

## Autocrine Metabolism of Vitamin D in Normal and Malignant Breast Tissue

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**Abstract Purpose:** Vitamin D seems to exert a protective effect against common cancers, although this does not correlate with circulating levels of active 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>], indicating a more localized activation of vitamin D. The aim of this study was to investigate the significance of this in breast cancer.

**Experimental Design:** Quantitative reverse transcription-PCR analysis of mRNA expression was carried out for the vitamin D – activating enzyme 1 $\alpha$ -hydroxylase, the catabolic enzyme 24-hydroxylase, and the vitamin D receptor in 41 tumors and paired nonneoplastic tissue as well as breast cancer cell lines. Immunohistochemistry was used to assess 1 $\alpha$ -hydroxylase protein expression, and enzyme assays were used to quantify vitamin D metabolism.

**Results:** Expression of mRNA for 1 $\alpha$ -hydroxylase (27-fold;  $P < 5 \times 10^{-11}$ ), vitamin D receptor (7-fold;  $P < 1.5 \times 10^{-8}$ ), and 24-hydroxylase (4-fold;  $P < 0.02$ ) was higher in breast tumors. 1 $\alpha$ -Hydroxylase enzyme activity was also higher in tumors ( $44.3 \pm 11.4$  versus  $12.4 \pm 4.8$  fmol/h/mg protein in nonneoplastic tissue;  $P < 0.05$ ). However, production of inactive 1,24,25-trihydroxyvitamin D<sub>3</sub> was also significantly higher in tumors ( $84.8 \pm 11.7$  versus  $33.6 \pm 8.5$  fmol/h/mg protein;  $P < 0.01$ ). Antisense inhibition of 24-hydroxylase *in vitro* increased antiproliferative responses to 1,25(OH)<sub>2</sub>D<sub>3</sub>.

**Conclusion:** These data indicate that the vitamin D – activating enzyme 1 $\alpha$ -hydroxylase is up-regulated in breast tumors. However, dysregulated expression of 24-hydroxylase seems to abrogate the effects of local 1,25(OH)<sub>2</sub>D<sub>3</sub> production in tumors by catalyzing catabolism to less active vitamin D metabolites. The enzymes involved in autocrine metabolism of vitamin D in breast tissue may therefore provide important targets for both the prevention and treatment of breast cancer.

Over the last 20 years, there has been increasing interest in the nonclassic actions of vitamin D and their potential impact on normal physiology and disease therapy. The active form of vitamin D, 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>], is a secosteroid intimately associated with the calcium homeostasis but which also has potent antiproliferative and immunomodulatory properties (1–3). This has led to the development of novel strategies for the clinical application of 1,25(OH)<sub>2</sub>D<sub>3</sub>, with cancer, psoriasis, autoimmune disease, and host-graft rejection being prominent therapeutic targets (4–7). However, the realization of these strategies *in vivo* has been compromised

by the potent hypercalcemic side effects of 1,25(OH)<sub>2</sub>D<sub>3</sub>, an issue that has been addressed with varying degrees of success by the generation of synthetic analogues of 1,25(OH)<sub>2</sub>D<sub>3</sub> with decreased calciotropic potential (8, 9). More recently, the complications associated with the therapeutic application of 1,25(OH)<sub>2</sub>D<sub>3</sub> have suggested an alternative role for non-calciotropic vitamin D. Specifically, in studies of common cancers and autoimmune disease, it has been proposed that vitamin D may be more important for disease prevention than disease treatment (10, 11).

The link between vitamin D and protection against breast cancer has been described at a variety of levels. For example, both normal and neoplastic breast tissues express the nuclear receptor for 1,25(OH)<sub>2</sub>D<sub>3</sub>, the vitamin D receptor (VDR; refs. 12–14). Gene ablation studies have implicated the VDR in normal breast development (15). Polymorphisms in the VDR gene have been proposed as risk markers for breast cancer (16). Other groups have assessed the impact of vitamin D intake and status on breast cancer risk. Exposure to sunlight, the principal mechanism involved in generating endogenous vitamin D, has been inversely correlated with breast cancer mortality (17–20), and reduced risk of breast cancer has been observed in women with high dietary intake of vitamin D (21, 22). As the active metabolite of vitamin D, the presumption is that the effects of dietary/environmental vitamin D restriction on breast cancer risk are due to changes in circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> levels

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Received 11/18/04; revised 1/17/05; accepted 1/26/05.

**Grant support:** Medical Research Council (United Kingdom) studentship (K. Townsend) and Breast Cancer Campaign (M. Guy, K.W. Colston, and J.L. Mansi). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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(23, 24). However, this does not seem to be the case, as serum concentrations of  $1,25(\text{OH})_2\text{D}_3$  are not specifically linked to vitamin D intake or status (25). Instead, the most direct correlate of vitamin D intake/status is the precursor metabolite 25-hydroxyvitamin  $\text{D}_3$  ( $25\text{OHD}_3$ ), the main circulating form of vitamin D (26). Although biologically inactive,  $25\text{OHD}_3$  is converted to active  $1,25(\text{OH})_2\text{D}_3$  by the enzyme 25-hydroxyvitamin  $\text{D}1\alpha$ -hydroxylase ( $1\alpha$ -hydroxylase) located classically in the proximal tubules of the kidney (27–29). In a series of recent studies, we and others have shown that  $1\alpha$ -hydroxylase is also expressed by a wide range of extrarenal tissues (28, 30), indicating that the nonclassic effects of vitamin D may be linked to tissue-specific expression of  $1\alpha$ -hydroxylase and autocrine/paracrine synthesis of  $1,25(\text{OH})_2\text{D}_3$ . Data suggest that extrarenal expression of  $1\alpha$ -hydroxylase is due to the same gene product as the proximal tubules, but it does not seem to be subject to same exquisite autoregulation characteristic of the renal enzyme (28, 30). Thus, tissue-specific expression of  $1\alpha$ -hydroxylase may act as the pivotal mechanism linking vitamin D status ( $25\text{OHD}_3$  levels) with the anticancer effects of  $1,25(\text{OH})_2\text{D}_3$ .

To investigate the possible role of  $1\alpha$ -hydroxylase in directing localized responses to vitamin D in breast cancer, we have assessed the expression of  $1\alpha$ -hydroxylase, VDR, and the vitamin D catabolic enzyme 24-hydroxylase in paired tumor and normal tissue from 41 women with breast cancer. Data indicate that each of these components of vitamin D metabolism and signaling are present in breast tissue with increased and dysregulated expression in tumors. Thus, local synthesis and action of  $1,25(\text{OH})_2\text{D}_3$  in breast tissue seems to be similar to that reported for other extrarenal tissues and may act as a key mechanism by which vitamin D exerts its protective effects against breast cancer.

## Materials and Methods

**RNA extraction from breast tissue.** Paired tumor and normal tissue were obtained from biopsies/resection specimens of Caucasian female patients who had undergone surgery for invasive ductal breast cancer at St. George's Hospital (London, United Kingdom). Age of diagnosis of primary tumor (range, 35–88 years) and estrogen receptor status were validated from histopathologic reports and patient medical records. The study received local ethical approval from St. George's Hospital Medical School Ethics Committee. Total RNA was extracted using the RNeasy Lipid Tissue Mini kit (Qiagen, West Sussex, United Kingdom). Briefly, a piece of breast tissue ( $\sim 2 \text{ mm}^3$ ) was excised from the relevant frozen surgical sample, which had been stored in liquid nitrogen. The tissue was placed directly into 1 mL lysis reagent and homogenized using a rotor-stator homogenizer (IKA-Werke, Staufen, Germany). RNA was then extracted according to the manufacturer's instructions, with one modification: before ethanol washes, DNA digestion was carried out using RQ1 RNase-free DNase (Promega, Hampshire, United Kingdom) by the addition of 10  $\mu\text{L}$  RQ1 DNase, 10  $\mu\text{L}$  of  $10\times$  RQ1 buffer, and 80  $\mu\text{L}$   $\text{H}_2\text{O}$  to each column followed by 15-minute incubation at room temperature. RNA was eluted in 30  $\mu\text{L}$  RNase-free water and stored at  $-70^\circ\text{C}$ .

**Analysis of  $1\alpha$ -hydroxylase and 24-hydroxylase activity in tissue samples.** Activity levels for  $1\alpha$ -hydroxylase and 24-hydroxylase in breast tumors and nonneoplastic breast tissue were assessed by quantifying the metabolism of  $25\text{OHD}_3$  in homogenates from these tissues. For each assay, [ $^3\text{H}$ ] $25\text{OHD}_3$  (10 nmol/L; specific activity, 152 Ci/mmol, Amersham, London, United Kingdom) was added to tissue homogenates prepared in PBS from snap-frozen tumor biopsies ( $n = 6$ ) or nonneoplastic tissue ( $n = 6$ ). Aliquots of homogenate used in

the assays contained 0.4 mg protein, 0.2 mol/L cofactor (NADPH), and 0.5 mmol/L protease inhibitor (phenylmethylsulfonyl fluoride). Homogenate/substrate mixtures were incubated for 5 hours at  $37^\circ\text{C}$  and the reaction was terminated by freezing at  $-20^\circ\text{C}$ . Vitamin  $\text{D}_3$  metabolites were then extracted from the reaction mixtures in 2.5 mL chloroform/methanol (4:1, v/v) and the conversion of [ $^3\text{H}$ ] $25\text{OHD}_3$  to [ $^3\text{H}$ ] $1,25(\text{OH})_2\text{D}_3$  and [ $^3\text{H}$ ] $1,24,25$ -trihydroxyvitamin  $\text{D}_3$  [ $1,24,25(\text{OH})_3\text{D}_3$ ] was quantified by scanning TLC as described previously (31). Results were expressed as mean  $\pm$  SD fmol [ $^3\text{H}$ ]vitamin D metabolite produced/h/mg of protein.

**Chemicals and vitamin D compounds.**  $1,25(\text{OH})_2\text{D}_3$  and  $25\text{OHD}_3$  were a kind gift from Dr. Lise Binderup (Leo Pharmaceuticals, Ballerup, Denmark) and stored at 1 mmol/L in ethanol at  $-20^\circ\text{C}$  in the dark.

**Cell culture.** The breast cancer cell lines T47-D, ZR-75-1, MCF-7, and MDA-MB-231 were obtained from the American Type Culture Collection (Rockville, MD). MCF-7Res, a variant of MCF-7 cells, generously supplied Dr. Christina Mork Hansen (University of Kuopio, Kuopio, Finland), were generated by long-term culture in the presence of  $1,25(\text{OH})_2\text{D}_3$ , resulting in the isolation of a clone that was resistant to the antiproliferative action of  $1,25(\text{OH})_2\text{D}_3$  (32). All cell lines were supplemented with 100 units/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin, and 10% fetal bovine serum (Life Technologies, Paisley, United Kingdom) in RPMI. MCF-12A cells were a generous gift of Prof. H. Phillip Koeffler (Cedars-Sinai Medical Center/University of California at Los Angeles School of Medicine, Los Angeles, CA). These cells are a nontumorigenic epithelial cell line established from tissue taken at reduction mammoplasty from a nulliparous patient with fibrocystic breast disease that contained focal areas of intraductal hyperplasia. They are not tumorigenic in immunosuppressed mice but do form colonies in semisolid medium. These cells were cultured in a 1:1 mixture of DMEM and Ham's F-12 medium, 20 ng/mL epidermal growth factor, 100 ng/mL cholera toxin, 0.01 mg/mL insulin, 500 ng/mL hydrocortisone, and 5% horse serum (33). All cells were grown at  $37^\circ\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$  in air.

**RNA extraction from cells.** For  $1,25(\text{OH})_2\text{D}_3$ -treatment, cells in culture were seeded as subconfluent conditions and treated with fresh medium or  $1,25(\text{OH})_2\text{D}_3$  (10 nmol/L for 12 hours or 100 nmol/L for 4 hours as indicated). Total RNA was extracted using the GenElute RNA extraction system (Sigma, Poole, United Kingdom) according to the manufacturer's instructions.

**Reverse transcription.** Aliquots (1.5  $\mu\text{g}$ ) of RNA from each DNase-treated sample were reverse transcribed using avian myeloblastosis virus reverse transcriptase (Promega, Southampton, United Kingdom). Briefly, RNA samples were incubated at  $70^\circ\text{C}$  for 5 minutes with random primers (500  $\mu\text{g}/\mu\text{L}$ , Promega) and molecular biology grade water. Primer extension and reverse transcription were done by the addition of deoxynucleotide triphosphates (10 mmol/L),  $10\times$  reaction buffer, RNasein (40 units/ $\mu\text{L}$ ), and avian myeloblastosis virus (10 units/ $\mu\text{L}$ ) in 30  $\mu\text{L}$  reaction volumes. Samples were then incubated at  $37^\circ\text{C}$  for 60 minutes and  $95^\circ\text{C}$  for 5 minutes before storage at  $-20^\circ\text{C}$ .

**Quantitative PCR analysis of gene expression.** Expression of specific mRNAs was quantified using an ABI 7700 sequence detection system (PE Biosystems, Warrington, United Kingdom) as described previously (34). Briefly, aliquots (25  $\mu\text{L}$ ) of PCR reactions were set up containing: Taqman Universal PCR Master mix in a  $2\times$  solution (PE Biosystems) 3 mmol/L manganese acetate, 200  $\mu\text{mol}/\text{L}$  deoxynucleotide triphosphates, 1.25 unit Ampliqaq Gold polymerase, 1.25 unit AmpErase uracil-N-glycosylase, 5 or 1.25 pmol/ $\mu\text{L}$  Taqman probe, and 5 or 9 pmol/ $\mu\text{L}$  primers. About 50 ng of cDNA were used per reaction. All reactions were multiplexed with the housekeeping gene 18S rRNA, provided as an optimized control probe labeled with VIC (PE Biosystems), enabling data to be expressed in relation to an internal reference to allow for differences in sampling. All fluorogenic probes for genes of interest were labeled with five-carboxyfluorescein. Data were obtained as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) according to the manufacturer's guidelines and used to determine  $\Delta\text{Ct}$  values (Ct of

target gene – Ct of housekeeping gene) as raw data for gene expression. Fold change in gene expression was determined by subtracting  $\Delta$ Ct values for tumor samples or treated cells from their respective control samples. The resulting  $\Delta\Delta$ Ct values were then used to calculate fold change in gene expression as  $2^{\Delta\Delta$ Ct}. All reactions were done in triplicate and expressed as a mean of these values from three separate experiments. Samples were amplified using the primers and probes outlined in Table 1 under the following conditions: 50°C for 2 minutes and 95°C for 10 minutes followed by 44 cycles of 95°C for 15 seconds and 60°C for 1 minute. Using the same thermal cycling variables eliminated further optimization of conditions and allowed multiple assays on the same plate.

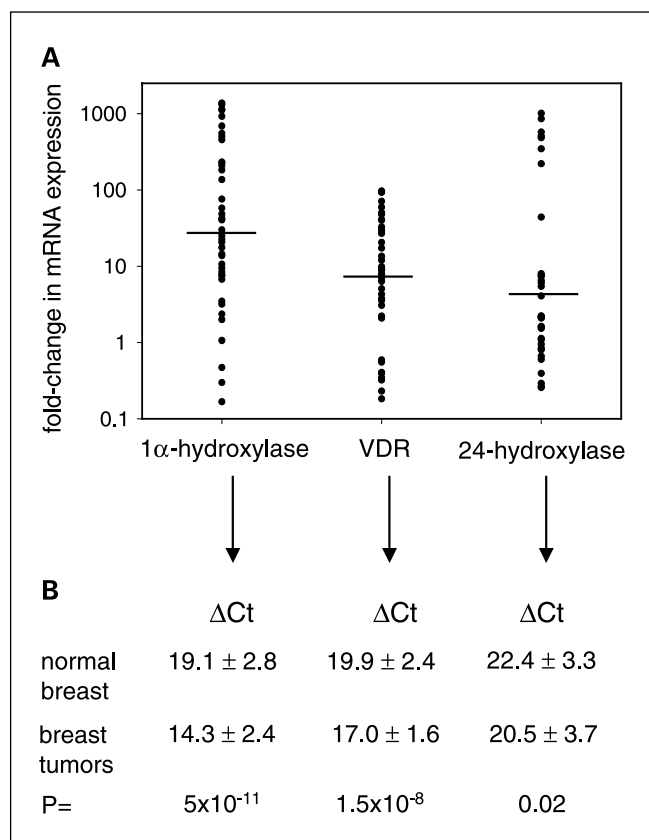
**Primers and probes for PCR reactions.** Real-time PCR primer and probe sequences for *1 $\alpha$ -hydroxylase*, *VDR*, and *24-hydroxylase* are shown in Table 1. In each case, the housekeeping gene used to derive  $\Delta$ Ct values was 18S rRNA, which was analyzed using primers and probes (PE Biosystems).

**Proliferation assays in the presence of 25-hydroxyvitamin D<sub>3</sub> and 1,25-dihydroxyvitamin D<sub>3</sub>.** Proliferation of cell lines in the presence of vitamin D compounds, either alone or in combination with antisense and control oligonucleotides, was measured by colony formation in soft agar and cellular ATP content. For colony formation 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  $1 \times 10^3$  cells in 400  $\mu$ L medium per well as described previously (35). The support layer was prepared with agar (1%) equilibrated at 42°C. Before 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<sub>50</sub> values were interpolated.

Cellular ATP was measured by a bioluminescent assay (Vialight HS, LumiTech, Nottingham, United Kingdom). Briefly, cells were plated ( $2 \times 10^3$  per well) into 96-well, white-walled, tissue culture-treated plates (Fisher Scientific Ltd. Loughborough, United Kingdom). The cells were exposed to treatments as indicated and the final volume of the well was made up to 100  $\mu$ L and incubated for 96 hours having been re-dosed with agent after 48 hours. After the incubation period, nucleotide releasing reagent (100  $\mu$ L) was added and the liberated ATP was quantitated by the addition of 20  $\mu$ L ATP monitoring reagent and using a microplate luminometer (Berthold Detection Systems, Fisher Scientific).

**Table 1.** Primer and probe sequences for real-time RT-PCR analyses

| Gene                                    | Primer probe sequence (5'-3')                                |
|---|--|
| <i>1<math>\alpha</math>-Hydroxylase</i> | Forward primer TTGGCAAGCGCAGCTGTAT                           |
|   | Reverse primer TGTGTAGGATCTGGGCCAAA                          |
|   | Taqman probe TTGCAATCAAGCTCTGCCAG-GCG                        |
| <i>VDR</i>                              | Forward primer CTTCAGGCGAAGCATGAAGC                          |
|   | Reverse primer CCTTCATCATGCCGATGTCC                          |
|   | Taqman probe AAGGCACTATTCACCTGCCCTTCAA                       |
| <i>24-Hydroxylase</i>                   | Forward primer CAAACCGTGAAGGCCTATC                           |
|   | Reverse primer AGTCTTCCCTTCCAGGATCA                          |
|   | Taqman probe ACTACCGCAAAGAAGGCTACGGGCTG                      |
| <i>CD14</i>                             | Assays-on-Demand (ABI) primer and probe mix ID Hs00169122.g1 |
| <i>Toll-like receptor 4</i>             | Assays-on-Demand (ABI) primer and probe mix ID Hs00152939.m1 |



**Fig. 1.** Increased expression of *1 $\alpha$ -hydroxylase*, *VDR*, and *24-hydroxylase* mRNA in breast tumors compared with paired normal tissue. **A**, fold increase in gene expression for individual tumor samples compared with matched normal samples with an arbitrary expression value of 1. Mean fold increase in expression ( $n = 3$  for separate RT-PCR analyses) is indicated by a horizontal line for each gene product. **B**, raw  $\Delta$ Ct data  $\pm$  SD for tumor and nonmalignant breast tissue for each gene product with statistical analysis of these data shown as *P*s. Higher  $\Delta$ Ct values represent lower levels of mRNA expression.

**24-Hydroxylase antisense oligonucleotide treatment of MDA-MB-231 cells.** Antisense and control oligonucleotides were synthesized by Morpholino (Oregon) and were as follows: antisense CYP24 5'-CGA-GGTTGGTACGAGGTGCTAGTGG-3', standard (control) 5'-CCTCTT-ACCCTAGTTACAATTATA-3', and a FITC-tagged control oligonucleotide. The oligonucleotides were diluted to a stock concentration of 500  $\mu$ mol/L using sterile distilled water and stored at  $-20^{\circ}$ C. Oligonucleotides were delivered to the cells by a scrape delivery system using MDA-MB-231 cells grown to 80% confluence. Growth medium was removed and replaced with PBS containing oligonucleotides at the desired concentration. The cells were then detached from the flask with a cell scraper, repeat pipetted to disaggregate clumps, counted, and reseeded into either 96-well or 24-well plates for ATP proliferation and colony formation assays, respectively. Treatment with FITC-conjugated control oligonucleotide was the same as the other oligonucleotides, but the cells were plated onto glass slides and grown for up to 96 hours before observation under a fluorescent microscope.

**Western blot analysis of 24-hydroxylase.** Briefly, total protein (20  $\mu$ g) for each sample was subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA) and blocked with PBS-Tween 20 containing 5% milk powder for 1 hour. The 24-hydroxylase sheep polyclonal antibody was a generous gift of Prof. Rajiv Kumar (Mayo Proteomics Research Center, Mayo Clinic and Foundation, Rochester, MN; ref. 36). For detection of 24-hydroxylase, sheep polyclonal antibodies were diluted 1:200 and 1:500, respectively, followed by secondary antibody

(anti-sheep horseradish peroxidase, the binding site). Proteins were detected using enhanced chemiluminescence (Amersham, Bucks, United Kingdom) and autoradiography. To ensure even loading and transfer of protein, membranes were subsequently probed with a 1:5,000 dilution of primary mouse monoclonal  $\beta$ -actin antibody (AC-15, Sigma) followed by anti-mouse horseradish peroxidase secondary antibody (binding site) and signals were developed with enhanced chemiluminescence and autoradiography as above. To quantify the relative changes in protein levels, densitometry analysis was done on triplicate sets of lysate and values were normalized to  $\beta$ -actin levels.

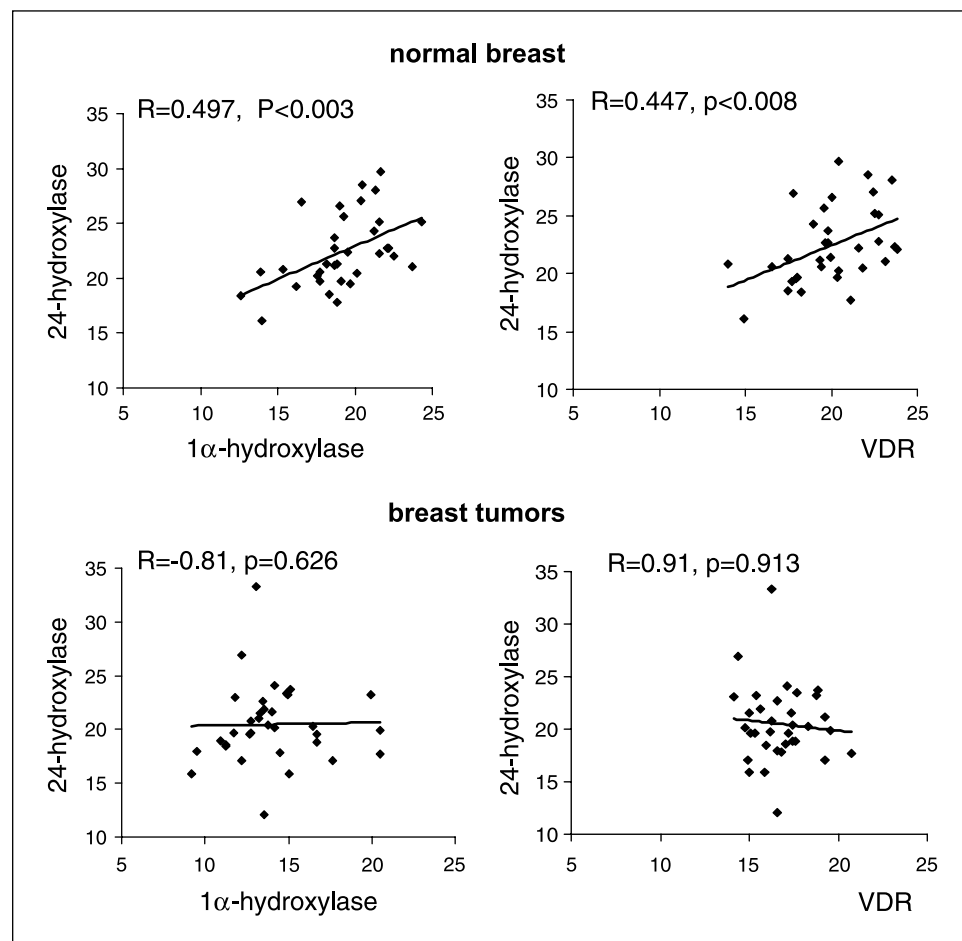
**Immunohistochemistry for  $1\alpha$ -hydroxylase.** Immunohistochemical analysis of  $1\alpha$ -hydroxylase in paraffin-embedded breast tumor biopsy sections was carried out using methods described previously (37).

**Data analysis.** Statistical analysis was done on the triplicate average raw  $\Delta$ Ct values using one-way ANOVA with Student's-Newman-Keuls' multiple comparison post-test or Pearson correlation (SigmaStat3 version 2.03).

## Results

**Dysregulated expression of  $1\alpha$ -hydroxylase, vitamin D receptor, and 24-hydroxylase in breast tumors.** Quantitative reverse transcription-PCR (RT-PCR) analysis of RNA from 41 paired tumor and normal breast tissue samples showed that expression of  $1\alpha$ -hydroxylase (mean 27-fold increase compared with normal breast tissue), VDR (mean 7-fold increase), and 24-hydroxylase (mean 4-fold increase) was up-regulated in tumors

compared with nonneoplastic tissue (Fig. 1). Although fold change values for all three genes ranged from 1 to 1,000, statistical analyses were carried out using raw  $\Delta$ Ct values to represent mean levels of mRNA expression for tumors and nonneoplastic tissue (Fig. 1B). Based on these values, expression of  $1\alpha$ -hydroxylase ( $P < 5 \times 10^{-11}$ ), VDR ( $P < 1.5 \times 10^{-8}$ ), and 24-hydroxylase ( $P < 0.02$ ) was significantly higher in tumors compared with normal breast tissue. Levels of mRNA for  $1\alpha$ -hydroxylase and VDR were closely linked, with 80% ( $n = 33$ ) of tumors showing elevated expression of both gene products. In a similar fashion, increased expression of 24-hydroxylase was only observed in tumors that had elevated  $1\alpha$ -hydroxylase and VDR (data not shown). However, further analysis of RT-PCR data showed that whereas in normal breast tissue expression of 24-hydroxylase correlated with both  $1\alpha$ -hydroxylase ( $r = 0.497$ ,  $P < 0.008$ ) and VDR ( $r = 0.447$ ,  $P < 0.003$ ; Fig. 2, top) there was no such correlation in breast tumors (Fig. 2, bottom). This suggested that in nonmalignant tissue 24-hydroxylase is transcriptionally modulated as part of a well-ordered feedback response to increased localized production of  $1,25(\text{OH})_2\text{D}_3$  and adequate VDR expression. In tumors by contrast, 24-hydroxylase is overexpressed in a dysregulated fashion. Changes in gene expression did not seem to be due to the estrogen receptor status of the tumors or the age at which tumor diagnosis was made (data not shown).



**Fig. 2.** Correlation among  $1\alpha$ -hydroxylase, VDR, and 24-hydroxylase mRNA levels in breast tumors and nonneoplastic breast tissue. Correlations between  $\Delta$ Ct values for 24-hydroxylase (Y axis) versus  $1\alpha$ -hydroxylase or VDR (X axis) for nonmalignant breast tissue (top) and breast tumors (bottom). Higher  $\Delta$ Ct values represent lower mRNA expression.

**1 $\alpha$ -Hydroxylase and 24-hydroxylase are functionally active in normal and malignant breast tissue.** Immunohistochemistry confirmed that protein for 1 $\alpha$ -hydroxylase was expressed in lobules and ducts within normal breast tissue but was more abundant in breast tumors, particularly invading tumor cells and associated inflammatory infiltrates (Fig. 3A-C). Enzyme activity assays were also carried out using homogenates from tumor-normal pairs ( $n = 6$ ) incubated with 10 nmol/L [ $^3\text{H}$ ]25OHD $_3$  as substrate. Data (Fig. 3D) confirmed that 1 $\alpha$ -hydroxylase activity was increased in tumors compared with nonneoplastic tissue ( $44.3 \pm 11.4$  versus  $12.4 \pm 4.8$  fmol [ $^3\text{H}$ ]1,25(OH) $_2\text{D}_3$  produced/h/mg protein;  $P < 0.05$ ). However, conversion of 1,25(OH) $_2\text{D}_3$  to inactive 1,24,25(OH) $_3\text{D}_3$  was also significantly higher in tumors ( $84.8 \pm 11.7$  versus  $33.6 \pm 8.5$  fmol [ $^3\text{H}$ ]1,24,25(OH) $_3\text{D}_3$  produced/h/mg protein;  $P < 0.01$ ). Thus, both normal and malignant breast tissues are able to activate vitamin D via 1 $\alpha$ -hydroxylase, but the efficiency of this mechanism in generating antiproliferative 1,25(OH) $_2\text{D}_3$  in tumors seems to be compromised by dysregulated expression of 24-hydroxylase.

**Expression of 24-hydroxylase is elevated in more aggressive breast cancer cell lines.** Quantitative RT-PCR analysis of RNA isolated from a panel of nonmalignant and tumor breast cell lines showed that 24-hydroxylase expression was increased in breast tumor cells compared with nonmalignant MCF-12A cells, particularly in hormone-resistant MCF-7Res and MDA-MB-231 cells (all  $P < 0.001$ ; Fig. 4). Data for fold change in mRNA relative to the MCF-12A normal cells indicated that both 1 $\alpha$ -hydroxylase and VDR expression was significantly decreased ( $P < 0.05$  and  $P < 0.01$ , respectively) in more aggressive cell types, with vitamin D-resistant MCF-7Res and

MDA-MB-231 cells showing the lowest expression of these genes.

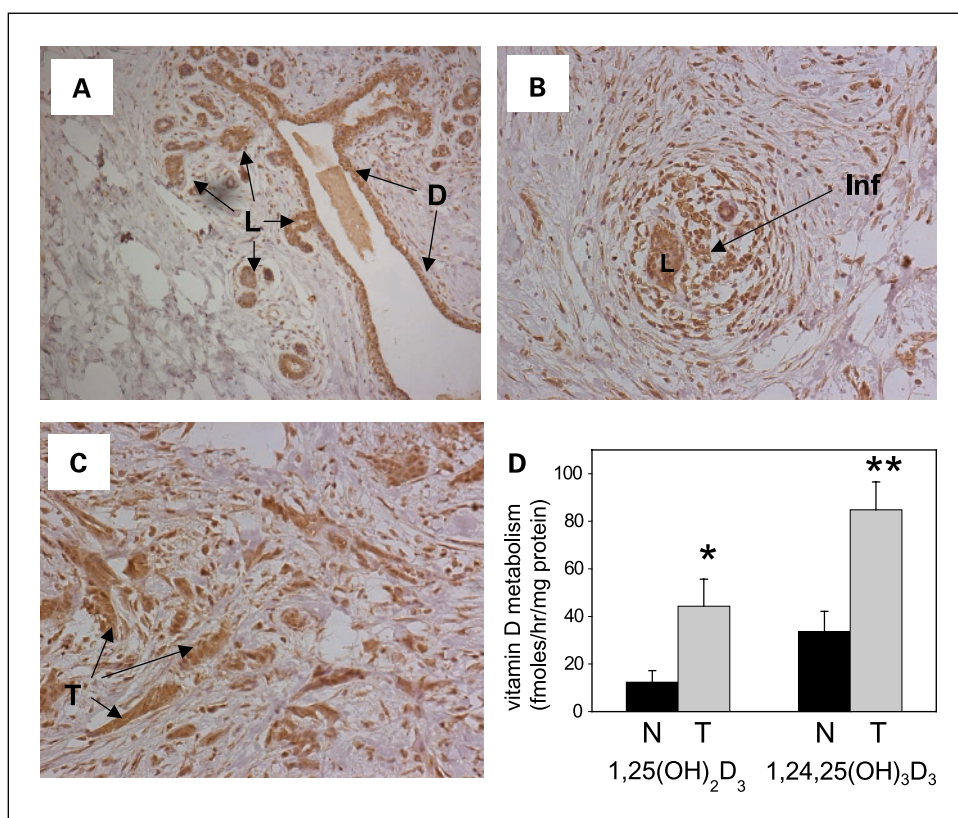
#### Antisense inhibition of 24-hydroxylase enhances sensitivity to 1,25-dihydroxyvitamin D $_3$ in MDA-MB-231 breast cancer cells.

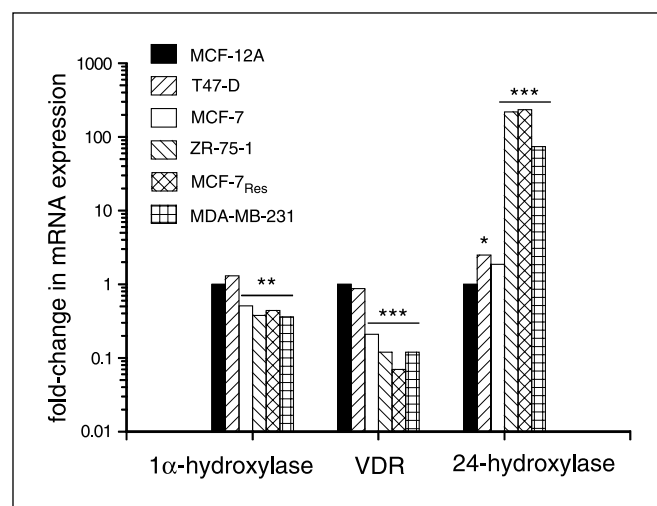
Further studies *in vitro* were carried out to determine the role of 24-hydroxylase in counteracting the anticancer effects of 1,25(OH) $_2\text{D}_3$  via catabolism to less active metabolites. MDA-MB-231 cells, which exhibit high endogenous levels of 24-hydroxylase, were incubated with antisense oligonucleotides to decrease expression of the enzyme (Fig. 5). Treatment with exogenous 1,25(OH) $_2\text{D}_3$  increased expression of 24-hydroxylase protein in MDA-MB-231 cells incubated with a nonspecific scrambled oligonucleotide. By contrast, antisense oligonucleotides to 24-hydroxylase decreased protein for the enzyme in both 1,25(OH) $_2\text{D}_3$ -treated and untreated cells (Fig. 5A). The functional consequence of this is illustrated by the data in Fig. 5B and C, which showed that antisense inhibition of 24-hydroxylase increased the sensitivity of MDA-MB-231 cells to the antiproliferative effects of exogenously added 1,25(OH) $_2\text{D}_3$ .

## Discussion

Recent studies have shown that responses to steroid hormones are modulated by crucial "preceptor" mechanisms involving tissue-specific activation or inactivation via locally expressed steroidogenic enzymes (38, 39). Prominent among these is vitamin D where synthesis of active 1,25(OH) $_2\text{D}_3$  via 1 $\alpha$ -hydroxylase is counterbalanced by 24-hydroxylase-mediated inactivation to 1,24,25(OH) $_3\text{D}_3$  in VDR-expressing target cells. In the kidney, these two enzymes form a classic feedback mechanism to allow the generation of adequate circulating

**Fig. 3.** Increased expression and activity of 1 $\alpha$ -hydroxylase protein in breast tumors. A-C, immunohistochemical analysis of 1 $\alpha$ -hydroxylase in breast tumors and nonneoplastic tissue. Analysis of 1 $\alpha$ -hydroxylase protein expression in breast tumors and adjacent normal tissue was carried out using a polyclonal antiserum to the enzyme. Staining (brown) for 1 $\alpha$ -hydroxylase: A, normal lobules (L) and ducts (D). Magnification,  $\times 100$ . B, inflammatory infiltrates (Inf) associated with breast tumor. Magnification,  $\times 200$ . C, invading epithelial breast tumor tissue (T). Magnification,  $\times 200$ . D, metabolism of [ $^3\text{H}$ ]25OHD $_3$  (10 nmol/L) in  $n = 6$  tumor (T) and nonneoplastic (N) breast homogenates. Synthesis of [ $^3\text{H}$ ]1,25(OH) $_2\text{D}_3$  and [ $^3\text{H}$ ]1,24,25(OH) $_3\text{D}_3$  was assessed by scanning TLC. Columns, mean fmol metabolite produced/h/mg protein; bars, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , statistically different from nonmalignant samples.

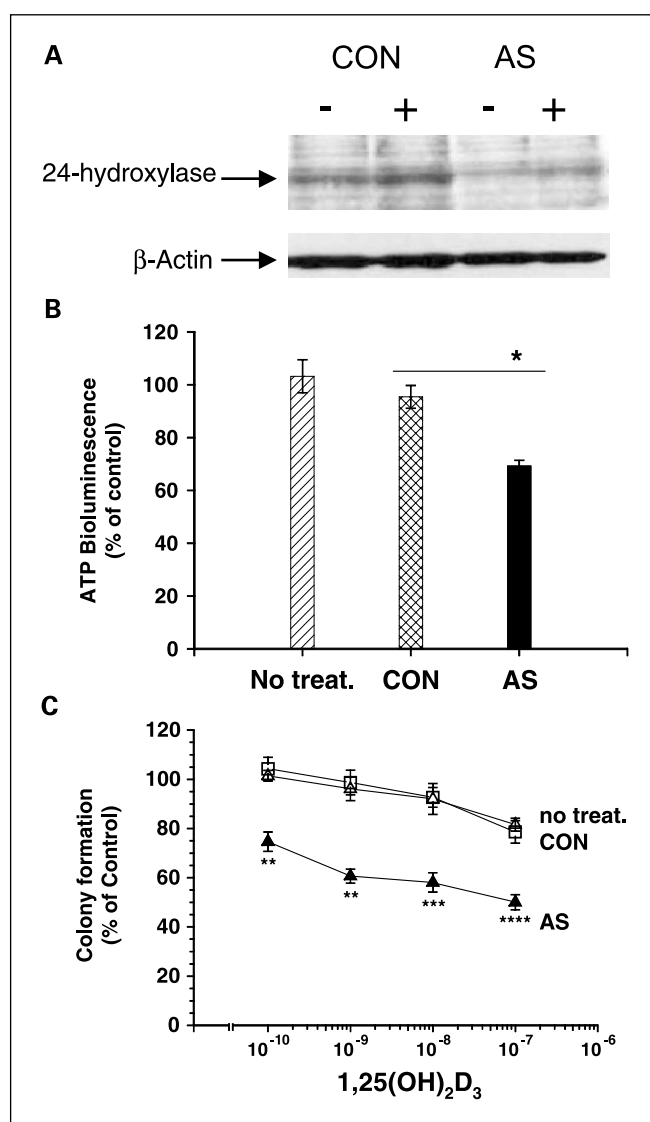




**Fig. 4.** Expression of  $1\alpha$ -hydroxylase, VDR, and 24-hydroxylase in breast cancer cell lines. Real-time RT-PCR analysis of gene expression in T47-D, MCF-7, ZR-75-1, MCF-7Res, and MDA-MB-231 breast cancer cells shown as fold change in expression when compared with nonneoplastic MCF-12A cells with an arbitrary expression value of 1. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , statistically different from nonneoplastic MCF-12A cells.

levels of  $1,25(\text{OH})_2\text{D}_3$  while protecting against the hypercalcaemic side effects of the hormone. However, both  $1\alpha$ -hydroxylase and 24-hydroxylase are expressed in a wide range of extrarenal tissues, suggesting functions for  $1,25(\text{OH})_2\text{D}_3$  that are quite distinct from its classic calciotropic effects. These include potential immunomodulatory effects mediated via macrophage/dendritic cell  $1\alpha$ -hydroxylase activity (30). Extrarenal  $1\alpha$ -hydroxylase activity may also play an important role in tumor pathophysiology by providing a mechanism for concentrating antiproliferative  $1,25(\text{OH})_2\text{D}_3$  in specific tissue. Studies of prostate (40, 41) and colon (42–44) cancer indicate that  $1\alpha$ -hydroxylase is expressed in both normal tissue and tumors, with increased expression of  $1\alpha$ -hydroxylase in differentiated tumors compared with paired normal tissue (42). Conversely, studies *in vitro* have shown that levels of the enzyme are lower in cultured prostate tumor cells compared with their nonneoplastic equivalents (45).

Although several studies have documented association between dietary/environmental intake of vitamin D and risk of breast cancer (17–20), relatively little is known about the autocrine metabolism of vitamin D in this type of malignancy. In data presented here, we have shown expression and activity of  $1\alpha$ -hydroxylase in normal and malignant breast tissues. The presence of the enzyme in nonneoplastic breast tissue highlights a potential conduit by which vitamin D can protect against cancer. Specifically, autocrine synthesis of  $1,25(\text{OH})_2\text{D}_3$  from circulating  $25\text{OHD}_3$  may provide a local supply of antiproliferative hormone within breast tissue. Of course,  $1,25(\text{OH})_2\text{D}_3$  may have additional functions within the nonmalignant breast, which would also be supported by local expression of  $1\alpha$ -hydroxylase. For example, recent studies using VDR-ablated mice have shown that VDR-mediated signaling is involved in the regulation of mammary cell turnover during the reproductive cycle (46). Importantly, this study also indicated that synthesis of the ligand for VDR,  $1,25(\text{OH})_2\text{D}_3$ , may occur at either an endocrine or an autocrine level, as  $1\alpha$ -hydroxylase was also detectable in mouse breast tissue.



**Fig. 5.** Inhibition of 24-hydroxylase sensitizes breast cancer cells to  $1,25(\text{OH})_2\text{D}_3$ . Estrogen receptor – negative,  $1,25(\text{OH})_2\text{D}_3$ -resistant MDA-MB-231 cells were incubated with standard [control (CON)] and antisense (AS) 24-hydroxylase oligonucleotides for 48 hours in the presence or absence of 100 nmol/L  $1,25(\text{OH})_2\text{D}_3$  after which time protein lysates were analyzed by SDS-PAGE. **A**, representative blots with the position of the proteins indicated on the left. Blots were subsequently stripped and reprobed for  $\beta$ -actin. **B**, bioluminescent ATP assays were used to measure the effect of oligonucleotide treatment on the response of MDA-MB-231 cells to  $1,25(\text{OH})_2\text{D}_3$ . Cells were plated into 96-well plates and treated with  $1,25(\text{OH})_2\text{D}_3$  and oligonucleotide as indicated. After 96 hours, total ATP was measured and compared with untreated control. Points, mean of three separate experiments undertaken in triplicate; bars, SE. Treatment with antisense oligonucleotides significantly enhances the antiproliferative action of  $1,25(\text{OH})_2\text{D}_3$ . \*,  $P < 0.05$ . **C**, effect of oligonucleotide treatment on colony formation response of MDA-MB-231 cells to  $1,25(\text{OH})_2\text{D}_3$ . Cells were plated into 24-well plates and treated with  $1,25(\text{OH})_2\text{D}_3$  and oligonucleotide as indicated. After 10 days, colonies were enumerated and expressed as a percentage of colonies in untreated control plates. Points, mean of three separate experiments undertaken in triplicate wells; bars, SE. Treatment with antisense significantly enhances the antiproliferative action of  $1,25(\text{OH})_2\text{D}_3$ . \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ .

Although expression of mRNA for  $1\alpha$ -hydroxylase was increased in 80% of the breast tumors studied, the magnitude of induction varied considerably. This may simply reflect the sensitivity of quantitative RT-PCR analyses: measurement of actual  $1\alpha$ -hydroxylase enzyme activity showed less variation despite a smaller sample number (Fig. 3D). Alternatively, these



data may reflect inherent differences in  $1\alpha$ -hydroxylase trans-activation: recent studies have characterized polymorphisms within the gene for  $1\alpha$ -hydroxylase (*CYP27B1*), which may confer variability in the regulation of  $1\alpha$ -hydroxylase expression (47). The underlying basis for up-regulation of  $1\alpha$ -hydroxylase in breast tumors is also clear, but we have observed a similar induction of  $1\alpha$ -hydroxylase in lymphomas (37) and dysgerminomas (48). In these cases, synthesis of  $1,25(\text{OH})_2\text{D}_3$  was mediated via expression of  $1\alpha$ -hydroxylase in tumor-associated macrophages, and a similar pattern of increased expression of  $1\alpha$ -hydroxylase has also been reported in alveolar macrophages associated with lung cancer (49). It was therefore interesting to note the immunolocalization of  $1\alpha$ -hydroxylase in the inflammatory infiltrate of breast tumors, suggesting that this cell type may be the source of at least some of the  $1,25(\text{OH})_2\text{D}_3$  in breast tumors. The possible contribution of macrophages to tumor vitamin D metabolism was further supported by the fact that we also able to correlate tumor expression of  $1\alpha$ -hydroxylase mRNA with toll-like receptor 4 ( $r = 0.417$ ;  $P < 0.007$ ;  $n = 41$ ) and CD14 ( $r = 0.629$ ;  $P < 0.01$ ;  $n = 18$ ), which together form part of the macrophage endotoxin response system (50).

The efficacy of  $1\alpha$ -hydroxylase as a system for concentrating active vitamin D within breast tissue is likely to be dependent on two key factors other than the level of enzyme expression. The first of these is substrate ( $25\text{OHD}_3$ ) availability, which is likely to vary considerably depending on season, latitude, and access to sunlight (17–20). In normal subjects, there is no clear correlation between  $25\text{OHD}_3$  and circulating levels of  $1,25(\text{OH})_2\text{D}_3$  as a consequence of endocrine interactions with parathyroid hormone (25, 26). However, there seems to be a closer link between serum  $25\text{OHD}_3$  and extrarenal  $1\alpha$ -hydroxylase activity, particularly in patients with inflammatory diseases, such as sarcoidosis, where there is significant macrophage synthesis of  $1,25(\text{OH})_2\text{D}_3$  (51).

The second factor that may influence the magnitude of  $1,25(\text{OH})_2\text{D}_3$  production in extrarenal tissues is coincident expression of the inactivating enzyme 24-hydroxylase, which is induced by  $1,25(\text{OH})_2\text{D}_3$  in all VDR-expressing tissues as part of a well-ordered system for modulating tissue responses to vitamin D. This is clearly illustrated by the correlation in expression among  $1\alpha$ -hydroxylase, VDR, and 24-hydroxylase in nonmalignant breast tissue and, in addition, provides further evidence for localized synthesis of  $1,25(\text{OH})_2\text{D}_3$  within normal breast tissue. Increased expression of 24-hydroxylase in breast tumors may be a response to the enhanced localized production of  $1,25(\text{OH})_2\text{D}_3$ . However, the lack of correlation between 24-hydroxylase and either VDR or  $1\alpha$ -hydroxylase in breast tumors suggests that the inactivating enzyme is independently dysregulated. This is supported by previous DNA array and positional cloning analyses of genomic amplification in breast tumors, which concluded that 24-hydroxylase was a potential oncogene (52). Irrespective of the molecular basis for increased 24-hydroxylase expression

observed in this study, the net effect in breast tumors was to increase the catabolism of  $1,25(\text{OH})_2\text{D}_3$  to  $1,24,25(\text{OH})_3\text{D}_3$ . Studies *in vitro* indicated that this is likely to have a significant impact on cellular responses to  $1,25(\text{OH})_2\text{D}_3$ . Firstly, cells with inherent resistance to  $1,25(\text{OH})_2\text{D}_3$  had higher background levels of 24-hydroxylase and antisense attenuation of 24-hydroxylase in one of these cell lines, MDA-MB-231, sensitized the cells to the growth-inhibitory effects of  $1,25(\text{OH})_2\text{D}_3$ . Furthermore, in unpublished studies, we have shown that  $1,24,25(\text{OH})_3\text{D}_3$  does not induce antiproliferative responses in breast cancer cells. This is consistent with previous reports (53) and has resulted in several studies, which have used cytochrome P450 inhibitors to suppress 24-hydroxylase activity and thereby increase sensitivity to  $1,25(\text{OH})_2\text{D}_3$ . The most commonly used example is the antibiotic ketoconazole (54), but the nonspecific nature of this compound means that it is also a potent inhibitor of  $1\alpha$ -hydroxylase activity. To alleviate this potentially detrimental effect, compounds that selectively inhibit 24-hydroxylase have been developed (55, 56) together with 24-hydroxylase-resistant vitamin D analogues (57). Studies to date suggest that this may be a fruitful avenue in the quest to potentiate the anticancer effects of  $1,25(\text{OH})_2\text{D}_3$  (58). An alternative strategy would be to use endogenous  $1\alpha$ -hydroxylase activity in breast tissue to increase localized concentrations of  $1,25(\text{OH})_2\text{D}_3$ . The tissue specificity of this approach has yet to be studied *in vivo*. Nevertheless, the ability of  $25\text{OHD}_3$  to induce potent antiproliferative effects in cancer cells that express  $1\alpha$ -hydroxylase activity (59) coupled with the apparent increased capacity for synthesis of  $1,25(\text{OH})_2\text{D}_3$  in breast tumors suggest that this may be a viable therapeutic option.

Data presented here show for the first time the interactions among the three main components of vitamin D metabolism and signaling in breast tissue and tumors. On the one hand, we have shown that normal breast tissue is able to synthesize active vitamin D, thereby providing a local source of antiproliferative hormone. That this mechanism is unsuccessful in preventing tumors may be attributed in part to inefficient substrate levels for  $1\alpha$ -hydroxylase—in other words, the vitamin D status of the individual. However, breast tumors also seem to exhibit vitamin D resistance and we have shown that dysregulation of 24-hydroxylase as part of the neoplastic transformation may play a crucial role in this process by converting  $1,25(\text{OH})_2\text{D}_3$  to less active vitamin D metabolites. These observations emphasize further the importance of extrarenal metabolism in directing the autocrine effects of vitamin D and, in particular, suggest that enzymes involved,  $1\alpha$ -hydroxylase and 24-hydroxylase, may be important targets for both the prevention and treatment of breast cancer.

## Acknowledgments

We thank Susan Hughes for technical assistance.

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