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Calcitriol and Lexicalcitol (KH1060) Inhibit the Growth of Human Breast Adenocarcinoma Cells by Enhancing Transforming Growth Factor-β Production

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ABSTRACT. The mechanisms involved in the antiproliferative action of calcitriol ($1\alpha,25(OH)_2D_3$) were investigated using human breast carcinoma epithelial cells (the MCF-7 cell line). Calcitriol and KH1060, a synthetic analog, inhibited cell growth in a time- and dose-dependent way. The substances similarly stimulated total TGF-β secretion after 24 hours, and Northern blot analyses showed that mRNA levels for TGF-β1 were increased, as well. When MCF-7 cells were co-incubated with calcitriol and a neutralizing antiTGF-β1,β2,β3 antibody, growth inhibition was completely abrogated. With KH1060, the antibody could only partly block growth inhibition. This study shows that TGF-β is involved in the growth response to calcitriol and KH1060 in MCF-7 cells. BIOCHEM PHARMACOL 52;3:505–510, 1996.

KEY WORDS. calcitriol; 1,25-dihydroxyvitamin-D_3; KH1060; TGF-β; MCF-7 cells

The TGF-βs† are dimeric polypeptides that have a wide variety of actions, depending on the type of target cells with which they interact. The biological effects of TGF-βs that have attracted the highest interest are the inhibition of the growth of epithelial cells (reviewed in [1]) and more recently, the promotion of apoptosis in several cell types [2–4]. Because of these actions, the role of this negative growth regulator in tumorigenesis has been primarily seen as the restriction of tumor progression. Conflicting results from recent studies have shown that, depending on the organ, this may or may not be the case. In colon carcinoma cells, repression of endogenous TGF-β synthesis enhances tumorigenic properties [5] and, in skin and skin tumors, loss of expression of TGF-β increases the risk of malignant conversions [6]. Other studies have reported a role of TGF-β in the maintenance and progression of transformed cells in a intact host, probably related to the effects of the cytokine on immune functions [7–9]. Although information is still too fragmentary to decide one way or the other, it remains important to define the actions on TGF-β production of drugs and molecules able to modify cell growth and differentiation.

Calcitriol, the active metabolite of vitamin D_3 ($1\alpha,25(OH)_2D_3$), can modify the growth and differentiation of many cell types [10], including breast carcinoma cells [11, 12]. The effects of calcitriol are mediated through binding to a specific nuclear receptor (VDR) that is present in a wide variety of breast carcinoma cells. Calcitriol, therefore, seems to possess therapeutic properties, and some vitamin D_3 analogs have proven efficient in the treatment of psoriasis and cancer [13]. Our interest lay in defining the possible relationship between vitamin D treatment and TGF-β synthesis in breast carcinoma epithelial cells (the MCF-7 cell line). Indeed, like a vast majority of cells, breast epithelial cells can produce and respond to TGF-βs. TGF-β1 and TGF-β2 are able to inhibit the growth of the majority of human breast cancer cells in culture [14–16]. In these cells, TGF-βs synthesis can be modulated by different hormones and molecules: estradiol treatment is associated with diminished TGF-β secretion in the medium [16]. TGF-β1 synthesis was increased in response to the synthetic progestin gestodene [17]. The antiestrogen tamoxifen has been reported to enhance TGF-β1 production [14, 18, 19], and it seems that the effect of this compound on the inhibition of cell growth or the induction of cell death is mediated by TGF-β in MCF-7 cells [19].

We show, in this report, that calcitriol and a vitamin D analog, KH1060 [20], act on MCF-7 growth by enhancing active TGF-β production.

MATERIALS AND METHODS

Reagents

TGF-β1 was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ^{125}I-TGF-β1 (120–180 μCi/mg) was

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† *Abbreviations:* TGF-β, transforming growth factor-β; $1\alpha,25(OH)_2D_3$, 1α25-dihydroxy-vitamin D_3; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.

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obtained from Dupont (Wilmington, DE, U.S.A.). Calcitriol was purchased from Roussel Uclaf Laboratories (Romainville, France). KH1060 was generously provided by Dr. Binderup, Leo Pharmaceutical Products (Ballerup, Denmark). Calcitriol and KH1060 were prepared as millimolar stock solutions in absolute ethanol. The solutions were aliquoted into glass vials and stored at −20°C. Mouse monoclonal IgG antiTGF-β1,β2,β3 (antiTGF-β Ab) and nonimmune mouse IgG were purchased from Genzyme (Cambridge, MA, U.S.A.) and Sigma Chemical Co., respectively.

Cell Culture

CCL 64 cells were purchased from the ECACC (Salisbury, U.K.). MCF-7 cells originated from the laboratory of Dr. Leclercq (Brussels, Belgium), and were given to us by the laboratory of Dr. L. M. Houdebine (Jouy en Josas, France). These cells produce TGF-β1 and not TGF-β2, and present only type I receptors when ^{125}I-TGF-β1 is cross-linked (T. Mercier, unpublished results). Both cell lines were maintained in continuous log phase growth in DMEM containing 10% FCS, 2 mM L-glutamine, 50 UI/mL penicillin, 50 mg/mL streptomycin, and 10 mg/mL gentamycin.

Cell Proliferation Experiments

MCF-7 cells were seeded in 35-mm diameter Petri dishes at 4×10^4 cells/well in DMEM, 10% FCS containing calcitriol, or KH1060 at the desired concentrations, and incubated overnight. Control dishes received a volume of ethanol similar to the dishes treated with the molecules. The final ethanol concentration was not higher than 0.1% (v/v). At the end of the incubations, the subconfluent cells were harvested with trypsin, and counted using a Coulter counter channelizer (Coultronics).

Determination of TGF-β Activity in Conditioned Medium

MCF-7 cells were grown in 75 cm^2 flasks. Exponentially growing cells (2×10^6 cells) were washed 3 times (2 × 15 min, 1 × 90 min at 37°C) in serum-free incubation buffer (DMEM medium, containing 0.5% bovine serum albumin (BSA) and 25 mM Hepes, pH 7.5). Cells were incubated in the same medium with or without the substances for 24 or 48 hr, and the media were collected at the end of the incubation. Samples were centrifuged (10000 × *g* for 10 min at 4°C) to remove any cellular debris, acidified to pH 2 with 1 M HCl to activate latent forms of TGF-β, and re-neutralized with 1 M NaOH [21]. The samples were, then, assayed for TGF-β activity according to the colorimetric method described by Absher *et al.* [22], using CCL64 cells in 96-well plates. Standard curves were obtained using pure TGF-β1 diluted in the incubation buffer at concentrations ranging from 2.5 to 1000 pg/mL. The IC_{50} of the assay was 300 pg/mL under our conditions.

RNA Extraction and Northern Blot Analysis

Total cellular RNA was extracted from exponentially growing cells using the guanidine isothiocyanate procedure of Chomczynski and Sacchi [23], and the extracts were centrifuged on cesium chloride gradient [24]. RNA (30 μg per lane) was loaded and electrophoresed in 1% agarose-formaldehyde gel, and blotted on nylon filter (NY 13, Schleicher and Schuell) by electroblotting. Hybridization was performed using the 715 bp SacI-PvuII restriction fragment of the porcine TGF-β1 cDNA [25] and the 604 bp PstI-HindIII restriction fragment of the simian TGF-β2 cDNA [26], radiolabeled by the multiprime DNA labeling system (Amersham, Buckingham, England). The specific activity of this probe was approximately 1×10^9 cpm/μg. Blots were stripped and rehybridized with the SalI-EcoRI restriction fragment of the murine 18S rRNA [27]. Bands on the autoradiograms were quantified by computer image analysis (Biocom, France), and the values for TGF-β1 mRNA levels were normalized against the corresponding 18S rDNA. Hybridization and washing conditions are described in Mercier *et al.* [28].

RESULTS

Effects of Calcitriol and KH1060 on MCF-7 Cell Growth

Calcitriol and KH1060 inhibited the growth of MCF-7 cells, as shown in Fig. 1. The effect was significant after 1 day of incubation with 10 nM calcitriol and 2 days with 100 pM KH1060. The growth inhibition was time- and dose-dependent for both compounds. After 6 days of treatment, the inhibitions were very similar: growth was impaired by

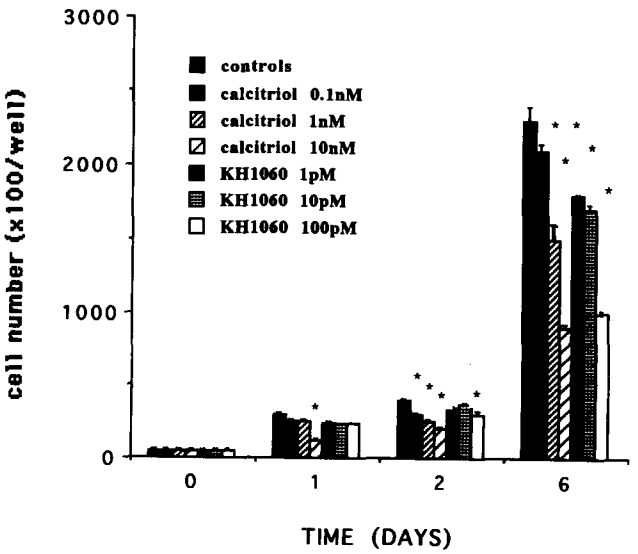

FIG 1. Effect of calcitriol and KH1060 on the growth of MCF-7 cells. Cells were incubated in the usual culture medium without or with calcitriol or KH1060 at the desired concentrations. Cells were harvested by trypsinization and counted at the times indicated. Each value is the mean of 3 wells ± SD. *$P < 0.05$ (Mann-Whitney U test).

TABLE 1. Total TGF-β secretion by the MCF-7 cell line

Compounds	Exposure (hr)	TGF-β secretion rate (pg/10^6 cells)
Control	24	770 ± 200
Calcitriol 1 nM	24	620 ± 250
Calcitriol 10 nM	24	1690 ± 170*
KH1060 10 pM	24	730 ± 60
KH1060 100 pM	24	1580 ± 340*
Control	48	700 ± 220
Calcitriol 1 nM	48	1340 ± 190*
Calcitriol 10 nM	48	1820 ± 60*
KH1060 10 pM	48	1280 ± 170*
KH1060 100 pM	48	2710 ± 1200*

TGF-β secretion was measured by a colorimetric bioassay using acid-activated conditioned media from the MCF-7 cell line. Data are the means ± SD of 3 independent experiments of at least 3 determinations. *Significantly higher than the control value ($P < 0.05$), Mann-Whitney U test.

60% in cells treated with 10 nM calcitriol and by 50% in cells incubated with 100 pM KH1060. Thus, under our experimental conditions, KH1060 was 100-fold more potent than calcitriol.

TGF-β Secretion and TGF-β1 mRNA Accumulation in MCF-7 Cells

We measured the total TGF-β activity in conditioned media. The results are summarized in Table 1. TGF-β secretion was significantly enhanced by calcitriol and KH1060, and the increases were very similar. The TGF-β secreted by the majority of cells in culture is in a biologically latent form that can be activated by transient acidification [21]. Under our assay conditions, the detection limit (5–10 pg/mL) did not enable us to observe any activity when the conditioned media were not acid-activated and, therefore, all the data in the table refer to activated media.

To further define the mechanism by which calcitriol and KH1060 enhance TGF-β secretion, we examined the effect of both compounds on the steady-state mRNA levels. In the different experiments, we never could detect any band corresponding to TGF-β2 mRNA, a result consistent with those of Arrick *et al.* [16], but different from those described by Madisen *et al.* [29] or Jeng *et al.* [30]. For both substances, the levels of TGF-β1 mRNA were markedly increased after 24 hr of incubation (Fig. 2). When the blots were normalized against the 18S rDNA, we observed that 10 nM calcitriol almost doubled the mRNAs for TGF-β1, and 100 pM KH1060 tripled them (Fig. 3). Thus, calcitriol and KH1060 probably acted at the transcriptional level, either by increasing transcription of by modifying the stability of the messengers.

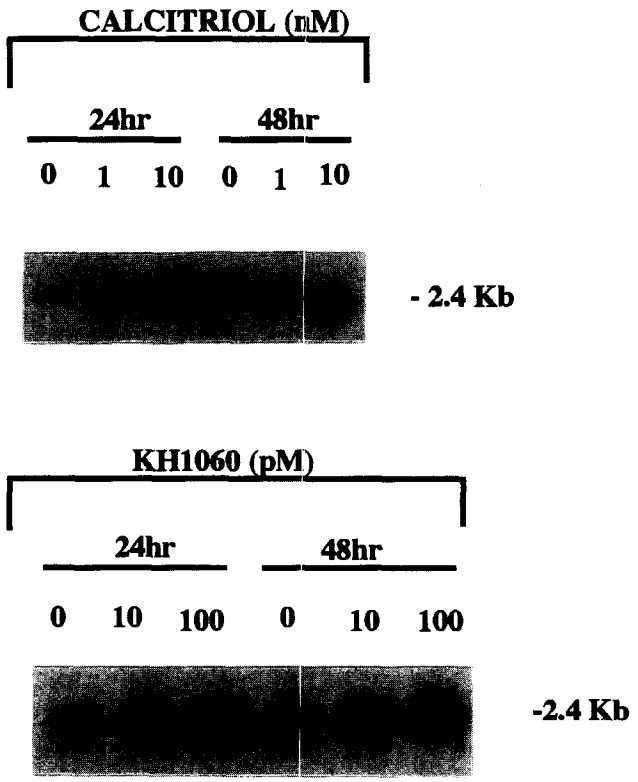

FIG. 2. TGF-β1 mRNA expression in cells treated or not with calcitriol or KH1060. Northern blots were performed using the TGF-β1 cDNA, after 24- and 48-hr incubations of the cells without or with the two compounds. The experiment was repeated twice with similar results.

Effect of Neutralizing Antibodies to TGF-β on the Growth of MCF-7 Cells

Because TGF-β1 gene expression and TGF-β secretion were increased under calcitriol treatment, the next question was if the antiproliferative effect of calcitriol was entirely mediated by TGF-β. We, therefore, used a neutralizing antiTGF-β1,β2,β3 antibody to block the endogenous TGF-βs. Figure 4A shows that, after 6 days of incubation, the

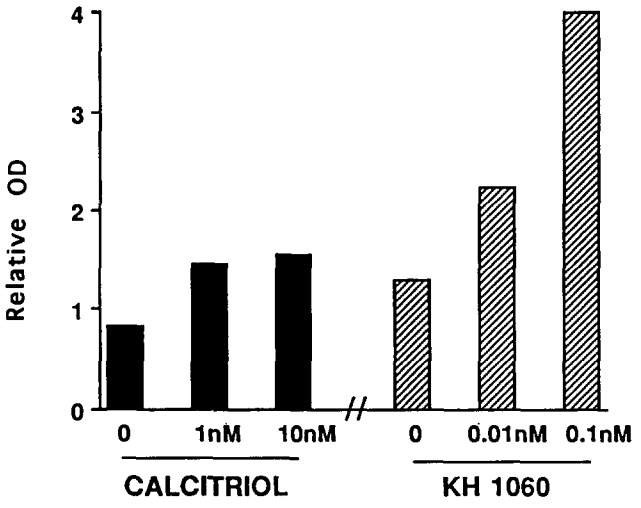

FIG. 3. Densitometry analysis of TGF-β1 mRNA expression. Cells were incubated for 48 hr with the compounds, and mRNAs were extracted. Northern blots were performed using the TGF-β1 cDNA and the 18S rDNA. TGF-β1 mRNA levels were quantified by densitometric scanning of the Northern blots. Each bar represents the value of the TGF-β1 mRNA level normalized against that of the 18S rRNA band.

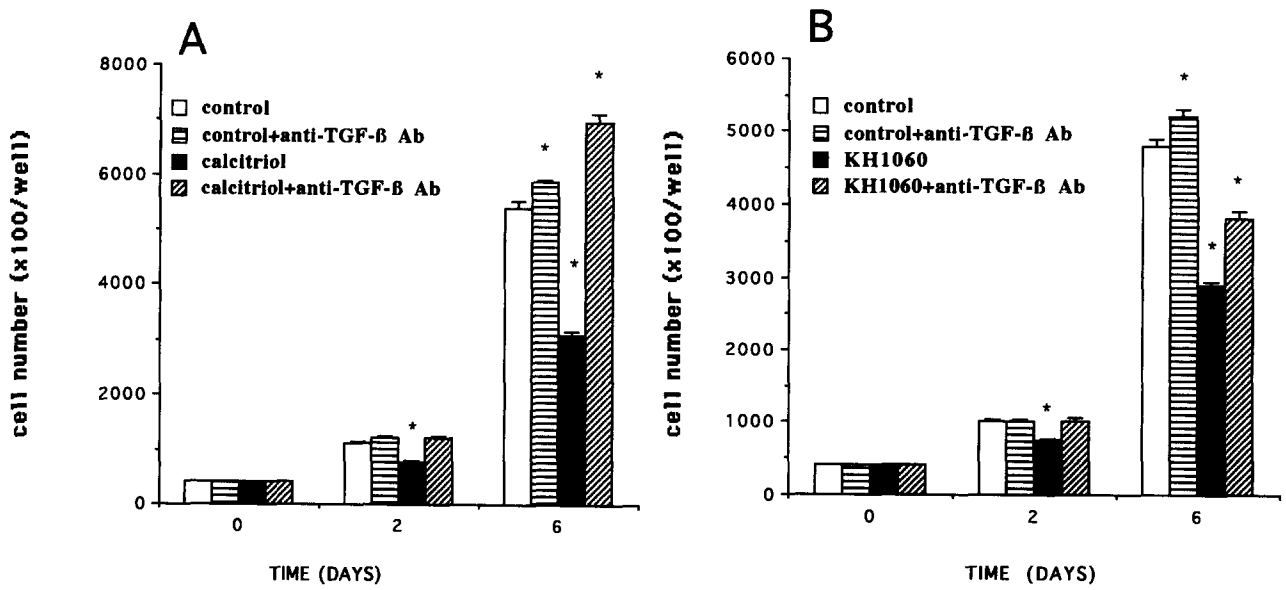

FIG. 4. Effect of the addition of a neutralizing antibody antiTGF-β on the growth of cells treated with calcitriol or KH1060. Cells were grown for the indicated times in the presence or absence of calcitriol (A) or KH1060 (B), and antiTGF-β Ab (50 μg/mL). Each value is the mean of 3 wells ± SD. *$P < 0.05$ (Mann-Whitney U test). The experiments were repeated twice with similar results.

inhibition of growth induced by calcitriol was completely reversed by the antibody. With KH1060, the antibody was not capable of entirely reversing growth inhibition (Fig. 4B). Nonimmune mouse IgG had no effect (data not shown). It can also be observed from these experiments that the growth of control cells was, also, slightly increased by addition of the antibody, indicating that TGF-β participates in cell growth regulation.

DISCUSSION

In the present study, we have examined the growth regulatory effects of calcitriol and KH1060 on the MCF-7 cell line, and established a link between growth inhibition and TGF-β production.

The antiproliferative activity of calcitriol has been demonstrated in several cell types [11, 19, 31], and our results support these observations. Under our conditions, KH1060 was 100-fold more potent than calcitriol. Using the T-47D cell line, Binderup *et al.* [20] reported that KH1060 was 800-fold more potent, although the affinities of both compounds for the VDR were very similar.

The literature shows that calcitriol and TGF-β induce many similar biological changes: they both modulate the expression of nuclear transcription factors such as c-myc or c-fos [32–34], or reduce the number of EGF receptors [35, 36]. In keratinocytes, the inhibition of growth induced by calcitriol has been related to the dephosphorylation of the retinoblastoma gene product [37], a result similar to that obtained with TGF-β in CCL-64 cells [38]. Therefore, it seemed possible that calcitriol could modulate TGF-β synthesis.

Our results showed that indeed, calcitriol enhanced TGF-β secretion and, moreover, in this cell line, the growth response was entirely mediated by TGF-β, because the addition of the neutralizing antibody completely abrogated growth inhibition. With KH1060, the addition of the antibody could not entirely block growth inhibition, showing that the effect of KH1060 was only partly mediated by TGF-β, and that the substances acted differently on cell growth.

The fact that calcitriol and KH1060 both bind to the same receptor (VDR) does not necessarily imply that they transactivate the same genes. Indeed, the pathways of nuclear signaling by VDR, which are being progressively disclosed, seem to be very complex. VDR can form homodimers and heterodimers with RXR, RAR, and T_3R. The complexes can, then, bind to different response elements; to date, 18 have been identified, are formed by direct repeats of hexameric core-binding motifs spaced by three (DR3s), four (DR4s) or six (DR6s) nucleotides. The complexes can also bind to inverted palindromes spaced by nine nucleotides (IP9s) (reviewed in [39]). It has been assumed that, in each protein-DNA complex, the VDR has a slightly different conformation, and that it may recognize calcitriol or its analogs differently [39]. Moreover, a recent study has reported that the analog EB1089 could bind preferentially to a IP9 and the analog KH1230 to a DR3, as compared to calcitriol [40]. As for KH1060, this compound did not display a promoter selectivity between DR3 or DR6, compared to calcitriol, but showed much higher ligand sensitivity on both response elements [41]. Given the complexity of the system, thus, it seems conceivable that both products act, in different ways, on cell growth.

Total TGF-β production was increased by both compounds in a time- and dose-dependent way. mRNAs for

TGF-β1 were also more abundant in cells treated with the substances. Under the conditions used for the TGF-β assay, it was not possible to determine if the proportion of active TGF-β was also increased by incubation with the molecules. Indeed, TGF-βs are released into the medium in latent forms that must be activated to interact with the receptors. The mechanisms for the activation of TGF-β are only partly known and seem to vary according to the cell type. Several enzymes, like plasmin or transglutaminase, have been implicated [42, 43] but, in MCF-7 cells, the mechanism of activation is not known. Interestingly, the presence of plasmin and transglutaminase has been described in MCF-7 cells [44, 45], and it would be interesting to determine if they can activate latent TGF-β. Our experiment with the antibody showed that TGF-β is, indeed, activated and participates in MCF-7 cell growth regulation, because addition of the antibody in control dishes stimulated cell growth significantly (Fig. 4). It would, therefore, be interesting to determine if calcitriol can also trigger activation in the same way as retinoic acid in bovine aortic endothelial cells [43].

Calcitriol and analogs are increasingly recognized and used as compounds with therapeutic value. It is, therefore, very important to know precisely the mechanisms underlying their action, especially as our knowledge of the good and the bad features of TGF-β is advancing rapidly [46].

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