

Growth inhibition of both MCF-7 and Hs578T human breast cancer cell lines by vitamin D analogues is associated with increased expression of insulin-like growth factor binding protein-3

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Abstract

The effects of two vitamin D analogues, EB1089 and CB1093, on insulin-like growth factor binding protein (IGFBP) expression have been examined in MCF-7 and Hs578T human breast cancer cell lines. Both vitamin D analogues inhibited IGF-I stimulated growth of MCF-7 cells and enhanced the production of IGFBP-3 as determined by Western-ligand blotting. Recombinant human IGFBP-3 inhibited the growth of MCF-7 cells over the concentration range 1-235 ng/ml. Hs578T cells were unresponsive to the mitogenic effects of

IGF-I but growth was inhibited by the two vitamin D analogues. Treatment of Hs578T cells with EB1089 and CB1093 (10 nM) as well as 100 nM 9-*cis* retinoic acid (9-*cis* RA) or all-*trans* retinoic acid (ATRA) was associated with increased accumulation of IGFBP-3 in conditioned medium. Furthermore, cotreatment of Hs578T cells with EB1089 and 9-*cis* RA led to augmented effects on both inhibition of cell growth and IGFBP-3 accumulation in conditioned medium as assessed by Western ligand blotting and radioimmunoassay. These findings suggest a role for IGFBP-3 in the growth inhibitory effects of vitamin D analogues.

Introduction

The active form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), has been shown to possess many properties unrelated to its classical functions as a regulator of bone and mineral metabolism. While the role of 1,25(OH)₂D₃ as an agent which inhibits proliferation of breast cancer cells is now well recognised, its use in cancer treatment is precluded by its potent calcaemic activity. Vitamin D analogues have been synthesised which retain the ability to control cell proliferation but display reduced calcaemic activity. Certain of these compounds markedly inhibit the growth of cultured breast cancer cells and cause regression of experimental mammary tumours *in vivo* (Abe *et al.* 1991, Colston *et al.* 1992). However, the precise mechanism of action of these new compounds remains unclear.

Breast cancer cells both secrete and respond to a variety of peptide growth factors. The growth in culture of many established human breast cancer cell lines is stimulated by the insulin-like growth factors (IGFs I and II): in the case of the MCF-7 cell line, IGF-I has been reported to be the most potent mitogen studied (Karey & Sirbasku 1988). Vitamin D analogues limit responsiveness of MCF-7 cells to the mitogenic effects of IGF-I (Vink-van Wijngaarden *et al.* 1996, Xie *et al.* 1997). Cellular effects of IGF-I are initiated by binding to the type I IGF receptor (IGF-IR) and the interaction between IGF-I and IGF-IR is modulated by a family of IGF binding proteins (IGFBPs). The IGFBPs act by either enhancing or by inhibiting the actions of IGFs (DeMellow & Baxter 1988, Cohen *et al.* 1993).

Human IGFBP-3 is a primary modulator of IGF-I actions by preventing IGF-I binding to its receptor and inhibiting IGF-I signalling pathways (Buckbinder *et al.* 1995). IGFBP-3 has been shown to inhibit the proliferation of oestrogen-dependent and -independent breast cancer cells (Oh *et al.* 1993a, Pratt & Pollak 1996) and its expression is increased by ATRA (Fontana *et al.* 1991), tumour growth factor β (Oh *et al.* 1995) and the pure anti-oestrogen ICI 182,780 (Huynh *et al.* 1996). Antisense oligonucleotide to IGFBP-3 abolishes ATRA-induced growth inhibition in MDA-MB-231 breast cancer cells (Gucev *et al.* 1996). The Hs578T breast cancer cell line is growth inhibited by retinoids but these cells are unresponsive to IGF-I. However, growth inhibitory effects of retinoids are associated with increased expression of IGFBP-3 which has been postulated directly to inhibit cell growth independently of IGF-I signalling pathways (Oh *et al.* 1993b). In order to further evaluate the association between modulation of IGF-I signalling pathways and vitamin D-induced growth inhibition of breast cancer cells, we have determined the effects of two vitamin D analogues, EB1089 and CB1093, on growth and IGFBP-3 expression in MCF-7 and Hs578T cells.

Materials and Methods

Compounds

Vitamin D compounds 1,25(OH)₂D₃, CB1093 and EB1089 (Mathiasen *et al.* 1993) were received as gifts from Leo Pharmaceutical Products, Ballerup, Denmark and 9-*cis* RA from Hoffmann-La Roche, Nutley, New Jersey, USA. All compounds were dissolved in absolute ethanol and the final

concentration of ethanol in tissue culture medium did not exceed 0.1%.

Peptides

Human recombinant IGF-I (R&D Systems, Abingdon, Oxon, UK) was diluted in 10 mM hydrochloric acid. Human recombinant IGFBP-3 (Upstate Biotechnology, Lake Placid, NY, USA) was diluted in 10 mM acetic acid. Tissue culture reagents were obtained from Gibco (Paisley, Scotland, UK). All other reagents were analytical grade and obtained from Sigma Chemical Co (Poole, Dorset, UK).

Cells and culture conditions

MCF-7 human breast cancer cells were routinely maintained in Dulbecco's modification of Eagle's minimal essential medium (DMEM), supplemented with 100 U penicillin/ml, 100 µg streptomycin/ml and 5% fetal calf serum (FCS) at 37°C with a humidified atmosphere of 5% CO₂. Hs578T cells were routinely maintained under the same conditions. For growth experiments, MCF-7 cells were trypsinized and 1×10⁴ cells were plated in 48 well plates in 0.5 ml of growth medium (R.p.m.I 1640 medium containing 2.5% charcoal-stripped fetal calf serum (SFCS) with the above mentioned supplements). Cells were cultured for 65-68 h before treatment with IGF-I, vitamin D derivatives or other test compounds in R.p.m.I 1640 medium containing 1% SFCS for 3-10 days with a medium change every 2-3 days. Control cultures were incubated in medium containing vehicle alone. Hs578T cells were incubated in R.p.m.I medium containing 0.1% bovine serum albumin (BSA) and the vitamin D derivatives, retinoids or ethanol vehicle.

Determination of cell proliferation

On selected days after removal of the incubation medium, the cell monolayer was washed twice with phosphate buffered saline (PBS). Cells were fixed and stained by adding 0.5 ml 0.5% crystal violet stain in 25% methanol for 10 min. After washing with distilled water, cultures were air-dried and incorporated dye was eluted with 0.1 M sodium citrate in 50% ethanol and cell number determined as previously described (Wosikowski *et al.* 1993).

Preparation of conditioned medium (CM)

MCF-7 and Hs578T cells were seeded into 24 well plates and grown for 2-3 days in serum containing medium and cell cultures were washed three times with serum-free medium. Cells were then incubated in serum-free R.p.m.I medium containing 0.1% BSA in the presence or absence of the test compounds for various times as indicated in the text. Conditioned media (CM) were collected and centrifuged at 1000 g for 10 min to remove all cell debris. The harvested CM from triplicate wells within each experiment were pooled and stored at -20°C until assay.

Western ligand blotting

Ligand and immunoblotting were performed as previously described (Coulson *et al.* 1991, Cwyfan-Hughes *et al.* 1995). Briefly, proteins from CM were concentrated 10-fold using Millipore Ultrafree-MC filter units, separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto a nylon membrane. This was then probed with an equal mixture of iodinated IGF-I and -II, washed, dried and exposed to X-ray film. The same membrane was then incubated with polyclonal antiserum to IGFBP-3 (SCH-2/6 at 1:15 000 dilution) overnight at room temperature. Following removal of excess unbound antibody, an anti-rabbit antibody conjugated to peroxidase (1:10 000 dilution) was added for 1 h. Binding of the peroxidase conjugate was visualised using enhanced chemiluminescence according to the manufacturer's instructions (Amersham International, Buckinghamshire, UK). The volume of sample loaded was normalized for mean cell number for each treatment group.

Radioimmunoassay

The levels of IGFBP-3 in CM were measured using a radioimmunoassay previously described (Yateman *et al.* 1993).

Statistical methods

Data were analysed by ANOVA using Statview program (Abacus Concept Inc., Berkeley, CA, USA).

Results

MCF-7 cells were incubated for 3, 7 and 10 days in the presence of 30 ng/ml IGF-I alone or in combination with 10 nM EB1089, CB1093 or 1,25(OH)₂D₃. Inhibition of IGF-I-stimulated cell growth was observed with the vitamin D analogues but the same concentration of 1,25(OH)₂D₃ was ineffective in limiting responsiveness of cells to IGF-I (Fig. 1a). The effect of human recombinant IGFBP-3 on MCF-7 cell growth was tested by incubating cells for 7 days in the presence of IGFBP-3 (0-235 ng/ml) before assessment of cell growth by crystal violet assay. IGFBP-3 inhibited cell growth in a dose-related manner such that 70% inhibition was achieved at a concentration of 235 ng/ml (Fig. 1b). We next determined the profile of expression of IGFFBPs present in CM from control cultures and MCF-7 cells treated with synthetic vitamin D analogues by Western ligand blotting. Basal CM showed three major proteins corresponding to IGFFBPs 2, 3 and 4 (Fig. 1c lane a). Immunoblotting confirmed the identity of the ~41 kD species as IGFBP-3 (data not shown). Increases in the accumulation of IGFBP-3 in CM from MCF-7 cells treated with EB1089 and CB1093 (1-100 nM) were observed after 3 days as assessed by Western ligand blots (Fig. 1c).

Unlike MCF-7 cells, Hs578T breast cancer cells were unresponsive to the mitogenic effect of IGF-I over the concentration range 1-150 ng/ml and for up to 7 days of treatment (data not shown). We have previously demonstrated

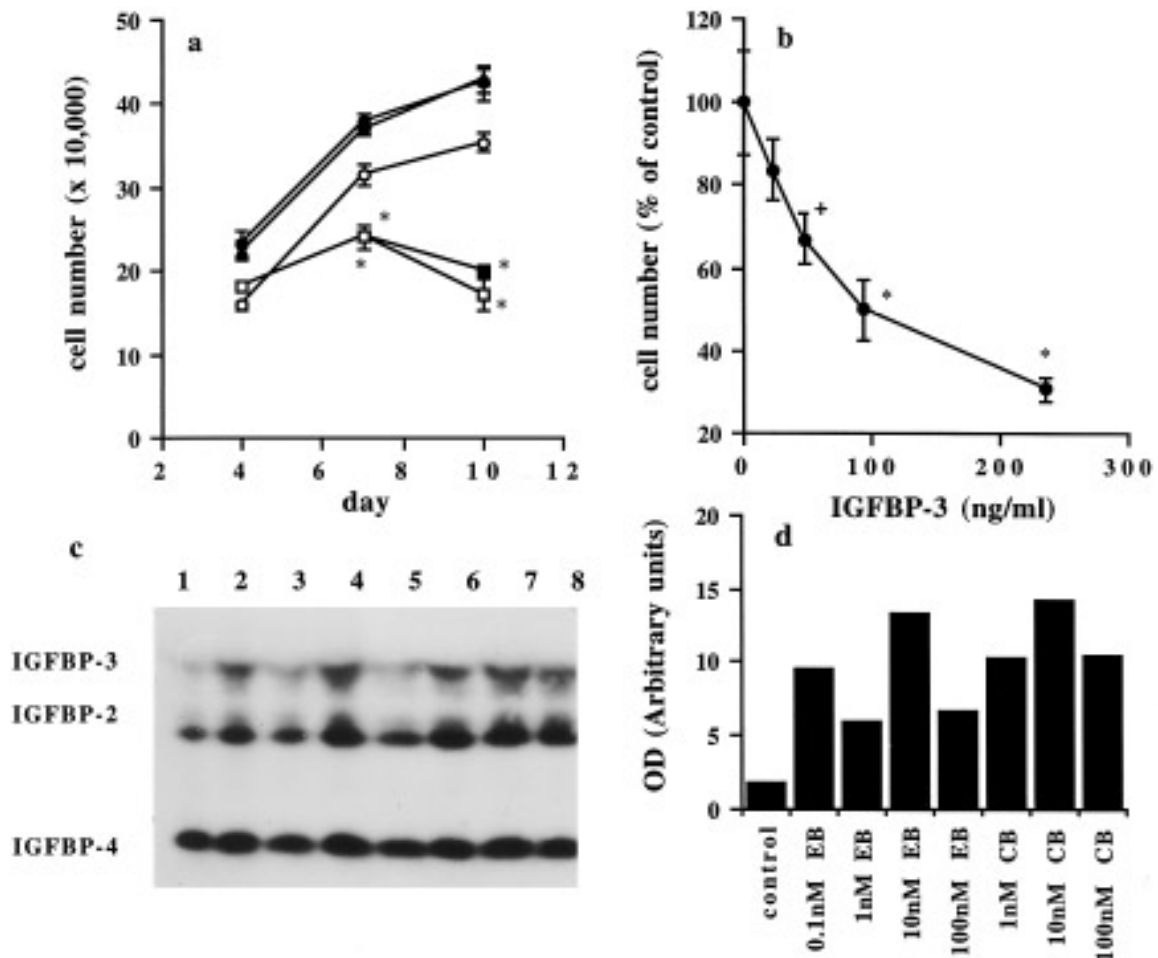


Figure 1 Effects of vitamin D analogues on growth and IGFBP expression in MCF-7 cells. (a) MCF-7 cells were incubated in multiwell plates with ethanol vehicle (○), 30 ng/ml human recombinant IGF-I alone (●) or IGF-I together with 10 nM 1,25(OH)₂D₃ (▲), EB1089 (■) or CB1093 (□). Results are mean ± S.E.M. (*n*=4). **P*<0.005 compared with IGF-I alone. (b) Inhibition of MCF-7 cell growth by increasing concentrations of human recombinant IGFBP-3 for 7 days. **P*<0.05, ***P*<0.005 compared with vehicle-treated control cultures. (c) Western-ligand blot of concentrated CM from cells cultured with vehicle (lane 1), 0.1-100 nM EB1089 (lanes 2-5) and CB1093 (1-100 nM, lanes 6-8) for 3 days. (d) Relative IGFBP-3 levels by scanning densitometry.

that Hs578T cells express specific receptors for 1,25(OH)₂D₃ (Colston *et al.* 1982) and our present studies demonstrate that these cells are inhibited in their growth by the vitamin D analogues EB1089 and CB1093 (Fig. 2a). Western ligand blots demonstrated that IGFBP-3 was the major binding protein secreted by Hs578T cells (Fig. 2c). Immunoblotting confirmed the identity of this protein as IGFBP-3. As expected, accumulation of IGFBP-3 in conditioned medium was increased in cultures treated with ATRA as well as 9-*cis* RA (Fig. 2c lanes 5 and 6). However, marked increases in IGFBP-3 secretion were also observed in cultures treated with the vitamin D analogues EB1089 and CB1093 (Fig. 2b and Fig. 2c lanes 2 and 4). Both the analogues produced an approximately 7-fold increase in IGFBP-3 accumulation in CM collected after

3 days treatment with the vitamin D derivatives. Effects of 1,25(OH)₂D₃ were less marked (Fig. 2b). Cotreatment of Hs578T cells with 10 nM EB1089 and 100 nM 9-*cis* RA led to augmented effects on cell growth (Fig. 3a) and IGFBP-3 accumulation in CM.

Fig. 3b shows the increase in IGFBP-3 levels as assessed by RIA in cotreated cells relative to control cultures. Significant effects on growth inhibition were seen after 3 days treatment with EB1089 and 9-*cis* RA in combination at which time a 3-fold increase in IGFBP-3 levels in CM was seen.

In cultures treated with EB1089 alone, the time course of change in IGFBP-3 accumulation was slower than in cotreated cultures but the magnitude of increase in IGFBP-3 levels was similar by 7-10 days treatment. 100 nM 9-*cis* RA

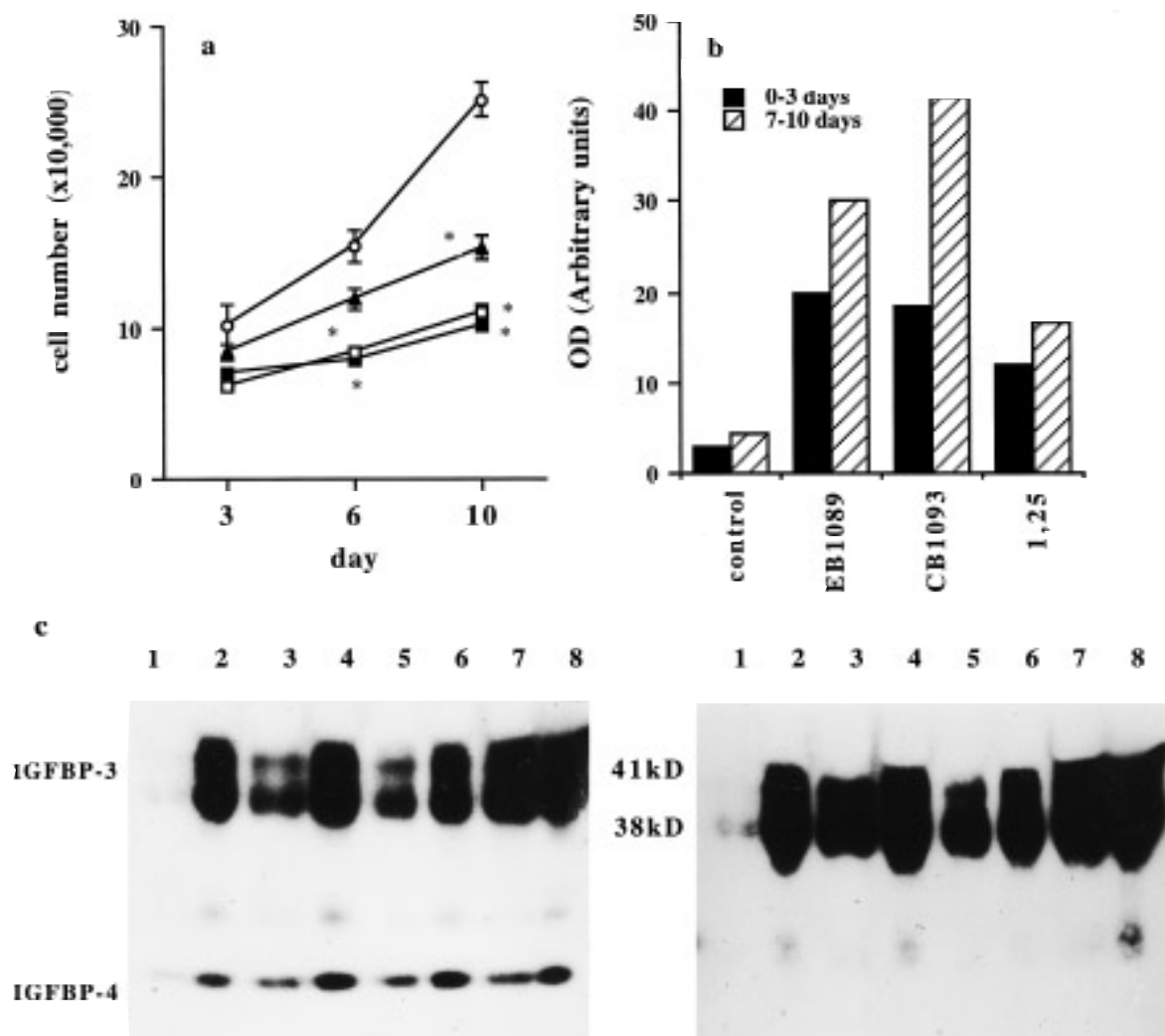


Figure 2 Effects of vitamin D analogues on growth and IGFBP expression in Hs578T cells (a) Effects of vitamin D analogues on cell growth. Hs578T cells were incubated with ethanol vehicle (○), 10 nM 1,25(OH)₂D₃ (▲), 10 nM EB1089 (■) or 10 nM CB1093 (□). Cell proliferation was assessed after 3, 6 and 10 days of treatment. Results are mean ± S.E.M. (n=4). *P<0.005 compared with vehicle-treated cultures. (b) Relative IGFBP-3 levels by scanning densitometry of Western ligand blots of concentrated CM collected from cultures treated with 10 nM vitamin D derivatives for up to 10 days. CM collected for days 0-3 (solid bars) or 7-10 (hatched bars) of treatment. (c) Western-ligand blot (left) and immunoblot with anti-IGFBP-3 antibody (right) of concentrated CM from cells cultured for 10 days with: ethanol vehicle (lane 1), 10 nM EB1089 (lane 2), 10 nM 1,25(OH)₂D₃ (lane 3), 10 nM CB1093 (lane 4), 100 nM ATRA (lane 5), 100 nM 9-cis RA (lane 6), EB1089 and ATRA (lane 7), EB1089 and 9-cis RA (lane 8). CM collected for days 7-10 of treatment.

alone induced only a modest increase in IGFBP-3 over this period.

Discussion

Insulin-like growth factors are believed to play an important role in the development and growth of breast tumours (Cullen *et al.* 1990, Osbourne *et al.* 1990, Rosen *et al.* 1991). The

mitogenic actions of IGF-I are initiated following binding to its membrane receptor and the interaction between IGF-I and IGF-IR is modulated by a family of at least 7 related IGFbps which have a tissue specific pattern of expression and which may potentiate or, more usually, inhibit the actions of IGF-1 by preventing receptor interaction. In human breast cancer cell lines, the pattern of IGFBP expression is correlated with oestrogen receptor status with ER positive cells producing

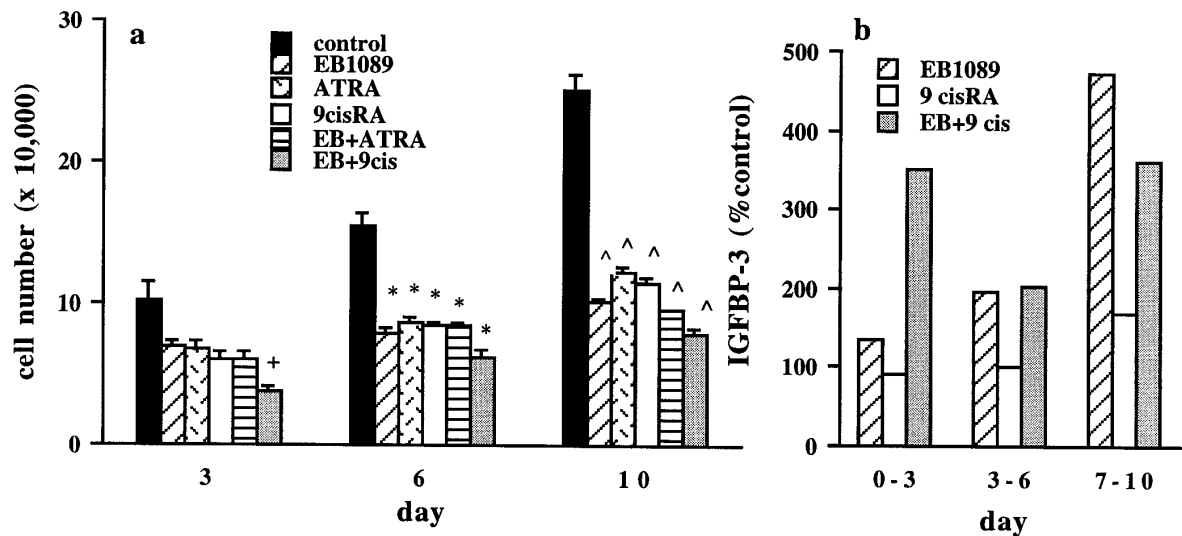


Figure 3 Effects of EB1089 and retinoids on growth and IGFBP-3 expression in Hs578T cells. Cells were cultured for up to 10 days with ethanol vehicle, 10 nM EB1089, 100 nM ATRA, 100 nM 9-*cis* RA or combinations of these agents. (a) Cell number was quantitated by crystal violet assay. Results are means \pm S.E.M. * P <0.01, * P <0.005, ^ P <0.001 compared with ethanol vehicle-treated cultures. (b) CM was collected from cultures treated with 10 nM EB1089, 100 nM 9-*cis* RA and these two agents in combination at days 0-3, 3-6 and 7-10 of treatment and IGFBP-3 concentration (ng/ml) determined by RIA. Results were adjusted for mean cell number in replicate cultures from each treatment group and are expressed as % of respective control cultures. Mean concentrations of IGFBP-3 in CM from control cultures were 41.1, 64.5 and 62.1 ng/ml at 3, 6 and 10 days of culture respectively.

IGFBP-2 as the major form whereas ER negative cells secrete mainly IGFBP-3. IGFBP-3 has been shown to inhibit the proliferation of both oestrogen dependent and independent breast cancer cells. As well as inhibiting IGF-I signalling, IGFBP-3 may have direct anti-proliferative activity as has been suggested by studies with the Hs578T breast cancer cell line which is unresponsive to IGF-I (Oh *et al.* 1993a) and expresses low or undetectable levels of IGF-IR (our unpublished observations). In this study we have demonstrated that two synthetic vitamin D analogues, EB1089 and CB1093, inhibited the growth of Hs578T cells as well as MCF-7 cells. In both cell lines, inhibition of cell growth was associated with increased production of IGFBP-3. It has previously been demonstrated that the potent inhibitory effects of the anti-estrogen ICI 182,780 are associated with increased secretion of IGFBP-3 by the cells (Huynh *et al.* 1996) and an association between growth inhibition by ATRA and modulation of IGFBP-3 has been demonstrated in both Hs578T and MCF-7 cells (Fontana *et al.* 1991). Our present findings indicate that effects of vitamin D analogues on breast cancer cells may be mediated in part by reduction in IGF-I responsiveness through increased expression of inhibitory binding proteins such as IGFBP-3. However, in comparison to effects in Hs578T cells, the magnitude of increase in IGFBP-3 expression in MCF-7 cells in response to vitamin D analogues was found to be modest and levels of the binding protein accumulating in media conditioned by the cells were low and beneath the detection

limit of our RIA (which detects concentrations of IGFBP-3 greater than 16 ng/ml).

We would speculate that, in Hs578T cells, increased production and secretion of IGFBP-3 plays a role in the growth inhibitory effects of vitamin D analogues which we have observed in this IGF-I-unresponsive cell line. However in MCF-7 cells, which produce smaller quantities of IGFBP-3 under basal conditions, although the expression of IGFBP-3 is increased by vitamin D analogues, the level of protein secretion is low and may only exert minimal effects on cell growth. Decreased expression of IGF-IR in response to vitamin D analogues (Xie *et al.* 1997) or changes in the production of other inhibitory IGFbps such as IGFBP-5 (Rozen *et al.* 1997) may also play a role in mediating the observed effects of vitamin D analogues on MCF-7 cell growth and IGF-I responsiveness. In breast cancer cells, vitamin D analogues may directly regulate IGFBP-3 gene expression as has been suggested for ATRA (Adamo *et al.* 1992). Alternatively, effects may be mediated via changes in p53. The gene encoding IGFBP-3 is induced by wild-type p53 (Buckbinder *et al.* 1995). We have previously shown that the vitamin D analogue EB1089 increases p53 protein expression in MCF-7 cells and that cotreatment with 9-*cis* RA enhances this effect (James *et al.* 1995). Furthermore, vitamin D analogues alone (Welsh 1994, James *et al.* 1996) and in combination with 9-*cis* RA (James *et al.* 1995) have been shown to induce apoptosis in MCF-7 cells. Attenuation of the cell survival signal generated

through the IGF-I receptor is a possible mechanism by which vitamin D derivatives could promote active cell death.

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