MCF-7/VD^R: A New Vitamin D Resistant Cell Line

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Abstract   Several in vitro and in vivo experiments have demonstrated potent cell regulatory effects of vitamin D compounds in cancer cells. Moreover, a promising phase I study with the vitamin D analogue Seocalcitol (EB 1089) in patients with advanced breast and colon cancer has already been carried out and more clinical trials evaluating the clinical effectiveness of EB 1089 in other cancer types are in progress (Mørk Hansen et al. [2000a]). However, only little is known about the mechanisms underlying the actions of vitamin D or about the possible development of drug resistance in the patients. Therefore, in an attempt to gain more insight into these aspects, we have developed the MCF-7/VD^R cell line, a stable subclone of the human MCF-7 breast cancer cell line, which is resistant to the growth inhibitory and apoptosis inducing effects of $1\alpha,25(OH)_2D_3$. Despite this characteristic, receptor studies on the VDR have clearly demonstrated that the MCF-7/VD^R cells contain fully functional VDRs, although in a lower number than seen with the parental MCF-7 cells. The regulation of the 24-hydroxylase enzyme appeared to be intact in the MCF-7/VD^R cells and no differences with regard to growth rate and morphological appearance between the MCF-7/VD^R cells and the parental MCF-7 cells were observed. Interestingly, however, the sensitivity of the MCF-7/VD^R cells to the pure anti-estrogen ICI 182,780 was found to be increased. The MCF-7/VD^R cell line shows characteristics different from those of previously described vitamin D resistant breast cancer cell lines but also some similarities. Together such vitamin D resistant cell lines therefore serve as a useful tool for studying the exact mechanism of action of vitamin D and the development of vitamin D resistance. J. Cell. Biochem. 82: 422–436, 2001.   © 2001 Wiley-Liss, Inc.

Key words: MCF-7/VD^R; MCF-7; vitamin D; resistance; breast cancer; apoptosis

The exact mechanism underlying the actions of $1\alpha,25(OH)_2D_3$ is not fully elucidated, but it is well accepted that the main effects of $1\alpha,25(OH)_2D_3$ are mediated through its binding to specific high affinity intracellular vitamin D receptors (VDR), which belong to the superfamily of steroid receptors. The receptor-ligand complex functions as a transcription factor by interacting with the vitamin D response elements (VDRE) present in the promoter regions of the primary responding genes, leading to either activation or suppression of gene transcription [Hannah and Norman, 1994; Carlberg, 1995, 1998; Freedman, 1999]. Substantial evidence suggests that the VDR binds these response elements either as a homodimer or as a heterodimer with other nuclear receptors, preferentially with the retinoid X receptor (RXR). In this way, multiple combinations and a number of different signalling pathways are possible, which may explain the great diversity of vitamin D actions [Carlberg, 1995; Haussler et al., 1998]. Recently, a number of receptor co-activators and co-repressors have been identified. These proteins appear to be interacting with the nuclear receptors and are thus capable of further modulating the transcriptional activity initiated by the receptor-ligand complex [Jurutka et al., 1997; Masuyama et al., 1997; Berghöfer-Hochheimer et al., 1998; Carlberg, 1998; Haussler et al., 1998; Aslam et al., 1999; Castillo et al., 1999; Yang and Freedman, 1999].

To date, more than 50 genes have been described to be sensitive to vitamin D, including 26 genes in which a natural VDRE has been identified [Hannah and Norman, 1994; Carl-

berg, 1995; Mørk Hansen et al., 2000a]. Among these vitamin D responsive genes are several genes which are known to play a key role in the regulation of cell growth and differentiation [Mørk Hansen et al., 1997a, 2000a]. This observation and the fact that VDRs have been demonstrated in a number of different cancer cells, make vitamin D compounds potentially useful agents in the future treatment of cancer [Hamada and Shinomiya, 1993; Hannah and Norman, 1994; Haussler et al., 1998; van den Bemd et al., 2000].

Several reports have already emerged demonstrating potent effects of vitamin D compounds on cancer cells in vitro and in vivo. In vitro, block of cell cycle progression, induction of apoptosis, inhibition of invasion and metastasis, and stimulation of differentiation have been observed in response to treatment of the cells with vitamin D compounds [Welsh, 1994; Colston, 1995; Mørk Hansen et al., 1997b, 2000]. Moreover, it has been shown that the administration of vitamin D compounds to tumor-bearing animals prevents the development of metastases, causes regression of tumors and prolongs survival time in tumor-bearing animals [Honma et al., 1983; Abe et al., 1991; Colston et al., 1992; Anzano et al., 1994; Colston et al., 1997; Lokeshwar et al., 1999; Nickerson and Huynh, 1999; Mørk Hansen et al., 2000a].

In an attempt to gain more insight into the mechanisms by which vitamin D exerts its anticancer effects, we have developed a subclone of the human MCF-7 breast cancer cell line which is resistant to the growth inhibitory effect of $1\alpha,25(OH)_2D_3$. The subclone is termed MCF-7/VD^R and has been selected by repeatedly passaging the cells in medium containing increasing concentrations of $1\alpha,25(OH)_2D_3$. To further characterise the MCF-7/VD^R cell line, basic properties such as growth rate, morphological appearance, expression of VDR, functionality of these receptors, and regulation of 24-hydroxylase activity have been studied. In addition, we investigated the effect of $1\alpha, 25(OH)_2D_3$ and one of its potent analogues, Seocalcitol (EB 1089), on induction of apoptosis, a process which is known to be involved in the growth reducing effects of vitamin D compounds in breast cancer cells [Peyrat et al., 1993; Welsh et al., 1995; Simboli-Campbell et al., 1996; Danielsson et al., 1997; Mathiasen et al., 1999; Pirianov et al., 1999; Xie et al., 1999]. Finally, since several investigations have

demonstrated that vitamin D compounds interfere with the ER signalling pathway in MCF-7 cells [James et al., 1994; Simboli-Campbell and Welsh, 1994; Nolan et al., 1998; Sun et al., 1998; Stoica et al., 1999], studies on the expression of ER as well as on the effect of two anti-estrogens, Tamoxifen and ICI 182,780 were also included.

MATERIALS AND METHODS

Chemicals

The vitamin D compounds were synthesised in the Department of Chemical Research at Leo Pharmaceutical Products (Ballerup, DK). Tumor Necrosis Factor-α (TNFα) and Camptothecin (CAM) were purchased from R&D Systems Europe Ltd. (Abingdon, UK). All-trans retinoic acid (all-trans RA) and Tamoxifen were purchased from Sigma-Aldrich (MO). 9-cis retinoic acid and ICI 182,780 were kindly provided by, respectively, Dr. A. Wakeling, Zeneca Pharmaceuticals (Macclesfield, UK) and Hoffmann-LaRoche (Basel, Switzerland).

Cell Culture

MCF-7, an estrogen receptor (ER) positive human breast cancer cell line derived from a differentiated adenocarcinoma, was purchased from Tumorbank, DKFZ, Heidelberg, Germany. The cells were grown in phenol red free DMEM supplemented with 5% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin (Life Technologies Ltd., Paisley, UK).

The vitamin D resistant cell line (MCF-7/ VD^R) was developed by growing the parental MCF-7 cells in the presence of increasing concentrations of $1\alpha,25(OH)_2D_3$. $1\alpha,25(OH)_2D_3$ was used in the dose range between $0.01-10\,\mu M$, and the cells were exposed to each concentration for three passages before increasing stepwise by half an order of magnitude. After the last three passages in the presence of 10 μM $1\alpha,25(OH)_2D_3$, the cells were grown in the same medium as described above for the parental MCF-7 cell line (without the addition of $1\alpha, 25(OH)_2D_3$) for up to 25 passages. During this time period, the responsiveness of the cells to vitamin D was tested regularly by assessment of DNA synthesis. The cells remained resistant to vitamin D during these 25 passages.

For both cell lines, the culture medium was changed every 2nd or 3rd day, the cells were pas-

saged once a week by trypsination (0.25% trypsin-1mM EDTA in Phosphate Buffered Saline (PBS)), and cultures were kept in a humidified atmosphere of 75% N_2, 20% O_2, and 5% CO_2 at 37°C.

Assessment of DNA Synthesis

The cells were seeded at a density of 7×10^3 cells/ml in 24-well plates (Nunc, Denmark) and the test compounds were added 2 h after seeding. After 5 days 1 µCi/ml ^{3}H-thymidine (Amersham, UK) was added to the cells and the plates were incubated for an additional of 4 h. The cells were then washed three times with NaCl + 25 mg/L unlabelled thymidine, solubilised for 30 min in 1 ml 0.5M NaOH and incorporated ^{3}H-thymidine was measured with a β-counter. Each sample was tested in quadruplicate.

Cell Cycle Analysis

The cells were seeded at a density of 1×10^4 cells/ml in T25 culture flasks (Nunc, Denmark) and grown in the presence of the test compounds for five days. Cell samples were then prepared using the CycleTest PLUS kit (Becton Dickinson, CA) and subsequently analysed on a Becton Dickinson FACSort flow cytometer. The samples were excited with a single 488 nm argon laser and analysed for light scatter and orange fluorescence (FL2, Orange 564–606 nm). 50,000 nuclei/sample were collected and the data were analysed with MODFit LT software from Becton Dickinson. The results were expressed as percent cells in G_0/G_1-, S-, and G_2/M-phase in relation to the total number of cells in the sample. Three experiments were performed and the results obtained were similar.

Determination of Cell Growth and Cell Size

The cells were seeded at a density of 7×10^3 cells/ml in T25 flasks (Nunc, Denmark). Prior to counting in a Coulter Counter, the cells were trypsinised as described above. The cell size was determined based on distribution curves obtained with the Coulter Counter.

Gel Electrophoresis and Western Blotting

To prepare whole cell extracts, the cells were lysed in lysis buffer containing 50 mM Tris HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40 and adjusted to a volume of 5×10^6 c/ml with Laemmli's SDS

sample buffer. The extracts were boiled for 5 min, centrifuged at 10,000 rpm for 15 sec and samples of 1.25×10^5 cells were subjected to Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis using 10% gels (SDS-PAGE) [Laemmli, 1970]. The proteins were transferred onto an Immobilon-PVDF Transfer Membrane (Millipore, MA) essentially by the method of Towbin et al. [1979] and the protein bands were identified immunochemically by the ECL-detection system (Amersham). The primary antibodies used included: anti-p21[WAF1/CIP1] (1:200, clone EA10, Oncogene Research Products, MA), anti-p27[kip 1] (1:2000, Transduction Laboratories, KY), anti-p53 (1:500, clone DO-7, DAKO A/S, Denmark), anti-VDR (1:750, clone 9A7γ, Chemicon International Inc., MA) and anti-ER (1:500, clone 1D5, DAKO A/S).

Northern Blotting

Total RNA was isolated from MCF-7/VD^R and parental MCF-7 cells using the TRIzol Reagent (Life Technologies, MD) according to the manufacturer's protocol, followed by poly(A)$^+$ RNA enrichment by Dynabeads Oligo (dT)$_{25}$ (Dynal, Norway). 4 µg of poly(A)$^+$RNA from each cell line was denatured by glyoxal/DMSO, separated on a 1.0% agarose gel and transferred onto a nylon membrane by Northern blotting. The Northern blot was hybridised with ^{32}P-labelled human VDR cDNA probe [Laborda, 1991]. As a control for differences in poly-(A)$^+$RNA loading, the Northern blot was rehybridised with a human 36B4 cDNA probe [Baker et al., 1988].

ER Ligand Binding Studies

The ER ligand binding studies were performed as previously described by McGuire and DeLaGarza [1973]. Briefly, the MCF-7 cells were treated with the test compounds over a 96 h period, after which the cells were washed in PBS and sonicated in homogenisation buffer (10 mM Tris-HCL, 15 mM EDTA, 10% glycerol, pH 8.0, and 1 mM dithiothreitol). The ER-containing cell cytosols were centrifuged at 25,000 rpm for 45 min at 4°C. The resulting cell extracts were then incubated with serial dilutions of 2, 4, 6, 7-[^{3}H]-estradiol (100 Ci/mmol, Amersham) at a concentration ranging from 1 µM to 31 nM, in the presence or absence of serially diluted DES (diethylstilbestrol), with a concentration range of 1 mM−31 µM. After an incubation period of 18 h at 4°C, bound from free

sterol separation was achived by using dextran coated charcoal, and radioactivity was detected using liquid scintillation counting.

Biochemical Assessment of DNA Fragmentation

The cells were seeded at a density of 1.5×10^4 cells/ml in 48-well plates and the test compounds were added 2 h after seeding. The fraction of apoptotic cells was determined using the Cell Death Detection ELISA; kit (Boehringer Mannheim, GmbH, Germany), which allows quantitative in vitro determination of cytoplasmic histone-associated DNA fragments after induced cell death. The optical density (OD) was read on an Elisa Reader (BioRad model 450), using a 415 and a 490 nm (reference) filter.

In order to correct for the antiproliferative effect of the compounds, the level of histone-associated DNA fragments obtained in the Cell Death Detection ELISA was normalised by counting cells that had been grown and treated in parallel using a Coulter Counter. The final results were calculated in relation to control cells and expressed as fold induction (OD/1000 cells±SD). Statistical analysis was carried out using Student's t-test. At least two separate experiments, each with four determinations/ sample, were performed, and the results were reproducible.

Morphological Assessment of DNA Fragmentation

The cells were seeded at a density of 1×10^4 cells/ml in two chamber glass coverslips (Nunc, DK) and grown in the presence of the test compounds for five days. The coverslips were washed twice with binding buffer (0.1 mM Hepes/NaOH, pH 7.4, 1.4M NaCl, 25 mM $CaCl_2$, 1 part to 9 parts distilled H_2O). 400 µl of binding buffer were added in each chamber. 20 µl of the primary antibody (Annexin V-FITC, Pharmingen, CA) were added to each chamber, and the coverslips were incubated for 60 min at rt in the dark. 10 µl of a secondary antibody (Alexa 488 anti-FITC, Molecular Probes, OR), 10 µl propidium iodide (PI) solution (50 µg/ml, Pharmingen, CA) and 40 µl Hoechst 33342 solution (10 µg/ml, Molecular Probes, MO) were added to each chamber and the coverslips were incubated for 45 min at 4°C in the dark. The coverslips were washed twice with binding buffer. Finally, the coverslips were mounted with fluoromount G (Southern Biotechnology

Ass., AL) to which 2.5 mg/ml n-propyl galate (Sigma-Aldrich, MO), was added, and they were then examined using a BH-2 fluorescence microscope. At least three separate experiments, each with two determinations/sample, were performed, and the results were reproducible.

Transcription Assay

The plasmids used were a gift from Dr. C. Carlberg, Duesseldorf, Germany. Cultures of MCF-7/VD^R and parental MCF-7 cells were transfected with a plasmid carrying a *CAT* (chloramphenicol acetyl transferase) gene, placed under the control of a VDRE fused to the *tk* promoter [Carlberg et al., 1993]. The VDR responsive element in the regulatory sequence of the *CAT* gene was the VDRE (nucleotide−514 to−486 from the transcription initiation start) from the human *osteocalcin* gene promoter [Ozono et al., 1990]. This plasmid was cotransfected with a control plasmid expressing a constitutive level of β-galactosidase activity. Both plasmids were introduced into semiconfluent cell cultures by the method of lipofection. The transfection was carried out in serum free medium and the cells were allowed to incubate for 4 h. Subsequently, treatment was initiated by the addition of the test compounds added to the medium containing charcoal stripped serum adjusted to a final concentration of 5% serum. After a two days incubation period, the cells were harvested and a protein stock was prepared from each cell culture. These stocks were then analysed for the presence of *CAT* activity using a commercially available kit (TRK 1012, Amersham) and for β-galactosidase activity using spectrophotometry and the o-nitrophenyl-b-D-galactopyranoside substrate. All samples were made in triplicate. The *CAT* activity was normalised in proportion to the β-galactosidase activity and the molar concentration resulting in 50% stimulation (EC$_{50}$) of gene transcription compared to untreated cells was calculated from a dose-response curve.

Assessment of 24-Hydroxylase Expression by RT-PCR Analysis

Total RNA was isolated from $1\alpha,25(OH)_2D_3$- or vehicle-treated MCF-7/VD^R and parental MCF-7 cells using TRIzol Reagent (Life Technologies), according to the manufacturer's protocol. Two microgram of total RNA was reverse transcribed into first-strand cDNA by 200 U/µl

of SuperScript II (Life Technologies) using oligo(dT)$_{16}$ as primer. The first-strand cDNA was PCR amplified using the following primers: 5′-CTGCTGCAGATTC-TCTGGAA-′3 and 5′-ATGATGAAGTTCACAGCTTC-3′ (coding for the 24-hydroxylase gene, GenBank accession no. L13286), and 5′-AGATTGGCTACCCAAC-TGTTGCA-3′ and 5′-CTTGGCTTCAACCTT-AGCTGGG-3′ (coding for the housekeeping gene 36B4, acidic ribosomal phosphopreotein P0, GenBank accession no. M17885). For each primer pairs the genes were amplified using 1U/25 μl AmpliTaq Gold (PE Applied Biosystems, CA) and 1.5 mM MgCl$_2$ by the following PCR profile: 95°C activation of AmpliTaq Gold for 12 min, and 28 cycles of 95°C for 20 sec, 60°C for 30 sec, and 72°C for 1 min. The PCR products were subjected to agarose gel electrophoresis and stained by SYBR Green I (BMA, ME) in a 1:10,000 dilution for 1 h. The stained gel was scanned in a STORM 860 (Molecular Dynamics, CA) using the blue fluorescence mode.

Fig. 1. Inhibition of DNA synthesis after treatment of the cells with increasing concentrations of the test compounds for 5 days. The cells were seeded in 24-well plates at a concentration of 7×10^3 cells/ml and the inhibition of DNA synthesis after a 5 days treatment period was measured by incorporation of ^{3}H-thymidine. Each sample was tested in quadruplicate. (■) parental MCF-7 cells treated with 1α,25(OH)$_2$D$_3$. (●) parental MCF-7 cells treated with EB 1089. (□) MCF-7/VD^R cells treated with 1α,25(OH)$_2$D$_3$. (○) MCF-7/VD^R cells treated with EB 1089.

RESULTS

Characterisation of Growth and Morphology of MCF-7/VD^R Vs. Parental MCF-7 Cells

Once established the MCF-7/VD^R cell line was grown in medium without 1α,25(OH)$_2$D$_3$. Under these conditions, the cells remained resistant to the growth inhibitory effect of 1α,25(OH)$_2$D$_3$ for at least 25 passages. DNA synthesis was assessed by ^{3}H-thymidine uptake, and as seen in Figure 1, a dose-dependent inhibition of DNA synthesis was observed with the parental MCF-7 cells after 5 days treatment with 1α,25(OH)$_2$D$_3$. In contrast, no significant decrease of DNA synthesis was seen with the MCF-7/VD^R cells. We also tested the analogue EB 1089, which is known to be more potent than 1α,25(OH)$_2$D$_3$ with respect to cell regulatory effects. As expected, EB 1089 was found to exert a more potent antiproliferative effect than 1α,25(OH)$_2$D$_3$ in the parental MCF-7 cell line, whereas no effect was observed in the MCF-7/VD^R cell line.

The evidence of the resistance of the MCF-7/VD^R cells to the two vitamin D compounds was further supported by counting the cells in a Coulter Counter and by results obtained in cell cycle analysis studies. With the parental MCF-7 cell line a time- and dose-dependent decrease in the number of cells in S-phase as well as a reduction of the total cell number were seen in
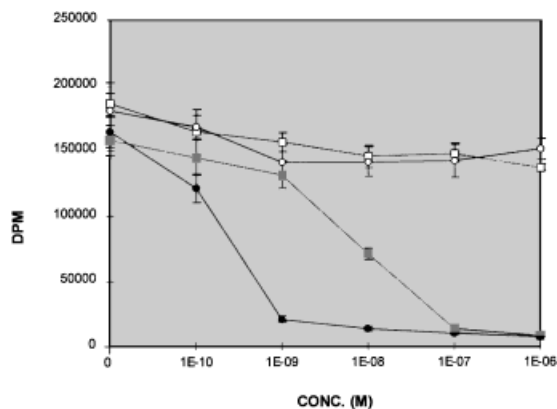
response to treatment of the cells with the test compounds. However, with the MCF-7/VD^R cells, no such effects could be detected (data not shown)

In order to see whether the development of resistance to vitamin D influenced the growth rate and morphological appearance, these two aspects were also investigated. The growth rates of MCF-7/VD^R and the parental MCF-7 cell line appeared to be identical with a doubling time of ∼24 h when the cells were grown in the absence of vitamin D (data not shown). Moreover, microscopic examination of such untreated cells, including determination of cell size and staining of the cells with anti-β-tubulin and anti-cytokeratin antibodies showed no major morphological differences (data not shown).

Regulation of Cell Cycle Regulatory Proteins

Since inhibition of cell growth in response to vitamin D compounds is known to be associated with modulation of important cell cycle regulatory proteins such as p21, p27, p53, and pRb, the expression of these proteins was next studied by Western blot analysis. As expected, treatment of the parental MCF-7 cells with 100 nM 1α,25(OH)$_2$D$_3$ for five days resulted in an up-regulation of p21 protein expression and an up-regulation of the hypophosphorylated form of
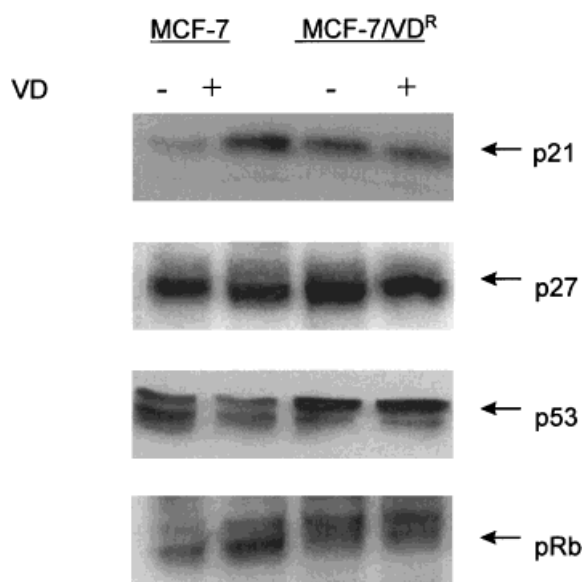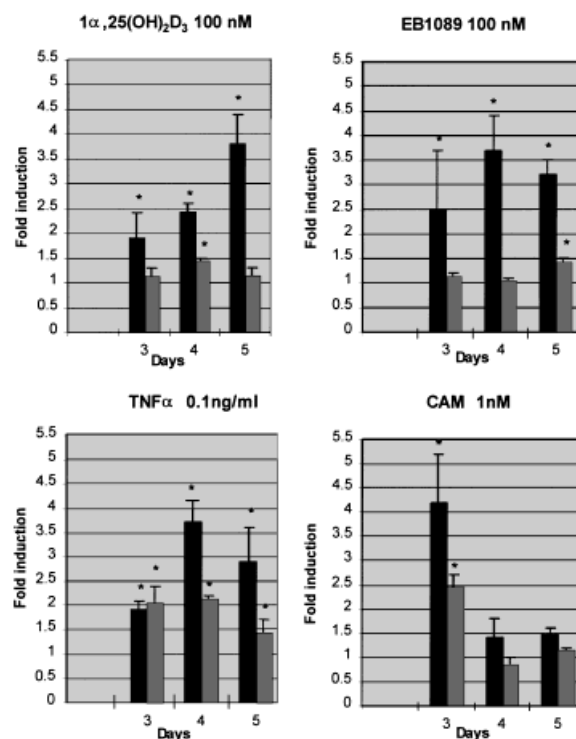
Fig. 2. Western blot analysis of cell extracts derived from MCF-7 cells and MCF-7/VD^R cells treated with 100 nM 1α,25(OH)$_2$D$_3$ (VD) or vehicle for five days. Samples of 1.25×10^5 cells were subjected to SDS-PAGE using 10% gels, transferred onto an Immobilon-PVDF Transfer Membrane and the protein bands were eventually stained with mononuclear antibodies against p21 (21 kDa), p27 (27 kDa), p53 (53 kDa), and pRb (pRb$_{hypophos}$ 110 kDa, and pRb$_{hyperphos}$ 116 kDa).

Fig. 3. Time-course study showing the induction of DNA fragmentation after treatment of the parental MCF-7 cells (■) and the MCF-7/VD^R cells (▨) with the two vitamin D compounds 1α,25(OH)$_2$D$_3$ and EB 1089 or with the reference compounds TNFα and CAM. The cells were seeded at a density of 1×10^4 cells/ml and the test compounds were added 2 h after seeding. The fraction of apoptotic cells was determined using the Cell Death Detection ELISA kit. Each sample was tested in quadruplicate. Statistical analysis was carried out using Student's, t-test. * $P < 0.05$.

pRb (Fig. 2). The expression of the p27 protein did not change significantly, while p53 protein expression appeared to be down-regulated. No major differences in p21, p27, p53, and pRb protein expression between untreated and treated cells were seen with the MCF-7/VD^R cells. However, interestingly, all four proteins seemed to be up-regulated in the MCF-7/VD^R cells compared to the parental MCF-7 cells.

### Induction of Apoptotic Features

To see whether other known effects of vitamin D were also abolished in the MCF-7/VD^R cell line, we investigated the induction of DNA fragmentation as a measurement of apoptosis. The cells were incubated with 1α,25(OH)$_2$D$_3$ or EB 1089 at a concentration of 100 nM for 3, 4, and 5 days, and the level of histone-associated DNA fragments in the cell cytoplasm was subsequently determined as described in Materials and Methods. Both compounds were found to cause a significant, time-dependent induction of DNA fragmentation in the parental MCF-7 cells, with EB 1089 being the most potent compound (Fig. 3). In contrast, only marginal

effects were seen in the MCF-7/VD^R cells with the two compounds. TNFα and CAM were included as references, as both compounds are known to induce apoptosis in MCF-7 cells. As seen in the lower part of Figure 3, both cell lines appeared to respond to the apoptotic effect of TNFα and CAM, although the effect was more pronounced in the parental MCF-7 cell line. The results on DNA fragmentation correlated well with the cell count data from cultures that had been grown and treated in parallel (data not shown).

To further substantiate these results the effect of 1α,25(OH)$_2$D$_3$, EB 1089, and TNFα on annexin-V staining (an early marker of apoptosis) was investigated. The cells were incubated with the test compounds for five days and were subsequently stained with annexin-V (green), PI (red), and the membrane permeable Hoechst
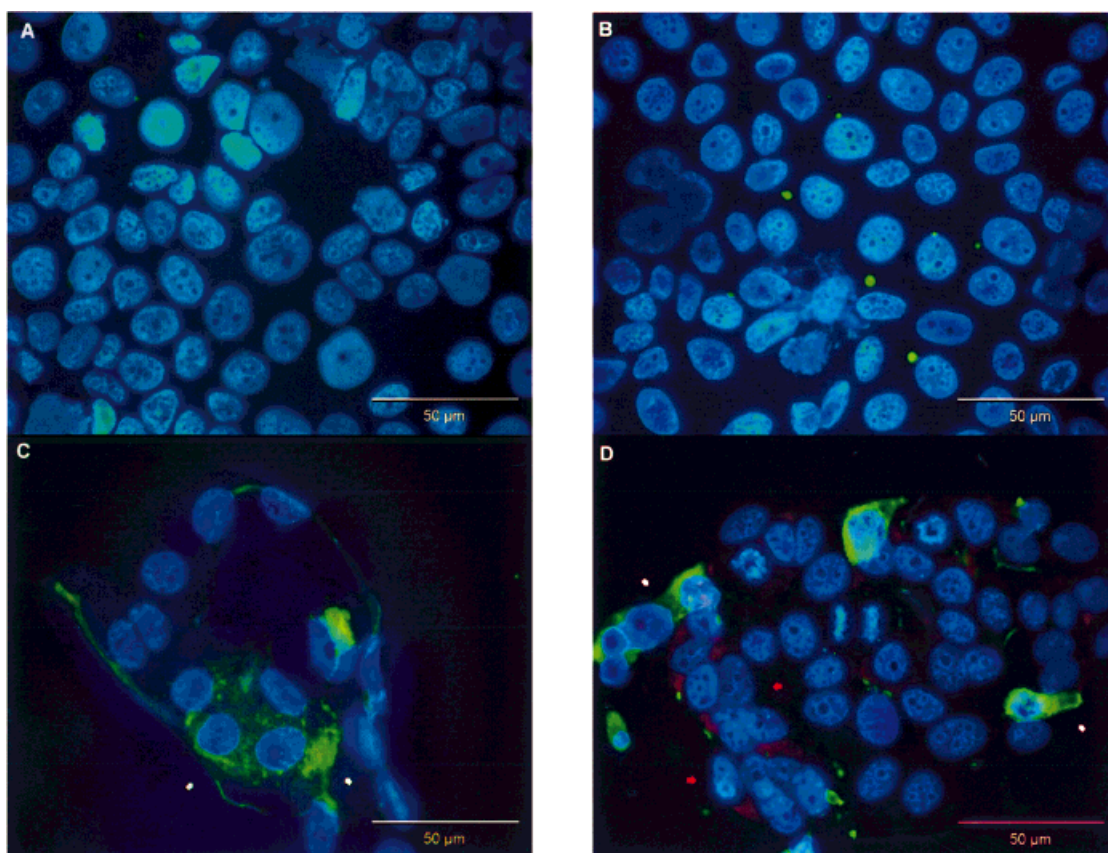
Fig. 4. Microscopic examination of apoptotic cells. **A:** vehicle treated MCF-7/VD^R cells, **(B)** MCF-7/VD^R cells treated with 100 nM EB 1089, **(C)** parental MCF-7 cells treated with 100 nM EB 1089, and **(D)** MCF-7/VD^R cells treated with 0.1 ng/ml TNFα. The cells were seeded at a density of 1×10^4 cells/ml in two chamber glass coverslips and grown in the presence of the test compounds for five days followed by staining with Annexin-V (green), PI (red) and Hoechst (blue). The white arrow indicates Annexin-V positive cells with intact plasma membranes. The red arrows indicate cells with leaky plasma membranes that allow for cellular uptake of PI. At least three separate experiments were performed and the photos shown are representative for the obtained results.

dye (blue). Figure 4 shows that no difference between MCF-7/VD^R cells in the control cultures (Fig. 4A) and MCF-7/VD^R cells treated with EB 1089 (Fig. 4B) could be seen. Only blue cells were detectable, representing viable cells in which the membrane permeable Hoechst stain has been taken up into the nucleus. No green, annexin-V positive cells were seen, indicating that EB 1089 does not induce apoptosis in these cells. In contrast, several annexin-V positive cells were detected in the EB 1089-treated parental MCF-7 cells (Fig. 4C) as well as in MCF-7/VD^R cells treated with TNFα (Fig. 4D). In the TNFα-treated cultures a few red cells appeared, indicative of cells in which the plasma membrane has become leaky, allowing the slow entry of PI into the cell. Similar results were obtained with the parental MCF-7

cells after treatment with TNFα (data not shown).

Sensitivity of MCF-7/VD^R and Parental MCF-7 Cells to Anti-Estrogens

Despite the resistance developed by the MCF-7/VD^R cells to the vitamin D compounds, their ability to undergo apoptosis is preserved. Since the ER signalling pathway is known to be involved in the growth-reducing effect of vitamin D compounds seen in the ER positive MCF-7 cells, we found it obvious to study the effect of the two anti-estrogens, Tamoxifen and ICI 182,780 on DNA fragmentation. Tamoxifen administrated at a concentration of up to 10 nM for 3, 4, and 5 days treatment did not induce DNA fragmentation in either the MCF-7/VD^R cells or the parental MCF-7 cells (data not
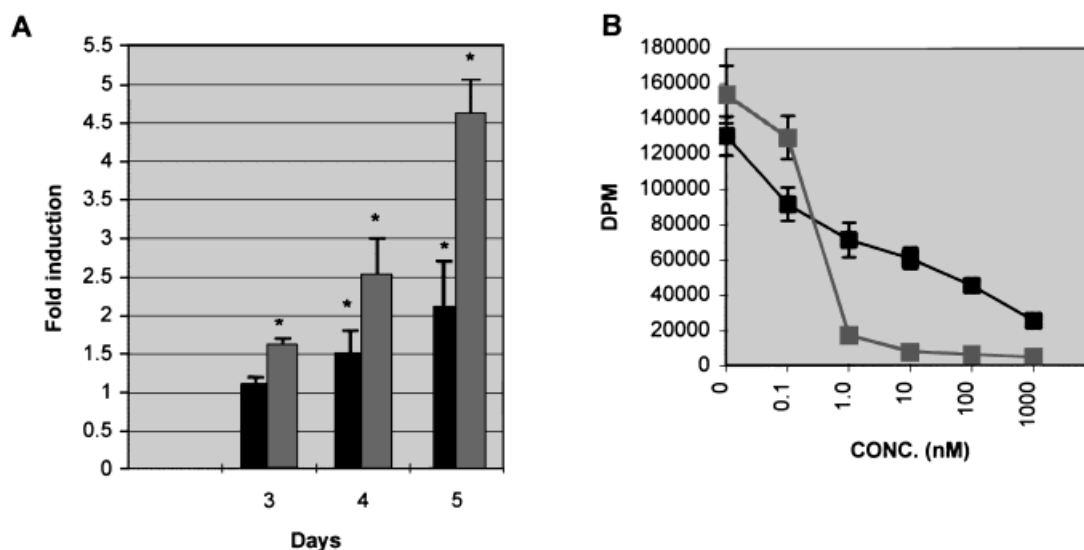
Fig. 5. **A:** Time-course study showing the induction of DNA fragmentation after treatment of the parental MCF-7 cells (■) and the MCF-7/VD^R cells (■) with the anti-estrogen ICI 182,780. **B:** Inhibition of DNA synthesis after treatment of the parental MCF-7 cells (■) and the MCF-7/VD^R cells (■) with increasing concentrations of ICI 182,780 for 5 days. The cells were seeded at a density of 1×10^4 cells/ml and the test compounds were added 2 h after seeding. The fraction of apoptotic cells was determined using the Cell Death Detection ELISA kit. Each sample was tested in quadruplicate. Statistical analysis was carried out using Student's, *t*-test. *$P < 0.05$.

shown). However, ICI 182,780 was found to cause a time-dependent increase of DNA fragmentation in both cell lines (Fig. 5A). Interestingly, the MCF-7/VD^R cells appeared to be much more sensitive to ICI 182,780 than the parental cell line. This observation was further supported by proliferation studies in which ICI 182,780 was shown to cause a greater inhibition of DNA synthesis in the MCF-7/VD^R cells than in the parental MCF-7 cells (Fig. 5B).

Presence of VDR and ER in MCF-7/VD^R and Parental MCF-7 Cells

The fact that most effects of vitamin D are known to be mediated by the binding of the compound to the VDR led us to speculate whether these receptors had been lost in the MCF-7/VD^R cell line. Northern and Western blot analysis demonstrated the presence of VDRs in both cell lines. However, the amount of receptor at both mRNA and protein level appeared to be lower in the MCF-7/VD^R cell line than in the parental MCF-7 cell line (Fig. 6).

Also, the presence of ER and its binding affinity in the two cells lines were investigated. As expected, Western blot analysis demonstrated a down-regulation of ER expression in response to treatment of the parental MCF-7 cells with $1\alpha,25(OH)_2D_3$ (Fig. 7A). However, no differences in the amount of ER protein were

observed between the MCF-7/VD^R cells and the parental MCF-7 cells. In addition, receptor ligand binding studies revealed no major difference in the apparent K_d value of the ER between the MCF-7/VD^R cells ($K_d = 0.41 \pm 0.11$ nM) and the parental MCF-7 cells ($K_d = 0.35 \pm 0.19$ nM) or in the total number of [^{3}H]-estradiol binding sites between the MCF-7/VD^R cells (89.5 ± 9.7 fmol/mg cytosol protein) and the parental MCF-7 cells (80.4 ± 5.8 fmol/mg cytosol protein) (Fig. 7B).

Functionality of the VDRs

Upon binding to the receptor, the receptor-ligand complex interacts with the VDRE on the genome, which eventually elicits the biological response. Thus, in order to test the functionality of the VDRs, the ability of $1\alpha,25(OH)_2D_3$ and EB 1089 to induce VDR mediated gene transcription from the human osteocalcin gene promoter was studied. As seen in Table I, an increased transcriptional activity was observed in both cell lines in response to treatment of the cells with the two test compounds, suggesting the presence of intact VDRs in both the MCF-7/VD^R and the parental MCF-7 cells.

Regulation of the 24-Hydroxylase Enzyme

Finally, the regulation of 24-hydroxylase, the major enzyme involved in the catabolism of
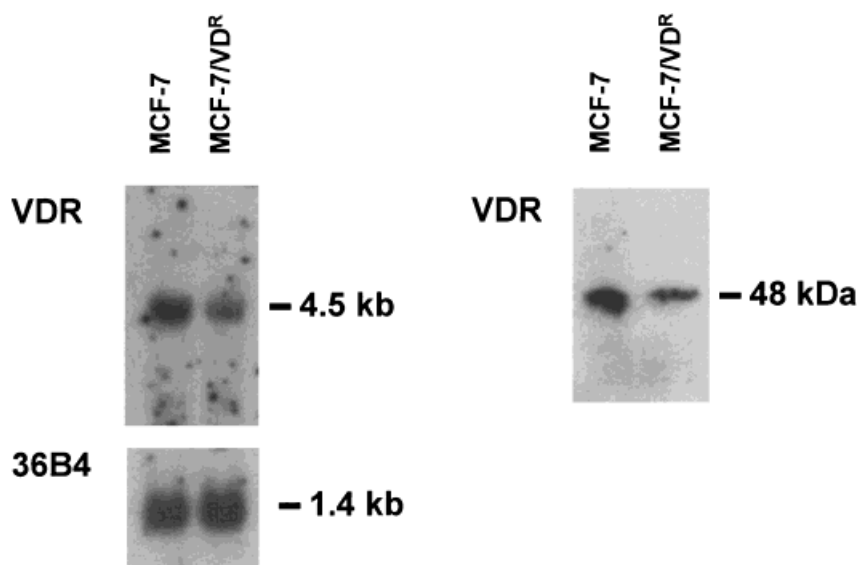
Fig. 6. Northern and western blots showing, respectively, the mRNA and protein levels of VDR (48 kDa) in MCF-7/VD^R cells and parental MCF-7 cells. mRNA levels (left): samples of 4 μg of denatured poly(A)$^+$RNA from each cell line were separated on a 1.0% agarose gel, transferred onto a nylon membrane and the membrane was subsequently hybridised with a ^{32}P-labelled human VDR cDNA probe. As a control for differences in poly(A)$^+$RNA loading, the Northern blot was rehybridised with a human 36B4cDNA probe. Protein levels (right): samples of 1.25×10^5 cells were subjected to SDS-PAGE using 10% gels, transferred onto an Immobilon-PVDF Transfer Membrane and the protein bands were eventually stained with a mononuclear antibody against VDR.

$1\alpha,25(OH)_2D_3$, was investigated. Since the amount of $1\alpha,25(OH)_2D_3$ available in the circulation depends on the rate of production as well as the rate of breakdown, the activity of the 24-hydroxylase enzyme might be a determinant factor for the effect of $1\alpha,25(OH)_2D_3$. The data in Figure 8 show that the level of 24-hydroxylase mRNA is markedly increased in both the MCF-7/VD^R and the parental MCF-7 cell line after treatment of the cells with $1\alpha,25(OH)_2D_3$ (100 nM) for 24 h, suggesting an up-regulation of the activity of the enzyme. The effect, however, was most pronounced in the parental cells.

DISCUSSION

The human ER positive MCF-7 breast cancer cell line was chosen as a source to develop a vitamin D resistant cell line. A substantial number of studies investigating the effect of vitamin D on breast cancer cells have been carried out using the MCF-7 cell line [Eisman et al., 1979; Findley et al., 1980; Abe et al., 1991; Mørk Hansen et al., 1996; Mathiasen et al., 1999; Mørk Hansen et al., 2000a], and a vitamin D resistant subclone derived from this cell line might therefore serve as a valuable tool to gain more insight into the mechanisms under-

lying the actions of vitamin D on breast cancer cells.

The vitamin D resistant cell line, named MCF-7/VD^R, was established by repeatedly passaging the cells in medium containing increasing concentrations of $1\alpha,25(OH)_2D_3$. After that, the cells were grown and treated similarly to the parental cells, i.e., in medium without $1\alpha,25(OH)_2D_3$. Initial growth studies demonstrated that the MCF-7/VD^R cells were resistant to the well known growth-reducing effect of $1\alpha,25(OH)_2D_3$ and its more potent analogue EB 1089 [Formigli et al., 2000; Mørk Hansen et al., 2000a] for up to at least 25 passages. However, despite this altered characteristic, the growth rate of the MCF-7/VD^R cells appeared to be identical to that of the parental MCF-7 cells, i.e., ~24 h. Also, no major morphological differences between the two cell lines were observed.

Several recent investigations have shown that $1\alpha,25(OH)_2D_3$ and some of its synthetic analogues are able to regulate cell cycle progression by modulating the expression of important cell cycle regulators [Mørk Hansen et al., 2000a; van den Bemd et al., 2000]. Increased expression of the cyclin-dependent kinase inhibitors (CKIs) results in a decreased

A

B

Parental MCF-7 cells
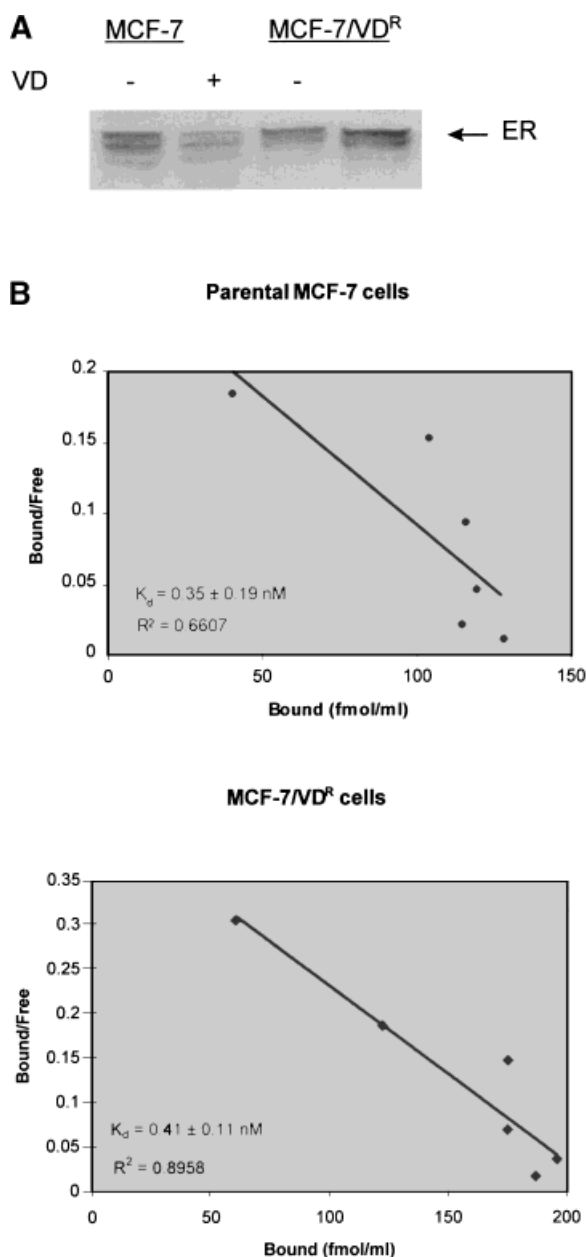
MCF-7/VD^R cells

Fig. 7. A: Western blot showing the level of ER (66 kDa) after treatment of the MCF-7/VD^R cells and the parental MCF-7 cells and the MCF-7/VD^R cells with 1α,25(OH)$_2$D$_3$ for five days. **B:** Scatchard analysis of the binding of [^{3}H]-estradiol to cell lysates from MCF-7/VD^R cells and parental MCF-7 cells. The receptor concentration was found to be 89.5±9.7 fmol/mg cytosol protein for the MCF-7/VD^R cells and 80.4±5.8 fmol/mg cytosol protein for the parental MCF-7 cells. K$_d$ values were calculated to be 0.41±0.11 nM and 0.35±0.19 nM for the MCF-7/VD^R cells and the parental MCF-7 cells, respectively.

activity of the cyclin-dependent kinases (Cdks), which are strongly implicated in the phosphorylation of the retinoblastoma protein (pRb). Under normal conditions, pRb is maintained

TABLE I. Induction of VDR-Mediated Gene Transcription From the Human Osteocalcin Gene (DR3 + DR6 VDRE) After Incubation of the Cells in the Presence of Increasing Concentrations of the Test Compounds for 48 h

Compound	MCR-7/VD^R (EC$_{50}$) (nM)	MCR-7 parental (EC$_{50}$) (nM)
1α,25(OH)$_2$D$_3$	21	20
EB 1089	2	5

The CAT activity was normalised in proportion to β-galactosidase activity and the molar concentration resulting in 50% stimulation (EC$_{50}$) of gene transcription compared to untreated cells was calculated from a dose-response curve.

in an hypo-phosphorylated state through most of G$_1$, but undergoes hyper-phosphorylation in late G$_1$, subsequently resulting in release of transcription factors and entry of the cells into the S-phase. Consequently, an up-regulation of CKIs such as p21 and p27 will lead to a block of cell cycle progression [Sclafani and Schauer, 1996; Shapiro and Harper, 1999]. In accordance with previous investigations [Fan and Yu, 1995; Mørk Hansen et al., 1997b; Wu et al., 1997; Bratland et al., 2000; Verlinden et al., 2000], we found a marked up-regulation of p21 and an enhanced expression of hypophosphorylated pRb, while no changes in p27 expression were observed in the parental MCF-7 cells in response to treatment of the cells with vitamin D. The amount of p53 protein, another important cell cycle regulator, appeared to be decreased rather than increased, as has been described in some reports on MCF-7 cells [James et al., 1996; Colston et al., 1997]. However, our results are in agreement with those of Narvaez and Welsh
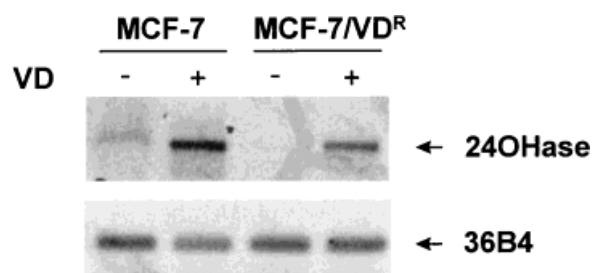
Fig. 8. Induction of 24-hydroxylase mRNA (24OHase) was analysed by RT-PCR. The cells were treated with 100 nM 1α,25(OH)$_2$D$_3$ (VD) or vehicle for 24 h followed by RNA extraction and RT-PCR amplification of the 24-hydroxylase. The RT-PCR amplification of acidic ribosomal phosphoprotein PO (36B4) was used as control for the differences in the amount of RNA included in the reaction.

[1997], who showed a down-regulation of p53 by $1\alpha,25(OH)_2D_3$ in MCF-7 cells and with results obtained by Fan and Yu [1995] in the CAMA-IEe breast cancer cell line. Moreover, we and others have previously shown that vitamin D compounds are capable of inducing block of cell cycle progression in MCF-7 cells as well as in other cancer cell lines without the involvement of p53 [Colston et al., 1997; Madsen et al., 1997; Mørk Hansen et al., 1997b; Matsumoto et al., 1998; Sundaram and Gewirtz, 1999].

In contrast to the results obtained with the parental MCF-7 cell line, no differences in p21, p27, p53, and pRb expression between untreated and treated cells were seen with the MCF-7/VD^R cells, further supporting the assertion that the MCF-7/VD^R cells are resistant to the growth inhibitory effect of vitamin D. Interestingly, the basal level of p21, p27, p53, and pRb appeared to be increased in the MCF-7/VD^R cells compared to the parental MCF-7 cells. Since the cell extracts used for Western blot analysis were adjusted to cell number and not protein concentration, we first speculated whether this observation was due to a generally increased cell size of the MCF-7/VD^R cells. However, distribution curves obtained from cell counting experiments using a Coulter Counter as well as microscopical examination of the cells proved that this explanation was unlikely to account for the phenomenon.

In some cell types, including MCF-7 cells, induction of apoptosis has been shown to contribute to the growth-reducing effect of vitamin D compounds [Welsh et al., 1995; James et al., 1996; Simboli-Campbell et al., 1996; Danielsson et al., 1997; Mathiasen et al., 1999; Pirianov et al., 1999; Xie et al., 1999]. In the present study, the level of DNA fragmentation in the cell cytoplasm was used as a parameter for induction of apoptosis. As expected, treatment of the parental MCF-7 cells with $1\alpha,25(OH)_2D_3$ or EB 1089 was found to result in a significant, time-dependent induction of DNA fragmentation accompanied by an concomitant decrease in cell number. Also, the two reference compounds, TNFα and CAM, were shown to significantly enhance the level of DNA fragmentation in the parental MCF-7 cells. In contrast, only marginal effects on DNA fragmentation were observed with the two vitamin D compounds in the MCF-7/VD^R cells. Likewise, almost no reduction in cell number was observed, which supports the results obtained in the initial growth studies. However, treatment of the MCF-7/VD^R cells with TNFα and CAM caused an increase in DNA fragmentation, clearly demonstrating that the MCF-7/VD^R cells have retained the ability to undergo apoptosis in response to other apoptosis-inducing compounds than vitamin D compounds.

Since morphological changes are generally accepted as a more reliable proof of apoptosis than the biological characteristics, induction of apoptosis was also investigated using a recently described immunofluorescence based microscopy technique [Mørk Hansen et al., 2000b]. Translocation of the phosphatidylserines from the inner to the outer leaflet of the plasma membrane is among the earliest detectable characteristics in the process of apoptosis [Gorczyca, 1999; Hall, 1999; Krahling et al., 1999]. Using a FITC-conjugated Annexin-V, a calcium-dependent anticoagulant protein that binds with high affinity to phosphatidylserine, early apoptotic cells were identified by green fluorescence [Cotter and Martin, 1996; Gorczyca, 1999; Hall, 1999]. In addition, the cells were stained with a mixture of Hoechst dye and PI. Since Hoechst is a membrane permeable interactive DNA dye, the nuclei of cells with an intact plasma membrane will fluoresce blue under ultraviolet excitation. PI also binds to the DNA, but in contrast to the Hoechst dye, this fluorochrome is charged and therefore excluded from cells that have preserved their plasma membrane integrity. Consequently, when the plasma membrane becomes leaky, PI starts entering the cells and slowly overwhelms the blue nuclear stain, giving rise to brightly red coloured cells [Cotter and Martin, 1996].

In agreement with the results obtained with the Cell Death Detection ELISA Kit, the microscopic examination of the cells clearly demonstrated that the MCF-7/VD^R cell line is resistant to the apoptosis-inducing effect of vitamin D, while apoptotic features could still be induced by treatment of the cells with other apoptosis-inducing compounds such as TNFα.

Thus, despite being resistant to the apoptosis-inducing effect of vitamin D, the ability to undergo apoptosis is retained by the MCF-7/VD^R cells. This was further substantiated by experiments showing a significant induction of DNA fragmentation in parental MCF-7 cells as well as in MCF-7/VD^R in response to treatment of the cells with the anti-estrogen ICI 182,780. Interestingly, the MCF-7/VD^R cells appeared to be more sensitive to ICI 182,780 than the

parental MCF-7 cells both with regard to induction of DNA fragmentation and inhibition of DNA replication. This fact was not due to modulation of the ER expression or changes in the binding affinity of the receptors in the MCF-7/VD^R cells compared to the parental MCF-7 cells, as both cell lines were shown to contain an equal amount of ER with similar K_d-values. However, it is well known that vitamin D compounds interfere with the ER signalling pathway in breast cancer cells, including MCF-7 cells [James et al., 1994; Simboli-Campbell and Welsh, 1994; Love-Schimenti et al., 1996], and VDREs have been identified within both the ER promoter and in the CYP19 gene (codes for the aromatase P450 enzyme that catalyses the biosynthesis of estrogens) [Sun et al., 1998; Stoica et al., 1999]. It is therefore obvious to assume that the increased sensitivity to ICI 182,780 of the MCF-7/VD^R cells may be due to the long-term exposure of the cells to $1\alpha,25(OH)_2D_3$ during the development of the cell line.

Tamoxifen, on the other hand, did not induce DNA fragmentation in either the MCF-7/VD^R cells or the parental MCF-7 cells. Conflicting reports have emerged on the ability of Tamoxifen and some of its analogues to induce apoptosis in breast cancer cells. Some showed induction of apoptotic features, while others, in agreement with the present results, failed to demonstrate DNA fragmentation [Bardon et al., 1987; Wakeling et al., 1988; Wilson et al., 1995]. This might be explained by the fact that unlike ICI 182,780, Tamoxifen is not a pure anti-estrogen and consequently does not provide a complete ablation of estrogen actions [Sutherland et al., 1983; Wakeling et al., 1991].

Detailed receptor studies on the VDR, including Western blot analysis, Northern blot analysis and assessment of VDRE-mediated transcription from both an exogenous (human osteocalcin gene) and an endogenous (human 24-hydroxylase) gene, clearly demonstrated that the MCF-7/VD^R cell line contains fully functional VDRs. However, the amount of receptors at both the mRNA and the protein level appeared to be lower in the MCF-7/VD^R cell line than in the parental MCF-7 cell line. This correlates with the slightly lower up-regulation of 24-hydroxylase mRNA in response to vitamin D treatment in the MCF-7/VD^R cells compared to the parental MCF-7 cells. Thus, a decreased number of functional VDRs in the

MCF-7/VD^R cells may in part account for the reduced sensitivity of these cells to vitamin D. However, these results also demonstrate that the resistance of the MCF-7/VD^R cells to the growth-reducing effect of $1\alpha,25(OH)_2D_3$ and EB 1089 is unlikely to be due to an enhanced stimulation of the 24-hydroxylase enzyme, which is involved in the catabolism of $1\alpha,25(OH)_2D_3$.

Most of the present results are in agreement with those of Welsh and colleagues, who have previously reported on a vitamin D resistant MCF-7 cell line, the MCF-7^{D3Res} cell line. [Narvaez et al., 1996; Narvaez and Welsh, 1997; Nolan et al., 1998]. However, in contrast to our MCF-7/VD^R cells, the MCF-7^{D3Res} cells were found to have a shorter doubling time and to express comparable or even higher levels of both VDR and ER. In addition, the MCF-7^{D3Res} cells seemed to display a sensitivity to anti-estrogens similar to that of the parental MCF-7 cell line, whereas our MCF-7/VD^R cell line appeared to be by far more sensitive to the anti-estrogen ICI 182,780. Thus, despite sharing the same overall defect, i.e., resistance to vitamin D, the MCF-7/VD^R and the MCF-7^{D3Res} cell lines are not fully identical, which makes these two cell lines useful as model systems for studying the development of vitamin D resistance in more detail. The MCF-7/VD^R cell line was slowly developed by growing the parental MCF-7 cells in the presence of increasing concentrations of $1\alpha,25(OH)_2D_3$. When resistance was obtained, the cells were propagated without the addition of $1\alpha,25(OH)_2D_3$. The MCF-7^{D3Res} cell line, on the other hand, was developed by selecting cells which were capable of surviving a 4 days incubation with 100 nM $1\alpha,25(OH)_2D_3$, and the cells were subsequently cultured in the presence of 100 nM $1\alpha,25(OH)_2D_3$ for one year. It is therefore likely to assume that the different characteristics of the two vitamin D resistant cell lines are due to the different methods by which these two cell lines were developed.

Similarly, to our MCF-7/VD^R cell line, the MCF-7^{D3Res} cells were shown to be resistant to the antiproliferative and the apoptosis-inducing effects of vitamin D compounds. Also, the MCF-7^{D3Res} cell line appeared to be sensitive to the growth reducing effects of other compounds such as anti-estrogens and phorbol esters, which argues against a complete abrogation of cell cycle control in vitamin D resistant cells. In

fact, TPA was found to be able to sensitise the MCF-7^{D3Res} cells to the growth reducing effect of 1α,25(OH)$_2$D$_3$ [Narvaez et al., 1996], indicating that even the vitamin D mediated growth regulation in vitamin D resistant cell clones is only partially blocked. This, together with the fact that functional VDRs are present in both the MCF-7/VD^R and the MCF-7^{D3Res} cell line, suggests involvement of a defect downstream of VDR complex formation as the main reason for the development of vitamin D resistance. This hypothesis is further supported by results obtained with vitamin D resistant human HL-60 leukemia cell variants, which showed that the cells possessed high levels of functional VDRs and that the initial steps in the vitamin D signalling pathway were intact [Studzinski et al., 1997].

In conclusion, the presently described MCF-7/VD^R cell line represents a stable subclone of human MCF-7 breast cancer cells which is resistant to the growth inhibitory and apoptosis inducing effects of vitamin D in passages for up to at least 25 passages. Despite these characteristics, the MCF-7/VD^R cells appear to contain functional VDRs and to be able to regulate 24-hydroxylase activity. The MCF-7/VD^R cell line, together with other vitamin D resistant cell lines, provides a valuable tool for identifying the basic mechanisms underlying the actions of vitamin D. Moreover, it might be useful for studying the development of drug resistance, which is a well know clinical problem with most of other available anticancer agents.

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