Vitamin D derivatives inhibit the mitogenic effects of IGF-I on MCF-7 human breast cancer cells

S P Xie, S Y James and K W Colston
Division of Gastroenterology, Endocrinology and Metabolism, St George’s Hospital Medical School, London SW17 0RE, UK
(Requests for offprints should be addressed to K W Colston, Division of Gastroenterology, Endocrinology and Metabolism, St George’s Hospital Medical School, Cranmer Terrace, Tooting, London SW17 0RE, UK)

Abstract
The effects of 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) and four novel synthetic analogues (EB1089, KH1060, KH1230 and CB1093) on IGF-I-stimulated growth of MCF-7 human breast cancer cells have been determined. A significant time- and dose-dependent inhibition of IGF-I-stimulated cell growth was seen with EB1089, such that after 7 days of treatment with $10^{-8}$ M EB1089, the mitogenic effect of IGF-I (30 ng/ml) was negated. Comparison with 1,25(OH)$_2$D$_3$ showed the synthetic analogues to be more potent. The anti-oestrogen ICI 182,780 similarly inhibited IGF-I-stimulated growth of these cells and in combination with EB1089 exerted additional inhibitory effects. Retinoids (all-trans-retinoic acid or the isomer 9-cis-retinoic acid) were less effective in limiting MCF-7 cell responsiveness to IGF-I but, in combination with EB1089, a co-operative effect was achieved. Using radioligand-binding techniques, we observed that 1,25(OH)$_2$D$_3$ and EB1089 down-regulated the levels of $^{125}$I-IGF-I binding to MCF-7 cell membranes. Scatchard analysis showed that EB1089 decreased maximal binding approximately 2-fold. Vitamin D derivatives were also demonstrated to reduce IGF-I receptor expression in MCF-7 cells by Western analysis.

Our findings demonstrate that vitamin D derivatives limit responsiveness of MCF-7 cells to the mitogenic effects of IGF-I, which may be mediated by reduction of IGF-I receptor expression.

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Introduction
The active hormonal form of vitamin D, 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) has potential as a therapeutic agent in hyperproliferative disorders, but is limited by its strong calcemic activity, leading to hypercalcemia (Koeffler et al. 1985). Therefore, new vitamin D analogues are being developed with increased growth-inhibitory effects and reduced calcemic activity. Most analogues synthesized have modifications in the C-17 side chain of the vitamin D molecule, which seems to be the most effective approach to separate calcemic from anti-proliferative activities. Vitamin D derivatives have been shown both to inhibit the proliferation of cultured human breast cancer cells and to cause regression of experimental mammary tumours in vivo (Colston et al. 1992a,b, Mathiasen et al. 1993, Saez et al. 1993).

Breast cancer is the most frequent malignancy of females in many western countries (van der Burg & de Laat 1991). Breast cancer cells both secrete and respond to a variety of autocrine/paracrine growth factors, including insulin-like growth factor-I (IGF-I) (Stewart et al. 1992), and are specifically dependent on the presence of these growth factors for their continual proliferation (Fontana et al. 1991). IGF-I is a potent stimulator of breast carcinoma proliferation (Cullen et al. 1990, Adamo et al. 1992) and is more potent than IGF-II (Osborne et al. 1990). In the case of estrogen receptor (ER)-positive MCF-7 cells, IGF-I has been reported to be the most potent mitogen studied (Karey & Sirbasku 1988). A recent study showed that total plasma IGF-I levels are higher in women with breast cancer (Peyrat et al. 1993), suggesting that enhanced IGF-I activity may play a significant role in the development of breast cancer (Kazer 1995). The mitogenic actions of IGF-I are initiated by interaction with its receptor (IGF-IR) located in the plasma membrane of the target cells, which exhibits a high affinity for IGF-I and a low affinity (about 1% of IGF-I) for insulin (Froesch et al. 1985). Breast cancer cells express higher levels of IGF-IR (Furlanetto & DiCarlo 1984), when compared with normal breast tissue cells (Papa et al. 1993).

The growth of many breast cancer cells is regulated by oestradiol. In ER-positive breast cancer cell lines, oestradiol stimulates cell growth by a receptor-mediated pathway. Oestradiol also induces an increase in IGF-IR expression (LeRoith et al. 1995). Anti-oestrogens are effective in controlling the growth of oestrogen-responsive tumours and cells (Wakeling & Bowler 1992, Vink-van Wijngaarden...
Retinoids have been shown to have synergistic activation or repression of specific responsive genes with 1,25(OH)2D3 in the inhibition of proliferation in human breast cancer cell lines T 47D (Bollag 1994).

As part of ongoing studies to elucidate the mechanisms underlying the antiproliferative effects of vitamin D, we have determined the effects of 1,25(OH)2D3 and its new synthetic analogues EB1089, KH1060, KH1230 and CB1093 on the IGF-I-stimulated growth of MCF-7 breast cancer cells. In addition, the effects of the vitamin D analogues on IGF-IR have been assessed.

Materials and Methods

Compounds

1,25(OH)2D3, EB1089 ((1S,3R)-dihydroxy-(20R)-(5'-ethyl-5'-hydroxyhepta-(1'E,3'E)-dien-1'-y1)-9,10-secopregna-(5Z,7E),10(19)-triene), KH1060, KH1230 and CB1093 were gifts from Dr L Binderup (Leo Pharmaceutical Products, Ballerup, Denmark). All-trans-retinoic acid (ATRA) was purchased from Sigma Chemical Co (Poole, Dorset, UK). 9-cis-RA was a gift from Dr M Uskokovic (Hoffmann-La Roche, Nutley, NJ, USA). ICI 182,780 (7a-[9-(4,4,5,5,5-pentafluoropentylsulphonyl)nonyl]oestra-1,3,5(10)-trien-3,17β-diol) was kindly provided by Dr A Wakeling (Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK). All the compounds were dissolved in absolute ethanol for in vitro studies. In all experiments the final concentration of ethanol in tissue culture medium did not exceed 0.1%.

Peptides

Human recombinant IGF-I from R&D Systems (Europe Ltd, Abingdon, Oxfordshire, UK) was diluted in 10 mM HCl. Bovine insulin was purchased from Sigma Chemical Co. 125I-IGF-I (2000 Ci/mmol) was obtained from Amersham International plc (Amersham, Bucks, UK). IGF-IR antibody (N-20; anti-IGF-IR α-subunit) was purchased from Santa Cruz Biotechnology, Inc. (Devizes, Wiltshire, UK).

Tissue culture medium and reagents were obtained from Gibco (Paisley, Strathclyde, UK). All other analytical grade reagents were obtained from Sigma.

Cell culture

The MCF-7 human breast cancer cell line was routinely maintained in Dulbecco's modified Eagle's medium, supplemented with 100 U penicillin/ml, 100 µg streptomycin/ml and 5% fetal calf serum at a constant temperature of 37 °C with a humidified atmosphere of 5% CO2. The cells were passaged once a week. For growth experiments, cells were trypsinized and 1 x 10^4 cells were plated in 12 mm diameter wells (48-well plate) in 0.5 ml of the growth medium (RPMI 1640 medium containing 2.5% charcoal-stripped fetal calf serum with the above mentioned supplements) or 2 x 10^4 cells were plated in 16 mm diameter wells (24-well plate) in 1 ml of the growth medium. The cells were cultured for 65–68 h before treatment. Cells were treated with IGF-I, the vitamin D derivatives or other test compounds in RPMI 1640 medium containing 1% charcoal-stripped fetal calf serum with the above mentioned supplements for 3–10 days with a medium change every 2–3 days. Control cultures were incubated in medium containing vehicle alone. Vehicle for IGF-I was 10 mM HCl (final concentration ≤ 30 µM), and for vitamin D derivatives it was ethanol.

Proliferation assay

A modification of the crystal violet assay was used to determine MCF-7 cell proliferation (Wosikowski et al. 1993). On selected days after removal of the incubation medium, the cell monolayer was washed twice with PBS. Cells were fixed and stained by adding 0.5 ml 0.5% crystal violet solution in 25% methanol. After 10 min, the excess dye was removed by washing three times with distilled water and the cells were then air-dried for at least 20 h. The incorporated dye was solubilized in 2 ml (16 mm well) or 1 ml (12 mm well) 0.1 M sodium citrate solution in 50% ethanol and 100 µl was transferred to a 96-well plate. In order to determine the optical density (OD) was measured directly at a wavelength of 550 nm in a microplate reader (Titertek Multiskan). The OD of each sample was then compared with a standard curve, in which the OD was directly proportional to known cell numbers.
IGF-I-binding assays were performed essentially as described by Stewart et al. (1990). IGF-I binding was measured with MCF-7 cell monolayers in 24-well plates. Cells were washed with PBS and incubated for 2.5 h at 4 °C with 50 000–80 000 c.p.m. of 125I-IGF-I (specific activity 2000 Ci/mm) in 0.2 ml PBS containing 1 mg/ml BSA. Non-specific binding was assessed by incubating cell cultures with 125I-IGF-I in the presence of 100 nm unlabelled IGF-I. After the incubation, the cell layer was washed three times with cold PBS and dissolved in 0.5 M NaOH. Radioactivity (c.p.m.) was measured in a gamma-counter (Packard, Cobra Auto-gamma). Results are expressed as c.p.m./5 × 10^5 cells. Cell numbers were assessed by crystal violet assay using replicate cell samples treated in the same way and cultured in the same microwell plates as those for the IGF-I-binding assay.

To determine the effect of vitamin D derivatives on 125I-IGF-I binding to cell membranes and to compare the effect with that of ICI 182,780, MCF-7 cells were treated for 6 days with EB1089 (10⁻⁹ or 5 × 10⁻⁹ M), 1,25(OH)₂D₃ (5 × 10⁻⁸ M) and ICI 182,780 (5 × 10⁻⁹ M) or ethanol vehicle, before the binding assay.

For Scatchard analysis, MCF-7 cells were incubated with increasing concentrations of 125I-IGF-I (20 000, 40 000, 80 000, 160 000 c.p.m.) for 2.5 h at 4 °C. Non-specific binding was determined in the presence of increasing concentrations of unlabelled IGF-I (12.5, 25, 50, 100 nm).

Western analysis

The expression of IGF-IR in MCF-7 cells was determined by Western analysis with a polyclonal antibody recognizing the α-subunit of IGF-IR. For the IGF-IR immunoblots, total protein was fractionated by SDS–PAGE on 7.5% gels, followed by electroblotting of the protein onto Hybond C-super nitrocellulose membrane (Amersham). The membrane was incubated (1 h) with 0.5 μg/ml of a rabbit polyclonal antibody (N-20). All subsequent steps were performed according to the protocol provided by Santa Cruz Biotechnology, Inc. Densitometry was performed on a MicroTek Scanmaker flat bed scanner (Redondo Beach, CA, USA) and quantified with NIH Image 1.52 software.

Statistical methods

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

Results

Effects of vitamin D derivatives on IGF-I-stimulated growth of MCF-7 cells

To investigate effects of vitamin D derivatives on IGF-I-stimulated growth of MCF-7 cells, cultures were incubated for 3, 7 and 10 days in the presence or absence of the vitamin D analogue EB1089 (10⁻⁸ M) alone or in combination with IGF-I (30 ng/ml). A significant time-dependent inhibition of IGF-I-stimulated cell growth was seen, which was evident after 3 days of treatment with EB1089 and was maintained until the end of the study at day 10. After 7 days of treatment, the combination of IGF-I and EB1089 inhibited MCF-7 cell growth to such an extent that the mitogenic effect of IGF-I was negated (Fig. 1a). EB1089 (10⁻⁸ M) alone produced a significant inhibition of cell growth compared with control cultures (7 and 10 days treatment). The effects of EB1089 on IGF-I-stimulated growth were dose-dependent over the range 10⁻¹⁰–10⁻⁸ M at 7 days of treatment (Fig. 1b). 10⁻⁹ M and 10⁻⁸ M EB1089 completely abrogated the mitogenic effect of IGF-I (1, 10 and 30 ng/ml).

The anti-IGF-I effects of EB1089 were further compared with those of the native hormone 1,25(OH)₂D₃ and other analogues (KH1060, KH1230 and CB1093) by treating MCF-7 cells with IGF-I (30 ng/ml) in the presence or absence of between 10⁻⁹ and 10⁻⁷ M vitamin D compounds for 7 days (Fig. 2). Over the dose range 10⁻⁹–10⁻⁸ M, 1,25(OH)₂D₃ was ineffective in inhibiting IGF-I-stimulated cell growth, while the analogues EB1089, KH1060, KH1230 and CB1093 each displayed significant antiproliferative actions. To identify whether 1,25(OH)₂D₃ was also effective in inhibiting IGF-I-stimulated MCF-7 cell proliferation, the maximal dose for this compound was increased to 5 × 10⁻⁸ M, and at this concentration 1,25(OH)₂D₃ was capable of decreasing the mitogenic effect of IGF-I. The maximal growth-inhibitory effect was similar (85%) for 1,25(OH)₂D₃, KH1060 and EB1089 and was reached at approximately 10⁻⁹ M KH1060, 10⁻⁸ M EB1089 and 5 × 10⁻⁸ M 1,25(OH)₂D₃. The concentrations of the analogues needed to achieve 50% inhibition of cell growth (IC₅₀) were assessed, and on the basis of these concentrations, the relative potencies with respect to 1,25(OH)₂D₃ were calculated. KH1230 displayed a small increased potency, whereas CB1093, EB1089 and KH1060 were clearly more potent than 1,25(OH)₂D₃: 18, 98 and 1000 times respectively (Table 1).

Comparison of the effects of vitamin D derivatives, retinoids and anti-oestrogens on MCF-7 cell growth in the presence or absence of IGF-I

Retinoids and anti-oestrogens are also known to inhibit MCF-7 breast cancer cell growth. In order to compare the efficacy of the vitamin D derivatives, retinoids and ICI 182,780 on IGF-I-stimulated and unstimulated growth, MCF-7 cells were cultured for 7 days with or without IGF-I (30 ng/ml) in the presence or absence of between 10⁻¹⁰ and 10⁻⁷ M concentrations of these compounds. The concentrations needed to achieve 50% inhibition of MCF-7 cell cultures relative to control (vehicle alone for
unstimulated growth and IGF-I alone for IGF-I-stimulated growth) were determined and are shown in Table 1. Results showed that EB1089 and ICI 182,780 inhibited IGF-I-stimulated growth of these cells similarly. In contrast, neither ATRA nor 9-cis-RA were effective in limiting the cellular responsiveness to IGF-I. The proliferation of cultures incubated with IGF-I (30 ng/ml) together with ATRA or 9-cis-RA (10^{-10} - 10^{-7} M) for 7 days was not significantly reduced compared with IGF-I alone; on the contrary, ATRA weakly increased the stimulatory effects of the growth factor (P<0.05). A similar trend was also seen with 9-cis-RA but the difference from control did not reach significance. The ratio of IC_{50} with and without IGF-I indicates the different effects of these compounds on the IGF-I-stimulated and unstimulated growth. The concentrations of the retinoids producing 50% inhibition for IGF-I-stimulated growth are much higher (about 30- and 60-fold for ATRA and 9-cis-RA respectively) than those for unstimulated growth (Table 1).

Interaction of vitamin D analogue with anti-oestrogen or retinoids on IGF-I-stimulated growth of MCF-7 cells

The interaction of vitamin D analogue EB1089 and the pure anti-oestrogen ICI 182,780 or retinoids in MCF-7 cell growth was studied in the presence of IGF-I (30 ng/ml) for 7 days. As shown in Fig. 3a, combination of 10^{-9} M EB1089 and ICI 182,780 exerted a distinct co-operative effect on inhibition of IGF-I-stimulated cell growth compared with the actions of either compound alone. Regarding treatment of cells with 10^{-9} M EB1089 and 10^{-8} M ATRA or 9-cis-RA alone or in combination in the presence of IGF-I (Fig. 3b), results showed that neither retinoid alone was effective in inhibiting IGF-I-stimulated cell growth. However, the combination of EB1089 with ATRA as well as the combination of EB1089 with 9-cis-RA produced additional inhibitory effects.
Effects on IGF-I-stimulated cell growth in comparison with that seen with EB1089 alone.

**Effects of vitamin D derivatives on IGF-I binding to MCF-7 cell membranes**

Experiments were undertaken to assess whether pretreatment of cells with vitamin D derivatives could diminish responsiveness to IGF-I via changes in IGF-I binding. Initial studies examined the specificity of 125I-IGF-I binding. Monolayers of MCF-7 cells were incubated with 125I-IGF-I in the presence and absence of various concentrations of competing unlabelled IGF-I and insulin. The non-specific binding was approximately 10% of the total binding and was subtracted from total binding values. Figure 4 shows the ability of radioinert IGF-I and insulin to displace 125I-IGF-I binding to MCF-7 cell membranes. 125I-IGF-I binding was completely abolished by incubation with unlabelled IGF-I (10^-7 M). A 50% displacement was obtained with IGF-I at 2 x 10^-9 M and with insulin at 5 x 10^-7 M.

MCF-7 cells were pretreated with EB1089 (10^-9 or 5 x 10^-9 M) for 6 days and then incubated with 125I-IGF-I alone or in the presence of excess IGF-I (10^-7 M). Figure 4a shows that EB1089 down-regulated the level of 125I-IGF-I binding to MCF-7 cell membranes in a dose-dependent manner. In cells cultured with 10^-9 M and 5 x 10^-9 M of EB1089, binding of 125I-IGF-I was reduced to 74 and 50% of control levels, respectively. 1,25(OH)2D3 (5 x 10^-8 M) and the pure anti-oestrogen, ICI 182,780 (5 x 10^-8 M), also decreased the IGF-I binding to MCF-7 cell membranes (Fig. 4b).

The number and affinity of 125I-IGF-I-binding sites on MCF-7 cell membranes were determined by Scatchard analysis (Fig. 6), which revealed that pretreatment of cells with EB1089 (5 x 10^-9 M) for 6 days reduced maximal binding from 10^-7 fmol/5 x 10^5 cells in control cultures to 5.0 fmol/5 x 10^5 cells in EB1089-treated cells with no marked change in affinity (Kd=0.38 and 0.26 nm for control and EB1089-treated cultures respectively).

**Effects of vitamin D derivatives on IGF-IR expression**

To further assess whether diminished responsiveness of the breast cancer cells to the mitogenic effects of IGF-I with vitamin D treatment occurred via modulation of IGF-IR, we determined the protein expression of IGF-IR in cells treated with vitamin D derivatives using immunoblot analysis. Figure 7 shows the effects of vitamin D derivatives on the IGF-IR expression in MCF-7 cells after 6 days of treatment. The protein (130 kDa) band of IGF-IR α-subunit was markedly diminished in cells treated with vitamin D derivatives, EB1089 (10^-8 M) and 1,25(OH)2D3 (5 x 10^-8 M) compared with control cultures. Since anti-oestrogens have been reported to reduce IGF-IR expression, we also examined and confirmed the modulation of receptor levels in MCF-7 cells treated with 10^-8 M ICI 182,780. Densitometric analysis of Western blots obtained from three separate experiments showed a significant difference between tests and control samples (Fig. 7).

**Discussion**

In this report we demonstrate that vitamin D derivatives limit the responsiveness of MCF-7 cells to the mitogenic actions of IGF-I. Treatment of cells with combinations of IGF-I and the synthetic vitamin D analogues resulted in a striking decrease in IGF-I-induced stimulation of cell proliferation.

A number of previous studies have demonstrated that 1,25(OH)2D3 and certain of its synthetic analogues inhibit the autonomous growth of cultured MCF-7 breast cancer cells and that vitamin D derivatives and retinoic acids reduce IGF-I-induced proliferation 

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**Table 1** Growth inhibition of MCF-7 cells by vitamin D derivatives, retinoic acids and anti-oestrogen. MCF-7 cells were cultured for 7 days in the absence or presence of various concentrations of the compounds alone or in combination with 30 ng/ml IGF-I. All compounds were tested in three separate experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Growth in absence of IGF-I</th>
<th>Growth in presence of IGF-I</th>
<th>Ratio of IC50</th>
</tr>
</thead>
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<tr>
<td></td>
<td>IC50 (M)</td>
<td>Relative to 1,25(OH)2D3</td>
<td>IC50 (M)</td>
</tr>
<tr>
<td>1,25(OH)2D3</td>
<td>1.6 x 10^-8</td>
<td>1</td>
<td>4.3 x 10^-8</td>
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<tr>
<td>EB1089</td>
<td>4.0 x 10^-10</td>
<td>40</td>
<td>4.4 x 10^-10</td>
</tr>
<tr>
<td>KH1060</td>
<td>3.6 x 10^-11</td>
<td>444</td>
<td>4.3 x 10^-11</td>
</tr>
<tr>
<td>KH1230</td>
<td>3.5 x 10^-8</td>
<td>0.5</td>
<td>2.1 x 10^-8</td>
</tr>
<tr>
<td>CB1093</td>
<td>3.7 x 10^-10</td>
<td>43</td>
<td>2.4 x 10^-9</td>
</tr>
<tr>
<td>ICI 182,780</td>
<td>4.5 x 10^-10</td>
<td>36</td>
<td>1.5 x 10^-9</td>
</tr>
<tr>
<td>9-cis-RA</td>
<td>2.7 x 10^-8</td>
<td>0.6</td>
<td>1.6 x 10^-6</td>
</tr>
<tr>
<td>ATRA</td>
<td>6.1 x 10^-8</td>
<td>0.3</td>
<td>1.8 x 10^-6</td>
</tr>
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</table>
cells (Saez et al. 1993, Demirpence et al. 1994, Colston et al. 1995). In comparing the potency of the various vitamin D analogues in MCF-7 cells, we found that EB1089 inhibits growth in the absence of IGF-I at about 40-fold lower concentration than 1,25(OH)2D3, with KH1060 being even more effective. The analogue CB1093 was similar in potency to EB1089, while KH1230 did not display enhanced potency compared with 1,25(OH)2D3. These vitamin D analogues inhibited IGF-I-stimulated growth with the same order of potency. These studies were carried out in low (1%)-serum medium. However, many novel vitamin D analogues have been demonstrated to have little affinity for vitamin D-binding protein compared with 1,25(OH)2D3 (Bikle 1992), which may increase the free fraction of these analogues in culture medium containing serum and may be associated with their increased potency.

A previous report showed that 1,25(OH)2D3 at 10−7 M inhibited IGF-I-stimulated growth in human osteosarcoma cells (Pirskanen et al. 1993). However, the mechanism has not been elucidated. Our results agree with those obtained by Vink-van Wijngaarden et al. (1996), who demonstrated that 1,25(OH)2D3 and EB1089 at 10−9 and 10−7 M blocked the mitogenic activity of an IGF-I analogue and insulin in MCF-7 cells and this was associated with inhibition of insulin-induced c-fos mRNA expression, a possible intracellular mediator of the insulin–IGF mitogenic signal.

9-cis-RA and ATRA have both been shown to inhibit the in vitro growth of ER-positive human breast cancer cells. The ability of the retinoids and vitamin D derivatives to limit the mitogenic effects of IGF-I was compared. Both 9-cis-RA and ATRA over the concentration range

**Figure 3** Co-operative effects of EB1089 and ICI 182,780 (a) and EB1089 and retinoic acids (b) on IGF-I-stimulated growth of MCF-7 cells. (a) Cells grown in 48-well plates were treated with IGF-I (30 ng/ml) alone or in combination with 10−9 M EB1089, the same dose of ICI 182,780 or EB1089 plus ICI 182,780. Cell proliferation was assessed after 7 days of treatment. Results are mean ± S.E.M. (n=4) representative of three separate experiments. ***P<0.005, compared with control; *P<0.005, compared with either EB1089 or ICI 182,780. (b) Cells were treated with IGF-I (30 ng/ml) alone or in combination with 10−8 M ATRA, 10−8 M 9-cis-RA, 10−9 M EB1089 or EB1089 plus ATRA or 9-cis-RA. *P<0.05, ***P<0.005, compared with control; *P<0.005, compared with either EB1089 or retinoic acids alone. Other experimental conditions were the same as in (a).

**Figure 4** Displacement of 125I-IGF-I from MCF-7 cells by unlabelled IGF-I and insulin. Cells were cultured until subconfluent and then incubated for 2.5 h at 4°C with 125I-IGF-I alone or in the presence of the indicated concentration of unlabelled IGF-I (●) or insulin (○). Non-specific binding was determined in the presence of 100 nM IGF-I and was subtracted from all the values to give specific binding. The bound 125I-IGF-I was determined as described in Materials and Methods. The results are expressed as the percentage of maximal binding.
10\(^{-10}\)–10\(^{-7}\) M produced a significant and dose-dependent inhibition of MCF-7 cell growth in the absence of IGF-I but were not effective in limiting responsiveness to this growth factor; indeed 10\(^{-10}\)–10\(^{-7}\) M ATRA weakly increased responsiveness to IGF-I, which is in agreement with results described previously (Bentel et al. 1995). Only at retinoid levels higher than 10\(^{-6}\) M was significant inhibition of IGF-I-stimulated growth achieved. Thus the concentration of both 9-cis-RA and ATRA producing 50% inhibition of growth in the absence of added IGF-I was insufficient to produce 50% inhibition of growth in the presence of the growth factor, whereas the concentrations of vitamin D derivatives required to achieve this degree of growth inhibition were similar for both experimental conditions. These data indicate that vitamin D derivatives are more effective than retinoids in inhibition of IGF-I mitogenic activity.

The present study shows that the anti-IGF-I activity of the vitamin D analogue EB1089 was strikingly increased by interaction with the pure anti-oestrogen, ICI 182,780. Combinations of 10\(^{-9}\) M EB1089 and ICI 182,780 in the presence of IGF-I for 7 days exerted an additional effect on inhibition of IGF-I-stimulated cell growth compared with the actions of either compound alone. Similar co-operative effects were also achieved by combination of EB1089 and the retinoids, ATRA and 9-\(\text{cis}-\text{RA}. The action of retinoids (10\(^{-8}\) M) in increasing IGF-I-stimulated growth was reversed by combination with EB1089 (10\(^{-9}\) M), and furthermore the inhibition of IGF-I-stimulated cell growth was augmented from 31% (EB1089 alone) to 59% by combined treatment with either retinoid.

The underlying mechanisms by which vitamin D derivatives are able to limit responsiveness to IGF-I were investigated by determining whether these compounds are capable of altering IGF-I receptor expression. Using ligand-binding techniques, effects of vitamin D derivatives on 125\(^{I}\)-IGF-I binding to MCF-7 cell membranes have
been determined. Initial studies showed that $^{125}\text{I}$-IGF-I binding to cell membranes was reduced by incubation with unlabelled IGF-I with 50% of inhibition of specific binding being achieved at a concentration of $2 \times 10^{-9}$ M. In contrast, 50% displacement was achieved with insulin at $5 \times 10^{-7}$ M. This pattern of potency indicates that binding was mainly to the IGF-IR, as previously reported for MCF-7 cells (Stewart et al. 1990) and not to the insulin receptor. IGF-I may also bind to IGF-IIR and a number of IGF-binding proteins (IGFBPs), but since insulin does not bind to these two classes of sites it will not compete with radiolabelled IGF-I for binding (Heath et al. 1993). The difference ($\sim 18\%$) in maximal displacement of $^{125}\text{I}$-IGF-I between radiinert IGF-I and insulin may indicate the amount of binding to IGFBPs and/or IGF-IIR.

Treatement of MCF-7 cells with increasing doses of EB1089 for 6 days led to a decrease in specific binding of $^{125}\text{I}$-IGF-I to cell membranes. Similar effects were seen with 1,25(OH)$_2$D$_3$ and the anti-oestrogen ICI 182,780. Scatchard analysis showed that treatment of cells with EB1089 reduced maximal binding to approximately 50% of control value. Vink-van Wijngaarden et al. (1996) showed that $^{125}\text{I}$-IGF-I binding to cell membranes was increased about 1.5- to 3-fold by vitamin D compounds. These divergent results may be attributable to different MCF-7 sublines and/or different experimental conditions, such as serum-free vs serum-containing medium or time and frequency of treatment with vitamin D compounds. Differences in cell density between treated and control cultures may also be a confounding factor (Larsen & Darbre 1996). However, our results obtained by Western analysis also demonstrated that the expression of IGF-IR was down-regulated by vitamin D derivatives, suggesting that effects of vitamin D derivatives in limiting responsiveness of breast cancer cells to the mitogenic IGF-I may be via reduction of IGF-IR. However, effects on expression and regulation of IGFBPs cannot be excluded.

Oestriadiol sensitizes the MCF-7 cell line to the mitogenic effects of insulin and IGF-I, which is mediated through the IGF-IR. A previous report has demonstrated that the levels of IGF-IR protein expression and its mRNA transcription are increased 7- and 6.5-fold respectively by oestriadiol in the MCF-7 cell line (Stewart et al. 1990). A positive correlation between ER levels and IGF-IR levels in breast tumour tissue (Peyrat et al. 1988) and breast cancer cells (Pollak et al. 1992) has been shown. Anti-oestrogen-induced down-regulation of IGF-IR has been reported in ER-positive breast cancer cells (Winston et al. 1994) and our results also show that the pure anti-oestrogen ICI 182,780 inhibits IGF-I-stimulated growth of these cells. The vitamin D analogue EB1089 down-regulates the expression of ER protein in MCF-7 cells and limits cellular responsiveness to 17$\beta$-oestradiol (James et al. 1994), both in terms of cell growth and expression of oestrogen-regulated genes. Vitamin D compounds may also modulate sensitivity of cells to IGF-I by directly modulating IGF-IR expression or indirectly via oestrogen-response pathways.

In summary, our findings demonstrate that vitamin D derivatives limit responsiveness of MCF-7 cells to the mitogenic effects of IGF-I. In addition we have demonstrated marked co-operative effects between the vitamin D analogue EB1089 and the anti-oestrogen ICI 182,780,

Figure 7 Western analysis of IGF-IR in MCF-7 cells treated with vitamin D and anti-oestrogen. (a) Cell cultures were treated for 6 days with ethanol vehicle (lane 1), $1 \times 10^{-8}$ M EB1089 (lane 2), $5 \times 10^{-8}$ M 1,25(OH)$_2$D$_3$ (lane 3) or $1 \times 10^{-8}$ M ICI 182,780 (lane 4). Total protein (30 µg for each well) from these cultures was fractionated by SDS–PAGE on 7.5% gel and blotted on to Hybond C-super nitrocellulose membrane. Immunoblotting was performed using rabbit polyclonal antibody against the $\alpha$-subunit of IGF-IR. (b) Densitometric analysis of the Western blot obtained from (a) and two other separate experiments. Results are expressed as the mean ± S.E.M. for relative IGF-IR levels in arbitrary units ($n$=3). $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$, compared with control.
indicating that combined treatment with vitamin D and anti-oestrogen may be an effective therapeutic regimen. Effects on IGF-I responsiveness may be mediated via decreased receptor expression.

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