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Original Paper

Cell Cycle Arrest and Induction of Apoptosis by β Galactoside Binding Protein (βGBP) in Human Mammary Cancer Cells. A Potential New Approach to Cancer Control

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Conflict between mitogenic pressure, as is the case in tumour cells and an imposed inability to proceed through the cell cycle may result in cell death. In the present study we examined the effect of β galactoside binding protein (βGBP), a negative growth factor which controls cell cycle transition from S phase into G_2, on three human mammary cell lines which differ for oncogenic potential, oestrogen receptor expression and expression of the EGF receptor family. We found that in all cases βGBP induced a cell cycle block prior to the cells' entry into G_2 and that this was followed by progressive apoptotic death. This evidence on epithelial cancer cells parallels previous data on tumour cells of mesenchymal origin and suggests that βGBP has potential therapeutic implications in the treatment of cancers. © **1999 Published by Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

In previous studies [1–4] we have demonstrated that in murine fibroblasts and in human T lymphocytes cell cycle transition from S phase to G_2 is regulated by β galactoside binding protein (βGBP), a monomeric 15 kDa cytokine whose regulatory activities extend to the immune system [3, 5]. Also we have demonstrated that at a dose range of 10^{-10}–10^{-8} M βGBP can enforce a reversible cell cycle arrest prior to the cells' entry into G_2 (S phase checkpoint block) which, when sustained, in leukaemic T cells but not in their normal counterparts, is followed by apoptotic death [4]. This evidence in cells of mesenchymal origin indicates that the S phase checkpoint may define a critical cell cycle regulatory window which in cancer cells can determine a shift from growth arrest to apoptosis and prompts the suggestion that the anticancer effect of βGBP may extend also to cells of epithelial origin.

For this study, we examined the effect of βGBP on three types of human mammary cell lines which differ for tumorigenic properties, oestrogen receptor status (ER^+; ER^-) and expression of the EGF family of receptors. We examined (ER^-) BT20 cells developed from an infiltrating primary ductal carcinoma [6]; (ER^+) T47D cells derived from cells isolated from the pleural effusion of a patient with metastatic carcinoma of the breast [7] and (ER^-) MTSV 1-7 luminal cells immortalised by the simian virus 40 large T antigen (S40TAg) which have no ability to cause tumours in SCID mice [8, 9]. We found that in all cases βGBP was able to inhibit cell replication via an S/G_2 cell cycle block and that cell arrest was followed by progressive apoptotic death.

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MATERIALS AND METHODS

Human mammary cell lines

BT20 cells were grown in Dulbecco's minimal essential medium (MEM) supplemented with 10 μg/ml insulin, 0.3 μg/ml glutamine and 15% fetal calf serum. Dulbecco's modified Eagle's medium supplemented with 0.3 μg/ml glutamine and 10% fetal calf serum was used for the T47D cells and the MTSV 1-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 μg/ml insulin and 5 μg/ml hydrocortisone, glutamine 0.3 μg/ml and 10% foetal bovine serum. Cells were seeded in 24-well plates and incubated at 37°C in a humidified atmosphere of 10% CO_2 in air. Cell viability was assessed by trypan blue exclusion.

Flow cytometry

Cytofluorimetric analysis of DNA content was performed on cells washed twice with phosphate buffered saline–bovine serum albumin (PBS–BSA), fixed with 70% ethanol at 4°C, washed with phosphate-citrate buffer and stained with 40 μg/ml propidium iodide. Apoptosis TUNEL analysis was evaluated by the fluorochrome labelling of DNA strand breaks by terminal deoxynucleotidyl transferase (TdT assay) using the Apo-Brdu kit (Phoenix Flow System, U.S.A.). Briefly, 1×10^6 cells for each sample were suspended in 0.5 ml of PBS, 5 ml of 1% paraformaldehyde in PBS was added and the suspension was placed on ice for 15 min. Cells were then washed twice in 5 ml of PBS, 4 ml of ice cold 70% ethanol was added and the samples were stored at − 20°C until use. Staining was performed according to the manufacturer's instructions. Each sample was incubated for 60 min at 37°C with TdT enzyme and FITC-DUTP in a reaction buffer. The cells were washed and resuspended in 1 ml of propidium iodide and RNase solution and then incubated for 30 min at room temperature. Samples were analysed by flow cytometry within 3 h of staining in a FACS Calibur (Becton Dickinson, San Jose, California, U.S.A.). Forward scatter was used as the trigger parameter and all doublets and clumps were excluded from analysis by pulse processing.

Assessment of mitogen activated protein (MAP) kinase

Cell cultures were lysed in 50 mM Tris–HCl pH 7.5, 150 mM NaCl and 0.1% Triton X-100 containing 1 mM phenylmethylsulphonylfluoride, 2.5 μg/ml leupeptin, 0.1 TIU aproteinin, 10 μM sodium fluoride, 1 mM sodium vanadate and 10 μM β glycerol phosphate at 4°C for 30 min. After centrifugation at 12 000 ***g*** at 4°C the supernatants were rapidly frozen in liquid nitrogen and stored at − 70°C. Following assessment of protein concentration (Pierce™ protein assay reagent), 30 μg of lysate samples were run on 10% sodium dodecyl sulphate (SDS) polyacrylamide gels using Rainbow molecular weight markers (Amersham International, Amersham, U.K.) to visualise protein migration. The gels were run until the 30 kDa marker had reached the bottom of the gel and they were then blotted on to nitrocellulose using standard methods. To reveal both non-phosphorylated and the more slowly migrating phosphorylated forms of both the p42 and p44 MAP kinases, blots were probed with an anti MAP kinase polyclonal rabbit antibody (a gift from C. Marshall, Institute of Cancer Research, London, U.K.) [10]. This was followed by an antirabbit horseradish peroxidase conjugated antibody (Amersham) and detection by enhanced chemiluminescence (ECL). Blots were also probed with an anti-MAP kinase antibody (Promega, Southampton, U.K.) which detects only the dually phosphorylated active form of MAP kinase, followed by a horseradish peroxidase conjugated secondary antibody and ECL for visualisation by exposure to X-ray film.

Recombinant human βGBP

The cDNA enclosing the full length coding sequence of the human βGBP protein was isolated by screening a human lung fibroblast cDNA library in λgt 11 (Stratagene, Cambridge, U.K.) using a full length murine βGBP cDNA as a probe. The human cDNA was first subcloned into pUC 19 vector using a Kpn-Sst 1 fragment. Subsequently the sequence encoding the human βGBP cDNA was amplified by polymerase chain reaction (PCR) using forward primer 5′ GT TCA ATC ATG GCT TGT GGT CTG GTC 3′ and reverse primer 5′ GT TCA GTC AAA GGC CAC AC 3′ and directly cloned into the eukaryotic cloning vector pCR3.1 using *Escherichia coli* TOP 10F′. Purified plasmid DNA was transfected in COS 1 cells and the recombinant protein purified by immunoaffinity chromatography following procedures described previously [1].

RESULTS

As in previous studies, the cells were first tested for a growth inhibitory response to βGBP used at concentrations ranging from 10^{-10} to 10^{-8} M. Sustained inhibition of growth was obtained with the latter dose, lower doses gave proportionally related responses (data not shown). The inhibitory effect of βGBP could be negated and growth could be re-established by the addition to the culture medium of monoclonal antibody clone B2 which neutralises βGBP [1].

In these experiments βGBP was added at a final concentration of 2.5×10^{-8} M from 6 h after seeding and left throughout the duration of the experiment in the BT20 and the MTSV 1-7 cells, but re-added at the same concentration to the T47D cells at day 4 and day 7 of culture. Figure 1(a) shows that the cells which originated from a primary mammary carcinoma (BT20) did not increase in number in the presence of βGBP and that the number of live cells diminished in time to values below those of the original seed. Parallel dual parameter TUNEL analysis under these conditions (Figure 1b) showed a prompt occurrence of apoptotic death which increased from approximately 11% at day 1 after βGBP addition to 73% by the end of the 7 day period of observation (right panels, boxed areas). Figure 1(b) also shows that in contrast to the βGBP arrested cells, cells which had not been exposed to βGBP showed a minimal degree of apoptosis (left panels, boxed areas). The histograms of Figure 1(b) (insets) demonstrate that where a subdiploid DNA population had developed (left of G_1 peak) as a consequence of apoptotic death, cells that had arrested showed a lack of development of a G_2M population, thus indicating, as in previous findings [1–4], that βGBP had enforced a cell cycle block prior to G_2 entry. Observation of the T47D cells which originated from a metastatic effusion showed that, although these cells responded promptly to the inhibitory effect of βGBP, re-addition of the protein (arrows) was necessary to prevent resumption of growth and, thus, to maintain the cell population at the basal level (Figure 2a). Figure 2(b) shows that under these conditions the cell arrest prior to entry into G_2 enforced by βGBP resulted in an apoptotic response (right panels, boxed areas and histograms) that, although less prompt than in the case of BT20 cells, extended in time to 87% of the cell population. The data of Figure 3 demonstrate that luminal cells immortalised and driven by SV40TAg responded to βGBP in a manner similar to that of cells originating from mammary cancers. However, in contrast to these, the MTSV 1-7 cells had an innate propensity to undergo apoptosis under unchallenged conditions (Figure 3(b), left panels). Figure 4 illustrates the pattern of occurrence and the extent of apoptotic death as a function of time in the three cell lines, both under unrestricted growth conditions and when exposed to βGBP.

Signalling pathways triggered by tyrosine kinase receptors. are of importance in many cancers and in particular in the case of cancers of the breast where the EGF family of receptors can be overexpressed and can form highly efficient hierarchical associations which can potentiate and prolong EGF

induced activation of MAP kinase [11]. We therefore examined whether βGBP affected MAP kinase activity in the three mammary cell lines under investigation. We found the p42 MAP kinase and the p44 MAP kinase to be strongly and equally expressed throughout the full time course of the experiment in all three cell lines, whether under restricted

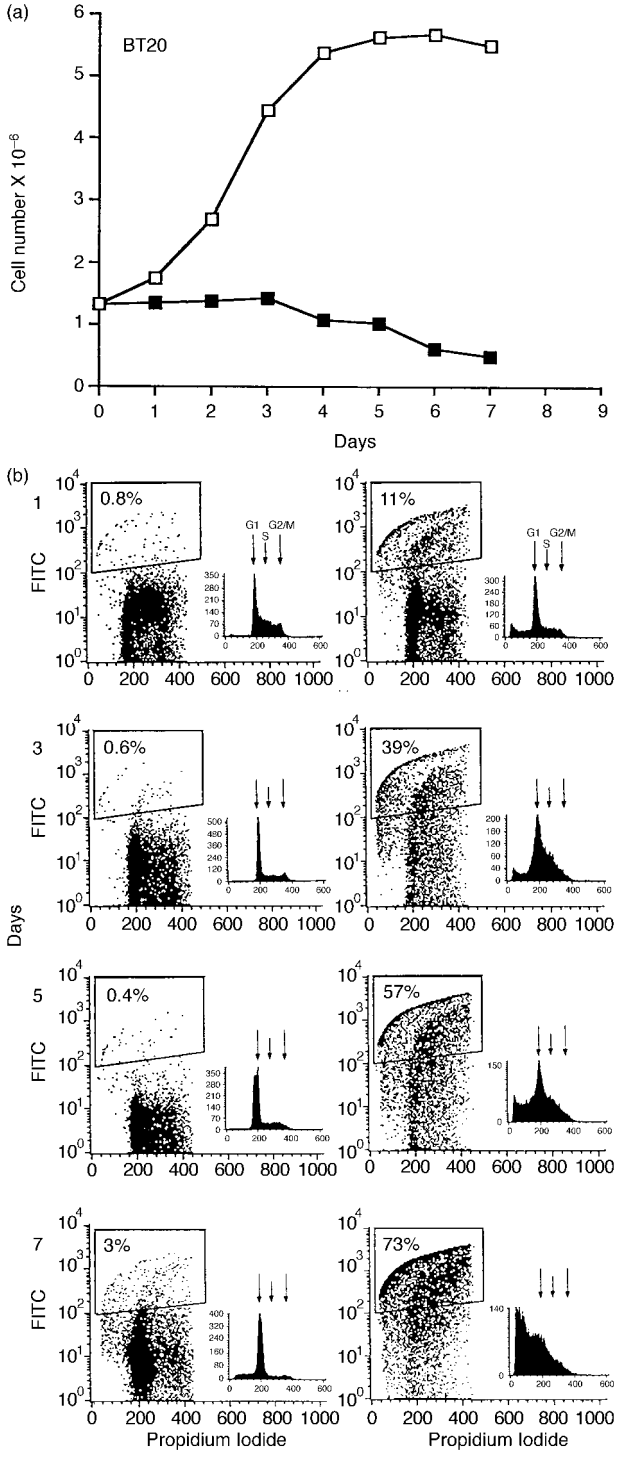

Figure 1. (a) Effect of β-galactoside binding protein (βGBP) on the proliferation of BT20 cells: –□–, untreated controls; –■–, cells treated with βGBP (2.5×10^{-8} M) from 6 h after seeding. Data plotted are the means of triplicate experiments. (b) Dual parameter TUNEL analysis from 10 000 events. The percentages give the proportion of cells in apoptosis. The histograms represent the cell cycle distribution of DNA content assessed by FACS analysis after propidium iodide staining. Plots are the result of 10 000 events. Control cells, left panels; treated cells, right panels.

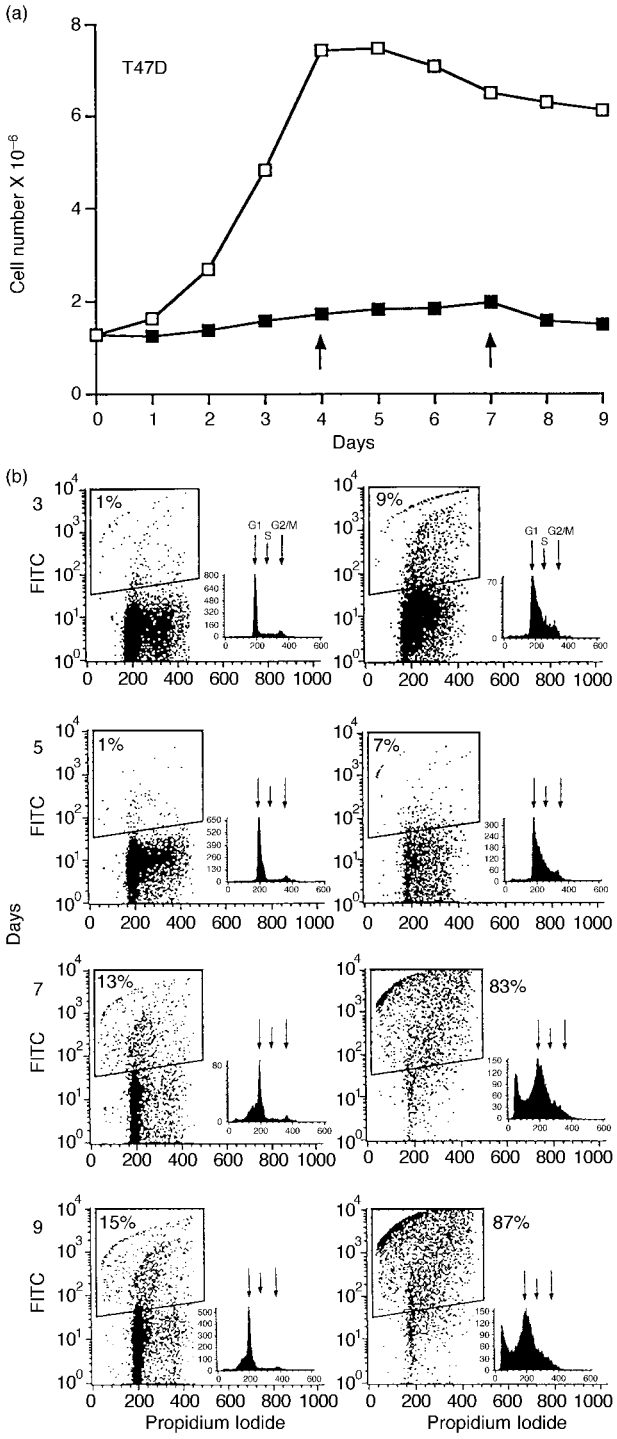

Figure 2. (a) Effect of β galactoside binding protein (βGBP) on the proliferation of T47D cells: –□–, untreated controls; –■–, cells treated with βGBP (2.5×10^{-8} M) at 6 h after seeding and 5 at day 4 and day 7 of culture (arrows). Data are the means of triplicate experiments. (b) Dual parameter TUNEL analysis from 10 000 events. The percentages give the proportion of cells in apoptosis. The histograms represent the cell cycle distribution of DNA content assessed by FACS analysis after propidium iodide staining. Plots are the result of 10 000 events. Control cells, left panels; treated cells, right panels.

growth conditions or when arrested by βGBP (data not shown). This is of importance as it demonstrates that βGBP can exert its effect by bypassing the impulse of mitogenic signals.

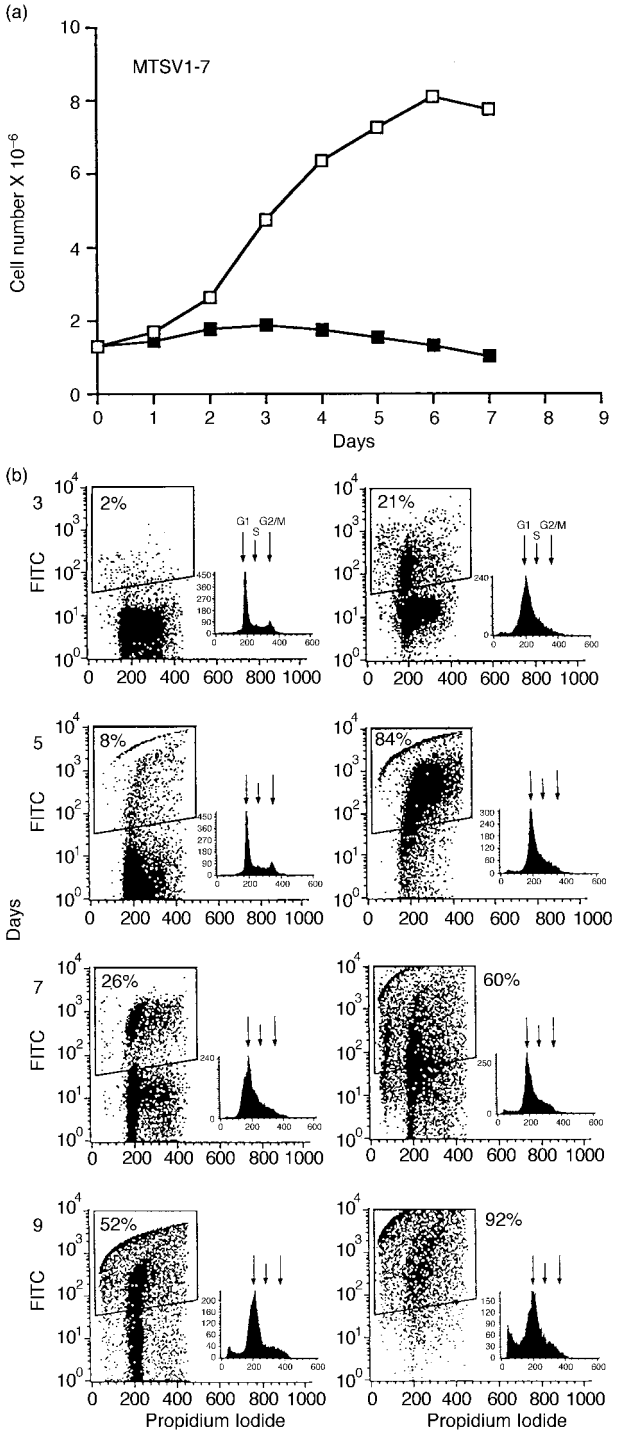

Figure 3. (a) Effect of β-galactoside binding protein (βGBP) on the proliferation of BT20 cells: –□–, untreated controls; –■–, cells treated with βGBP (2.5×10^{-8} M) from 6 h after seeding. Data plotted are the means of triplicate experiments. (b) Dual parameter TUNEL analysis from 10 000 events. The percentages give the proportion of cells in apoptosis. The histograms represent the cell cycle distribution of DNA content assessed by FACS analysis after propidium iodide staining. Plots are the result of 10 000 events. Control cells, left panels; treated cells, right panels.

DISCUSSION

We have shown previously in fibroblastic cells and in T lymphocytes [1–4] that βGBP is an endogenous negative regulator of the cell cycle which physiologically controls passage from S phase into G_2 and that at nanomolar concentrations βGBP can act as a growth suppressor by arresting cells during transition from S phase to G_2. Possibly relevant to the cell cycle regulatory role of βGBP is that the gene which encodes this cytokine [12, 13] maps in the sis/PDGFB homology region (murine chromosome 15E and human chromosome 22 q12-q13), a syntenic group which is deleted or translocated in a variety of human tumours [14–17]. In the present study we examined cancer cells of epithelial derivation, focusing on mammary cells of luminal origin, as the commonest phenotype in breast cancer is the luminal type. In particular, we compared cells originating from a primary carcinoma of the duct (line BT20) and cells from an invasive, metastatic breast carcinoma (line T47D). Also we examined cells which did not originate from a cancer but instead had been immortalised in the laboratory (MTSV 1-7) and which were not tumorigenic [9]. The high degree of spontaneous death of these latter cells as they increase in number (Figure 3) is a possible explanation for their inability to produce tumours, an inability which remains when Bcl-2 is

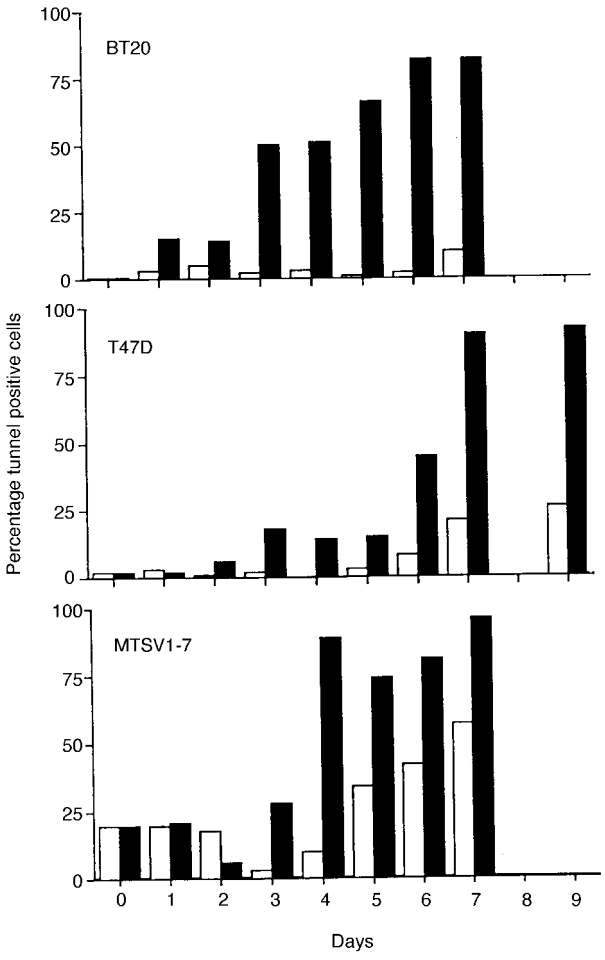

Figure 4. Patterns of occurrence and percentage of apoptotic death in untreated cells (unshaded areas) and in cells treated with β galactoside binding protein (βGBP) (shaded areas). βGBP 2.5×10^{-8} M at 6 h after seeding in BT20 and MTSV 1-7 cells and at 6 h, day 4 and day 7 in T47D cells.

overexpressed and spontaneous death is inhibited [18]. By contrast, the low degree of spontaneous death in the BT20 and the T47D cells, even under crowded cultural conditions (Figures 1 and 2), is an indication of the ability of these cells to form and expand as solid tumours. Our data show that the effect of βGBP on cancer and immortalised cells of epithelial origin parallels that already demonstrated in tumour cells which originate from the mesenchyma [4]. Regardless of their oncogenic potential the three cell lines examined behaved similarly. They were promptly arrested by βGBP during transition from S phase to G_2 and were then led into apoptosis. The requirement for re-addition of βGBP to the T47D cells could relate to the presence of oestrogens in the medium which would stimulate these ER^+ cells or/and to the abundance of proteolytic enzymes that metastatic cells may produce, a fact which would accelerate βGBP degradation and consequent shortening of its 24–28 h operational half-life (V. Wells and L. Mallucci, data not shown). It is of interest that in normal luminal cells, obtained from cosmetic reduction mammoplasties, in short-term culture growth arrest by βGBP is not followed by apoptosis and growth is instead resumed (M. O'Hare, UCL Breast Cancer Laboratory and the Ludwig Institute for Cancer Research, London, U.K.) adding to the evidence that treatments which induce apoptosis in tumour cells fail to do so in their normal counterparts [4, 19, 20] and endorsing the view that apoptosis is the response of transformed cells to an unresolved conflict between sustained mitogenic signals and the cells' inability to progress through the cell cycle [21, 22].

The different properties of the cells examined, such as the oncogenicity of the BT20 and T47D cells and the lack of oncogenicity of the MTSV 1-7 cells plus the dissimilarities in their molecular make up, such as the difference in EGF receptor expression [11, 23] and in ER expression [24], point to the enforced cell cycle arrest as the common feature in all three cell types and as the critical occurrence which may determine a shift from growth arrest to apoptosis. Which molecular events are controlled by βGBP in the transition from S phase to G_2 and may, therefore, be responsible for the apoptotic response of tumour cells, have to be elucidated, but it is conceivable that the activation of the death programme is the result of an alteration of S phase regulatory events. Our emerging view is that high levels of ectopic expression of the E2F1 transcription factor during S phase arrest may be linked to the apoptotic death induced by βGBP [4, 25]. This view is supported by the finding that unscheduled binding of E2F1 to specific DNA sequences during S phase can activate an S phase check point and cause cell cycle arrest, accumulation of cells in S phase and apoptosis [26], by the finding that overexpression of E2F1 can be an initiator of apoptosis [27] and by the finding that an E2F1 block by dominant negative mutants in HBL-100 breast cancer cells inhibits apoptosis and induces tumour growth in SCID mice [28].

Taken together the results of the observations reported above and those of previous studies [4] indicate that βGBP is a potent cell growth suppressor of potential therapeutic value. The present study based on mammary cell lines with different properties now calls for an extended survey of mammary derived cells to determine whether βGBP induced apoptosis is a common property of mammary cancer. What seems of particular interest is that βGBP appears to be effective regardless of EGF receptor and ER expression. Also the evidence presented and its analysis provide reasons to assume that an understanding of the molecular mechanism of action of this novel negative regulator of the cell cycle may permit the identification of molecular phenotypes that are predisposed to βGBP induced cell death.

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