

# The increase in bladder carcinoma cell population induced by the free beta subunit of human chorionic gonadotrophin is a result of an anti-apoptosis effect and not cell proliferation

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**Summary** Ectopic production of free beta human chorionic gonadotrophin (hCG $\beta$ ) by bladder carcinoma is well described and occurs in approximately 35% of cases. hCG $\beta$  secreting tumours are more aggressive, radioresistant and have a greater propensity to metastasize. We proposed that the ectopic production of hCG $\beta$  was contributing in an autocrine fashion to the radioresistance and metastatic potential of such secreting tumours. Though we demonstrated that the addition of hCG $\beta$  to the culture media of bladder, cervical and endometrial carcinoma cell lines brought about an increase in cell populations this was not accompanied by a significant increase in the rate of replication. Since a cell population size is a balance of mitosis and mortality, we proposed that hCG $\beta$  was inhibiting apoptosis. Here we have demonstrated that following incubation with recombinant hCG $\beta$ , bladder carcinoma cells refrain from undergoing apoptosis. Quantitation of apoptotic bodies was carried out by immunoassay and corrected to cell number as determined by MTT assay. In each cell line, addition of hCG $\beta$  reduced the number of apoptotic bodies dose-dependently, indicating a diminished apoptotic rate. Furthermore, TGF $\beta$ 1-induced apoptosis could be dose-dependently inhibited by co-incubation with hCG $\beta$ . We propose, therefore, that such a decline in apoptosis may account for the cell population increase previously reported. It may also explain the radioresistance and aggressive nature of hCG $\beta$ -secreting tumours and the poor prognosis associated therein. © 2000 Cancer Research Campaign

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The beta subunit of human chorionic gonadotrophin (hCG $\beta$ ) confers the structural and functional identity of the biologically active glycoprotein heterodimer of hCG. The alpha subunit is common to all members of the glycoprotein hormone family, which includes thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH) and luteinizing hormone (LH). Only the intact  $\alpha$ - $\beta$  arrangement is hormonally functional; the free subunits themselves do not exhibit gonadotrophic or thyrotrophic activity, and the beta subunit alone is not sufficient to stimulate the LH/hCG receptor (Pierce and Parsons, 1981). Therefore, any growth factor activity exhibited is generally regarded to be occurring via a novel pathway.

The ectopic production of free hCG $\beta$  – in the absence of the alpha subunit – is a well-recognized phenomenon in many epithelial tumours (Braunstein, 1983; Iles and Chard, 1989). It was originally regarded as an epiphenomenon, with no biological significance given the absence of hCG $\alpha$  and a functional heterodimeric structure. More recently, however, it has been shown that the beta subunit may have its own unique functions. Lunardi-Iskander et al (1995) and Gillott et al (1996) have independently shown growth inhibitory and growth stimulatory effects of free

hCG $\beta$ . Elucidation of the crystal structure of hCG revealed surprising topological similarities with the known 'cystine knot' growth factors – transforming growth factor beta (TGF- $\beta$ ), platelet derived growth factor beta (PDGF $\beta$ ) and nerve cell growth factor (NGF). In particular, hCG $\beta$  and its urinary breakdown product, the hCG $\beta$  core fragment (hCG $\beta$ cf), bear a striking similarity to TGF $\beta$  and PDGF $\beta$  respectively (Lapthorn et al, 1994). Furthermore, the growth factor family also share a common feature of only functioning as hetero- and/or homodimers and investigations into hCG $\beta$  as a cystine knot growth factor have shown that, along with the established heterodimeric structure of the hormone hCG, hCG $\beta$  and hCG $\beta$ cf also form homodimers (Butler et al, 1999; Iles et al, 1999).

These surprising new findings support the hypothesis that the ectopic production of hCG $\beta$  by epithelial tumours is not an epiphenomenon, but rather a significant release of a possible autocrine/paracrine growth factor which is contributing directly to the poor prognosis of these tumours. Further studies into the stimulation of bladder carcinoma cells with recombinant hCG $\beta$  continue to bring about an increase in cell number when quantified by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) reduction. However, cell replication – as quantified by thymidine incorporation (unpublished data) – is not consistent with the same increase in population, suggesting that the hCG $\beta$  is not, in fact, increasing the growth rate, but rather is slowing the rate of apoptosis. Here we have investigated apoptosis in bladder carcinoma cell lines in response to hCG $\beta$  stimulation.

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## MATERIALS AND METHODS

### Growth factors

Recombinant hCG $\beta$  and recombinant TGF $\beta$ 1 (Sigma, Poole, UK) were reconstituted according to the manufacturer's recommendations. Aliquots of reconstituted proteins were diluted to working concentrations in RPMI-1640 cell culture medium (Sigma) containing 10% fetal calf serum (FCS) and 1% antibiotic/antimycotic solution (Gibco-BRL, Life Technologies Ltd, Paisley, UK).

### Cell lines

Bladder carcinoma cell lines T24 (Dr J Masters, Institute of Urology, London, UK), SCaBER, J82, 5637 and RT112 (American type Culture Collection, Rockville, MD, USA) were used in the incubation studies. T24 has been shown to be the most sensitive to the addition of hCG $\beta$  believed to be due to the fact that it produces no endogenous hCG $\beta$  of its own (Gillott et al, 1996). SCaBER and RT112 are high level hCG $\beta$  producers and 5637 and J82 low level hCG $\beta$  producers (Iles and Chard, 1989).

### MTT assay

A variation on the MTT assay (Mosman, 1983; Twentyman and Luscombe, 1987) was used to determine cell numbers following an incubation with a growth factor or combination of growth factors.

Ninety-six-well plates (Flat-bottomed, Nunc, Gibco-Life Technologies) were seeded with  $5 \times 10^4$  cells per well, in RPMI-1640/10% FCS/1% antibiotic antimycotic and incubated at 37°C in the presence of 5% carbon dioxide for 24 h. The media were then replaced with new media containing the growth factor material (0–400 pmol ml<sup>-1</sup>), in replicates of 6 and incubated as before, for a period of 48 h. Control plates were treated with the same concentrations of TGF- $\beta$ 1, a known inducer of epithelial cell apoptosis and a member of the same family of growth factors as hCG $\beta$ . In the co-incubation experiments apoptosis was induced by incubation in 100 pmol ml<sup>-1</sup> TGF- $\beta$ 1 in addition to increasing concentrations (0–400 pmol ml<sup>-1</sup>) of hCG $\beta$ . Following this, the cells were subjected to MTT assay. Media was again replaced with RPMI without phenol red and further incubated for a period of 1 h, upon which 20  $\mu$ l of sterile filtered MTT (Sigma) (5 mg ml<sup>-1</sup> in phosphate-buffered saline) was added to each well. After a 3-h incubation all solutions were removed from the cells and replaced with 200  $\mu$ l of dimethylsulphoxide (DMSO, Sigma, UK) and the formazan crystals allowed to dissolve. Each well was then read for absorbance at 570 nm in a Microplate autoreader (Bio-Tek instruments).

Prior to MTT assay an aliquot of incubation media was removed from each well and assayed for the presence of mono- and oligonucleosomes using the Boehringer Mannheim apoptosis cell death detection enzyme-linked immunosorbent assay. Nucleosome concentration was then determined from the spectrophotometric data and corrected to cell number as subsequently determined by tetrazolium salt reduction.

## RESULTS

Absorbance data were corrected to percentage change from untreated control. The percentage change in cell number and percentage change in nucleosome concentration clearly indicate a

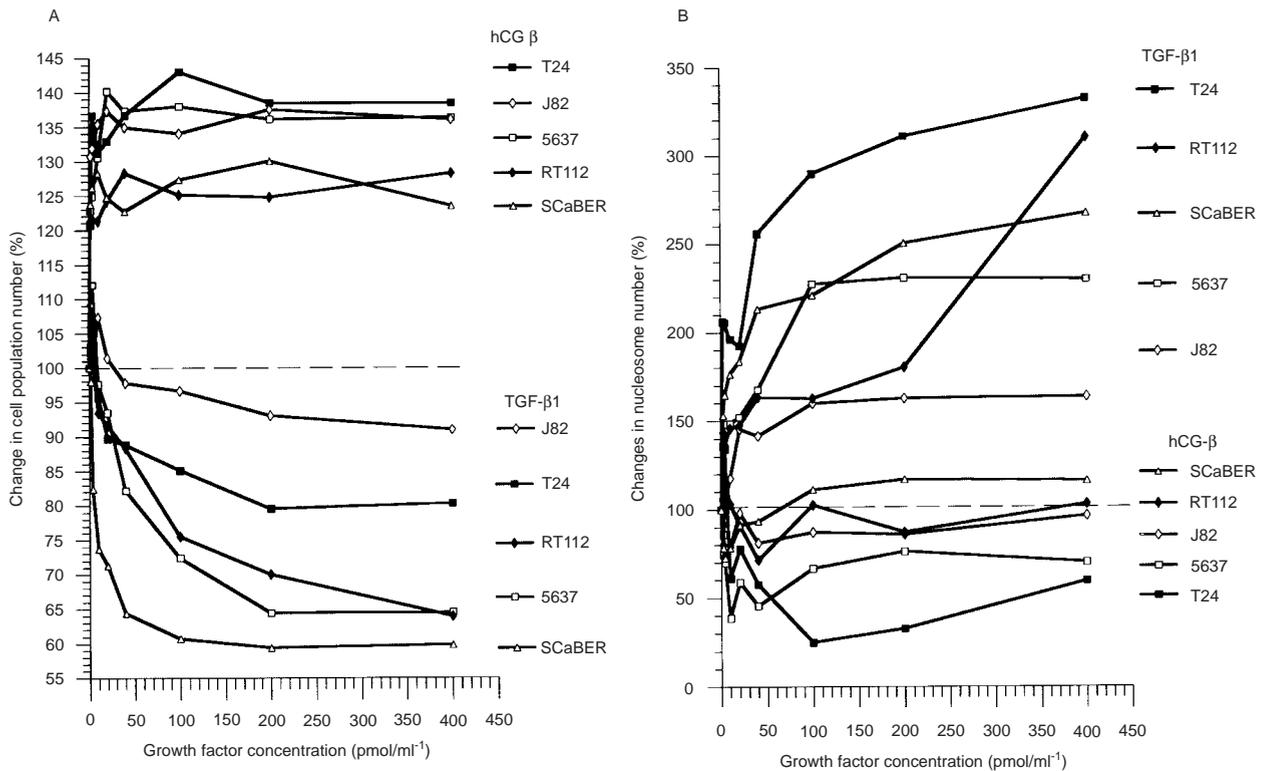
rise in cell number accompanied by a corresponding fall in nucleosome concentration in response to hCG $\beta$ . Conversely TGF- $\beta$ 1 brings about a dose-dependent fall in cell number and rise in nucleosome concentration (Figure 1 A, B). Figure 2 indicates the results of the co-incubation study, a sharp rise in the percentage number of nucleosomes (and hence increase in apoptosis) following incubation with 100 pmol ml<sup>-1</sup> TGF- $\beta$ 1. The effect gradually diminishes to below 100% as the concentration of hCG $\beta$  increases from 0 to 400 pmol ml<sup>-1</sup>, despite the continued presence of TGF $\beta$ 1.

## DISCUSSION

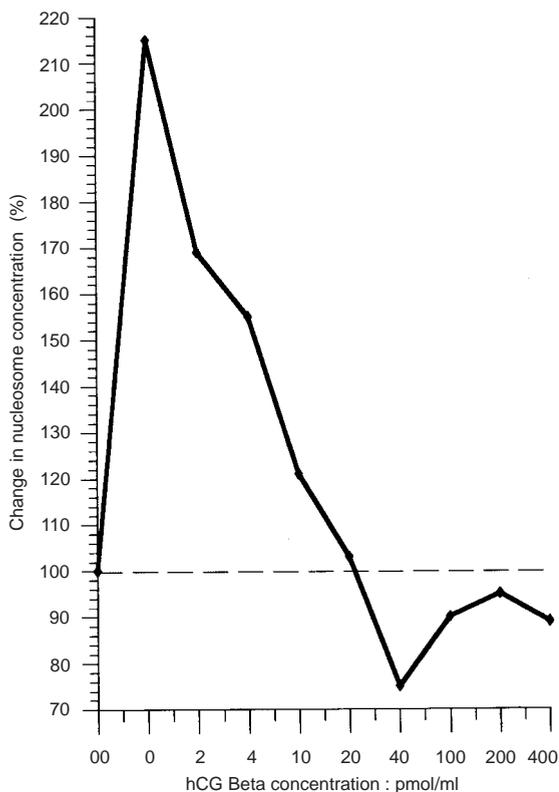
The ectopic production of free hCG $\beta$  is a well-recognized phenomenon in many epithelial tumours (Cole et al, 1988). It is now apparent that this event may significantly affect tumour development given the growth effects recently described (Lunardi-Iskander et al, 1995; Gillott et al, 1996). It is accepted that free hCG $\beta$  is unable to activate the LH/hCG receptor and stimulate the subsequent second messengers. Therefore the recently reported growth factor activities of hCG $\beta$  are assumed to proceed via novel, and as yet unidentified, pathways. Following the elucidation of hCG's crystal structure, its subunits were grouped with the family of cystine knot growth factors which includes TGF, PDGF and NGF (Laphorn et al, 1994). The topological similarities observed – particularly the presence of the central cystine knot – suggest a common growth factor function. This concept has recently been reinforced by data which clearly indicate the presence of hCG $\beta$  homodimers that, like TGF, PDGF and NGF, may be required to bring about cellular growth responses.

In this study we have reconfirmed the apparent growth stimulation of bladder carcinoma cells by hCG $\beta$  in comparison to the effect brought about by TGF- $\beta$ 1, a cystine knot growth factor and known inducer of epithelial apoptosis (Sporn et al, 1986; Sun and Davies, 1995). Figure 1A shows the contrasting effects of TGF- $\beta$ 1 and hCG $\beta$  on cell number in five bladder carcinoma cell lines. The change in apoptosis in response to hCG $\beta$  or TGF- $\beta$ 1 can be seen in Figure 1B and could certainly account for the difference in cell number observed in the previous figure (Figure 1A). We propose that the increase in cell number brought about by hCG $\beta$  could be solely accounted for by a reduction in apoptosis. The sharp rise in cell numbers in response to low concentrations of hCG $\beta$  followed by a plateau indicates that the molecule may be acting in an inhibitory fashion given that there is a sudden change in percentage cell number at a given hCG $\beta$  concentration and not a gradual dose-dependent response. The results following co-incubation of 5637 cells with constant TGF- $\beta$ 1 and increasing hCG $\beta$  indicate that hCG $\beta$  can reverse the apoptotic effect brought about by the addition of TGF- $\beta$ 1. An increase in nucleosome concentration in the presence of 100 pmol ml<sup>-1</sup> TGF- $\beta$ 1 is consistent with that seen for 5637 in Figure 1B. However, as the hCG $\beta$  concentration increases the number of nucleosomes reduces to below that seen in the negative control despite the presence of the TGF- $\beta$ 1.

In light of the particular topological similarities between hCG $\beta$  and TGF- $\beta$  and the opposing nature of their effect on epithelial cells, it could be suggested that hCG $\beta$  may be interacting with the TGF- $\beta$  receptor complex. Interactions between the cystine knot growth factors is not uncommon, and TGF- $\beta$  and PDGF in particular, cooperate to bring about their designated responses. The hCG $\beta$  homodimer recently described could be such a candidate for



**Figure 1** (A) The percentage change in cell number from the control (100%, and indicated by the dashed line) as a result of incubation with hCG $\beta$  (top) and TGF- $\beta$ 1 (bottom) on the five different bladder cancer cell lines. (B) The percentage change in nucleosome concentration from the control (100%, and indicated by the dashed line) as a result of incubation with hCG $\beta$  (bottom) and TGF- $\beta$ 1 (top) on the five different bladder cancer cell lines



**Figure 2** Shows the percentage change in nucleosome concentration from the control following coincubation of the bladder carcinoma cell line 5637 with TGF- $\beta$ 1 (to initiate apoptosis) and increasing concentrations of hCG $\beta$  (to negate the TGF- $\beta$ 1 effect while the apoptotic factor was still present). 00 contained no hCG $\beta$  or TGF- $\beta$ 1 and represents the control to which the other values were corrected and further indicated by the dashed line

TGF- $\beta$  receptor binding, given that it requires a dimeric species to align the receptor subunits and initiate the apoptotic cascade. The marked similarity in topology may be enough to allow binding but not to activate second messengers in addition and thus prevent the action of the autocrine TGF- $\beta$  regulation on cell growth. The results here do not provide the information to determine the route by which hCG $\beta$  is acting. Given the complexity of an apoptotic cascade any cross-talk occurring may do so at any number of locations following stimulation by hCG $\beta$  and warrants further investigation.

In conclusion it appears that the recently described growth effect attributed to ectopic free hCG $\beta$  production is not due to growth stimulation but an anti-apoptosis effect which may be brought about in an antagonistic manner via a known receptor or receptor-mediated cascade. Whatever the mode of action occurring here the evidence for hCG $\beta$  as a growth modulator is increasing and its involvement in an apoptotic cascade opens up a new field for investigation.

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