Antiproliferative Action of Vitamin D-Related Compounds and Insulin-Like Growth Factor-Binding Protein 5 Accumulation

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Background and Purpose: **Vitamin D-related compounds can inhibit cancer cell growth, but the biologic mechanism of this inhibition remains to be determined. We investigated the possibility that these compounds interfere with the activity of insulin-like growth factors. Such activity can be suppressed or otherwise modulated by specific insulin-like growth factor-binding proteins.** ***Methods:*** **The human breast cancer cell line MCF-7 was used in this study. The effects of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] and two related compounds, EB1089 and KH1060, on cell proliferation were assessed by monitoring cell numbers and by measuring cellular incorporation of [^{3}H]thymidine. Changes in the accumulation of insulin-like growth factor-binding proteins in cell-conditioned media (i.e., culture fluids) were assessed by means of standard protein blotting techniques; ligand blots were probed with [^{125}I]insulin-like growth factor I, and immunoblots were probed with antibodies raised against specific binding proteins. Binding protein messenger RNA levels were determined by use of RNA blotting methods and complementary DNA probes.** ***Results:*** **At concentrations of 10^{-8} *M* and 10^{-9} *M*, EB1089 and KH1060 exhibited stronger antiproliferative activity than 1,25$(OH)_2D_3$. When each of the vitamin D-related compounds was used separately at a concentration of 10^{-9} *M*, a 20- to 25-fold increase in the concentration of insulin-like growth factor-binding proteins in MCF-7 cell-conditioned media was observed; this binding capacity was increased ninefold, ninefold, and threefold, respectively, in the presence of 10^{-10} *M* EB1089, KH1060, and 1,25$(OH)_2D_3$. Immunoblotting experiments demonstrated that all three vitamin D-related compounds induced the accumulation of insulin-like growth factor-binding protein 5 in cell-conditioned media. The accumulation of this binding protein was associated with an increase in cellular expression of its messenger RNA. EB1089 and 1,25$(OH)_2D_3$ attenuated the growth-promoting activity of insulin-like growth factor I on MCF-7 cells; however, these compounds did not inhibit the growth-promoting activity of long R^3 IGF-I, an insulin-like growth factor I analogue with greatly reduced affinity for insulin-like growth factor-binding proteins.** ***Conclusions and Implications:*** **Our results indicate that vitamin D-related compounds stimulate production of insulin-like growth factor-binding protein 5, thereby indirectly suppressing cell proliferation.**

It has been recognized for more than a decade that, apart from its effects on calcium homeostasis, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] exhibits potent antiproliferative and differentiating activities (*1-4*). However, clinical investigation of this antineoplastic activity is limited by the hypercalcemia associated with high-dose vitamin D administration. More recently, novel vitamin D analogues with potent antiproliferative activity but little effect on calcium homeostasis have been described (*5-12*). These compounds are presently considered drug candidates for cancer prevention and/or treatment.

The effects of vitamin D and its analogues are mediated by the vitamin D receptor (VDR), which is a member of the nuclear steroid hormone receptor family (*13*). VDRs affect gene expression by binding to specific vitamin D response elements in the promoter region of target genes (*14,15*). Interactions between the VDR and its target genes are complex: for example, certain response elements of target genes preferentially bind VDR-retinoid X receptor heterodimers (*16*). VDRs have been detected in intestine, kidney, bone, skin, pancreas, and the pituitary gland (*17*) as well as in many neoplastic cell lines, including MCF-7 breast cancer cells (*1,2,18*). VDRs have been reported to be present in 80% of human breast cancer specimens (including some that are estrogen receptor negative), and patients with VDR-positive tumors have been reported to experience significantly longer disease-free survival than those with VDR-negative tumors (*3,19*).

Insulin-like growth factors I and II (IGF-I and IGF-II) are well recognized as potent mitogens and antiapoptotic agents for many normal and neoplastic cell types [reviewed in (*20,21*)]. Their bioactivity is modulated in a complex fashion by specific IGF-binding proteins (IGFBPs), six of which have been described to date (IGFBP-1 through IGFBP-6) (*22*). Since the antiproliferative actions of retinoids, transforming growth factor-β, and antiestrogens on MCF-7 cells have recently been shown to involve modulation of IGFBP physiology (*23-26*), we sought to determine whether the antiproliferative effects mediated by vitamin D are also related to effects on IGFBPs.

Materials and Methods

Cell Culture

MCF-7 human breast cancer cells were obtained from American Type Culture Collection (Rockville, MD) and maintained as monolayer cultures in Alpha Modified Eagle Medium (α-MEM; Life Technologies, Inc., Gaithersburg, MD) that was supplemented with 5 μg/mL bovine insulin (Sigma Chemical Co., St. Louis, MO) and 10% fetal calf serum (FCS; Life Technologies, Inc.) in a humidified incubator at 37 °C and 5% CO_2.

Proliferation and Thymidine Incorporation Assays

Confluent stock cultures of cells were treated with trypsin and plated in phenol red-free α-MEM supplemented with 5% FCS at 5×10^3 cells per well in 24-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ). After 48 hours, the cells were washed twice with serum- and phenol red-free α-MEM, and then they were incubated for 7 days in this medium supplemented with 2.5% FCS in the presence or absence of various concentrations of 1,25$(OH)_2D_3$ (Biomol Research Laboratories, Plymouth Meeting, PA) or the analogues EB1089 or KH1060. The media were replaced every second day. The cells were counted using a hemocytometer.

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See ''Notes'' following ''References.''

The cell-number and thymidine-incorporation end points for MCF-7 cells have been previously shown to be well correlated in our cell culture system (*27*). Thymidine incorporation was measured as described previously (*27*). Experiments were carried out in triplicate. In some experiments, IGF-I (Celltrix Pharmaceuticals, Santa Clara, CA) or the IGF-I analogue, long R^3 IGF-I (GroPep, Adelaide, Australia), was added at a concentration of 1.4×10^{-9} *M*.

Western Ligand Blot Analysis

The proteins in 10×-concentrated cell-conditioned media were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under nonreducing conditions and electroblotted onto nitrocellulose membranes. The membranes were blocked, labeled with [^{125}I]IGF-I, and exposed to x-ray films (X-Omat AR; Kodak, Rochester, NY) as previously described (*28*).

Western Immunoblot Analysis

A synthetic IGFBP-5 fragment corresponding to amino acids 120-144 of human IGFBP-5 (*29*) was conjugated to keyhole-limpet hemocyanin (KLH). Rabbits were inoculated with this conjugate to generate a polyclonal anti-human-IGFBP-5 antiserum. Anti-human-IGFBP-5 antibodies were purified using an IGFBP-5 affinity column. The purified antibodies were specific for IGFBP-5 and exhibited no cross-reactivity to other IGFBPs. Protein samples were resolved by SDS–PAGE under reducing conditions and then transferred to nitrocellulose membranes as described above for ligand blotting. The membranes were blocked overnight (or for 2 hours) with 7% skim milk, incubated for 2 hours with IGFBP-5 antibodies at a 1:1000 dilution in 1% TTBS (1% skim milk, 0.05% Tween-20, 20 m*M* Tris-HCl [pH 7.5], and 200 m*M* NaCl) or an IGFBP-2 antiserum (Upstate Biotechnology Inc., Lake Placid, NY) at 1:1000 dilution in 1% TTBS, and then incubated for 1 hour with a goat anti-rabbit immunoglobulin G–horseradish peroxidase conjugate (Amersham, Buckinghamshire, U.K.) at a 1:2000 dilution in 1% TTBS. Antigen–antibody reactions were visualized by means of enhanced chemiluminescence (Amersham). A human T986 glioblastoma cell-culture supernatant (a positive control for IGFBP-2) was provided by Upstate Biotechnology Inc., and recombinant human IGFBP-5 was purchased from Austral Biologicals (San Ramon, CA).

Northern Blot Analysis

For RNA extraction, 5×10^6 MCF-7 cells were plated in 100-mm tissue culture dishes and treated with $1,25(OH)_2D_3$ or vitamin D analogues under conditions similar to those described above for the proliferation experiments or in serum- and phenol red-free medium. After 48 hours, cytoplasmic RNA was extracted using RNAZOL B (Tel-Test, Friendswood, TX), and northern blotting of 50-μg samples of total RNA was performed as described previously (*30*). The RNA blots were hybridized with a 317-base-pair human IGFBP-5 complementary DNA probe as well as with a human glyceraldehyde-phosphate dehydrogenase complementary DNA probe (CLONTECH Laboratories, Inc., Palo Alto, CA) to control for equal loading of wells and equivalence of RNA transfer.

IGF-I Receptor-Binding Assay

Cell membrane extracts were prepared from MCF-7 cell monolayers (*31*). One hundred micrograms of membrane protein were incubated with 250 000 cpm [^{125}I]IGF-I and cross-linked as previously described (*31*).

Results

Fig. 1 shows the chemical structures of $1,25(OH)_2D_3$ and the vitamin D analogues EB1089 and KH1060. The potent antiproliferative activity of various concentrations of $1,25(OH)_2D_3$, EB1089, and KH1060 on MCF-7 cells, in keeping with previous reports (*5,7-9*), is also shown in Fig. 1. Measurements of [^{3}H]thymidine incorporation were consistent with the end point of cell numbers (data not shown).

The ligand blot shown in Fig. 2 indicates the major effects of $1,25(OH)_2D_3$ and its analogues on IGFBP accumulation in MCF-7 cell-conditioned media. The

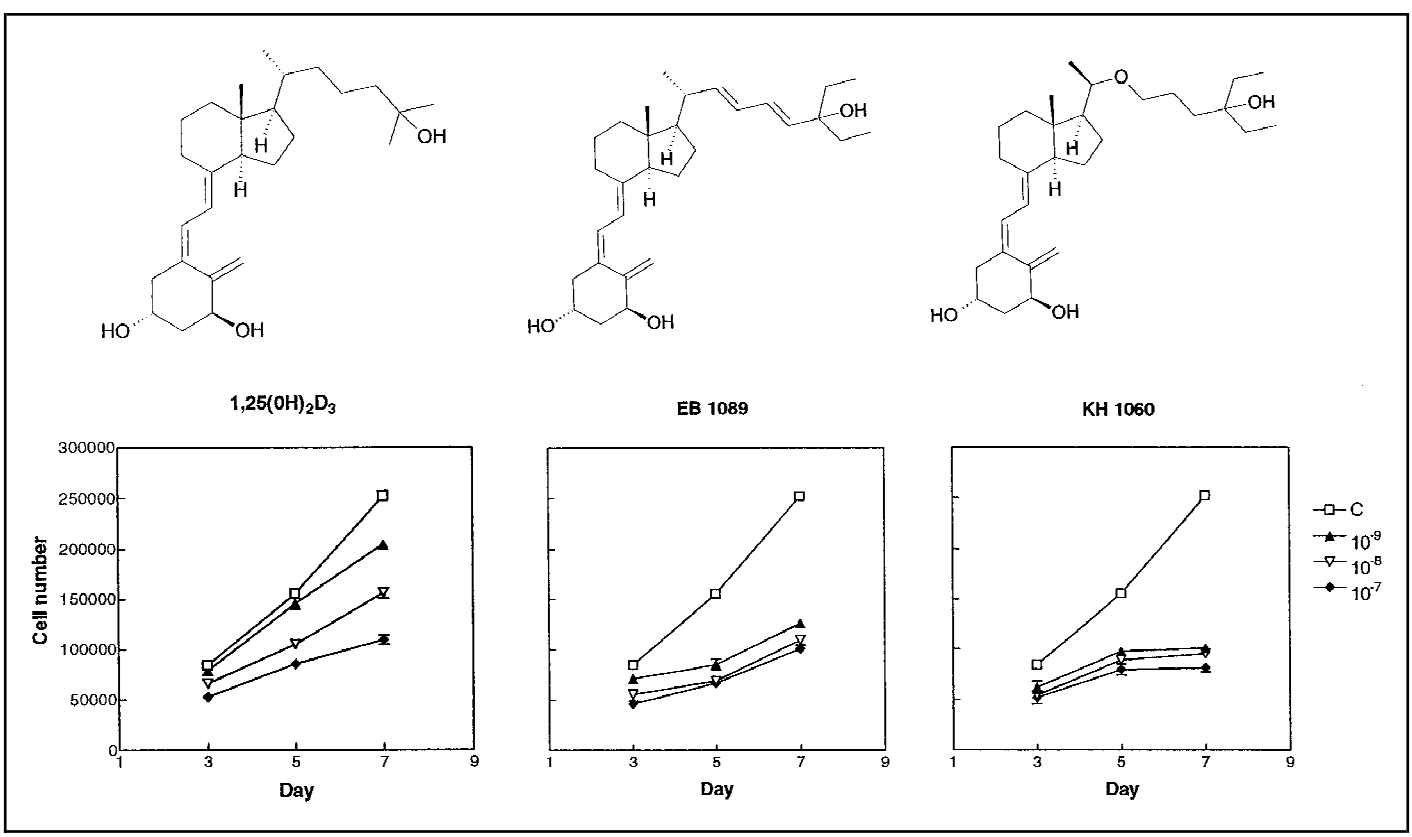

Fig. 1. Chemical structures of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] and its analogues EB1089 and KH1060 and their effects on MCF-7 cell proliferation. Chemical structures of $1,25(OH)_2D_3$, EB1089, and KH1060 are indicated. MCF-7 cells were cultured as described in the ''Materials and Methods'' section. The concentrations of $1,25(OH)_2D_3$, EB1089, and KH1060 used were as follows: □ = C = control = none; ▲ = 10^{-9} *M;* ▽ = 10^{-8} *M;* and ◆ = 10^{-7} *M*. Standard error at each point was less than or equal to 10%.

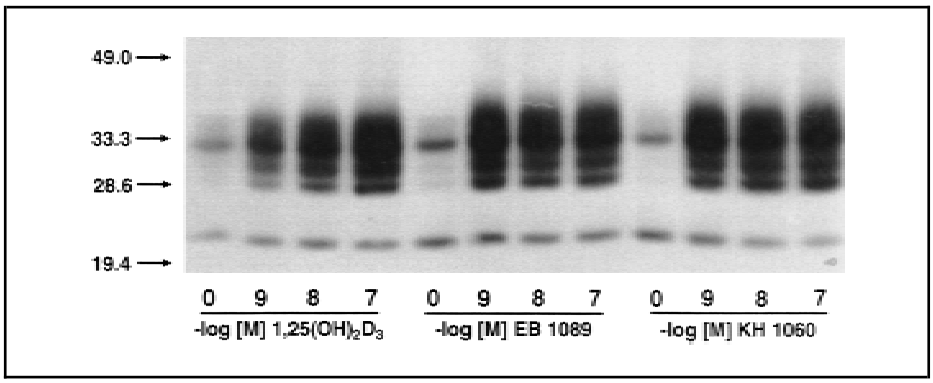

Fig. 2. Effects of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] and its analogues EB1089 and KH1060 on insulin-like growth factor-binding protein accumulation in MCF-7 cell-conditioned media. Cells were cultured as described in the ''Materials and Methods'' section and treated with or without 10^{-9} *M,* 10^{-8} *M,* or 10^{-7} *M* $1,25(OH)_2D_3$, EB1089, or KH1060 for 72 hours in the presence of 2.5% fetal calf serum. The cells were washed, and the media were replaced with fresh, serum-free media in the presence or absence of the vitamin D derivatives for 24 hours before the collection of cell-conditioned media for western ligand-blot analysis as described in the ''Materials and Methods'' section. Note: −log[M] = −log concentration in terms of molarity; therefore, 0, 9, 8, and 7 equal 0 *M,* 10^{-9} *M,* 10^{-8} *M,* and 10^{-7} *M,* respectively.

IGF-binding capacity of conditioned media (estimated by densitometric scanning of all bands) increased 20-fold over control levels in the presence of 10^{-9} *M* $1,25(OH)_2D_3$ and 25-fold in the presence of 10^{-9} *M* EB1089 or 10^{-9} *M* KH1060. At concentrations of 10^{-10} *M,* EB1089, KH 1060, and $1,25(OH)_2D_3$ increased the IGF binding capacity of conditioned media ninefold, ninefold, and threefold, respectively (data not shown), indicating that the analogues are more potent inducers of IGFBP accumulation than $1,25(OH)_2D_3$. This finding is consistent with the higher antiproliferative activity of the analogues relative to the activity of $1,25(OH)_2D_3$, as shown in Fig. 1.

Immunoblotting experiments were performed to identify the IGFBP species affected by $1,25(OH)_2D_3$ treatment. IGFBP-4 (24 kd) and IGFBP-3 (46 kd doublet on ligand blotting) were excluded from consideration on the basis of their molecular weights, since these proteins are not in the molecular weight range of proteins affected by $1,25(OH)_2D_3$ treatment. In addition, IGFBP-1 (29 kd) and IGFBP-6 (30 kd) were considered to be unlikely candidates for modulation by $1,25(OH)_2D_3$, since previous investigations have failed to detect these species in MCF-7 cell-conditioned media (*32*). Immunoblotting with an antiserum to IGFBP-2 (36 kd) demonstrated the presence of an immunoreactive species in the conditioned media, but no effect of $1,25(OH)_2D_3$, EB1089, or KH1060 on the accumulation of this species was revealed (Fig. 3, A). In contrast, immunoblots with antibodies to IGFBP-5 demonstrated $1,25(OH)_2D_3$-, EB1089-, and KH1060-induced accumulation of immunoreactive species with molecular weights ranging from 29-34 kd (Fig. 3, B), similar in size to the $1,25(OH)_2D_3$-induced bands in the ligand blot. Other investigators (*32-34*) have observed IGFBP-5 species ranging in size from 28 to 32 kd. This heterogeneity in molecular size is likely due to variations in O-glycosylation and/or phosphorylation (*22,34*).

To determine whether the accumulation of IGFBP-5 in conditioned media was associated with IGFBP-5 messenger RNA (mRNA) abundance, we performed northern blot analysis of total cellular RNA isolated from control MCF-7 cells or MCF-7 cells treated with 10^{-7} *M* $1,25(OH)_2D_3$, EB1089, or KH1060 for48 hours. As shown in Fig. 3, C, a complementary DNA probe for human IGFBP-5 hybridized to a 6-kilobase (kb) mRNA species. Treatment of cells with $1,25(OH)_2D_3$ or its analogues in serum-free medium dramatically increased IGFBP-5 mRNA abundance.

It has been shown that IGFBP-5 accumulation can be influenced by changes in both its synthesis and its proteolysis (*33,34*). To determine whether vitamin D suppresses the secretion of an IGFBP-5 protease and/or induces the secretion of an IGFBP-5 protease inhibitor, we performed ligand blots on samples of conditioned media from $1,25(OH)_2D_3$-treated cells and control cells as well as on mixtures of these conditioned media. No evidence for a major effect of $1,25(OH)_2D_3$ on IGFBP proteolysis was seen (data not shown). Furthermore, affinity labeling experiments indicated no major influence of vitamin D-related compounds on IGF-I receptor levels on MCF-7 cells (data not shown).

To determine whether vitamin D-

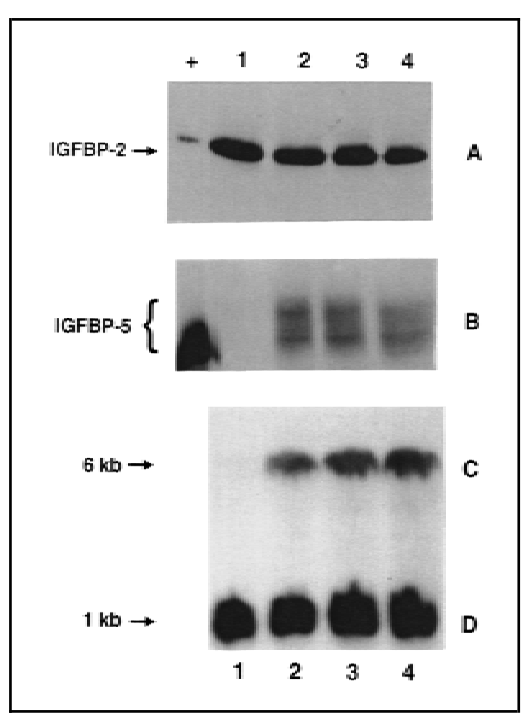

Fig. 3. Western immunoblotting of MCF-7 cell-conditioned media and northern blot analysis of total RNA from control MCF-7 cells and MCF-7 cells exposed to 10^{-7} *M* 1,25-dihydroxyvitamin D_3 or its analogues EB1089 and KH1060. Lane 1 = control, lane 2 = 1,25-dihydroxyvitamin D_3, lane 3 = EB1089, and lane 4 = KH1060. **A)** Conditioned media from control and treated cells were immunoblotted with anti-insulin-like growth factor-binding protein-2 (IGFBP-2) antiserum as described in the ''Materials and Methods'' section. Ten microliters of 10×-concentrated human T986 glioblastoma culture fluid was used as a positive control for IGFBP-2 in the lane marked '+'. **B)** Conditioned media from control and treated cells were immunoblotted with anti-IGFBP-5 antibodies as described in the ''Materials and Methods'' section. Recombinant human IGFBP-5 (200 ng) was used as a positive control in the lane marked '+'. **C** and **D)** RNA (50-μg samples) from control and treated cells was hybridized with a 317-base-pair human IGFBP-5 complementary DNA probe as described in the ''Materials and Methods'' section. The positions of the 6-kilobase IGFBP-5 messenger RNA (C) and the 1-kilobase glyceraldehyde-phosphate dehydrogenase messenger RNA (D) are indicated.

induced IGFBP accumulation is functionally related to its growth inhibitory action, we carried out experiments with long R^3 IGF-I, an IGF-I analogue that exhibits greatly reduced affinity for IGFBPs but similar affinity for IGF-I receptors (*35*). We observed that 1.4×10^{-9} *M* IGF-I increased cell numbers to 282% ± 3.1% (mean ± standard error) of control values on day 4, and that this major growth-stimulatory action was completely attenuated in the presence of 10^{-7} *M* $1,25(OH)_2D_3$ or 10^{-7} *M* EB1089 (Fig. 4). In contrast, $1,25(OH)_2D_3$ had no substantial inhibitory effect on proliferation stimulated by long R^3 IGF-I, indicating a functional role for IGFBPs in the antiproliferative action of $1,25(OH)_2D_3$ on MCF-7 cells.

Discussion

Our data implicate IGFBPs in the growth-inhibitory action of vitamin D-related compounds on MCF-7 breast cancer cells. Recent evidence suggests that the growth inhibitory actions of p53 (*36*), transforming growth factor-β (*24*), retinoids (*23*), and antiestrogens (*26*) all involve modulation of IGFBP physiology and, presumably, modulation of IGF bioactivity in the microenvironment of target cells. In view of the central role of IGFs in regulating proliferation (*20*) and apoptosis (*37*), it is not unexpected that physiologic growth inhibitors act, in part, by influencing IGF responsiveness. While we have observed that treatment with antiestrogens reduces IGF-I receptor numbers (*31*), the dominant mechanism by which antiestrogens influence IGF responsiveness in the MCF-7 model system appears to involve stimulation of IGFBP-3 and IGFBP-5 expression (*26*,*38*). The results reported herein suggest a convergence in effector pathways for the antiproliferative actions of vitamin D-related compounds and antiestrogens. Analogous to antiestrogens, the effects of vitamin D-related compounds on IGFBPs are substantially greater than their effects on IGF-I receptor levels.

The molecular mechanism by which vitamin D-related compounds increase the abundance of IGFBP-5 mRNA is currently under investigation. It is of interest in this context that the promoter region of the human IGFBP-5 gene contains at position −182 to −175 the sequence CGAAGGGG (*39*), which has a 2-base-pair mismatch with the proximal element (TGAACGGG) in the vitamin D-response element of the human osteocalcin gene (*15*).

A physiologic context for an inhibitory effect of IGFBP-5 on breast epithelial cell proliferation is provided by recent evidence (*40*) that postlactational involution of the mammary gland is mediated, at least in part, by increased local expression of IGFBP-5, which presumably acts by antagonizing the antiapoptotic effect of IGFs in the gland. The stimulus for increased IGFBP-5 expression in this physiologic setting remains unclear.

It is possible that a subset of breast neoplasms remains susceptible to growth regulation by measures that reduce IGF bioactivity. This possibility may be clinically relevant. There are data to suggest that antiestrogens act, in part, by increasing the secretion of IGFBP-3 and IGFBP-5 (*26*,*38*). Novel therapeutic approaches that also target IGF bioactivity have been proposed. These approaches include the direct administration of IGFBPs (*32*) as well as the use of somatostatin analogues (*41*,*42*) or antagonists to the receptors for growth hormone, growth hormone-releasing hormone, or IGF-I itself [reviewed in (*21*)]. Vitamin D analogues, alone or in combination with other regulators of IGFBP expression, may provide additional therapeutic opportunities for targeting IGF-stimulated neoplastic proliferation. The efficacy of EB1089 in cancer treatment is currently under clinical investigation (Binderup L: personal communication). Finally, our results, taken together with the antiapoptotic action of IGFs [reviewed in (*37*)], are consistent with the observed induction of apoptosis by vitamin D (*43*) and suggest a role for IGF-binding proteins in the regulation of apoptosis.

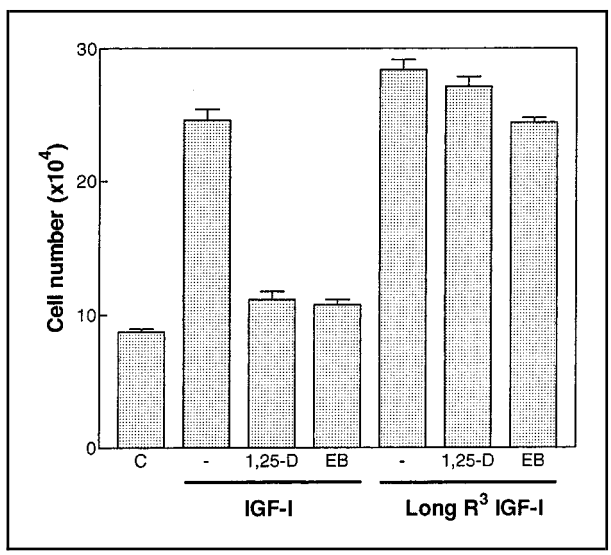

Fig. 4. Effects of 1,25-dihydroxyvitamin D_3 and its analogue EB1089 on insulin-like growth factor-I (IGF-I) and long R^3 IGF-I-stimulated growth of MCF-7 cells. Cells were cultured for 96 hours in 0.5% fetal calf serum with or without 10^{-7} *M* 1,25-dihydroxyvitamin D_3 (1,25-D) or EB1089 (EB) in the presence or absence of 1.4×10^{-9} *M* IGF-I or long R^3 IGF-I. Data are presented as mean ± standard error of triplicate cultures. C = control cells not exposed to IGF-I or long R^3 IGF-I.

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Notes

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