Pergamon

European Journal of Cancer, Vol. 35, No. 12, pp. 1717–1723, 1999
© 1999 Elsevier Science Ltd. All rights reserved.
Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(99)00200-2

Original Paper

Vitamin D Analogues Suppress IGF-I Signalling and Promote Apoptosis in Breast Cancer Cells

S.P. Xie, G. Pirianov and K.W. Colston

Department of Oncology, Gastroenterology, Endocrinology and Metabolism, St George's Hospital Medical School, Cranmer Terrace, London SW17 ORE, U.K.

Survival factors are known to promote cell viability, and factor deprivation can be a potent apoptotic signal. Insulin-like growth factors are potent mitogens and inhibitors of apoptosis for many normal and neoplastic cells with insulin-like growth factor-I (IGF-I) being the most effective in many breast cancer cell lines. 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and its analogues inhibit IGF-I-stimulated growth of MCF-7 human breast cancer cells. The aim of this study was to determine the relationship between inhibition of IGF-I responsiveness and induction of apoptosis by vitamin D analogues in breast cancer cells. Vitamin D analogues EB1089 and CB1093 inhibited autonomous and IGF-I-stimulated growth of MCF-7 and T47D cells and autonomous growth of IGF-I-insensitive Hs578T cells. In MCF-7 cells, IGF-I alone (4 nM) protected against apoptosis mediated by serum deprivation. Co-treatment with vitamin D analogues prevented the anti-apoptotic effects of IGF-I. In T47D cells, IGF-I treatment provided only partial protection against apoptosis induced by serum deprivation and co-incubation of serum-deprived cells with 100 nM CB1093 and IGF-I abrogated this partial protection. In Hs578T cells, addition of IGF-I did not prevent apoptosis induced by serum deprivation. However, treatment with CB1093 attenuated the protective effect of the serum in these cells. Our findings suggest that vitamin D analogues inhibit IGF-I signalling pathways to promote apoptosis in breast cancer cells. **© 1999 Elsevier Science Ltd. All rights reserved.**

Key words: apoptosis, breast cancer cells, 1,25-dihydroxyvitamin D_3, EB1089, insulin-like growth factor-I, insulin-like growth factor-I receptor, MCF-7 cells, vitamin D analogues
Eur J Cancer, Vol. 35, No. 12, pp. 1717–1723, 1999

INTRODUCTION

INSULIN-LIKE GROWTH factor I action has been implicated in the pathogenesis of many different malignancies including breast cancer. Insulin-like growth factor receptors are over-expressed in virtually all breast cancer cell lines [1, 2] and a recent report has suggested that high circulating IGF-I concentrations may be associated with an increased risk of breast cancer [3]. There is substantial experimental evidence to suggest that the insulin-like growth factor I receptor (IGF-IR) plays an important role in cellular transformation, mitogenesis and the inhibition of apoptosis [4]. IGF-I is a potent mitogen and survival factor for many cell types including normal breast epithelium and breast cancer cells [5]. The influence of IGF-I on cell survival is determined by extracellular concentrations of the factor and by the levels of several IGF-I binding proteins which influence the availability of IGF-I to interact with its specific receptor (IGF-IR). Excessive IGF-I levels or IGF-IR activity may be tumorigenic because apoptosis is blocked [4]. There is evidence that IGF-I can prevent or delay apoptosis induced by a variety of stimuli and conversely, a decrease in IGF-IR abundance or function induces apoptosis and inhibits the tumorigenicity of metastatic breast cancer cells [6]. The active metabolites of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and its analogues have been demonstrated to inhibit the growth of human breast cancer cells and induce apoptosis *in vitro* and *in vivo* [7-9]. However, the mechanism of action of these vitamin D compounds remains to be elucidated. It has been demonstrated that vitamin D compounds limit the responsiveness of MCF-7 human breast cancer cells to the

Correspondence to K.W. Colston, e-mail: k.colston@sghms.ac.uk
Received 20 Feb. 1999; revised 6 Jul. 1999; accepted 16 Jul. 1999.

mitogenic effects of IGF-I [10, 11]. In order to identify the mechanisms involved in this anti-IGF-I activity, we have examined the regulatory effects of $1,25(OH)_2D_3$ and its low calcaemic analogues EB1089 and CB1093 on the expression of IGF-IR. The relationship of reduced IGF-I responsiveness to induction of apoptosis by the vitamin D analogues was determined in MCF-7 cells in comparison with T47D cells which are less responsive to the growth factor [12] and the IGF-I-independent cell line Hs578T [13].

MATERIALS AND METHODS

Materials

Vitamin D derivatives, including $1,25(OH)_2D_3$, EB1089 and CB1093 were received as gifts from Leo Pharmaceutical Products (Ballerup, Denmark). All the compounds were initially dissolved in absolute ethanol, dilutions made in full culture medium (the final concentration of ethanol in tissue culture medium did not exceed 0.1%). Human recombinant IGF-I from R&D Systems Europe Ltd (Abingdon, Oxon, U.K.) was diluted in 10 mM acetic acid. Polyclonal antibodies recognising α and β subunits of IGF-IR were purchased from Santa Cruz Biotechnology, Inc. (Devizes, Wilts, U.K.). Tissue culture medium and reagents were purchased from Life Technologies (Paisley, Strathclyde, U.K.). All other analytical grade reagents were obtained from Sigma (Poole, Dorset, U.K.).

Cell lines and culture conditions

All human breast cancer cell lines (MCF-7, T47D and Hs578T) were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 5% fetal calf serum. For growth experiments, cells were trypsinised and 2×10^4 cells were incubated in 24 well plates in the growth medium (RPMI-1640 containing 5% fetal calf serum (FCS)). Following 24 h incubation a fresh medium containing 1% FCS and test compounds was added. Control cultures were incubated in medium containing appropriate vehicle.

Proliferation assay

Cell growth was determined by a crystal violet assay as previously described [14]. Briefly, cells were fixed and stained by 0.5% crystal violet in 25% methanol for 10 min. After washing with water, cells were air-dried and incorporated dye was eluted with 0.1 M sodium citrate in 50% ethanol. The absorbance at 550 nm of each sample was measured in a microplate reader (Titertek Multiskan) and then compared with a standard curve of known cell numbers, from which cell growth was calculated.

Cell viability and DNA fragmentation assays

Neutral red assay. Neutral red is a vital dye which accumulates in the lysosomes of living, uninjured cells [15]. To assess the effects of serum deprivation and vitamin D analogue treatment on breast cancer cell viability, cells were seeded into 24-well plates (Nunc, Oxford, U.K.) at a density of 2×10^4 cells/well in 1% FCS in RPMI-1640 medium. In other experiments, cells were washed and switched to serum-free RPMI-1640 (supplemented with bovine serum albumin 0.5 mg/ml and transferrin 0.01 mg/ml) and vitamin D analogues or IGF-I alone or in combination were added for up to 4 days of culture. At the end of the incubation period, medium was removed and cells were incubated with neutral red solution (Sigma; 40 μg/ml in phenol-red and serum-free DMEM) for 2 h at 37°C. After removal of the neutral red solution, wells were rinsed once with 1 ml 4% formal saline containing 0.5% $CaCl_2$. Plates were inverted on paper towels to drain and 200 μl of elution fluid (1% acetic acid in 50% ethanol) was added. Following incubation at room temperature for 30 min with gentle shaking, absorbance at 550 nm was determined using a Titertek plate reader.

DNA fragmentation assay. Breast cancer cells were incubated with [^{3}H]-thymidine (0.5 μCi/ml) for 24 h to label the DNA and then washed before exposure to the indicated agents. Cells were lysed and [^{3}H]-thymidine incorporated into both soluble and unfragmented DNA was determined by liquid scintillation counting using the formula: % fragmented DNA = 100(fragmented/fragmented + intact chromatin) as previously described [16].

Immunoblotting

For the IGF-IR, cells were harvested and total protein was extracted. Equivalent protein from each sample was fractionated on a 7.5% SDS-PAGE gel and transferred onto a nitrocellulose membrane. This was then incubated with 0.5 μg/ml of rabbit polyclonal antibodies recognising the α-subunit of IGF-IR (N-20) or the β subunit of IGF-IR (C-20). All subsequent steps were performed according to the protocol provided by Santa Cruz Biotechnology. The protein bands obtained were quantified using densitometric analysis that was performed on a MicroTek Scanmaker flat bed scanner with NIH Image 1.52 software.

Statistical methods

Data were analysed by ANOVA using the software programme, Statview (Abacus Concept, Inc., Berkeley, California, U.S.A.). Statistical significance was calculated at $P<0.001$, $P<0.005$ and $P<0.01$ levels.

RESULTS

Vitamin D analogues suppress IGF-I signalling in MCF-7 breast cancer cells

Effects on IGF-I-stimulated cell proliferation. MCF-7 cells were incubated for up to 10 days in the presence of 4 nM IGF-I alone or in combination with 10 nM EB1089 or CB1093. Both vitamin D analogues produced a significant inhibition of IGF-I-stimulated growth indicating that these compounds limit responsiveness to the growth-stimulatory effects of the growth factor (Figure 1a). Cell proliferation was also assessed by a crystal violet assay after 7 days treatment with 4 nM IGF-I or vehicle in combination with increasing concentrations of CBI093. 50% growth inhibition was achieved with 2.4 nM CB1093 in the presence of IGF-I and 0. 37 nM CB1093 in the absence of the cytokine.

Downregulation of IGF-IR expression. To study whether diminished responsiveness of MCF-7 cells to the mitogenic effects of IGF-I with vitamin D treatment occurred via modulating the expression of IGF-IR, immunoblot analysis was carried out. Figure 1(c) shows the effects of vitamin D analogue EB1089 on IGF-IR expression in MCF-7 cells after 4, 7 and 10 days of treatment. Results revealed that expression of both the α and β subunit was diminished with vitamin D analogue treatment. However, downregulation of the β subunit appeared to be more rapid than that of the α subunit, being evident by 4 days of treatment.

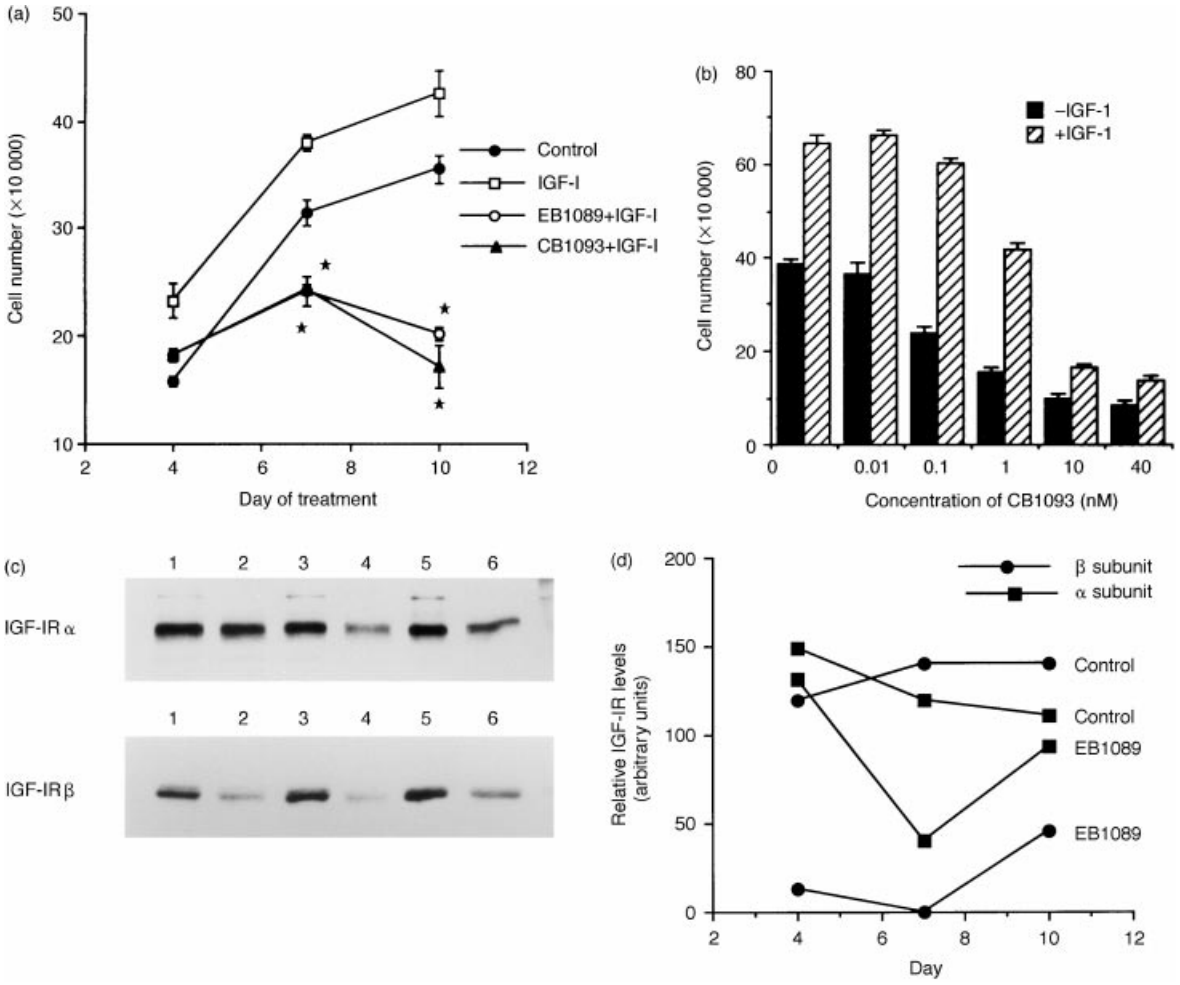

Figure 1. Effects of vitamin D analogues on IGF-I-stimulated growth and IGF-IR expression in MCF-7 cells. (a) Inhibition of IGF-I-stimulated growth of MCF-7 breast cancer cells. Results are mean ± SEM ($n = 4$) and are representative of three separate experiments. *$P < 0.005$, compared with IGF-I alone. (b) Inhibition of autonomous and IGF-I-stimulated MCF-7 cell growth by increasing concentrations of CB1093. 4 nM IGF-I (hatched bars) or vehicle (solid bars). Results are ± SEM (standard error of the mean) ($n = 4$) and are representative of three separate experiments. (c, d) Effects of EB1089 on IGF-IR expression in MCF-7 cells. MCF-7 cells were treated for 4, 7 or 10 days with vehicle (lanes 1, 3, 5) or 10 nM EB1089 (lanes 2, 4, 6). The α and β-subunits of IGF-IR were determined by Western immunoblots. Densitometric analysis of the bands is shown in (d).

Interaction of vitamin D analogues with IGF-I on induction of apoptosis in MCF-7 breast cancer cells. We have already demonstrated that both EB1089 and CB1093 induce apoptosis in MCF-7 cells [17]. Serum deprivation is a potent stimulus for induction of apoptosis. MCF-7 cells were induced to undergo apoptosis by switching cultures from medium containing 1% serum to serum-free medium. MCF-7 cells incubated in 1% serum-containing medium and treated with CB1093 (100 nM) for 4 days (S + CB1093) showed induction of apoptosis similar to that obtained by serum deprivation (SF) (Figure 2a,b). IGF-I (4 nM) protected cells from apoptosis due to serum deprivation as assessed by neutral red cell viability assay (Figure 2c) and quantitation of DNA fragmentation (Figure 2d). To determine if vitamin D analogues may, in part, induce apoptosis by inhibiting responsiveness to this cell survival factor, we determined if cotreatment with vitamin D analogues could attenuate the anti-apoptotic effects of IGF-I. Our results show that co-incubation of serum-deprived cells with 100 nM CB1093 and IGF-I for 4 days significantly decreased the effectiveness of IGF-I to promote cell survival (Figure 2c) and to protect against induction of DNA fragmentation (Figure 2d).

Interactions of vitamin D analogues and IGF-I in T47D cells

The (oestrogen receptor) ER-positive T47D cell line has been shown to be growth stimulated by IGF-I but the concentration required to achieve half-maximal stimulation is approximately 10-fold higher than for MCF-7 cells [12]. The vitamin D analogues EB1089 and CB1093 inhibited both autonomous and IGF-I-stimulated growth of T47D cells (Figure 3). Our results also indicate that vitamin D analogues induce apoptosis in these cells. As with MCF-7 cells, treatment of T47D cells with CB1093 in medium containing 1% FCS led to loss of cell viability and induction of DNA fragmentation (Figure 4a,b). Serum deprivation also induced T47D cells to undergo apoptosis, and levels of DNA fragmentation seen in serum-deprived cells were similar to values seen in cultures incubated with the vitamin D analogue in serum-containing medium (Figure 4b). However, IGF-I (4 nM) only partially protected cells from apoptosis due to serum deprivation as assessed by neutral red cell viability assay (Figure 4a) and quantitation of DNA fragmentation (Figure 4b). Co-incubation of serum-deprived cells with 100 nM CB1093 and IGF-I abrogated this partial protection (Figure 4a,b).

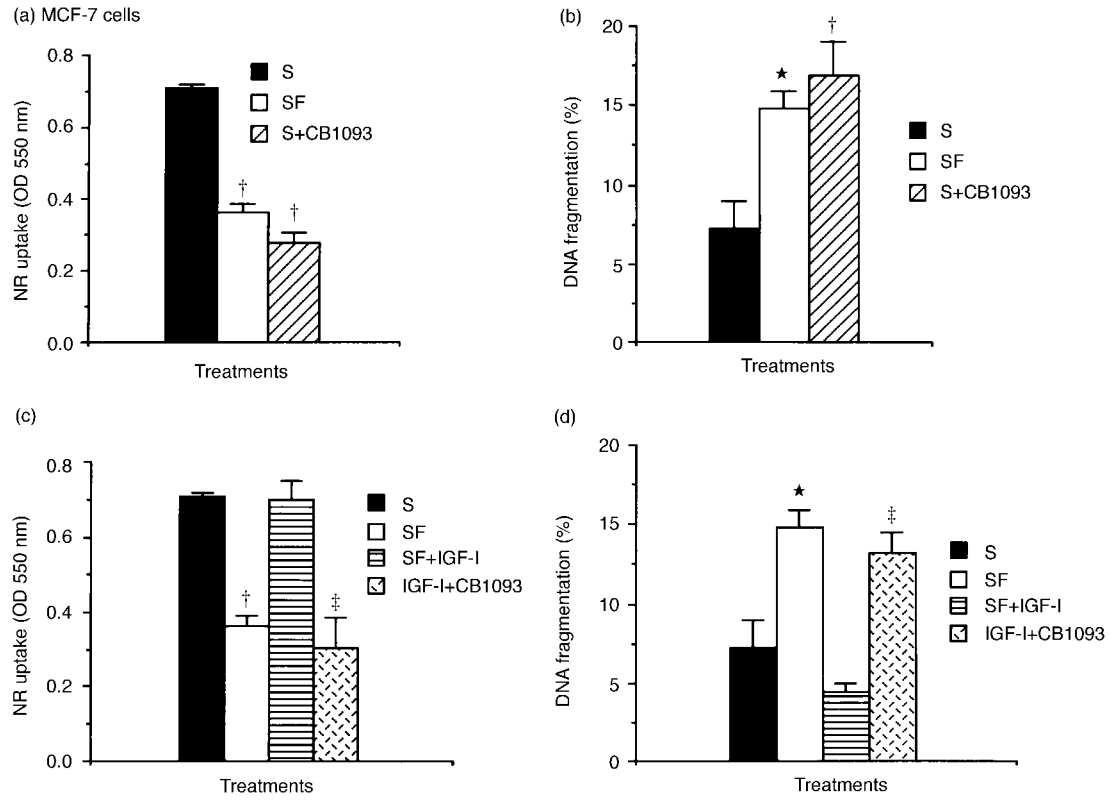

Figure 2. Interaction of CB1039 with IGF-I on apoptosis in MCF-7 cells. MCF-7 cells were cultured in 1% fetal calf serum (FCS) medium (S) or in serum-free medium (SF) and treated for 4 days with vehicle, 4 nM IGF-I, 100 nM CB1093 alone or together with IGF-I. Cell viability (a, c) and DNA fragmentation (b, d) were assessed. Results are expressed as ± SEM (standard error of the mean) ($n = 4$). *$P<0.01$, †$P<0.001$ compared with control (vehicle treated) cultures maintained in 1% FCS containing medium and ‡$P<0.01$ compared cotreatment of CB1093 and IGF-I versus IGF-I alone in serum-free medium.

Effects of vitamin D analogues on IGF-I-independent Hs578T cells

We have previously demonstrated that vitamin D analogues inhibit the growth of Hs578T cells which are non-responsive to the mitogenic effects of IGF-I [18]. Using Western analysis, no bands corresponding to either the α or β subunit of IGF-IR could be visualised with lysates from Hs578T cells (data not shown). To determine the effects of serum deprivation in these cells and its relation to apoptosis, Hs578T cells were changed from medium containing 1% serum to serum-free medium and loss of cell viability and DNA fragmentation were assessed. This study revealed that serum deprivation induces apoptosis in these cells but that IGF-I (4 nM) is not protective (Figure 5). We therefore conclude that IGF-I is an important cell survival factor in MCF-7 cells but other serum factors mediate cell survival in Hs578T cells. To determine if vitamin D analogues can induce apoptosis in an IGF-I-independent manner, Hs578T cells were incubated with CB1093 and cell viability and DNA fragmentation were assessed in 1% serum-containing medium. Results show that CB1093 induces a modest loss of cell viability accompanied by an increase in DNA fragmentation (Figure 5).

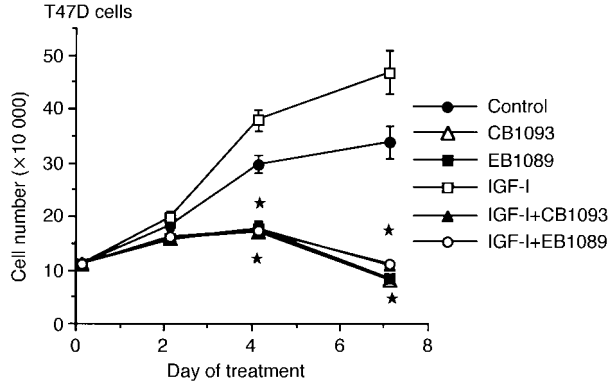

Figure 3. Anti-IGF-I activity of vitamin D analogues EB1089 and CB1093 in T47D breast cancer cells. T47D cells were treated for 7 days with 4 nM IGF-I alone or together with 100 nM CB1093 or EB1089. Results are expressed as ± SEM (standard error of the mean) ($n = 4$) and are representative of three separate experiments. *$P<0.001$, compared with IGF-I alone.

DISCUSSION

In previous studies, we and others have demonstrated that vitamin D derivatives inhibit the mitogenic effects of IGF-I on ER-positive MCF-7 breast cancer cells [10, 11]. In this paper, we have investigated further the effects of these compounds on two other breast cancer cell lines, T47D which display reduced responsiveness to IGF-I, and Hs578T cells which are IGF-I non-responsive. The results show that vitamin D analogues also inhibit IGF-I-stimulated growth in T47D cells (Figure 3). Treatment of these cells with IGF-I in combination with EB1089 or CBI093 produced a significant and time-related inhibition of cell growth compared with IGF-I alone. It has been previously demonstrated the Hs578T cells are non-responsive to the mitogenic actions of IGF-I [11] but are growth inhibited by EB1089 and CB1093

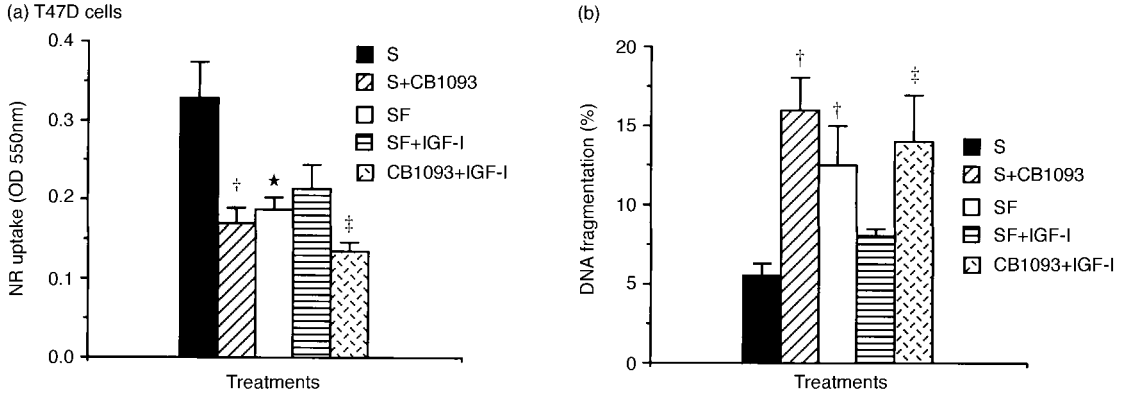

Figure 4. Interaction of CB1039 with IGF-I on apoptosis in T47D breast cancer cells. Cells were cultured in 1% fetal calf serum (FCS) medium (S) or in serum-free medium (SF) and treated for 4 days with vehicle, 4 nM IGF-I, 100 nM CB1093 alone or together with IGF-I. Cell viability (a) and DNA fragmentation (b) were assessed. Results are expressed as mean ± SEM (standard error of the mean) ($n = 4$). *$P < 0.01$. †$P < 0.001$ compared with control (vehicle treated) cultures maintained in 1% FCS containing medium. ‡$P < 0.01$ compared co-treatment of CB1093 and IGF-I versus IGF-I alone in serum-free medium.

[18]. We were unable to identify the IGF-IR (α or β-subunits) in using Hs578T cell lysates by Western analysis indicating that this cell line does not express a functional receptor (or it is expressed at levels too low to detect by immunoblotting) and is consistent with a previous report by De Leon and colleagues, showing a lack of Hs578T cell proliferation in response to IGF-I [19]. The ability of vitamin D analogues to inhibit cell growth and promote cytotoxicity in Hs578T cells demonstrates that these compounds can modulate responsiveness of breast cancer cells to different growth factors.

The growth of breast cancer depends not only on the proliferation of cells but also on the rate of cell death. Apoptosis is an important mechanism of cell death and it has already been proposed as the major target for cancer therapy [20]. Excessive levels of IGF-I or IGF-IR activity may be tumorigenic because apoptosis is blocked and several observations suggest a direct role for inhibition of apoptosis by IGF-I receptor activation [4, 21].The ability of anticancer drugs to induce apoptosis in breast cancer cells may be modulated by the availability of survival factors and IGF-I has been shown to be effective against apoptosis induced by diverse chemotherapeutic agents and tamoxifen [22]. Serum deprivation is a potent promotor of apoptosis and our results demonstrate that IGF-I can prevent cell death induced by this signal in MCF-7 cells. The effectors of IGF-IR signalling which lead to inhibition of apoptosis are poorly understood. Stimulation of the IGF-IR leads to activation of both the Ras/Raf/MAP-kinase cascade and the phosphoinositol-3-kinase (P13K) signalling pathway. PI3K has been implicated in the anti-apoptotic pathway since inhibitors of this kinase have been shown to prevent IGF-I-mediated cell survival in MCF-7 cells and human fibroblasts [23, 24]. Inhibition of the SAPK/JNK cascade has also been suggested as an effector of the anti-apoptotic actions of IGF-I [25]. Our present results clearly demonstrate that vitamin D analogues prevent the anti-apoptotic effects of IGF-I. Incubation of cells maintained in serum-free medium with the vitamin analogue CB1093 inhibited the protective effect of IGF-I suggesting that IGF-I is the most important serum factor promoting survival of these cells and that vitamin D analogues may induce apoptosis via attenuation of the survival signal generated through the IGF-IR. Mechanisms involved may include modulation of bioavailability of IGF-I via changes in expression of inhibitory binding proteins, decreased expression of IGF-IR, and/or modulation of downstream effectors in the IGF-I anti-apoptotic signalling pathway, such as P13K and/or JNK.

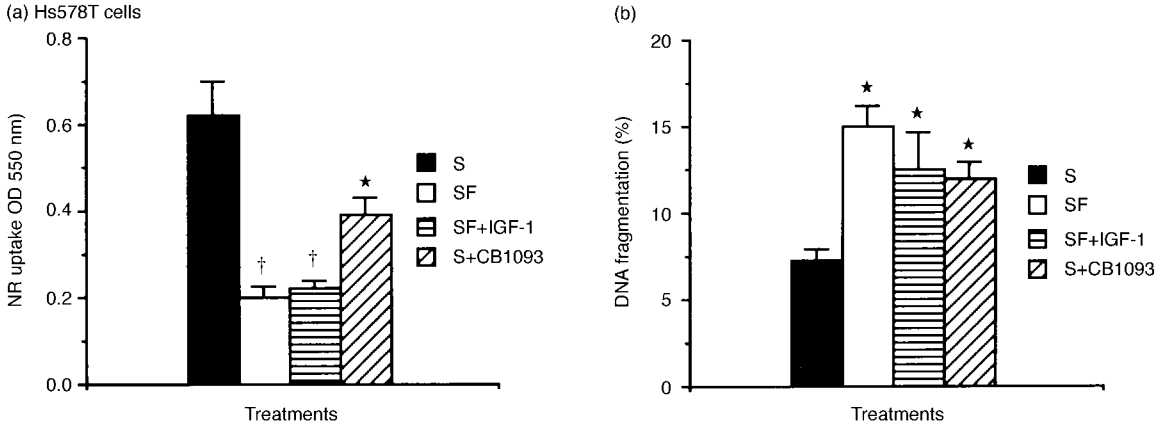

Figure 5. Effect of serum deprivation, IGF-I and CB1093 in Hs578T breast cancer cells. Cells were cultured in 1% fetal calf serum (FCS) medium (S) or in serum-free medium (SF) and treated for 5 days with vehicle, 4 nM IGF-I, 100 nM CB1093 alone or together with IGF-I. Cell viability (a) and DNA fragmentation (b) were assessed. Results are expressed as ± SEM (standard error of the mean) ($n = 4$). *$P < 0.01$. †$P < 0.001$ compared with control (vehicle treated) cultures maintained in 1% FCS containing medium.

The interaction between IGF-I and IGF-IR is modulated by a family of related binding proteins (IGFBPs). The major circulating form, IGFBP-3, binds IGF-I with high affinity, and inhibits IGF-I signalling pathways by limiting interaction with IGF-IR. Modulation of IGFBP-3 expression in MCF-7 cells by anti-oestrogens may promote apoptosis by reducing the bioavailability of IGF-IR agonists [26]. We have previously demonstrated that inhibition of MCF-7 cell growth by vitamin D analogues is associated with increased IGFBP-3 expression [18]. Furthermore, Rozen and colleagues reported that vitamin D-related compounds suppress MCF-7 cell proliferation by stimulation of the production of IGFBP-5 [27].

Effects of vitamin D derivatives on limiting the anti-apoptotic effects of IGF-I in breast cancer cells may be related to diminished expression of IGF-IR. The IGF-IR belongs to the family of tyrosine kinase receptors. The α subunit of IGF-IR (130 kDa) is required for ligand binding with IGF-I [28]. Intracellular regions of the β subunit possesses several binding sites (ATP, insulin receptor substrate-1, 2 and 3) and a tyrosine kinase domain, required for activation of downstream effectors in IGF-IR signalling [4]. Previous studies have shown that antibodies to IGF-IR inhibit the growth of breast cancer cells, both *in vivo* and *in vitro* [21, 29]. Furthermore, these studies have shown that the specific domains in the C-terminus of the β subunit required for the anti-apoptotic function are distinct from those required for mitogenesis [29]. Our present findings demonstrate that the vitamin D analogue EB1089 decreases expression of both the α and β subunits of the IGF-IR with different kinetics (Figure 1c,d). Treatment with EB1089 decreased levels of the β subunit at day 4, while for the α subunit the decrease in expression was evident at day 7. Downregulation of the β subunit of the IGF-IR by the vitamin D analogue may contribute to the observed suppression of anti-apoptotic signals generated by IGF-I. Possible interactions between vitamin D and downstream effectors in IGF-IR signalling warrant further investigation.

The ER-positive T47D cell line has been shown to be growth stimulated by IGF-I but the concentration required to achieve half-maximal stimulation is higher than for MCF-7. The vitamin D analogues EB1089 and CB1093 inhibited both autonomous and IGF-I-stimulated growth of T47D cells (Figure 3). Our results also indicate that vitamin D analogues induce apoptosis in these cells. T47D cells also undergo apoptosis following serum deprivation but IGF-I was only partially effective in promoting cell survival in these cells indicating that other serum factors are also important for maintaining viability of these cells (Figure 4). Nevertheless, levels of DNA fragmentation seen in cultures maintained in serum-containing medium and treated with the vitamin D analogues were similar to values in serum-deprived cells indicating that vitamin D analogues may also limit the responsiveness of breast cancer cells to survival factors other than IGF-I.

Our present studies confirm that vitamin D analogues inhibit the proliferation of Hs578T cells, which are not growth stimulated by IGF-I and lack functional IGF-IR. Our studies also reveal that serum deprivation induces apoptosis in these cells but that IGF-I is not protective, indicating that other serum factors mediate cell survival in Hs578T cells. Treatment of these cells with CB1093 attenuated the protective effect of serum indicating that vitamin D analogues may modulate responsiveness to other serum growth factors. Our previous studies have revealed that vitamin D analogues increase expression of IGFBP-3 in Hs578T breast cancer cells [18]. It has been suggested that IGFBP-3 may directly inhibit the growth of this IGF-I-independent cell line [13]. In contrast to MCF-7 cells, IGFBP-3 does not appear to promote apoptosis directly in Hs578T cells but potentiates the effects of C_2-ceramide, a known inducer of apoptosis [30]. Our preliminary studies indicate that the vitamin D analogue CB1093 promotes ceramide-induced apoptosis and we speculate that this could be due, in part, to increased IGFBP-3 expression. Further studies are required to determine the nature of the serum factors which mediate cell survival in this IGF-I-independent cell line and the mechanisms by which vitamin D analogues and IGFBP-3 can promote apoptosis.

1. Dews M, Nishimoto I, Basgra R. IGF-I receptor protection from apoptosis in cells lacking the IRS proteins. *Receptor Signal Transduct* 1997, **7**, 231–240.
2. Jung Y, Miura M, Yuan J. Suppression of interleukin-1 beta-converting enzyme-mediated cell death by IGF-I. *J Biol Chem* 1996, **271**, 5112–5117.
3. Hankinson SE, Willett WC, Colditz GA, *et al.* Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. *Lancet* 1998, **351**, 1393–1396.
4. Baserga R, Hongo A, Rubini M, Prisco M, Valentinis B. The IGF-I receptor in cell growth, transformation and apoptosis. *Biochem Biophys Acta Rev Cancer* 1997, **1332**, F105–F106.
5. Pollack MN. Endocrine effects of IGF-I on normal and transformed breast epithelial cells: potential relevance to strategies for breast cancer treatment and prevention. *Breast Cancer Treat Res* 1998, **47**, 209–217.
6. Dunn SE, Ehrlich M, Sharp NJ, *et al.* A dominant negative mutant of the insulin-like growth factor-I receptor inhibits the adhesion, invasion and metastasis of breast cancer. *Cancer Res* 1998, **58**, 3355–3361.
7. Colston KW, Chander SK, Mackay AG, Coombes RC. Effects of synthetic vitamin D analogues in breast cancer cell proliferation *in vivo* and *in vitro*. *Biochem Pharmacol* 1992, **44**, 693–702.
8. Saez S, Falette N, Guillot C, Meggouh F, Lefebvre M, Crepin M. $1,25(OH)_2D_3$ modulation of mammary tumor cell growth *in vitro* and *in vivo*. *Breast Cancer Res Treat* 1993, **27**, 69–81.
9. Van Weelden K, Flanagan L, Binderup L, Tenniswood M, Welsh J. Apoptotic regression of MCF-7 xenografts in nude mice treated with the vitamin D3 analog, EB1089. *Endocrinology* 1998, **139**, 2102–2110.
10. Vink-van Wijngaarden T, Pols HAP, Buurman CJ, Birkenhäger JC, van Leenwen JPTM. Inhibition of insulin- and insulin-like growth factor-I-stimulated growth of human breast cancer cells by 1,25-dihydroxyvitamin D_3 and the vitamin D_3 analogue EB1089. *Eur J Cancer* 1996, **32A**, 842–848.
11. Xie SP, James SY, Colston KW. Vitamin D derivatives inhibit the mitogenic effects of IGF-I on MCF-7 human breast cancer cells. *J Endocrinol* 1997, **154**, 495–504.
12. Karey KP, Sirbasku DA. Differential responsiveness of human breast cancer cell lines MCF-7 and T47D to growth factors and 17β-estradiol. *Cancer Res* 1988, **48**, 4083–4992.
13. Oh Y, Muller HL, Larnson G, Rosenfeld RG. Insulin-like growth factor (IGF)-independent action of IGF-binding protein-3 in Hs578T human breast cancer cells. *J Biol Chem* 1993, **268**, 14964–14971.
14. Wosikowski K, Eppenberger U, Küng W, Nagamine Y, Mueller H. c-fos, c-jun and c-myc expressions are not growth rate limiting for the human MCF-7 breast cancer cells. *Biochem Biophys Res Commun* 1992, **188**, 1067–1076.
15. Skehan P. Assays of cell growth and cytotoxicity. In Studzinski GP, ed. *Cell Growth and Apoptosis*. IRL Press, 1995, 169–191.
16. Duke R, Cohen J. In Coligan J, ed. *Current Protocols in Immunology* (Sppl. 3), New York, Green/Wiley, 1992, 3.17.1–3.17.16.
17. Danielsson C, Mathiasen IS, James SY, *et al.* Induction of apoptosis in breast cancer cells by a novel 1,25-dihydroxyvitamin D analogue is related to promoter selectivity. *J Cell Biochem* 1997, **66**, 552–565.

18. Colston KW, Perks CM, Xie SP, Holly JMP. 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 binding protein-3. *J Mol Endocrinol* 1998, **20**, 157–162.
19. De Leon DD, Wilson DM, Powers M, Rosenfield RG. Effects of IGFs and IGF receptor antibodies on the proliferation of human breast cancer cells. *Growth Factors* 1992, **6**, 327–336.
20. Fisher DE. Apoptosis in cancer therapy: crossing the threshold. *Cell* 1994, **78**, 539–542.
21. Resnicoff M, Abraham D, Yutanawiboonchai W, *et al.* Insulin-like growth factor I receptor protects tumor cells from apoptosis *in vivo*. *Cancer Res* 1995, **55**, 2463–2469.
22. Dunn SE, Hardman R, Kari F, Barrett JC. Insulin-like growth factor I (IGF-I) alters drug senstivity of HBL100 human breast cancer cells by inhibition of apoptosis induced by diverse anticancer drugs. *Cancer Res* 1997, **57**, 2687–2693.
23. Kulik G, Klippel A, Webber M. Antiapoptotic signalling by the IGF-I receptor, PI3K, and Atk. *Mol Cell Biol* 1997, **17**, 1595–1606.
24. Dufournay B, Alblas J, van Teeffelen H, *et al.* Mitogenic signalling of IGF-I in MCF-7 cells requires P13K and is independent of MAPK. *J Biol Chem* 1977, **272**, 31163–31171.
25. Cheng HL, Feldman EL. Bidirectional regulation of p38 kinase and c-Jun N-terminal protein kinase by insulin-like growth factor-I. *J Biol Chem* 1998, **273**, 14560–14565.
26. Nickerson T, Huynh H, Pollak M. IGFBP-3 induces apoptosis in MCF-7 breast cancer cells. *Biochem Biophys Res Commun* 1997, **237**, 690–693.
27. Rozen F, Yang XF, Huynh H, Pollak M. Antiproliferative action of vitamin D-related compounds and IGFBP-5 accumulation. *J Natl Cancer Inst* 1997, **89**, 652–656.
28. Schumacher R, Soos MA, Schlessinger D, Brandenburg K, Siddle EK, Ullrich A. Signalling-competent receptor chimeras allow mapping of major insulin receptor binding domain determinants. *J Biol Chem* 1993, **268**, 1087–1094.
29. O'Connor R, Kauffmann-Zeh A, Lui Y, *et al.* Identification of domains of the insulin-like growth factor receptor that are required for protection from apoptosis. *Mol Cell Biol* 1997, **17**, 427–435.
30. Gill Z, Perks C, Newcomb P, Holly JMP. IGFBP-3 predisposes breast cancer cells to programmed cell death in a non-IGF-dependent manner. *J Biol Chem* 1977, **272**, 25602–25607.

Acknowledgements—We are grateful to Dr L. Binderup (Leo Pharmaceutical Products, Denmark) for CB1093 and EB1089. This study was supported by The Association for International Cancer Research.