamination. Seven-micron thick sections were mounted onto specialised membrane-covered slides (PALM Technologies, Bernried, Germany) and immediately fixed in ice-cold 70% ethanol. The sections were then dehydrated in an ethanol series (75, 95 and 100% on ice), xylene treated and left to air dry briefly. Sections were laser dissected within 1 h. Slides were observed using a Zeiss Axiovert 200 M inverted laser-capture microscope and visualised on a monitor using the PALM Robo-Software. Individual crypts were identified (minimum of 200) and cut out along pre-drawn lines (hand drawn circles around the crypts) using the laser. Then using a separate catapult function (LPC), the individual crypts

were catapulted up into specialised PALM adhesive caps. Approximately equal areas of stromal tissue and crypts were laser dissected and collected separately. The individual caps were then placed onto RNase-free Eppendorf tubes and tissue was immediately lysed with lysis buffer. RNA was extracted using the RNeasy Microkit (Qiagen) and microdissection protocol. Both a poly-A RNA carrier and an equal volume of 70% ethanol were added to the lysate and mixed thoroughly. The lysate was transferred to a MinElute spin column and centrifuged at $8000g$ for 15 s. The column was washed with buffer, centrifuged and the bound RNA was DNase treated (15 min at room temperature). The column was washed again with buffer and 80% ethanol and centrifuged at $8000g$ for 2 min. The column was centrifuged for a further 5 min to allow the silica gel membrane to dry. To elute the RNA, 25 μl elution buffer were put directly onto the silica gel membrane and left to incubate for 10 min at room temperature. The sample was then spun at $8000g$ for 1 min; this step was repeated to maximise yield. All samples were put at -70°C for long-term storage.

Immunocytochemistry

Four-micron sections were cut from paraffin-embedded blocks of the cancers and paired normal colonic tissue, including all those used for laser microdissection, and deparaffinised by standard techniques. For antigen retrieval, sections were microwave treated under pressure for 4 min at 900 W in preheated 0.01 M citrate buffer pH 6.0. Sections were then blocked with horse serum for 5 min to block non-specific binding before incubating with the primary rabbit anti-human affinity-purified polyclonal IGFBP-3 antibody (GroPep, Adelaide, Australia) at a 1/50

dilution for 90 min at 37°C. After washing off the primary antibody in Tris-buffered saline, slides were incubated with a secondary biotinylated antibody (Vectastain Universal Elite ABC kit; Vector Labs, Peterborough, Cambs, UK) at room temperature for 30 min and rinsed again in Tris-buffered saline. A tertiary peroxidase-labelled avidin was subsequently applied at room temperature for 20 min before the sections were visualised both with a Tyramide signal amplification fluorescein system (NEN Life Science Products, Boston, MA, USA) for 5 min and with activated 3,3'-diaminobenzidine tetrahydrochloride solution (Kentec DAB Tablets 4170; Biostat, Stockport, UK) for 10 min. Sections of human liver were used as a positive control in each run. Specificity of the antibody was confirmed by the absence of immunoreactivity after pre-incubation of the antibody with IGFBP-3. Assessment was performed in a blinded manner and a score determined in both the epithelial cells and the stroma of the intensity of staining (1 = absent/weak, 2 = moderate, or 3 = strong) and the percentage of positive staining cells (1 = less than 5%, 2 = 5–50%, or 3 = more than 50%). A total score (maximum of 6) was then generated for the epithelial and stromal component of each section.

Tissue culture

Two human colorectal carcinoma cell lines, HT-29 and CaCo-2 (American Type Culture Collection, ATCC), were grown to near confluence in 150 ml flasks in 5% CO₂ at 37°C in McCoy's modified medium containing 10% foetal calf serum, 1% glutamine, 25 mM HEPES buffer, 1% penicillin and streptomycin. Cells were used between passage 14 and 40. Prior to experiments, cells were trypsinised, washed and incubated in serum-free medium for 24 h. The serum-free medium was then replaced with that containing the experimental stimuli. Negative controls were serum-free medium only and positive controls were 20 ng/ml IGF-I only (gift from Pfizer, UK).

Assessment of proliferation was carried out in 96-well plates at an initial cell density of 1×10^4 cells/well. The final concentration of IGFBP-3 (GroPep) investigated was 200 ng/ml (4 nM), with and without IGF-I (20 ng/ml). Sodium butyrate (5 mM) was used as a known apoptotic agent. The final volume was 100 µl/well. Each stimulus was assessed in 12 separate wells and each experiment repeated on at least three occasions. Cell number was assessed after 24 and 48 h by the MTS assay (a modification of the MTT assay — CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega) and calculated as a

proportion of that obtained from medium only. Results are expressed as means \pm S.E.M. of three experiments.

Assessment of apoptosis was performed on cells incubated in T25 flasks at the same initial density (10^5 cells/ml) with and without the addition of IGFBP-3 (200 ng/ml), IGF-I (20 ng/ml) and sodium butyrate (5 mM). Apoptosis was assessed using FACS analysis at 24 h by annexin staining and at 48 h by propidium iodide (PI). Results are expressed as means \pm S.E.M. of three experiments. For annexin staining, cells were trypsinised and re-suspended in 1 ml annexin-binding buffer (Pharmingen) to which were added 5 µl Annexin V-FITC (2.5 µg/ml; Pharmingen). After incubation in the dark at room temperature for 15 min, 50 µl PI (50 µg/ml; Sigma) were added to discriminate dead cells and the samples were analysed on a FACS Calibur (Becton Dickinson) (Vermes *et al.* 1995). For sub-G1 analysis, cells were trypsinised, washed in PBS and fixed in cold 70% alcohol for at least 30 min. After washing twice in phosphate-citrate buffer, cells were treated with 50 µl RNase A (100 mg/ml; Sigma) and 250 µl PI (50 µl/ml) and analysed on a FACS Calibur with appropriate pulse processing to exclude cell doublets (Gong *et al.* 1994).

Inhibition of MAP kinase and phosphatidylinositol 3 (PI3)/AKT signalling

The effects of UO126 (Sigma), an inhibitor of the MAP kinase pathway, acting at the level of ERK-1 and ERK-2 and wortmannin (Sigma), a selective PI3 kinase inhibitor, were tested on HT-29 cells. Cells were grown as described above with cell number measured after 48 h incubation in response to IGFBP-3, butyrate and IGF-I. The inhibitors were added to the incubation medium at final concentrations of 10 µM and 50 nM respectively.

Statistical analysis

The data were analysed using the Stats Direct Biostatistics package (Camcode, Ashwell, Herts, UK). The Wilcoxon test was used for comparison of mRNA expression between paired normal and malignant samples and the Mann-Whitney U test for cell proliferation studies. The chi-squared test was used for comparison of the number of samples with detectable expression in the normal colon and carcinomas. Fisher's exact test was used where expected values were less than 5. Spearman rank correlation was used for comparison between members of the IGF-I axis. A *P* value of <0.05 was taken as statistically significant.

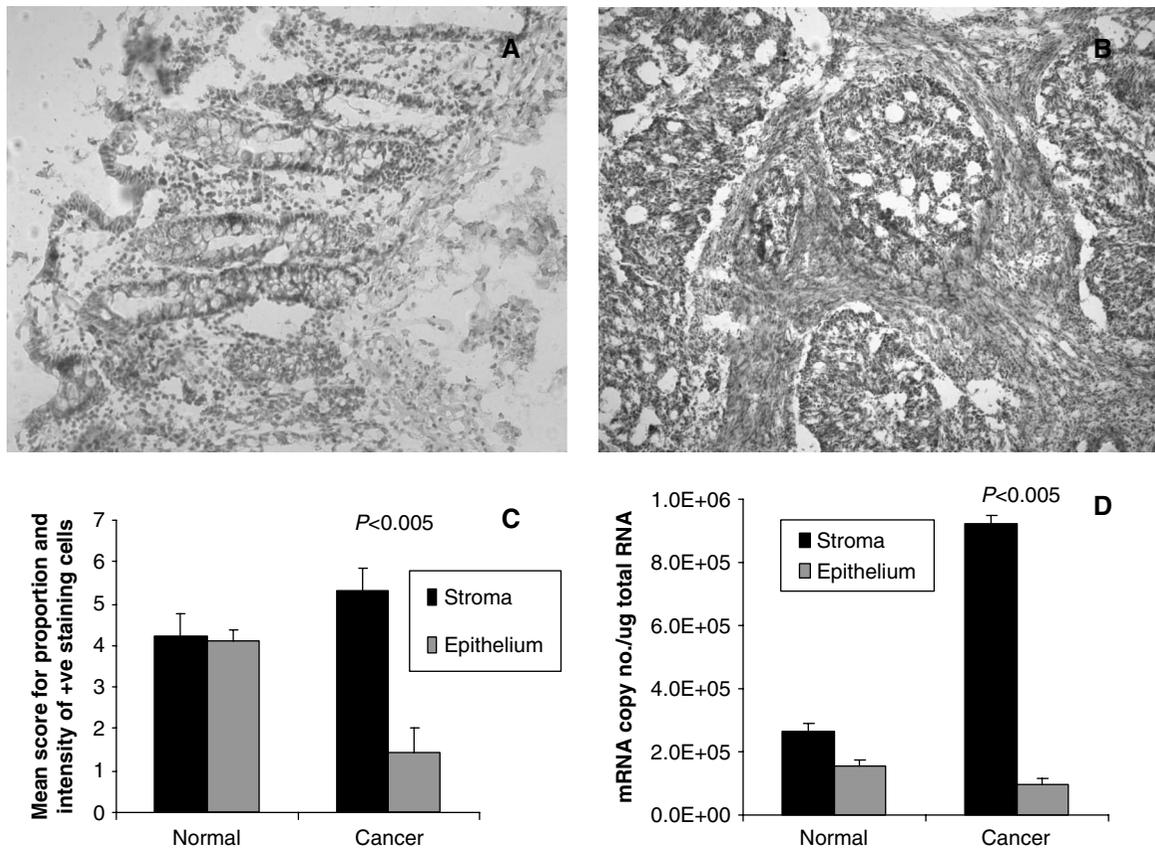


Figure 1 Immunohistochemistry against IGFBP-3 protein expression in a representative section of colonic tissue: (A) normal colon, (B) colonic cancer, (C) mean scores of all samples for proportion and intensity of positive-staining cells in stroma and epithelium and (D) mean \pm s.e.m. of 12 per group. IGFBP-3 mRNA copy number/ μ g total RNA in stroma and epithelium obtained from laser-captured microscopy.

Results

Expression of IGFBP-3 mRNA in normal and malignant colon

IGFBP-3 mRNA was detected in all samples. Median copy number was higher in the malignant samples, 1.3×10^7 (range 2.1×10^6 to 2.0×10^8) copy number/ μ g total RNA compared with the normal colon samples, 2.6×10^6 (range 4.8×10^4 to 4.2×10^7) copies/ μ g total RNA ($P < 0.0001$). A direct comparison of paired normal and malignant samples shows that mRNA levels were higher (>3-fold) in 31 of 46 (67%) tumour samples and lower in 2 of 46 (2%). IGFBP-3 mRNA levels could not be detected in either HT-29 or CaCo-2 cells.

Stromal vs epithelial expression of IGFBP-3

Expression of IGFBP-3 protein was confirmed by immunocytochemistry. Expression in the normal

colon was in both the stroma and epithelial cells (mean score \pm s.e.m. 4.2 ± 0.5 and 4.1 ± 0.6 respectively), while in the tumours it was predominantly confined to the stromal component with significantly less expression in the epithelium (mean score \pm s.e.m. 5.3 ± 0.3 and 1.4 ± 0.6 respectively; $P < 0.005$; Fig. 1). Analysis of extracted RNA from the laser-capture microdissected tissue samples confirmed this differential expression of IGFBP-3 between normal and malignant colon. mRNA levels were approximately equal in the stromal and epithelial components of normal colon, but significantly higher in the stromal component of the cancers compared with the epithelial cells (Fig. 1).

Expression of IGF-I, IGF-IR and GH-R mRNA

All samples expressed mRNA for receptors for both IGF-I and GH (Table 2). Thirty-one (67%) normal and 19 (41%) tumour samples expressed IGF-I, but

Table 2 Median (range) mRNA copy number/ μg total RNA of IGF-I, IGF-IR and GH-R in 46 paired samples of normal and malignant human colon and the correlation with IGFBP-3 mRNA levels

	Normal tissue	Correlation with IGFBP-3 (<i>r</i> value)	Cancers	Correlation with IGFBP-3 (<i>r</i> value)
IGF-I	4.7×10^5 ($6.9 \times 10^2 - 1.5 \times 10^7$)	0.6***	2.6×10^5 ($1.9 \times 10^3 - 3.4 \times 10^6$)	n.s.
IGF-IR	6.3×10^6 ($1.5 \times 10^5 - 2.5 \times 10^7$)	0.44**	1.1×10^7 ($3.0 \times 10^6 - 9.5 \times 10^7$)	n.s.
GH-R	7.4×10^5 ($1.2 \times 10^4 - 1.9 \times 10^7$)	0.38*	4.2×10^5 ($1.3 \times 10^3 - 1.7 \times 10^7$)	n.s.

* $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

with median copy numbers significantly lower than those for IGFBP-3 (Table 2). IGF-I-positive normal samples expressed significantly higher IGFBP-3 levels than IGF-I-negative normal samples (4.3×10^6 vs 8.4×10^5 ; $P < 0.02$). Overall, there was a significant correlation between IGFBP-3 mRNA levels and those of IGF-I, IGF-IR and GH-R mRNA in normal tissue. In contrast, IGF-I -positive and -negative tumours recorded similar levels of IGFBP-3 mRNA and there was no correlation between IGFBP-3 expression and IGF-I, IGF-IR and GH-R mRNA levels. IGF-I expression was absent from both HT-29 and CaCo-2 cells.

Comparison with clinical phenotype

There was no association between expression of IGFBP-3 mRNA in the tumours and age of patient, Dukes' staging, Microsatellite instability status, TNM classification, or location (proximal or distal colon).

Effect of IGFBP-3 on cell proliferation and apoptosis

Incubation with IGFBP-3 alone resulted in a significant decrease in proliferation of CaCo-2 cells compared with medium only at 24 h, but there was no effect on HT-29 cells (Fig. 2*ii*). Addition of IGF-I to the incubation medium largely relieved the inhibitory effects of IGFBP-3. As expected, butyrate exerted significant anti-proliferative effects on both cell lines, especially at 48 h, but these were markedly enhanced by the concomitant addition of IGFBP-3 (Fig. 2*ii*). This effect was largely relieved by IGF-I.

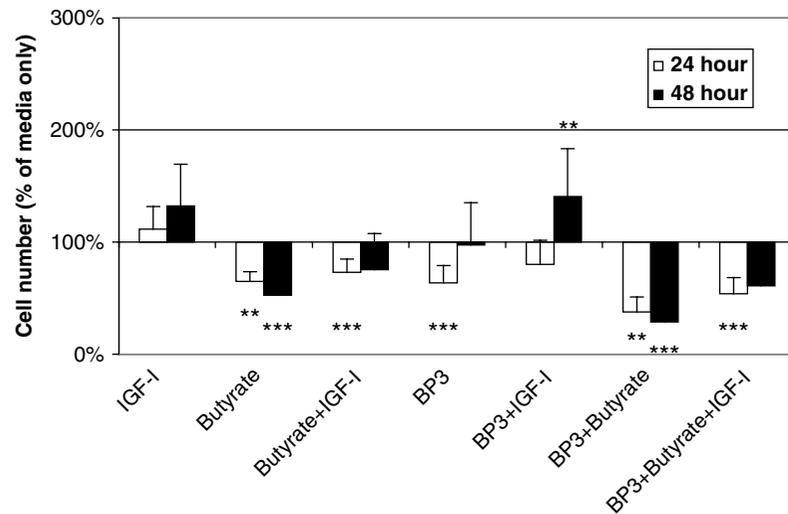
Cell morphology suggested that the inhibitory effects of IGFBP-3 were predominantly due to apoptosis, as demonstrated by the small shrunken appearance of the cells, but with intact membranes (Fig. 2*iii*). This was supported by FACS analysis, which demonstrated IGFBP-3 by itself caused a

significant increase in late (PI) apoptosis of CaCo-2 cells compared with medium only, but had no effect on HT-29 cells (Fig. 3). Butyrate alone caused a significant increase in both early (annexin binding) and late apoptosis in both cell lines. This was markedly enhanced by the addition of IGFBP-3. Co-incubation with IGF-I significantly reduced these apoptotic responses, particularly for HT-29 cells, but did not completely prevent them. Interestingly, cell morphology suggested that the proliferative response to IGF-I alone was accompanied by an increase in cell-cell aggregation (Fig. 2*iii*).

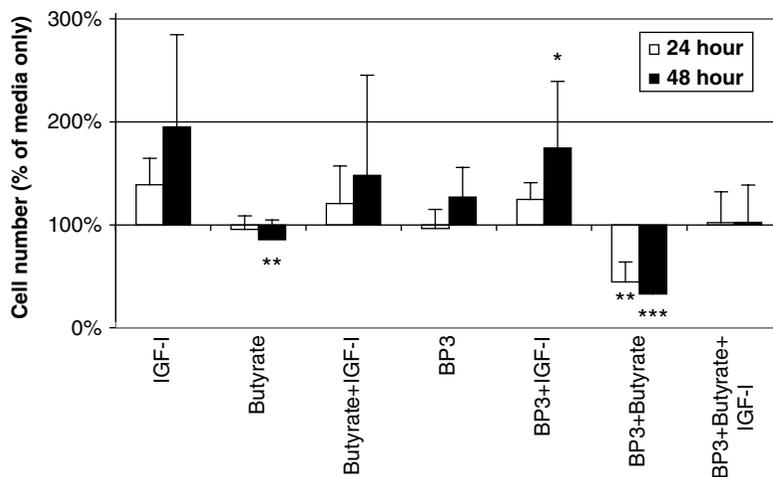
Discussion

Intestinal epithelial cells form a single layer of laterally adherent cells that are organised into crypts. Stem cells are found towards the base of the crypts and give rise to daughter cells, which cease dividing and differentiate as they migrate towards the top of the crypt. As they approach the lumen of the gut, they undergo apoptosis and are sloughed off. IGFBP-3 is emerging as a key regulator of these processes, both in its role as an IGF-I antagonist and as a potent independent anti-proliferative agent where it appears to function by both cell cycle blockade and the induction of apoptosis (Baxter 2001). This study has clarified its expression within the colon: using quantitative RT-PCR analysis it is the first to demonstrate universal but differential expression of IGFBP-3 in human normal and malignant colon. IGFBP-3 mRNA was detected in all samples, with its median mRNA levels significantly higher in cancers compared with paired normal tissue. A 3-fold or greater increase was recorded in nearly 70% of cancers, whereas significant downregulation was observed in only two cancers.

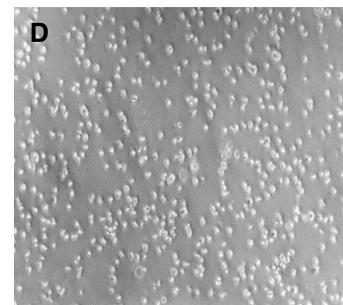
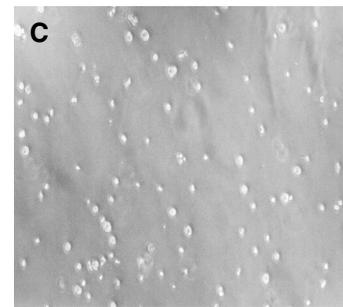
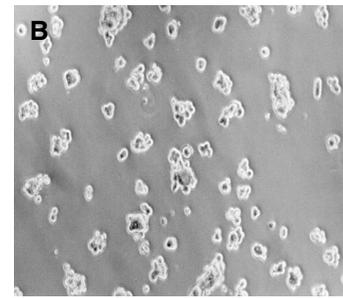
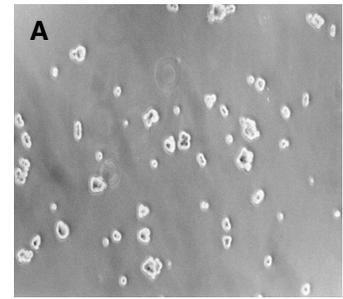
Our finding of increased IGFBP-3 levels in cancers is initially counter-intuitive given the reported anti-proliferative and pro-apoptotic effects of IGFBP-3.



(i)



(ii)



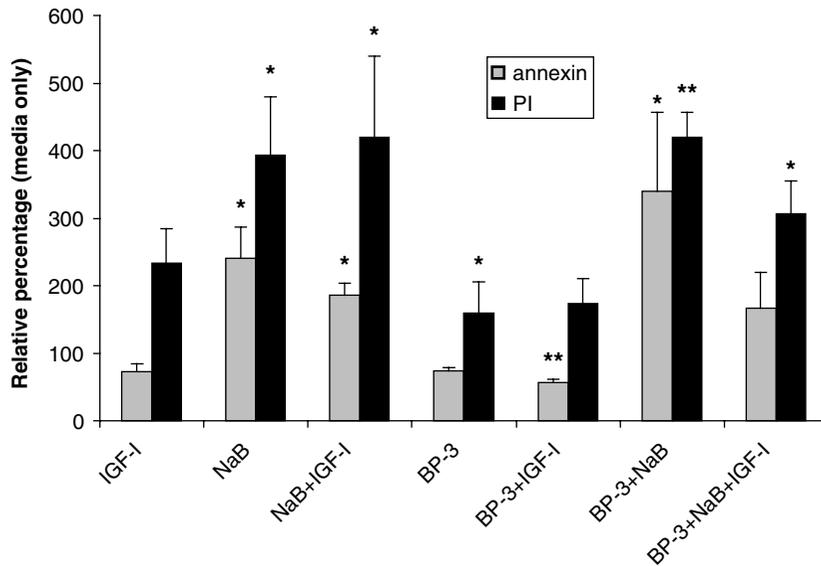
(iii)

Figure 2 Effect of IGFBP-3, IGF-I and sodium butyrate, alone and in combination on (i) CaCo-2 and (ii) HT-29 cell number, assessed by the MTS assay, after 24 and 48 h incubation compared with medium only; the results are a mean of three experiments with $n=12$; (iii) cell morphology ($\times 200$ magnification) of CaCo-2 cells incubated with (A) medium only, (B) IGF-I (20 ng/ml), (C) IGFBP-3 (200 ng/ml) and (D) IGFBP-3 + sodium butyrate (5 mM). * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$.

However, localisation of IGFBP-3 expression within the colonic samples by immunohistochemistry and laser-capture microdissection offers a solution to this paradox. The pattern of expression between normal and cancer tissue is clearly different. In normal tissue,

IGFBP-3 is expressed in both the stroma and the epithelial cells. This is in agreement with a proposed regulatory role for IGFBP-3 on cell proliferation, and its induction by wild-type p53 (Buckbinder *et al.* 1995). In marked contrast, expression of IGFBP-3 is virtually

(i) CaCo-2 cells



(ii) HT-29 cells

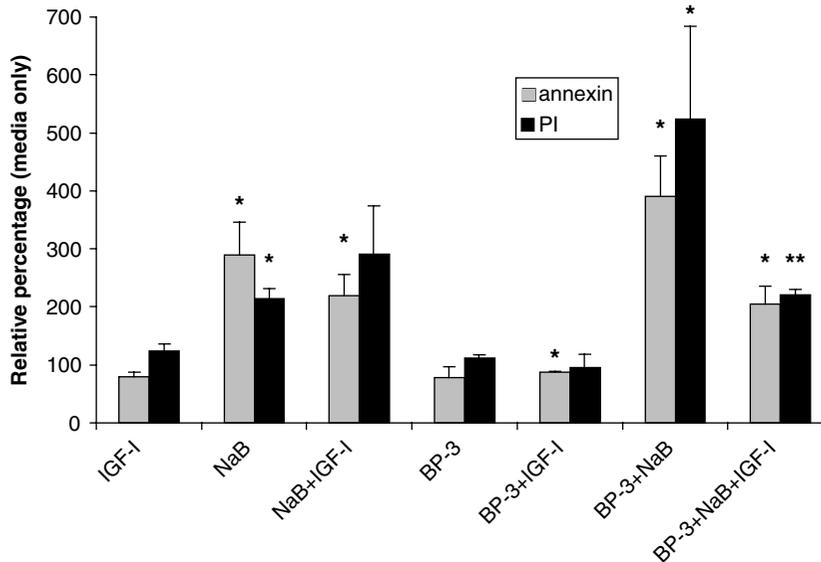


Figure 3 Effects of IGFBP-3, IGF-I and sodium butyrate, alone and in combination on early (annexin binding) and late (PI binding) apoptosis of CaCo-2 and HT-29 cells. Results are means \pm S.E.M. (of three experiments) proportion of apoptotic cells compared with controls (medium only). * $P < 0.05$; ** $P < 0.001$.

absent from malignant epithelial cells, whereas it is expressed at high levels by the non-malignant stromal component of the tumours. We hypothesise that the marked upregulation of IGFBP-3 expression in the stromal cells of cancers is the result of a physiological

response to uncontrolled proliferation of the epithelial cancer cells. The cancer cells themselves have switched off the synthesis of IGFBP-3, and have additionally developed a mechanism to prevent IGFBP-3 protein from diffusing towards them from the stroma. This

suggests that they may be still responsive to its anti-proliferative and apoptotic effects. This theory is supported by our finding that IGFBP-3 mRNA is absent from both HT-29 and CaCo-2 cancer cells, yet they respond appropriately to the anti-proliferative and apoptotic effects of exogenous IGFBP-3. The exact mechanisms responsible for the absence of expression in the cancer component remain undetermined but may involve differences in extracellular matrix, components of which clearly influence IGFBP-3 expression, binding and apoptotic actions (McCaig *et al.* 2002). The IGF-I axis has also been implicated in the invasion and metastasis of colorectal cancer. These include its direct effects on location, stability and transcriptional activity of β -catenin (Playford *et al.* 2000) and the increase in IGF-I bioavailability through the proteolysis of IGFBP-3 by matrix metalloproteinase-19 (Sadowski *et al.* 2003).

A previous study using northern blot analysis detected IGFBP-3 mRNA in only five of ten cancers; however, this technique is significantly less sensitive than real-time RT-PCR. Two previous studies report *in vivo* expression of IGFBP-3 protein in colon cancers. The first recorded expression in seven out of ten cancers but no details were given as to its localisation (Michell *et al.* 1997). The second study reported that in normal tissue the predominant expression was in the epithelial cells, while in cancers it was predominantly maintained in the differentiated epithelial cells (Williams *et al.* 2000), with some expression in the stroma. Our results agree with the predominant expression of IGFBP-3 in normal epithelium, while in cancers they show that IGFBP-3 expression is virtually exclusive to the stromal component. The reason for this discrepancy is not clear, although it may relate to the known correlation between IGFBP-3 expression and p53 status of the studied tissue (Buckbinder *et al.* 1995, Collard *et al.* 2003). Wild-type p53 is present in differentiated cells but lost in malignant cells.

Our studies have also clarified the function of this local expression of IGFBP-3. It is now well established that IGFBP-3 exerts both IGF-I -dependent and -independent effects on cell proliferation and apoptosis (Baxter *et al.* 2000). It is probable that some local IGFBP-3 in the colon is involved in modulating the activity of IGF-I at a tissue level and a linkage between the regulation of expression of both genes is suggested by our finding of significantly less IGFBP-3 mRNA in the IGF-I negative samples and a significant correlation between the mRNA expression of both genes. However, the expression of IGFBP-3 mRNA and protein in the absence of local IGF-I in 60% of our colon cancers also suggests an IGF-I-independent role

for the colonic IGFBP-3 gene product. In order to confirm these effects we used HT-29 and CaCo-2 cells, neither of which express IGF-I, and incubated the cells in medium lacking this peptide. The possibility of residual IGF-I activity was excluded by washing the cells and incubation for 24 h before adding the peptide. IGFBP-3 on its own exerted modest apoptotic actions in CaCo-2 cells, in contrast to a previous study using an adenoma cell line, which failed to show an effect (Williams *et al.* 2000). This difference may be explained by either a physiological difference between the different cell lines or the higher dose of IGFBP-3 which we used. A dose-dependent effect of IGFBP-3 has also been reported in breast and prostate cancer cell lines (Nickerson *et al.* 1997, Rajah *et al.* 1997). What is clear is that IGFBP-3 markedly enhances the apoptosis of colonic epithelial cells in response to sodium butyrate, a known differentiating and apoptotic agent for colorectal cancer cells. A similar enhancement by IGFBP-3 of irradiation-induced apoptosis of colonic epithelial cells was previously reported and shown to be p53-dependent (Williams *et al.* 2000). The mechanisms underlying our results are likely to be different, as neither HT-29 or CaCo-2 cells express wild-type p53. While this suggests p53 independence, we cannot exclude some contribution by the mutant p53 gene in these cells. However, other studies using different tissues have shown pro-apoptotic p53-independent effects by IGFBP-3 (Rajah *et al.* 1997, Butt *et al.* 2000). This influence of p53 status has also been confirmed in the colon with IGFBP-3 expression occurring in adenoma cells expressing wild-type p53 but absent or very low in p53 mutant cancer epithelial cells (Collard *et al.* 2003). Our *in vitro* and *in vivo* findings agree with this as IGFBP-3 expression was absent in both cell lines and the p53 mutant malignant epithelial cells from the cancer tissues but present in the normal epithelial samples containing wild-type p53. This correlation with p53 status may have important therapeutic implications, as administration of IGFBP-3 to malignant p53 mutant cells may enhance the p53-independent apoptotic responses to chemotherapeutic agents, while having no effect on normal cells. Such a differential effect has been reported in breast cancer cells (McCaig *et al.* 2002).

In conclusion, these findings suggest an important paracrine role of IGFBP-3 in mediating colonic tissue homeostasis and a clear difference between normal and malignant tissue. The pro-apoptotic effects of IGFBP-3 also help explain some of the recent epidemiological studies linking low levels of IGFBP-3 coupled with high IGF-I levels in the colon to a higher risk of colorectal cancer (Giovannucci *et al.* 2000,

Palmqvist *et al.* 2002). Finally, strategies to increase cellular expression of IGFBP-3 may offer the opportunity of a novel powerful adjunctive therapy for colorectal cancer.

Acknowledgements

This work was supported by grants from Bowel & Cancer Research and from the Joint Research Board and Cancer Research Committee of St Bartholomew's Hospital. In addition, PJJ is supported by an unrestricted grant from Pfizer UK for research into growth hormone and growth factors. The authors are grateful for the assistance of Professor S Dorudi in the obtaining of surgical samples. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Baxter RC 2001 Signalling pathways involved in antiproliferative effects of IGFBP-3: a review. *Molecular Pathology* **54** 145–148.
- Baxter RC, Butt AJ, Schedlich LJ & Martin JL 2000 Antiproliferative and pro-apoptotic activities of insulin-like growth factor-binding protein-3. *Growth Hormone and IGF Research* **10** (Suppl A) S10–S11.
- Buckbinder L, Talbott R, Velasco-Miguel S, Takenaka I, Faha B, Seizinger BR & Kley N 1995 Induction of the growth inhibitor IGF-binding protein 3 by p53. *Nature* **377** 646–649.
- Bustin SA 2000 Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology* **25** 169–193.
- Bustin SA, Gyselman VG, Williams NS & Dorudi S 1999 Detection of cytokeratins 19/20 and guanylyl cyclase C in peripheral blood of colorectal cancer patients. *British Journal of Cancer* **79** 1813–1820.
- Bustin SA, Dorudi S, Phillips SM, Feakins RM & Jenkins PJ 2002 Local expression of insulin-like growth factor-I affects angiogenesis in colorectal cancer. *Tumour Biology* **23** 130–138.
- Butt AJ, Firth SM, King MA & Baxter RC 2000 Insulin-like growth factor-binding protein-3 modulates expression of Bax and Bcl-2 and potentiates p53-independent radiation-induced apoptosis in human breast cancer cells. *Journal of Biological Chemistry* **275** 39174–39181.
- Collard TJ, Guy M, Butt AJ, Perks CM, Holly JM, Paraskeva C & Williams AC 2003 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. *Carcinogenesis* **24** 393–401.
- Gill ZP, Perks CM, Newcomb PV & Holly JM 1997 Insulin-like growth factor-binding protein (IGFBP-3) predisposes breast cancer cells to programmed cell death in a non-IGF-dependent manner. *Journal of Biological Chemistry* **272** 25602–25607.
- Giovannucci E, Pollak MN, Platz EA, Willett WC, Stampfer MJ, Majeed N, Colditz GA, Speizer FE & Hankinson SE 2000 A prospective study of plasma insulin-like growth factor-1 and binding protein-3 and risk of colorectal neoplasia in women. *Cancer Epidemiology, Biomarkers and Prevention* **9** 345–349.
- Gong J, Traganos F & Darzynkiewicz Z 1994 A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. *Analytical Biochemistry* **218** 314–319.
- Le Roith D, Bondy C, Yakar S, Liu JL & Butler A 2001 The somatomedin hypothesis: 2001. *Endocrine Reviews* **22** 53–74.
- Ma J, Pollak M, Giovannucci E, Chan JM, Tao Y, Hennekens CH & Stampfer M 1999 Prospective study of colorectal cancer risk in men and plasma levels of insulin like growth factor (IGF)-1 and IGF-binding protein-3. *Journal of the National Cancer Institute* **91** 620–625.
- McCaig C, Perks CM & Holly JM 2002 Intrinsic actions of IGFBP-3 and IGFBP-5 on Hs578T breast cancer epithelial cells: inhibition or accentuation of attachment and survival is dependent upon the presence of fibronectin. *Journal of Cell Science* **115** 4293–4303.
- Michell NP, Langman MJ & Eggo MC 1997 Insulin-like growth factors and their binding proteins in human colonocytes: preferential degradation of insulin-like growth factor binding protein 2 in colonic cancers. *British Journal of Cancer* **76** 60–66.
- Miraki-Moud F, Jenkins PJ, Fairclough PD, Jordan S, Bustin SA, Jones AM, Lowe DG, Monson JP, Grossman AB, Besser GM, et al. 2001 Increased levels of insulin-like growth factor binding protein-2 in sera and tumours from patients with colonic neoplasia with and without acromegaly. *Clinical Endocrinology* **54** 499–508.
- Nickerson T, Huynh H & Pollak M 1997 Insulin-like growth factor binding protein-3 induces apoptosis in MCF7 breast cancer cells. *Biochemical and Biophysical Research Communications* **237** 690–693.
- Oh Y 1997 IGFBPs and neoplastic models. New concepts for roles of IGFBPs in regulation of cancer cell growth. *Endocrine* **7** 111–113.
- Palmqvist R, Hallmans G, Rinaldi S, Biessy C, Stenling R, Riboli E & Kaaks R 2002 Plasma insulin-like growth factor 1, insulin-like growth factor binding protein 3, and risk of colorectal cancer: a prospective study in northern Sweden. *Gut* **50** 642–646.
- Playford MP, Bicknell D, Bodmer WF & Macaulay VM 2000 Insulin-like growth factor 1 regulates the location, stability, and transcriptional activity of beta-catenin. *PNAS* **97** 12103–12108.
- Rajah R, Valentinis B & Cohen P 1997 Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-beta 1

- on programmed cell death through a p53- and IGF-independent mechanism. *Journal of Biological Chemistry* **272** 12181–12188.
- Renehan AG, Zwahlen M, Minder C, O'Dwyer ST, Shalet SM & Egger M 2004 Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. *Lancet* **363** 1346–1353.
- Sadowski T, Dietrich S, Koschinsky F & Sedlacek R 2003 Matrix metalloproteinase 19 regulates insulin-like growth factor-mediated proliferation, migration, and adhesion in human keratinocytes through proteolysis of insulin-like growth factor binding protein-3. *Molecular Biology of the Cell* **14** 4569–4580.
- Vermes I, Haanen C, Steffens-Nakken H & Reutelingsperger C 1995 A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. *Journal of Immunological Methods* **184** 39–51.
- Williams AC, Collard TJ, Perks CM, Newcomb P, Moorghen M, Holly JM & Paraskeva C 2000 Increased p53-dependent apoptosis by the insulin-like growth factor binding protein IGFBP-3 in human colonic adenoma-derived cells. *Cancer Research* **60** 22–27.

