ANTICANCER RESEARCH *26*: 2573-2580 (2006)

Vitamin D Status and Breast Cancer Risk

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Abstract. *Background: Local synthesis of 1α,25(OH)D_3 in breast tissue may contribute to maintenance of normal cell function and could be impaired with low circulating levels of the precursor 25hydroxyvitamin D. The aims of this study were to: i) assess the association between breast cancer risk and plasma 25OHD_3 concentration and ii) define the significance of expression of the 25OHD activating enzyme CYP27b1 in non-malignant and malignant models of breast epithelial cells. Materials and Methods: Breast cancer patients and control women were recruited and their 25OHD levels measured by enzyme-linked immunosorbent assay (ELISA). MRNA expression of CYP271b and the 1,25(OH)$_2D_3$ inactivating enzyme CYP24 were measured in breast cancer cell lines by RT-PCR and correlated with immunoblotting approaches to the translated proteins. Results: For women with 25OHD <50 nM the odds ratio for breast cancer compared with women with 25OHD >50 nM was 3.54 (CI 1.89-6.61, p<0.001). CYP271b and CYP24 were detected in non-malignant and malignant cell models. Protein levels of 24OHase but not 1αOHase were decreased at confluence in the cell lines. Conclusion: Impaired local generation of 1,25OHD_3 may contribute to the development of breast cancer.*

There are approximately 1 million new cases of breast cancer diagnosed in the world each year. Until recently, it was the leading cause of cancer deaths in women and still comprises 18% of all cancers in women. It has been estimated that at least 1 in 10 women in the UK will develop breast cancer at some time in their lives and there are more than 14,000 breast cancer deaths in the UK annually.

The underlying causes for the development of breast cancer are still not understood, but many of the major risk factors are unavoidable *e.g.*, being a woman, having a strong family history of breast cancer and increasing age (12). Other risk factors include aspects of reproductive history such as age at menarche, menopause and first live birth, use of the contraceptive pill and hormone replacement therapy. A strong family history of breast cancer is associated with a significant increase in a woman's risk of developing the disease. However, familial breast cancers, linked to high penetrant genetic mutations, account for only about 5% of cases (3, 4). The vast majority of cases are sporadic and the underling causes are poorly understood.

The relative importance of environmental, life-style and dietary factors to risk of breast cancer is still a matter of debate. Dietary factors associated with risk reduction for breast cancer include a high ratio of vegetable derived to total energy intake, a high fish intake (28), as well as a high dietary vitamin D intake (40). Another observed risk factor is latitude as it relates to UVB irradiation. Garland and associates (24, 25) reported that the risk of fatal breast cancer in major urban areas of the United States appears to be inversely proportional to the intensity of sunlight and that synthesis of vitamin D from sunlight exposure may be associated with low risk of fatal breast cancer.

Experimental studies have provided evidence for a link between vitamin D and protection against breast cancer. In addition to its well-established role in maintenance of calcium homeostasis, the active form of the hormone vitamin D, 1α,25 dihydroxyvitamin D (1α,25(OH)$_2D_3$), is known to have potent antiproliferative effects in many cancer cell types, including breast (reviewed in (13, 14). Both normal and neoplastic breast tissues express the nuclear vitamin D receptor (VDR) through which 1α,25(OH)$_2D_3$ exerts both its anticancer effects and its effects on calcium homeostasis. Gene ablation studies have implicated the VDR in normal breast development (46, 53, 57). Furthermore, a number of polymorphisms have been identified in the gene encoding the VDR, some of which have been linked to risk of certain cancers including breast (8, 26, 30, 33).

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Key Words: Vitamin D, *CYP27b1, CYP24,* 1α,25 dihydroxyvitamin D_3, breast cancer.

Evidence suggests that any effects of dietary/environmental vitamin D restriction on risk of breast cancer are not directly related to changes in the circulating levels of $1\alpha,25(OH)_2D_3$. The most direct correlate of vitamin D status is the serum 25 hydroxyvitamin D_3 ($25OHD_3$) level, the major circulating metabolite of vitamin D. $1\alpha,25(OH)_2D_3$ is produced by hydroxylation of $25OHD_3$, a reaction catalyzed by the renal enzyme 25-hydroxyvitamin D-1α hydroxylase (1αOHase, encoded by the *CYP27b1*). The activity of the renal enzyme is regulated such that circulating concentrations of $1\alpha,25(OH)_2D_3$ are maintained within a relatively narrow range in the face of substantial changes in serum $25OHD_3$ levels (11).

Until recently mRNA expression of the $25OHD_3$ activating enzyme, *CYP27b1*, was thought to be restricted to the classical sites for endocrine synthesis of $1\alpha,25(OH)_2D_3$. However, the extra renal expression of *CYP27b1* in non-classical target tissues has raised the possibility of an autocrine/paracrine role for the local synthesis and signalling of $1\alpha,25(OH)_2D_3$ (7, 15, 17, 20, 22, 23, 55). Recent studies have indicated that breast cancer cells express *CYP27b1*, although its role in modulating local responses to vitamin D remains to be defined (51). Thus, $25OHD_3$ may enter into an intracellular VDR signalling axis that co-ordinates the local synthesis, metabolism and signal transduction of $1\alpha,25(OH)_2D_3$. The components of the axis have been shown to be dynamically regulated as the activity of 1αOHase is repressed by $1\alpha,25(OH)_2D_3$ and, correspondingly, *CYP24,* which encodes the catabolic $25(OH)D_3$-24-hydroxylase (24OHase) is positively regulated by $1\alpha,25(OH)_2D_3$. Thus, elevated levels of $1\alpha,25(OH)_2D_3$ appear to block its synthesis and induces its own inactivation (34, 47, 50) and, therefore, represents a classic endocrine axis.

There is a possibility that local synthesis of $1\alpha,25(OH)_2D_3$ in normal breast tissue may promote innate antiproliferative effects which, in turn, are repressed in cancer, possibly as a result of altered vitamin D status and specifically the circulating levels of 25OHD, the substrate for the 1αOHase enzyme. Therefore, we have hypothesised that the development of breast carcinoma is associated with both reduced local $1\alpha,25(OH)_2D_3$ availability, which is determined by an altered balance of *CYP27b1* and *CYP24* expression and activity, and VDR polymorphisms associated with disease progression.

To explore these possibilities, a series of studies were undertaken, with cell line models and primary tumour material, to assess the relationship between $25OHD_3$ levels and breast cancer risk in a UK population. Secondly, we aimed to further define the significance of the expression of 1αOHase in non-malignant and malignant breast epithelial cell models in order to develop a more comprehensive model with which to predict responsiveness towards $1\alpha,25(OH)_2D_3$.

Materials and Methods

Volunteers and measurement of circulating 25OHD. Ethical approval for this study was received from the St. George's Hospital Ethics Committee, UK. Breast cancer patients and control volunteers were recruited as part of a study investigating the *VDR* genotype and risk of breast cancer. Only Caucasian women were recruited. All gave written informed consent before providing a 10-ml blood sample and answering a brief questionnaire. The questionnaire was used to determine personal information such as hormone replacement therapy (HRT) use and menopausal status. In addition to this, volunteers were asked whether they were vegetarians or vegans. No detailed dietary information was requested.

One hundred and thirty-one control volunteers were recruited from the Duchess of Kent breast-screening clinic on hospital grounds after having a negative mammogram. Forty-eight control volunteers were recruited from the same clinic where they were being investigated for benign breast disease. The breast cancer patients (n=179) were recruited from the Combined Breast Clinic in St. George's Hospital. Breast cancer patients were matched to one control volunteer each. The main criteria for matching was the time of year the blood sample was taken, since vitamin D is derived from cutaneous synthesis in response to sunlight exposure. The year was divided into summer (April - September) and winter months (October - March). Each case was matched to a control subject where the sample was obtained in the same half of the year and also within 2 months of each other. The majority of patients were matched to a control that had the same menopausal status although 14 were not. The majority were also matched closely for age at sampling. The median difference in age at sampling was 2 years (range 0 - 12 years). None of the patients or control volunteers were vegetarians or vegans.

Aliquots of whole blood were taken for *VDR* genotyping as previously described (27) and the remaining blood was separated into plasma and buffy coat by centrifugation. The plasma was then used to measure the circulating 25OHD concentration by enzyme immunoassay (ImmunoDiagnostics Services, UK). This assay detects both $25OHD_3$ and $25OHD_2$.

Analyses of VDR BsmI polymorphism. Genomic DNA was extracted from whole blood samples using a GenElute blood genomic DNA kit (Sigma). The *Bsm*I genotype was determined by performing a PCR reaction followed by a restriction digest. PCR was carried out using a forward primer (5' CAACCAAGACTACAAGTACCGCGT CAGTGA 3') and a reverse primer (5' AACCAGCGGGAAGAG GTCAAGGG 3'). The PCR product was digested using the *Bsm*I enzyme (BioLabs). Restriction digest products were separated on a 1.5% agarose gel and visualised using ethidium bromide.

Expression of components of the vitamin D signalling axis in breast cancer cell lines. To address the contribution of the local generation and catabolism of $1\alpha,25(OH)_2D_3$, the expressions of *CYP27b1* and *CYP24* were determined in established breast cancer cell lines. The cell lines T-47D, ZR-75-1, MCF-7, MDA-MB-231 and MCF-12A were cultured as previously described (51). Total RNA was extracted from sub-confluent cultures using the GenElute RNA extraction system (Sigma, Poole, UK) according to the manufacturer's instructions. RNA was eluted in 30 μl of RNase-free water and stored at –70˚C. Quantitative RT-PCR analysis of mRNA expression for *CYP27b1*, *CYP24* and *VDR* was carried out as previously described (10).

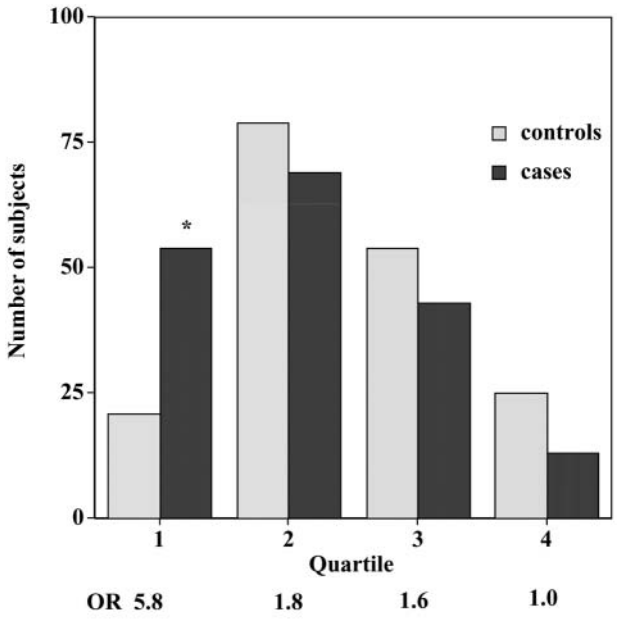

Figure 1. *Plasma 25OHD concentration (by quartile) and breast cancer risk. 25OHD values were separated into quartiles (quartile 1, <50 nM; quartile 2, 50-100 nM; quartile 3, 100-150 nM; quartile 4, 150-200 nM). The number of controls and cases in each 25OHD quartile and the odds ratio (OR) for breast cancer risk for each quartile are shown. (OR=5.8 (95% CI 2.31-14.7, p<0.001).*

Western immunoblot analysis. The cells were seeded at low and high density ($2x10^4$/ cm^2 and $8x10^4$/ cm^2, respectively) and cultured until the high-density cultures reached confluency (72 h), to give sub-confluent and confluent cultures, respectively, and whole cell lysates prepared for Western immunoblot analysis. Briefly, 30 μg of total protein for each sample was subjected to SDS-PAGE and transferred onto PVDF membrane (Immobilon-P; Millipore, Bedford, MA, USA) and blocked with TBS-T containing 5% milk powder for 1 h. $25OHD_3$-24- hydroxylase sheep polyclonal antibody was a generous gift from Prof. Rajiv Kumar (Mayo Proteomics Research Center, Mayo Clinic and Foundation, Rochester, MN, USA). For detection of $25OHD_3$-1α-hydroxylase or $25OHD_3$-24-hydroxylase, sheep polyclonal antibodies were diluted 1:200 and 1:500, respectively, followed by secondary antibody (anti-sheep-HRP) (The Binding Site, Birmingham, UK) (55). Proteins were detected using ECL (Amersham, Amersham, Bucks, UK) and autoradiography. To ensure even loading and transfer of protein, the membranes were subsequently probed with a 1/5000 dilution of primary mouse monoclonal β-actin antibody (AC-15, Sigma), followed by anti-mouse-HRP secondary antibody (Binding Site) and the signals developed with ECL and autoradiography as above. To quantify the relative changes in protein levels, densitometry analysis was performed on triplicate sets of lysate and the values normalised to β-actin levels.

Proliferation assays in the presence of 1α,25(OH)$_2$D$_3$ and 1α,24(R),25(OH)$_3$D$_3$. 1α,25$(OH)_2D_3$ was a kind gift from Dr. Milan R. Uskokovic, (Hoffman-La Roche, Nutley, NJ, USA). 1α,24(R),25$(OH)_3D_3$ was a generous gift from Prof. Satya G. Reddy (Brown University School of Medicine, Providence, USA). All compounds were stored at 1 mM in ethanol at –20˚C in the dark. The proliferation of the cell lines in the presence of vitamin D compounds was measured by colony formation in soft agar. Trypsinized and washed, single-cell suspensions were prepared from 80% confluent cultures, counted and plated onto 24-well flat-bottomed plates using a two-layer soft agar system with $1x10^3$ cells in 400 μl of media per well, as described previously (9). The support layer was prepared with agar (1%) equilibrated at 42˚C. Prior to the addition of this layer to the plate, the various treatments were pipetted into the wells. After 10 days of incubation, the colonies (>50 cells) were counted using an inverted microscope. Dose response curves were constructed and the ED_{50} values were interpolated.

Table I. *Odds ratios (OR) for breast cancer risk by BsmI genotype and 25OHD levels.*

Group	OR (95% CI)	*P*-value
quartile 1, all genotypes	3.54 (1.89 – 6.61)	<0.001
quartiles 2-4, all genotypes	1.00	
quartile 1 and *bb*	6.82 (2.57 – 17.1)	<0.001
quartile 1 and *Bb*/*BB*	4.01 (1.89 – 8.58)	<0.001
quartile 2-4 and *bb*	2.66 (1.48 – 4.79)	0.001
quartile 2-4 and *Bb*/BB	1.00	

CI, confidence interval.

Results

25hydroxyvitamin D levels in breast cancer patients and controls. The mean plasma 25OHD concentrations were significantly lower in breast cancer patients than matched controls (80.1 *vs.* 97.8 nM, $p<0.001$). Overall, for women with low or insufficient 25OHD levels (<50 nM), the odds ratio (OR) for breast cancer compared with those with levels >50 nM was 3.54 (95% CI 1.89-6.61, $p<0.001$, ref. 41). When the 25OHD values were separated into quartiles (quartile 1, <50 nM; quartile 2, 50-100 nM; quartile 3, 100-150 nM; quartile 4, 150-200 nM) analysis showed that, compared to women with the highest levels of 25OH-D (quartile 4), women with insufficient levels (quartile 1) had more than five times the risk of breast cancer (OR=5.83 (95% CI 2.31- 14.7, $p<0.001$, ref. 41). These data are summarised in Figure 1, which shows the distribution of plasma 25OHD levels between quartiles and the indicated ORs.

Of the 179 patients, 47 were newly diagnosed. It is therefore possible that altered sunlight exposure as a consequence of treatment could account for this difference. When statistical analysis was repeated on patients recruited at diagnosis and their matched controls, the OR for breast cancer risk for women with 25OHD levels <50 nM compared to those with levels >50 nM (3.67) was similar to

Table II. Expression and activity of components of the VDR signalling axis in breast cancer cell lines and primary material.

Cell type	ED_{50}[1]	ED_{50}[2]	*CYP27b1*[3]	1αOHase[4]	Met[5]	*VDR*[6]	VDR[7]	*CYP24*[9]	24OHase[10]	Met[11]
MCF-12a	5	Nd	1	Nd	Nd	1	Nd	1	Nd	Nd
T47-D	15	>1000	1.3	+++	Nd	0.87	Nd	2.2	++	Nd
MCF-7	70	>1000	0.51	+++	+	0.21	Nd	1.87	++	+
ZR-75-1	70	>1000	0.38	++	Nd	0.12	Nd	74	++	Nd
MCF-7Res	>1000	>1000	0.44	Nd	Nd	0.07	Down[8]	234.0	Nd	Nd
MDAMB-231	>1000	>1000	0.36	++	Nd	0.12	Nd	220.0	++	Nd

[1]ED_{50} is the dose of 1α,25$(OH)_2D_3$ required to inhibit colony formation by 50% (1, 5, 9, 10, 14, 18, 37, 45).
[2]ED_{50} is the dose of 1α,24(R),25$(OH)_3$-D_3 required to inhibit colony formation by 50%.
[3]Measurement of relative *CYP27b1* mRNA levels compared to MCF-12A cells (22, 51).
[4]Detection of 1αOHase protein by immunoblotting analyses in breast cancer cell lines (22, 51).
[5]Measurement of 1αOHase activity by examining conversion of radio-labelled 25(OH)D precursor (51).
[6]Measurerement of relative *VDR* mRNA levels compared to MCF-12A cells (32).
[7]Detection of VDR protein by immunoblotting analyses in breast cancer cell lines or by immunohistochemistry (IHC).
[8]Levels of VDR protein are down-regulated in resistant variants of MCF-7 compared to parental MCF-7 cells (21).
[9]Measurerement of relative *CYP24* mRNA levels compared to MCF-12A cells (22, 51).
[10]Detection of 24OHase protein by immunoblotting analyses in breast cancer cell lines.
[11]Measurement of 24OHase activity by examining conversion of radiolabelled 25(OH)D precursor (51).
Nd = Not determined, + = detected.

that for the group as a whole. However, this was of only borderline significance due to the small number of cases.

VDR polymorphisms and breast cancer risk. We have previously reported that the VDR gene *Bsm*I polymorphism (which in Caucasians is in linkage disequilibrium with the polyA repeat sequence) is associated with breast cancer risk in a UK population (11). Results from the present study are in agreement with our previous findings such that the odds ratio for breast cancer risk for women with the *bb* genotype was 2.02 (95% CI 1.03-3.97, $p=0.04$) compared with the *BB* genotype. Furthermore, we found that women with the *bb* genotype in combination with low/insufficient 25OHD levels had an even greater risk for breast cancer than low 25OHD levels alone. While women of all genotypes with 25OHD concentrations <50 nM had an OR for breast cancer risk of 3.54, the OR for women with 25OHD levels <50 and the *bb* genotype was 6.82 (95% CI 2.57-17.1, $p<0.001$, (41)). These data are summarised in Table I.

Expression of 1αOHase in cell lines. There is evidence that breast cells contain the 1αOHase enzyme required for the autocrine production of 1α,25$(OH)_2D_3$ from circulating 25OHD_3. Previously we detected *CYP27b1* mRNA in breast cancer cell lines and, using immunohistochemical approaches, confirmed readily detectable staining in primary tumours and, importantly, demonstrated enzyme activity (51) (Table II). Parallel studies by Dr. Heide Cross and co-workers found that 24OHase expression was dependent upon the proliferation status. Thus, in CaCo2 colon cancer cells the levels of 24OHase were reduced upon confluence, which was associated with differentiation (6). Together, these studies support the concept that the components of the VDR signalling axis are dynamically expressed and that these processes may be corrupted in cancer.

To compliment both of these studies, we examined 1αOHase and 24OHase in a panel of cancer cell lines either during exponential proliferation or at confluence. The 1αOHase appeared relatively constant in cells which were either actively cycling or undergoing confluence-induced G1 arrest (Figure 2A). Equally, the levels appeared to be modestly reduced in the cancer cell lines, compared to T47-D cells, which reflects our previous mRNA studies (51). By contrast, in the most rapidly proliferating cells, for example in MCF-7 and MDA-MB-231 cells, there was evidence that 24OHase was indeed down-regulated upon confluence (Figure 2B). The pattern reflected the expression of Cyclin E, as a marker of S-phase cells, and the percentage of cells out of G1 of the cell cycle. For example, sub-confluent growing populations of MDA-MB-231 cells were associated with approximately 2- fold and 3-fold higher expressions of Cyclin E and 24OHase levels, respectively, and 62% of cells in S- and G_2/M-phases of the cell cycle (Figure 2B). Similar levels of down-regulation were seen in MCF-7 cells. Thus, elevated 24OHase expression appeared to correlate well with cell proliferation.

1α,25$(OH)_2D_3$ and 1α,24(R),25(OH)3-D3 demonstrate a range of antiproliferative potencies against breast epithelial cells. The antiproliferative effects of 1α,25$(OH)_2D_3$ upon MCF-12A and a series of breast cancer cell lines were titrated across a broad concentration range (0.001 to 100 nM) in clonogenic assays to generate dose-response curves. An example for

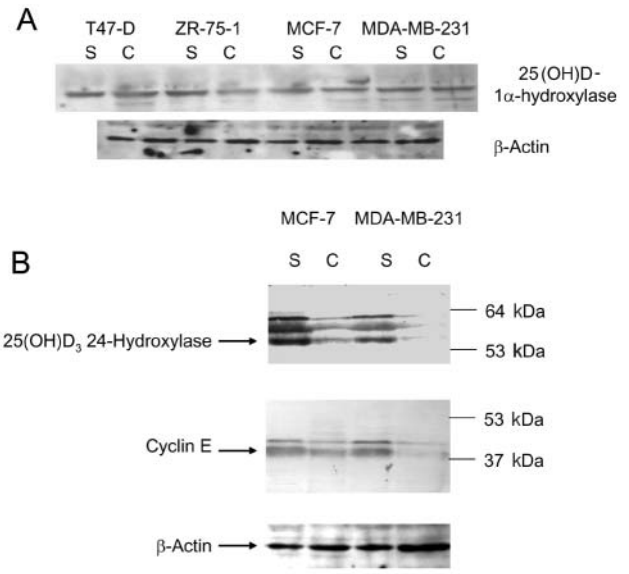

Figure 2. *Regulation of 25(OH)D_3-1α-hydroxylase and 25(OH)D_3-24-hydroxylase. Panel A. Protein was isolated from parallel sub-confluent (S) and confluent (C) cells (Materials and Methods) and resolved by SDS-PAGE and probed with antibodies to 25(OH)D_3-1α-hydroxylase, Panel B. Proteins from MDA-MB-231 cells were resolved and probed with antibody to 25(OH)D_3-24-hydroxylase or Cyclin E. Representative blots are shown with the position of the proteins indicated on the left. The blots were subsequently stripped and re-probed for β-actin.*

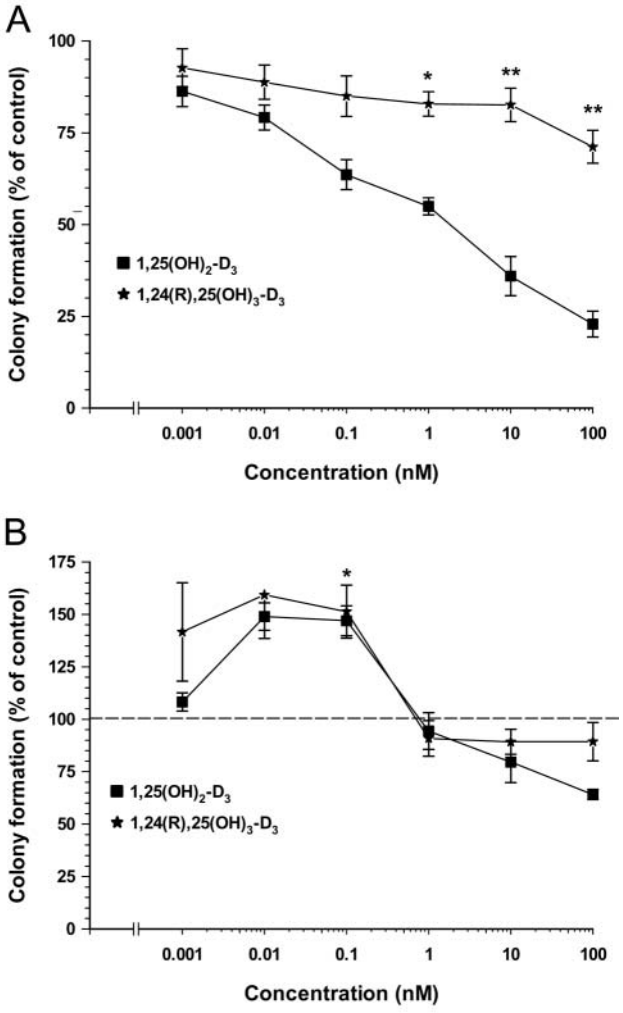

Figure 3. *Inhibition of breast cell proliferation by 1α25(OH)$_2D_3$ and 1α,24(R),25(OH)$_3$-D_3. Clonal growth of T-47D (Panel A) and MDA-MB-231 (Panel B) in the presence or absence of vitamin D_3 compounds was examined using a two-layer soft-agar colony assay (Materials and Methods). After 10 days, the colonies were enumerated and expressed as a percentage of colonies in untreated control plates. Each data-point represents the mean of three separate experiments undertaken in triplicate wells ± S.E.M. *$p<0.05$, **$p<0.001$.*

T-47D cells is shown in Figure 3A and the ED_{50} values are summarised in Table II. The responses of the cancer cell lines to 1α,25(OH)$_2$D$_3$ were in accordance with previous studies (9, 18, 31). In the current study, these responses were compared to those for MCF-12A [ED_{50} 5 nM], which represents an *in vitro* model of non-malignant breast epithelial cells. As shown in Table II, each of the cancer cell lines had higher ED_{50} values than MCF-12A. However, there remained considerable variation between the sensitivity of these malignant cells with ED_{50} values ranging from 15 nM in the case of T-47D cells to >1000 nM in MDA-MB-231 cells (Figure 3B and Table II).

In contrast to the range of antiproliferative responses to 1α,25(OH)$_2$D$_3$, there was a uniform absence of antiproliferative response to 1α,24(R),25(OH)$_3$-D$_3$, its 24OHase-generated metabolite product (Figure 3 and data not shown). Thus, the addition of an OH group to C 24 of 1α,25(OH)$_2$D$_3$ appears to ablate the antiproliferative potency of the parent compound. Interestingly, in MDA-MB-231 cells, a range of proproliferative responses were observed only at low concentrations of 1α,25(OH)$_2$D$_3$ and 1α,24(R),25(OH)$_3$-D$_3$ (Figure 3B). Thus colony growth was stimulated significantly at concentrations of 0.1 nM 1α,25(OH)$_2$D$_3$ and 1α,24(R),25(OH)$_3$-D$_3$ by 152±12% (±SEM) and 146±7% ($p<0.05$) relative to respective control cultures.

Discussion

The central hypothesis of the current study is that the proliferation of breast epithelial cells is regulated by the local synthesis, signalling and metabolism of 1α,25(OH)$_2$D$_3$. The initiation and/or progression of breast cancer involves suppressed VDR signalling, which can occur by either dysregulation of the balance of synthesis and metabolism of 1α,25(OH)$_2$D$_3$ and/or specific polymorphisms of the VDR.

In vivo data now support a physiological role for VDR-mediated signalling to promote differentiation of normal breast epithelial cells. Prodifferentiating hormones such as

cortisol, prolactin and insulin up-regulate the VDR, which is initially present at low levels, in mammary gland explants. The up-regulation of the VDR is essential for gland differentiation and function as there is reduced ductal differentiation and development in the mammary gland of VDR knockout mice compared to wild-type litter-mates (46, 56, 58). Breast cancer cell lines display a spectrum of sensitivities, including complete insensitivity towards $1\alpha,25(OH)_2D_3$, suggesting these functions are targeted in malignancy. The mechanisms underpinning the basis of this apparent hormonal insensitivity are unclear, but it seems likely that their resolution will facilitate the clinical application of $1\alpha,25(OH)_2D_3$ as an anticancer agent.

Vitamin D is derived both from dietary sources and cutaneous synthesis in response to UV irradiation. Circulating concentrations of 25OHD serve as a useful index of vitamin D status and in vitamin D sufficiency serum concentrations range from 50-200 nM (14). Although several studies have documented an association between dietary/environmental intake of vitamin D and risk of breast cancer, the precise relationship between serum 25OHD levels and cancer risk has not been documented in a large cohort. Our present study has shown a significant association of low 25OHD levels with breast cancer risk such that women with the lowest levels of 25OH D (<50 nM) had over five times the risk of breast cancer compared with those with the highest levels (>150 nM). It was also found that the risk associated with low 25OHD levels was increased in combination with the 'at risk' VDR polymorphism such that women with the *bb* genotype in combination with low/insufficient 25OHD levels had an even greater risk of breast cancer than those with low 25OHD levels alone. Low vitamin D status and VDR polymorphisms have also been associated with the risk of prostate cancer (42).

The observed expression of 1αOHase in normal breast tissue suggests that paracrine production of $1\alpha,25(OH)_2D_3$ could be important in maintenance of normal breast cell function (51). Thus, it could be postulated that low circulating concentrations of 25OHD, arising either as a result of reduced exposure to sunlight or to dietary patterns, impair the generation of $1\alpha,25(OH)_2D_3$ within breast tissue, increasing the risk of tumour development. Elevated *CYP24* (2, 51) may lead to overexpression of 24OHase which, in turn, attenuates the capacity for $1\alpha,25(OH)_2D_3$ to activate antiproliferative *VDR* target genes. Certainly the current data fit with a model of autocrine production of vitamin D compounds, the antiproliferative actions of which are determined in part by precursor availability and perhaps other dietary and environmental factors, as has been proposed for the colon (16).

These results of the current study, and our recent parallel ones (26, 27, 51) together indicated that $1\alpha,25(OH)_2D_3$ may be generated locally in breast tissue and may play a role in the maintenance of normal breast cell function. The capacity to generate $1\alpha,25(OH)_2D_3$ locally in the breast will be dependent on the availability of 25OHD as substrate for the 1αOHase enzyme. Little is known about the metabolism or half-life of $1\alpha,25(OH)_2D_3$ in normal breast or breast cancer cells. Catabolism of $1\alpha,25(OH)_2D_3$ is initiated *via* hydroxylation at the 24 position in the side-chain to produce $1\alpha,24(R),25(OH)_3\text{-}D_3$, a reaction catalyzed by 24OHase. The current study demonstrated that this compound has a different activity profile to the parental $1\alpha,25(OH)_2D_3$ and support further the significance of the concept of inappropriate inactivation of locally generated $1\alpha,25(OH)_2D_3$. This concept is supported further by a number of parallel studies. We targeted 24OHase *via* an antisense strategy in MDAMB- 231 cells, which have high basal *CYP24* levels (Table II) and sustained 24OHase expression in proliferating cells. These studies demonstrated a significant enhancement of the growth inhibitory effects of $1\alpha,25(OH)_2D_3$. Equally the $MCF\text{-}7_{Res}$ cells validate further the important contribution of elevated *CYP24* to predict $1\alpha,25(OH)_2D_3$ insensitivity as these cells demonstrate significantly elevated levels of *CYP24* expression relative to their parental MCF-7 cell line (132 fold, $p<0.001$) (Table II).

Expression and activity of 1αOHase and *CYP27b1* has been detected in various extrarenal tissues and is reduced in transformed cells (19, 29, 35, 36, 38, 39, 43, 44, 48, 49, 52, 54). The current study extends these findings to breast cancer cells and supports the concept that loss of *CYP27b1* expression, possibly resulting in local, tumour-specific $1\alpha,25(OH)_2D_3$-insensitivity, is associated with transformation. The enzymes involved in activation and catabolism of vitamin D in breast tissue may provide novel targets for prevention and treatment of breast cancer.

Acknowledgements

The authors gratefully acknowledge their stimulating discussions with Dr. Martin Hewison, Research Scientist, Division of Endocrinology, Diabetes and Metabolism, Room 3088 Davis Building, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA. We are grateful to Judith Bliss and Clare Peckitt, the Institute of Cancer Research UK for statistical analyses. This research was funded by the Breast Cancer Campaign (BCC), the Breast Cancer Research Trust (BCRT) and the World Cancer Research Fund (WCRF).

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Received December 29, 2005
Accepted January 14, 2005