Analysis of 25-Hydroxyvitamin D_3-1α-Hydroxylase in Normal and Malignant Breast Tissue

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Abstract. *Background: The presence of extra-renal 25-hydroxyvitamin D_3 [25(OH)D_3]-1α-hydroxylase (1α-OHase) has been reported in several cell types including prostate and colon cancer cells. Additionally, alterations in the local production of 1α,25-dihydroxyvitamin D [1α,25(OH)$_2D_3$] have been implicated in the tumorigenesis of these malignancies. The aim of this study was to analyze whether normal breast tissue or breast cancer cells expressed 1α-OHase and to evaluate whether breast tissue possessed the capacity to produce 1α,25(OH)$_2D_3$ from 25(OH)D_3. Materials and Methods: Total RNA was extracted from normal breast tissue (n=11), breast carcinomas (n=12) and cultured MCF-7 breast cancer cells for real-time (LightCycler using specific hybridization probes) and conventional PCR analysis. Results: mRNA for 1α-OHase was detected in breast cancer tissue and in MCF-7 breast cancer cells. Interestingly, the mRNA levels for 1α-OHase were significantly increased in breast cancer compared to normal breast tissue. When the MCF-7 cells were treated with 1α,25(OH)$_2D_3$, cell proliferation was inhibited in a dose-dependent manner. Incubation of the MCF-7 cells with [^{3}H]-25(OH)D_3 resulted in its conversion to [^{3}H]-1,25(OH)$_2D_3$. The 1α-OHase activity in the MCF-7 cells was blocked by a specific cytochrome P450 inhibitor, clotrimazole. Conclusion: The data suggest that at least breast cancer cells expressed 1α-OHase mRNA and, therefore, might have the ability to synthesize 1α,25(OH)$_2D_3$ within the cells. The local production of 1α,25(OH)$_2D_3$ might play an important role in regulating the proliferation and differentiation of breast cells. We hypothesize that alterations in the local production of 1α,25(OH)$_2D_3$ may be involved in the tumorigenesis of breast cancer. Additionally, breast cancer may be a target for treatment with precursors of biologically-active vitamin D analogs.*

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In vitro studies have demonstrated that 1α,25(OH)$_2D_3$, the hormonally-active metabolite of vitamin D_3, suppressed proliferation and induced differentiation in various cell types, including MCF-7 and T-47 breast cancer cells (1, 2, rev. in 3). It is generally accepted that the antiproliferative effect is the result of vitamin D receptor (VDR)-mediated action on the genome (4). In addition, studies with animal cancer models demonstrated that treatment with 1α,25(OH)$_2D_3$ or its analogs prolonged the survival of leukemic mice and suppressed the growth of breast tumors (rev. in 3). However, the therapeutic efficacy of systemically-applied vitamin D analogs for cancer treatment has not yet fulfilled its promise. One main reason for these disappointing results is that the use of systemically-applied vitamin D analogs is limited by severe side-effects, mostly hypercalcemia, at the supra-physiological doses needed to be clinically effective (rev. in 3). Thus, new strategies, possibly enabling the use of vitamin D analogs that exert selective (growth-inhibitory activity combined with a reduced effect on calcium and bone metabolism) or tissue-specific activity, are currently being employed. Several of these new concepts are based on recent laboratory results demonstrating that various cell types have the capacity for tissue-specific local production of calcitriol (5-8).

Two principal enzymes are involved in the formation of circulating 1α,25(OH)$_2D_3$ from vitamin D; the hepatic microsomal or mitochondrial vitamin D-25-hydroxylase (25-OHase) and the renal mitochondrial 25-hydroxyvitamin D_3- 1α-hydroxylase for vitamin D and 25(OH)D_3, respectively (9, 10). These hydroxylases belong to a class of proteins known

as the cytochrome P450 mixed-function mono-oxidases. Recently, extra-renal activity of 1α-hydroxylase (1α-OHase) for $25(OH)D_3$ has been reported in various cell types including macrophages, keratinocytes, prostate and colon cancer cells (5-8).

The aim of this study was to analyze the expression and enzyme activity of 1α-OHase in MCF-7 breast cancer cells and the expression of the enzyme in normal and breast cancer tissues to evaluate whether breast tissue possesses the capacity to produce $1\alpha,25(OH)_2D_3$ from $25(OH)D_3$, indicating that: (i) $1\alpha,25(OH)_2D_3$ may be a locally produced hormone that controls proliferation in breast tissue, (ii) alterations in the local production of $1\alpha,25(OH)_2D_3$ may be involved in the tumorigenesis of breast cancer and (iii) breast tissue represents a target for the therapeutic or preventive use of vitamin D precursors.

Materials and Methods

Breast specimens. Biopsies from normal breast tissue (n=11) were obtained from patients who had experienced reductive surgery for macromasty of the breast. Therefore, the biopsies consisted of fat as well as epithelial tissue. Histological examination by a certified pathologist confirmed that the biopsy samples were of normal breast tissue with no sign of malignancy. Biopsies from breast carcinomas (n=12) were obtained from patients who had undergone surgery for breast tumors. All the patients were postmenopausal. Biopsies were taken from macroscopically-visible tumor areas and from the centre of the tumor, to minimize the risk of obtaining biopsies with an excess of non-malignant material such as fibroblasts or fat. Analysis for immune cells was not performed. Histological examination, by a certified pathologist, confirmed the diagnosis and assured that the specimens were 100% tumor tissue. All the breast specimens were immediately embedded in OCT-Tissue-Tek II (Miles Laboratories, Naperville, IL, USA), snap-frozen in melting isopentane, pre-cooled in liquid nitrogen and stored at –80˚C. The histological types are shown in Table I.

Table I. *Histological types and values for 1α-OHase mRNA content in breast specimens.*

Specimen no.	1α-OHase/GAPDH	Histological type
Breast cancer		
1	0.0064	ductal carcinoma
2	196.1401	ductal carcinoma
3	0.7397	ductal carcinoma
4	298.8774	lobular carcinoma
5	8.9439	lobular carcinoma
6	0.5705	ductal carcinoma
7	57.2374	ductal carcinoma
8	15.4201	lobular carcinoma
9	$1.0x10^{-13}$	medullar carcinoma
10	62.5385	ductal carcinoma
11	0.2703	ductal carcinoma
12	$1.0x10^{-13}$	lobular carcinoma
median	4.8	
mean	53.4	
standard deviation	95.9	
Normal breast tissue		
1	0.052040516	normal tissue
2	0.000035472	normal tissue
3	0.000009621	normal tissue
4	0.000034028	normal tissue
5	0.000000001	normal tissue
6	$1.0x10^{-13}$	normal tissue
7	0.000000002	normal tissue
8	0.000000003	normal tissue
9	0.000000005	normal tissue
10	0.000000002	normal tissue
11	0.000000019	normal tissue
median	0.00000000535	
mean	0.004738	
standard deviation	0.01569	

Real-time reverse transcription polymerase chain reaction (RT-PCR) for analysis of 1α-OHase mRNA expression in breast cancer and normal breast tissue.

RNA extraction and cDNA synthesis: Total RNA was isolated using the TRI reagent (Fa. Sigma)/ chloroform method. One μl random primer (Random Hexamer Primer; concentration 0.5 μg/μl, Promega, Mannheim, Germany) was mixed in a sterile PCR reaction cup with a volume of 7 μl RNA and 17 μl of a mix containing 5 μl five-fold RT buffer, 2.5 μl dNTP4 (4 deoxyribonucleotides) mix 2.5mM, 0.5 μl MMLV-reverse transcriptase (Moloney Murine Leukemia Virus Reverse Transcriptase; 200 U/μl, Promega), 1 μl RNasin (Ribonuclease inhibitor 40U, Promega) and 8 μl RNase-free H_2O.

The RNA and primers were denaturated at 65˚C for 5 min and, after the addition of the mix, were incubated at 37˚C for 1 h before transcription was stopped by incubation at 65˚C for 10 min. The samples were stored at 4˚C until further processing.

Real-time reverse transcriptase polymerase chain reaction (LightCycler). The expression of 1α-OHase was analyzed using a real-time quantification method (LightCycler) according to the manufacturer's recommendations (Roche Diagnostics, Grenzach, Germany). The target gene was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard using specific hybridization probes (LCRed 640 and fluorescein] for 1α-OHase and GAPDH as a detection format. Purified PCR products were used as external standards (High Pure PCR Purification Kit, Roche Diagnostics). External standards were produced with a ten-fold serial dilution of a PCR product of the cDNA HaCaT cell line, which highly expresses the vitamin D receptor (VDR) and the enzymes of the vitamin D metabolism. The PCR sequence-specific primers used were as follows: Forward primer: 5'-TgTTTgCATTTgCTCAgA-3'. Reverse primer: 5'-CCgggAgAg CTCATACAg-3' (PCR product size 226 bp). For GAPDH the primers were: Forward primer: 5'-TTggTATCgTggAAggACTCA-3'. Reverse primer: 5'-TgTCATCA TATTTggCAggTTT-3' (PCR product size 269 bp). Two μl cDNA were added to 18 μl master mix containing (specific for primers) 9.6 μl/ 9.8 μl/ 10.4 μl H_2Odd, $MgCl_2$ (2.5 mM for 1α-OHase, 3 mM for GAPDH and 25-OHase, 4 mM for VDR and 25-hydroxyvitamin D_3-24-hydroxylase (24-OHase)), primer pairs 0.5 μM 2 μl and 4 μl for 1α-OHase, respectively, and hybridization probes 0.25 μM (LCRed 640 and fluorescein) 1 μl, respectively. The PCR protocol was: i) 95˚C for 10 min, ii) 95˚C for 10 sec (5 sec for 1α-OHase), iii) 60˚C

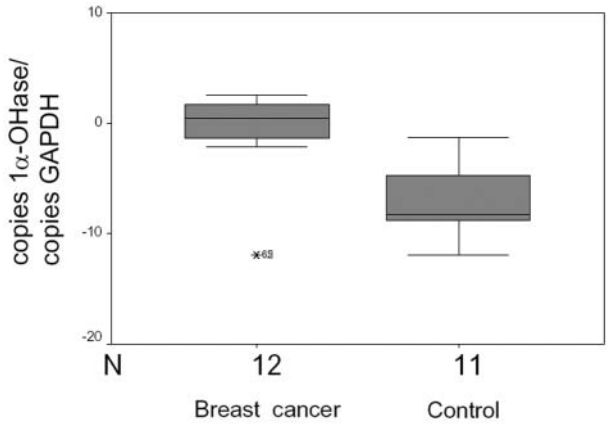

Figure 1. *Analysis of 1α-OHase mRNA expression in breast cancer and normal breast tissue using real-time PCR, as described.*

for 10 sec (55˚C for 8 sec for 1α-OHase), iv) 72˚C for 5 sec (12 sec for 1α-OHase) for 50 cycles followed by cooling at 40˚C for 30 sec. Quantification of the target gene expression was obtained by direct comparison with external standards amplified in parallel reactions in the same run. The target load in the unknown samples was quantified by measuring the C_t (threshold cycle) value and a standard curve to determine the starting target message quantity. The amplification efficiency of the target (1α-OHase) and housekeeping gene (GAPDH) did not differ >±0.05. Finally, the target gene/GAPDH ratio was calculated in order to normalize the data.

Statistical analysis. The non-parametric Wilcoxon-Mann-Whitney *U*-test was used to evaluate the target gene/GAPDH ratios in the breast tissue samples.

Proliferation analysis in MCF-7 cells. The cells were subcultured to 24-well plates for the proliferation assay studies. Pre-confluent MCF-7 cells were deprived of fetal bovine serum (FBS) for 24 h and subsequently treated with increasing concentrations of $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ (10^{-7} to 10^{-9} M) in the presence of epidermal growth factor (EGF) 25 ng/ml for 18 h, as published previously (12). Eighteen hours later, the dosing medium was replaced with 0.5 ml of fresh basal medium containing [methyl-^{3}H]thymidine and incubated for 3 h at 37˚C. ^{3}H-Thymidine incorporation into the DNA was stopped by placing the 24-well plates on ice. Unincorporated ^{3}H-thymidine was then removed and the cells washed three times with ice-cold phosphate-buffered saline (PBS). DNA, labeled with ^{3}H-thymidine and other macromolecules, was first precipitated with ice-cold 5% perchloric acid for 20 min and then extracted with 0.5 ml of 5% perchloric acid at 70˚C for 20 min, as described previously (13). The radioactivity in the extracts was determined by a liquid scintillation counter. The results were expressed as percent of radioactivity incorporated in the absence of $25(OH)D_3$ or $1\alpha,25(OH)_2D_3$ (control).

1α-Hydroxylase activity in MCF-7 breast adenocarcinoma cells. The 1α-OHase activity was determined in the monolayer primary cultures grown on 35-mm dishes. When the cells had reached 80% confluency, 50 nM $25(OH)D_3$ containing 0.1 μCi of ^{3}H-$25(OH)D_3$ was added to the cultures containing 1 ml of fresh medium without FBS. To prevent the free radical, non-enzymatic auto-oxidation, 10 μM N, N'-diphenyl-p-phenylenediamine (DPPD), an antioxidant, was also added during the incubation (13). After a 2 h-incubation at 37˚C, the cultures were placed on ice and the media were removed. Immediately, 1 ml of methanol was added to extract $25(OH)D_3$ and its metabolites, including $1\alpha,25(OH)_2D_3$ and $24,25(OH)_2D_3$. After extraction, the solvent was dried down under nitrogen gas and redissolved in methylene chloride/isopropanol (19:1) for HPLC analysis using an Econosphere silica column (5 μ particle size, 250 x 4.6 mm) with a flow rate of 0.5 ml/min. The mobile phase was methylene chloride/isopropanol (19:1). Thirty fractions were collected at 0.5-min intervals from each run. The fractions were air-dried, followed by the addition of scintillation fluid and counting with a beta counter. The retention times for $25(OH)D_3$, $24,25(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$ were calibrated by applying standard $25(OH)D_3$, $24,25(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$ to the HPLC before, during and after unknown sample application.

Table II. *Effect of $25(OH)D_3$ or $1,25(OH)_2D_3$ on the proliferation of MCF-7 breast adenocarcinoma cells. Note inhibition of growth that was not significant when the cells were treated with $25(OH)D_3$ at 10^{-9} to 10^{-7} M. However, there was a significant inhibition of cell growth when the MCF-7 cells were treated with $1\alpha,25(OH)_2D_3$ at 10^{-7} M and 10^{-8} M ($p<0.01$).*

Conc.	$25(OH)D_3$ % of control	$1,25(OH)_2D_3$ % of control
10^{-9}M	98±5%	97±6%
10^{-8}M	95±11%	89±5%
10^{-7}M	93±6%	77±3%

Reverse transcription-PCR in MCF-7 breast adenocarcinoma cells. MCF-7 and HaCaT cells were cultured to 80% confluency at which time the cells were washed once with 1X PBS, trypsinized for 10 min in 1X trypsin-EDTA, and spun for 10 min at 5000 rpm. Total RNA isolation was performed using the SV Total RNA Isolation System (Promega, Madison, WI, USA). Two μg of total RNA isolated from the treated cells were reverse transcribed into single-stranded cDNA at 37˚C for 2 h using Superscript II RNase H Reverse Transcriptase (Life Technologies, Inc., Rockville, MD, USA). Two hundred ng of single-stranded cDNA template was used in each PCR reaction. The following sequence specific primers were designed: 1α-OHase sense primer was 5'-GAA GGC GGT GGT CAA GGA A-3' and antisense primer was 5'-GGC AGG GTC CCT TGA AGT G-3'. The PCR protocol was: 95˚C for 10 min and then for 40 cycles, denaturation at 95˚C for 15 sec and annealling/extension at 60˚C for 1 min. One-fifth of the product was analyzed on a 4% agarose gel.

Statistical analysis. Comparisons of the antiproliferative activities of the control and treated groups were performed using one-way ANOVA. The differences between groups were considered statistically significant when the *p*-values were ≤0.05.

Results

Polymerase chain reaction (PCR) analysis of 1α-hydroxylase expression in normal breast tissue, breast cancer and MCF-7 cells. The mRNA of 1α-OHase for $25(OH)D_3$ was detected

in normal breast and in breast cancer samples (Figure 1, Table I). Real-time PCR analysis showed 1α-OHase gene expression in breast cancer (median: 4.842; S.D.±95.865; range from 10^{-13} to 298.88) that was significantly increased ($p<0.02$, Wilcoxon-Mann-Whitney *U*-test) as compared to normal breast tissue (median: 5.35^{-9}; S.D.±0.157; range from 10^{-13} to 0.05) (Figure 1). The lowest measurable expression was 10^{-13} copies. Using conventional PCR, mRNA for 1α-OHase was detected in the breast cancer cell line MCF-7. The HaCaT cell line served as a positive control for these experiments (Figure 2).

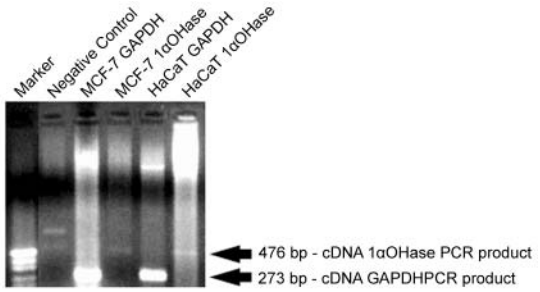

Figure 2. *RT-PCR analysis of 25-hydroxyvitamin D_3-1α-hydroxylase in MCF-7 breast adenocarcinoma cells.*

1α-Hydroxylase activity in MCF-7 breast adenocarcinoma cell. Figure 3 demonstrates typical HPLC chromatograms of the MCF-7 cells incubated with [^{3}H]-25(OH)D_3 in the presence and absence of clotrimazole. Incubation of MCF-7 cells with [^{3}H]-25(OH)D_3 resulted in the formation of [^{3}H]-1α,25(OH)$_2D_3$. Clotrimazole completely inhibited the synthesis of ^{3}H-1α,25(OH)$_2D_3$ (Figure 3).

Proliferation analysis in MCF-7 cells. Non-significant inhibition of growth resulted when the cells were treated with 25(OH)D_3 at 10^{-9} to 10^{-7} M (Table II). However, there was significant inhibition of cell growth when the MCF-7 cells were treated with 1α,25(OH)$_2D_3$ at 10^{-7} M and 10^{-8} M ($p<0.01$).

Discussion

Epidemiological studies have suggested that low levels of 25-(OH)D_3 represent an increased risk for the development of various malignancies including breast cancer (14). However, the production of 1,25(OH)$_2$D is tightly regulated by the kidney and, therefore, it remained unexplained how increases in the pro-hormone (25-(OH)D) in the circulation could result in inhibition of growth when the circulating concentrations of 1,25(OH)$_2D_3$ were unchanged. One possible explanation for the protective effect of increased 25-(OH)D from the diet and sunlight could lay in the ability of tissues to produce the active form of vitamin D (1,25(OH)$_2D_3$) for local control of growth.

The 25-(OH)D_3-1α-hydroxylase belongs to a class of proteins known as the cytochrome P450 mixed function monooxidases (9, 10). The cytochrome P450 enzymes are involved in the oxidative metabolism of steroids such as sex hormones, vitamin D, retinoids, fatty acids, prostaglandins, leukotrienes and biogenic amines (9, 10). Additionally, the cytochrome P450 enzymes have been shown to metabolize drugs, chemical carcinogens, mutagens and other environmental agents (9, 10). This dual functionality allows their characterization as having "mixed functions" (9, 10).

The expressions of mRNA for extra-renal 25-(OH)D_3-1α-hydroxylase in normal breast tissue, breast carcinomas and cultured MCF-7 breast adenocarcinoma cells were described. Additionally, 1α-OHase enzyme activity was shown in MCF-7 cells, which could be blocked *in vitro* by incubation of the cells with clotrimazole. In a recently published study by Segersten *et al.* (18) 25-(OH)D_3-1α-hydroxylase protein and mRNA was demonstrated in breast cancer as well as in benign breast tissue. However, this study group found less 25-(OH)D_3-1α-hydroxylase protein and mRNA expression in malign as compared to benign tissue (18), while our results showed an increased expression in breast cancer, which may be due to the heterogenity of breast cancer. As has been demonstrated in microarrays of triplet biopsies of primarily endocrine- or cytostatic- treated breast cancer patients, the gene expression patterns of the patients varied tremendously. It may be speculated that cellular synthesis of 1,25(OH)$_2D_3$ contributes (in an autocrine/paracrine fashion) to the defence strategy of breast cells against the multistep process of transforming events in carciongenesis.

Recently, the extra-renal activity of 25-hydroxyvitamin D_3-1α-hydroxylase was reported in various cell types including alveolar macrophages, placenta, keratinocytes and prostate, colon and lung carcinoma cells (5-8). It has been shown that the mRNA for extra-renal 25-hydroxyvitamin D_3-1α-hydroxylase in keratinocytes as well as in prostate, colon and lung cancer cells was of the same size as that previously characterized in the kidney, being the product of the same gene (6). This indicates that there is only one form and gene of the enzyme in the kidney and extra-renal tissues, including breast tissue.

These results strongly indicated that breast cells may not depend completely on a 1,25(OH)$_2D_3$ supply from systemic sources, having themselves a high intrinsic capacity to generate 1,25(OH)$_2D_3$ locally. Incubation of MCF-7 cells with [^{3}H]25(OH)D_3 yielded a metabolite identified as 1,25(OH)$_2D_3$ by its HPLC characteristics. This chromatographic system separated the 10-keto-25-hydroxyvitamin D_3 from 1,25(OH)$_2D_3$. Alterations in the local production of 1,25(OH)$_2D_3$ in breast tissue, *i.e.*, *via* a functional defect in 25(OH)D_3-1α-hydroxylase, may be

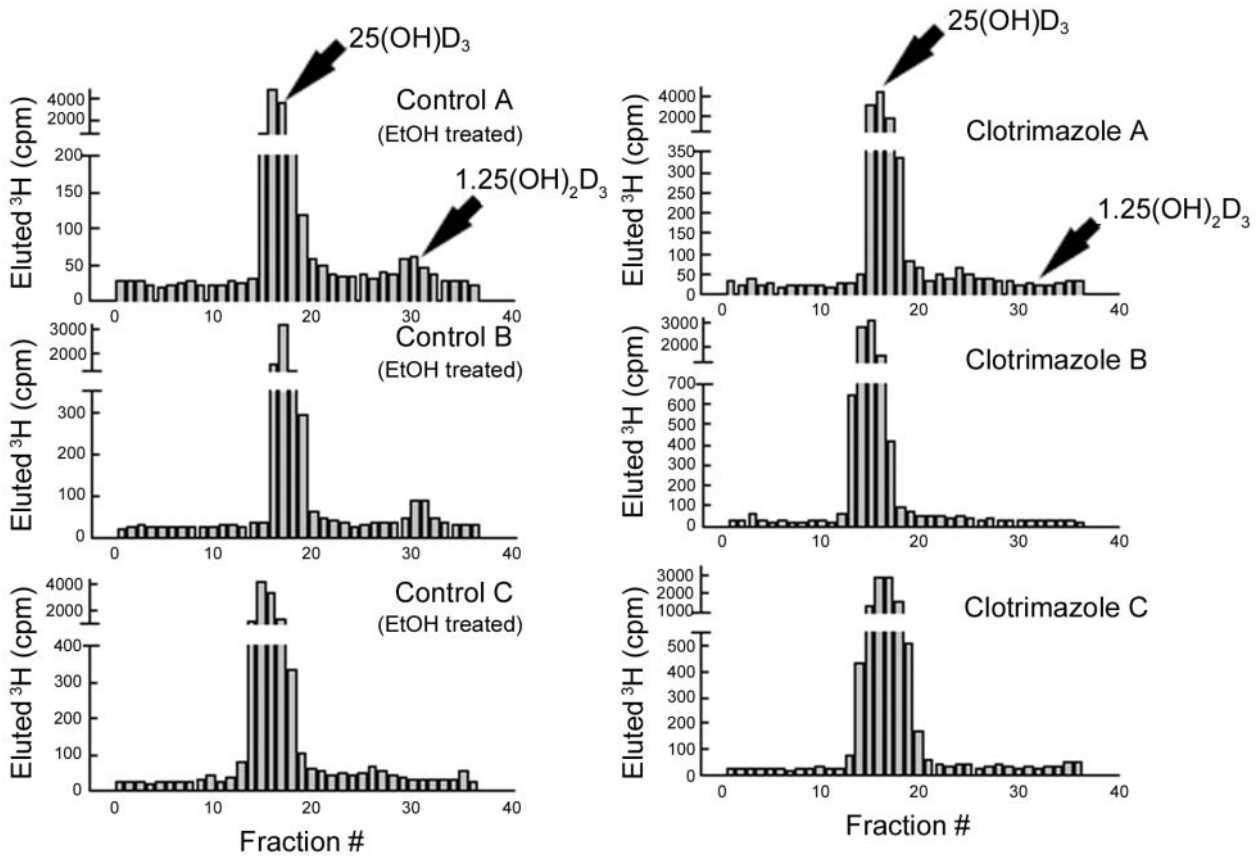

Figure 3. *Activity of 1α-OHase in MCF-7 breast adenocarcinoma cells. Incubation of MCF-7 cells with ^{3}H-25(OH)D_3 resulted in the formation of ^{3}H-1α,25(OH)$_2D_3$. Clotrimazole completely inhibited the synthesis of ^{3}H-1α,25(OH)$_2D_3$. In the figure, "control" stands for ethanol-treated cells.*

involved in the tumorigenesis of breast cancer. The proliferation of MCF-7 breast adenocarcinoma cells was markedly reduced by 1,25(OH)$_2D_3$ while the reduction of cell proliferation was observed when MCF-7 cells were treated with 10^{-7} to 10^{-8} M 25-hydroxyvitamin D3, though to a very low extent with 10^{-9} M. However, this effect was less pronounced as compared to 1,25(OH)$_2D_3$.

Another implication of this work is the role of extra-renal 25-(OH)-D_3-1α-hydroxylase in the pathogenesis of malignant hypercalcemia. Jones *et al.* (6) described, in their study on cell lines randomly obtained from tumor banks, that presumably reflect the incidence of 25-(OH)D_3-1α-hydroxylase expression in the general pool of such tumors, a surprisingly high incidence of 25-(OH)D_3-1α-hydroxylase expression compared with that observed by Mawer *et al.* (7). The authors implied that the extra-renal production of 1,25(OH)$_2D_3$ might be more common than currently believed, reflecting the technical difficulty that existed for proving the presence and activity of 25-(OH)D_3-1α-hydroxylase. However, it was shown that the levels of 1,25(OH)$_2D_3$ in the blood of breast cancer patients are usually low (17). Therefore, one cannot draw the conclusion that malignant hypercalcemia in breast cancer patients is caused by 25-(OH)D_3-1α-hydroxylase activity in breast cancer tumor cells.

New findings in molecular biology and cell physiology have substantially increased our understanding of the biological mechanisms of vitamin D analogs in the target tissues (rev. in 3). Besides studies to clarify the role of vitamin D analogs in the chemoprevention of cancer, current investigations aim to identify vitamin D analogs with selective anticancer activity that reveal no or few systemic side-effects (rev. in 3).

Thus, it can be concluded that breast cells have the enzymatic machinery to produce 1α,25(OH)$_2D_3$ which acts as an autocrine/paracrine growth inhibitor. Increasing local tissue levels of 1α,25(OH)$_2D_3$ by using vitamin D analogs or the manipulation of the 1α-OHase enzyme by gene therapy may represent novel approaches for the prevention and treatment of breast carcinomas.

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