Human Mammary Epithelial Cells Express CYP27B1 and Are Growth Inhibited by 25-Hydroxyvitamin D-3, the Major Circulating Form of Vitamin D-3

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ABSTRACT  1α,25-dihydroxycholecalciferol [1α,25(OH)2D3], the active form of cholecalciferol, is a negative growth regulator of breast cancer cells. CYP27B1 is a cytochrome P450-containing hydroxylase expressed in kidney and other tissues that generates 1α,25(OH)2D3 from an inactive vitamin D precursor 25-hydroxycholecalciferol [25(OH)D3]. In these studies, we tested the hypothesis that mammary cells express CYP27B1 and locally produce 1α,25(OH)2D3, which acts in an autocrine manner to regulate cell turnover. Using Western blot and quantitative real-time PCR, CYP27B1 mRNA and protein were detected in immortalized, nontumorigenic human mammary epithelial cell (HMEC) cultures. Furthermore, HMEC cultures were dose dependently growth inhibited by physiological concentrations of 25(OH)D3, suggesting that CYP27B1 converts this precursor cholecalciferol metabolite to 1α,25(OH)2D3, the ligand for the vitamin D receptor (VDR). In support of this suggestion, both 1α,25(OH)2D3 and 25(OH)D3 activated VDR in HMEC cultures, as measured by induction of a vitamin D responsive reporter gene and upregulation of CYP24, an endogenous VDR target gene. No induction of CYP24 by 25(OH)D3 was observed in mammary cells derived from CYP27B1 null mice. Similar results were observed in 2 independently derived immortalized HMEC lines as well as in primary cultures derived from human breast epithelium. These are the first studies to demonstrate that nontransformed human mammary cells express CYP27B1, that they are growth inhibited by physiologically relevant concentrations of 25(OH)D3, and that they provide a biological mechanism linking vitamin D status to breast cancer risk. J. Nutr. 136: 887–892, 2006.

KEY WORDS: vitamin D • mammary • breast cancer • CYP27B1

The vitamin D steroids ergocalciferol (D-2) and cholecalciferol (D-3) modulate calcium homeostasis, cell turnover, and immune responses in a variety of tissues. Vitamin D-2 and D-3 can be obtained from natural foods, fortified products, and supplements, and vitamin D-3 can be synthesized from 7-dehydrocholesterol in skin exposed to UVB radiation (sunlight). Regardless of source, vitamins D-2 and D-3 exert biological activity only after a series of hydroxylations catalyzed by cytochrome P450 (CYP)-containing enzymes. The first of these conversions is catalyzed by CYP27A, a vitamin D 25-hydroxylase, which metabolizes cholecalciferol to 25-hydroxycholecalciferol [25(OH)D3], a circulating metabolite present in the pmol/L range that correlates with vitamin D-3 status (1). Although 25(OH)D3 is the major circulating form of cholecalciferol, its only known function is to serve as a precursor to 1,25-dihydroxycholecalciferol [1α,25(OH)2D3], the biologically active metabolite generated by CYP27B1 [25(OH)D3 1α-

hydroxylase], a mitochondrial enzyme present in renal proximal tubules (2). Renal CYP27B1 activity is inversely correlated with calcium status, and serum concentrations of 1α,25(OH)2D3 are kept in the pmol/L range through classical negative feedback mechanisms. Thus, under conditions of normocalcemia, renal CYP27B1 activity is inhibited and 25(OH)D3 is instead metabolized by CYP24 (a vitamin D 24-hydroxylase) to 24,25-dihydroxyvitamin D3 [24,25(OH)2D3], a biologically inactive metabolite that is ultimately converted to calcitroic acid and excreted (3).

In addition to its role in calcium homeostasis, epidemiologic studies suggest that optimal vitamin D status has a protective effect against the formation and progression of several common cancers (4–6). 1α,25(OH)2D3 interacts with the vitamin D receptor (VDR) to inhibit directly the growth of prostate, colon, and breast cancer cells (7–10); however, these growth regulatory effects are observed at concentrations (100 nmol/L) well above the physiologic range that are in fact toxic in vivo. Based on these considerations, it is unlikely that 1,25(OH)2D3 acts at the systemic level to regulate cell growth in vivo. The identification of CYP27B1 in skin, colon, prostate, and breast (11–15) suggests that locally generated 1,25(OH)2D3 could act in an autocrine manner to protect cells against transformation. In support of this concept, low circulating levels of the CYP27B1 substrate, 25(OH)D3, are positively (1) correlated with
b biomarkers and/or risk for prostate, colon, and breast cancer (5,16–18). Moreover, extrarenal expression of CYP27B1 appears to be of biological significance because locally generated 1,25(OH)₂D₃ inhibited growth and induced differentiation of transformed keratinocytes in a xenograft model (19). In addition, loss of CYP27B1 in prostate cancer cells correlated with reduced sensitivity to 25(OH)D₃ (14).

The hypothesis that CYP27B1 in extrarenal tissues may generate sufficient 1,25(OH)₂D₃ to affect cell transformation predicts that normal epithelial cells would express both VDR and CYP27B1, a prediction that has already held true for normal keratinocytes, colonocytes, and prostate epithelial cells (12,14). Although CYP27B1 is expressed in human and murine mammary tissue and breast cancer cell lines (20,21), little is known about vitamin D metabolism in nontumorigenic mammary epithelial cells. In this study, we demonstrate that both immortalized and primary cells derived from human breast express CYP27B1, and that 25(OH)D₃ regulates expression of VDR target genes and inhibits cell growth at physiologic concentrations. These data suggest that the concentration of circulating 25(OH)D₃ may dictate the ability of mammary cells to synthesize 1,25(OH)₂D₃, which could affect cell turnover, thus providing a biological basis for the epidemiologic data linking vitamin D status to breast cancer risk.

MATERIALS AND METHODS

Cell culture. Primary human mammary epithelial cells (PHMEC) and a telomerase-immortalized, nontumorigenic human mammary epithelial cell (HMEC) line were obtained from Cambrex. The data obtained in the HMEC cultures were confirmed in an independently derived telomerase-immortalized HMEC line donated by Dr. Robert Weinberg (MIT, Cambridge, MA) (22); however, for simplicity, only data from the commercially available cells are shown. Although capable of infinite population doublings in vitro, telomerase-immortalized HMEC cultures retain morphology and growth characteristics of normal mammary epithelial cells, and are not tumorigenic in the absence of additional genetic mutations (22). MCF-7 breast cancer cells were obtained from the American Type Culture Collection, and HK-8 SV-40 immortalized human proximal kidney cells were provided by Dr. Lorraine Racusen (Johns Hopkins University, Baltimore, MD). All cell lines were grown in DMEM/F-12 containing 5% fetal bovine serum. MCF-7 cells were obtained from the late Dr. Jack Omdahl (University of Colorado, Denver, CO). MCF-7 cells immortalized by telomerase were obtained by Dr. Robert Weinberg and provided by Dr. Lorraine Racusen (Johns Hopkins University, Baltimore, MD). All cell lines were grown at 37°C in a humidified atmosphere and replenished every 3 or 4 d. Growth assays were performed using reagents from Promega and pGL3-24OH values were normalized to pRL-TK. Data are presented as relative luciferase units (RLU) where control values were set to 1 for each cell line.

Growth assay. Exponentially growing HMEC, PHMEC, and MCF-7 cultures were treated 1 d after plating with ethanol vehicle, 1,25(OH)₂D₃ or 25(OH)D₃ at the concentrations indicated in the figure legends. For time-course studies, cells were harvested before treatment and after 24, 48, 72, and 96 h with no media changes; for all other studies, media were replenished after 72 h. Cultures were fixed with 1% glutaraldehyde and stained with 0.1% crystal violet. After solubilization of the stain in 0.1% Triton X-100, absorbance was measured at 590 nm as an indicator of cell density.

Immunoblotting. Subconfluent cultures of HMEC, PHMEC, MCF-7, HK-8, and primary murine cells were treated with ethanol vehicle, 1,25(OH)₂D₃ or 25(OH)D₃, for 48 h. Monolayers were scraped into lysis buffer containing protease and phosphatase inhibitors (10 mM/L benzamidine, 10 mM/L sodium fluoride, 100 mM/L sodium vanadate, 25 µg/µL aprotinin, 25 µg/µL pepstatin, 25 µg/µL leupeptin, and 1 mM/L phenylmethylsulfonyl fluoride) and sonicated. After separation on 10% SDS-PAGE, transfer to nitrocellulose, and blocking with 20% skim milk, primary antibodies were applied at 1:40 (VDR Clone 9A7, NeoMarkers), 1:200 (CYP24, donated by Cytochrome) or 1:500 (CYP27B1, The Binding Site) dilutions. After being washed in PBS/0.1% Tween, horseradish peroxidase conjugated anti-rat (Santa Cruz Biotechnology), anti-mouse (Amersham Biosciences) or anti-rabbit (Jackson ImmunoResearch) secondary antibodies were applied and VDR, CYP24, and CYP27B1 abundance was detected by chemiluminescence. Specific bands were detected at 52 kDa (VDR), 50 kDa (CYP24), and 56 kDa (CYP 27B1). Ponceau staining was used to confirm equal loading, and in some cases, blots were stripped and reprobed with anti-GAPDH.

Quantitative real-time PCR. Subconfluent HMEC, MCF-7, and HK-8 cells were treated with ethanol vehicle, 100 nmol/L 1,25(OH)₂D₃, or 100 nmol/L 25(OH)D₃ for 24 h, and total RNA was extracted with RNeasy Mini Kit (Qiagen). cDNA synthesized with TaqMan Reverse Transcription Reagents (Applied Biosystems) was analyzed by real-time PCR using TaqMan PCR Core Reagent Kit (Applied Biosystems) and primers and probes specific for CYP27B1 (forward: AGTTGCTAAGTTGAGGAGTC; reverse: GTGCCGGAGAGCTCATACA, probe: ACTACCCCGAAGGAGGCGCAGCTACGCTG), and CYP24 (forward: CAAACGCTGAAAGGCCTATG, reverse: AGTCTTCCAGAGTATCA, probe: ACTACCCGAAAGGGCTACTAAC, probe: CCACCCTGCTGACTAAGGATTG) expression, which was analyzed in parallel.

Transient transfections. Subconfluent HMEC, PHMEC, MCF-7, and HK-8 cells were co-transfected in serum-free medium with a 300-bp vitamin D-responsive region of the CYP24 promoter driving the firefly luciferase reporter gene (pGL3–24OH, 0.64 µg, obtained from the late Dr. Jack Omdahl) and the thymidine kinase promoter driving the renilla luciferase reporter gene (pRL-TK, 0.16µg, Promega) using a 3:2 (v:w) ratio of FUGENE:DNA. After 4 h, the cells were treated with ethanol vehicle, 100 nmol/L 1,25(OH)₂D₃ or 100 nmol/L 25(OH)D₃ in complete media conditions for 24 h. Dual luciferase assays were performed using reagents from Promega, and pGL3-24OH values were normalized to pRL-TK. Data are presented as relative luciferase units (RLU) where control values were set to 1 for each cell line.

Statistical analyses. Data are expressed as means ± SE, with the number of replicates indicated in each figure legend. Student’s t test and 1-way ANOVA followed by Tukey’s multiple comparison test were used [Instat Software (GraphPad)]. Differences were considered significant at P < 0.05.

RESULTS

VDR is expressed and functional in non-transformed mammary cells. The growth inhibitory effects of 1,25(OH)₂D₃, the VDR ligand, in breast cancer cells are well recognized; however, little is known about vitamin D signaling in nontumorigenic mammary cells. Therefore, we compared VDR expression and function in HMEC, MCF-7, and HK-8 cells (Fig. 1). VDR was detected in all cell lines, but was highest in the noncancerous HMEC cultures and lowest in HK-8 renal cells (Fig. 1A). In all cell lines, 100 nmol/L 1,25(OH)₂D₃ significantly (P < 0.05) induced the vitamin D responsive reporter gene containing the CYP24 promoter (Fig. 1B). Similarly, 100 nmol/L 1,25(OH)₂D₃ significantly (P < 0.05) enhanced CYP24 mRNA and protein expression in HMEC, MCF-7 and HK-8 cells (Fig. 1C,D). Based on the magnitude of CYP24 induction, MCF-7 cells were more sensitive to 1,25(OH)₂D₃ than HMEC and HK-8 cultures. Collectively, these data indicate that the VDR is expressed and functional in HMEC cultures, and that this signaling pathway remains intact in MCF-7 breast cancer cells.

Comparative effects of 1,25(OH)₂D₃ on growth of HMEC and MCF-7 cultures. Because 1,25(OH)₂D₃ induces differentiation, cell cycle arrest, and apoptosis in VDR positive breast cancer cells, we next compared the effects of 1,25(OH)₂D₃ on HMEC and MCF-7 cell growth (Fig. 2). HMEC cultures were dose
dependently growth inhibited by 1–100 nmol/L 1,25(OH)2D3 over 96 h of treatment (P, 0.05). In agreement with previous reports (24), MCF-7 cells were also growth inhibited by 10–100 nmol/L 1,25(OH)2D3 after 96 h (P, 0.05). These data indicate that 1,25(OH)2D3 induces growth inhibition in both immortalized and transformed mammary epithelial cell types.

**CYP27B1 expression and regulation in HMEC, MCF-7, and HKC-8 cultures.** The presence of CYP24 (Fig. 1) indicates that HMEC cultures have the potential to convert 25(OH)D3 to 24,25(OH)2D3, an inactive metabolite that does not bind VDR. To determine whether mammary cells also have the potential for bioactivation of vitamin D to 1,25(OH)2D3, we measured CYP27B1 expression by quantitative real-time PCR and immunoblotting (Fig. 3A, B). CYP27B1 mRNA was detected in all 3 cell lines, but expression was higher in HMEC and MCF-7 cultures compared with HKC-8 renal cells, which were characterized previously as an in vitro model of vitamin D metabolism (25). CYP27B1 protein was detected at 56 kDa in all cell lines, with the highest relative expression in MCF-7 cells. Consistent with known negative feedback of 1,25(OH)2D3 on its own synthesis (3), CYP27B1 mRNA expression in HMEC cultures was inhibited by 24 h of treatment with 100 nmol/L 1,25(OH)2D3 (P < 0.1, Fig. 3C). In contrast, 1,25(OH)2D3 did not reduce CYP27B1 mRNA expression in MCF-7 or HKC-8 cells (Fig. 3C).

**Effects of 25(OH)D3 on VDR target genes and growth in HMEC cultures.** We hypothesized that the presence of CYP27B1 would enable mammary cells to metabolize 25(OH)D3 to 1,25(OH)2D3, which could activate VDR in an autocrine fashion. This hypothesis predicts that 25(OH)D3 would exert similar effects on growth and VDR-mediated gene expression in HMEC cultures as 1,25(OH)2D3 at concentrations ≥ 10 nmol/L (Fig. 4). As would be predicted if metabolic conversion is a prerequisite for activity, HMEC were 10-fold less sensitive to 25(OH)D3 than 1,25(OH)2D3 (compare Figs 2A and 4A). In addition, 100 nmol/L 25(OH)D3 mimicked the effects of 1,25(OH)2D3 on the VDR target genes CYP24 (induction) and CYP27B1 (inhibition) (Fig. 4B). To exclude the possibility that 25(OH)D3 could directly activate VDR in the absence of metabolism, the effects of 25(OH)D3 and 1,25(OH)2D3 on CYP24 protein expression were measured in cells isolated from the mammary gland of CYP27B1 knockout mice (Fig. 4C). In these cells, 1,25(OH)2D3 clearly induced CYP24 (indicating the presence of functional VDR) but 25(OH)D3 did not, indicating that functional CYP27B1 is required for VDR target gene induction by 25(OH)D3. Collectively, these data strongly suggest that CYP27B1 in
mammary cells is capable of bioactivating 25(OH)D3 to 1,25(OH)2D3 which in turn activates VDR and inhibits cell proliferation.

Expression and function of VDR and CYP27B1 in primary mammary cell cultures. To exclude the possibility that the expression of CYP27B1 and VDR in telomerase-immortalized mammary cells could be secondary to the immortalization process, we utilized PHMEC. PHMEC cultures, derived from normal breast, are telomerase negative, have a finite lifespan, and express markers of mammary cell differentiation. Immunoblotting indicated that PHMEC cultures also express VDR, CYP27B1, and CYP24 proteins (Fig. 5A, top panels). For these cells, both 100 nmol/L 1,25(OH)2D3 and 100 nmol/L 25(OH)D3 induced CYP24 protein (Fig. 5A, bottom panels) and activated the VDR responsive reporter gene (Fig. 5B). As for HMEC cultures, 25(OH)D3 was less effective than 1,25(OH)2D3 in inducing the vitamin D responsive reporter gene and CYP24 protein expression. Time-course studies indicated that the growth of PHMEC cultures was significantly reduced within 72 h of treatment with either 1,25(OH)2D3 or 25(OH)D3 (Figure 5C). These data indicate that, like HMEC cultures, PHMEC cultures express functional VDR and CYP27B1, allowing cellular responses to both precursor (25(OH)D3) and active (1,25(OH)2D3) metabolites of vitamin D3.

DISCUSSION

In these studies we demonstrated that nontumorigenic human mammary epithelial cells express transcriptionally active VDR and are growth inhibited by its ligand, 1,25(OH)2D3. Furthermore, these cells express the vitamin D metabolizing enzymes CYP27B1 and CYP24, which act on the same substrate, 25(OH)D3, to generate either 1,25(OH)2D3 or 24,25(OH)2D3 respectively. Treatment of mammary cells with 25(OH)D3 activates VDR transcription, regulates VDR target genes, and inhibits growth, effects that mimic those of 1,25(OH)2D3. These observations suggest that, under basal conditions, CYP27B1 activity predominates over CYP24 activity, resulting in net conversion of 25(OH)D3 to 1,25(OH)2D3. Although confirmatory studies are required to measure CYP27B1 and CYP24 activity in HMEC cultures with accuracy, our data suggest that normal mammary cells are capable of 1,25(OH)2D3 biosynthesis.
from 25(OH)D₃. This suggestion is consistent with reports of 1,25(OH)₂D₃ production in homogenates of human breast tumors and adjacent normal breast tissue (20). Furthermore, CYP27B1 mRNA and protein are present and developmentally regulated in normal murine mammary gland (21).

These are the first studies to demonstrate that concentrations of 25(OH)D₃ within the range found in the human circulation (35–100 nmol/L) are growth inhibitory to nontumorigenic mammary cells. Further work will be necessary to define how 25(OH)D₃, the majority of which circulates bound to vitamin D binding protein (DBP), gains access to mammary cells. In particular, the relative contributions of free 25(OH)D₃ (which enters cells via diffusion) and DBP-bound 25(OH)D₃ (which enters cells via receptor mediated endocytosis) to the intracellular 25(OH)D₃ pool require clarification. Regardless of the specific mechanism, it is likely that low circulating 25(OH)D₃ subsequent to vitamin D deficiency would reduce substrate availability to CYP27B1 and limit 1,25(OH)₂D₃ production in the mammary gland. A suboptimal supply of 1,25(OH)₂D₃ could result in deregulation of both VDR-mediated gene expression and growth control, a concept supported by data from VDR knockout mice, which exhibit accelerated mammary gland development during puberty and pregnancy (21,26). Furthermore, inhibitory effects of dietary vitamin D and VDR agonists were reported in animal models of breast cancer (27–29). Collectively, these observations provide a potential biological basis for the epidemiologic observations linking vitamin D status in general, and 25(OH)D₃ in particular, to breast cancer risk (17,30).

Particularly relevant to the potential role of vitamin D in breast cancer, both aging and estrogen deficiency are associated with low vitamin D status. Aging reduces vitamin D production in the skin; therefore, elderly individuals are more dependent on dietary and supplemental vitamin D than younger individuals. Estrogen stimulates renal CYP27B1 activity, and estrogen deficiency is associated with low circulating 1,25(OH)₂D₃ (31). Thus, postmenopausal women, the population most at risk for breast cancer, have a high prevalence of marginal vitamin D status (32). Furthermore, it should be noted that the definitions of “adequate,” "low," and “deficient” circulating levels of 25(OH)D₃, as well as the intake necessary to sustain appropriate vitamin D status, are currently being reevaluated (1,33).

Our studies also examined the regulation of CYP27B1 and CYP24 gene expression by 1,25(OH)₂D₃ in mammary cells. In all breast-derived cell lines studied, 1,25(OH)₂D₃ induced CYP24, an expected finding because this gene promoter contains a well-characterized VDR responsive region. Consistent with the known negative feedback regulation of 1,25(OH)₂D₃ on its own production, 1,25(OH)₂D₃ downregulated CYP27B1 in nontumorigenic HMEC cultures (Fig. 3C). Surprisingly, 1,25(OH)₂D₃ did not inhibit CYP27B1 gene expression in MCF-7 cells despite the ability of this metabolite to activate VDR and inhibit growth in these cells. This finding suggests that CYP27B1 may be deregulated during transformation, a suggestion that is consistent with data indicating elevated expression of vitamin D–metabolizing enzymes in human breast tumors compared with adjacent normal tissue (20). Follow-up studies on the molecular regulation of CYP27B1 in a defined model of mammary cell transformation are currently in progress to test this hypothesis.

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LITERATURE CITED


