Microenvironment and Immunology

Vitamin D Deficiency Promotes Human Breast Cancer Growth in a Murine Model of Bone Metastasis

Li Laine Ooi1,2, Hong Zhou1, Robert Kalak1, Yu Zheng1, Arthur D. Conigrave2, Markus J. Seibel1, and Colin R. Dunstan1,3

Abstract

Vitamin D exerts antiproliferative, prodifferentiation, and proapoptotic effects on nonclassic target tissues such as breast. Blood levels of 25-hydroxyvitamin D [25(OH)D], the most sensitive indicator of vitamin D status, are inversely correlated with breast cancer risk; however, a causal relationship between vitamin D deficiency and breast cancer growth has not been assessed. We examined the effect of vitamin D deficiency on the intraskeletal growth of the human breast cancer cell line MDA-MB-231-TxSA in a murine model of malignant bone lesions. Subsets of mice were treated concurrently with osteoprotegerin (OPG) to abrogate bone resorption. Outcomes were assessed by repeated radiographic and end-point microcomputed tomography and histologic analyses. Mice weaned onto a vitamin D–free diet developed vitamin D deficiency within 4 weeks [mean ± SE serum 25(OH)D: 11.5 ± 0.5 nmol/L], which was sustained throughout the study and was associated with secondary hyperparathyroidism and accelerated bone turnover. Osteolytic lesions appeared earlier and were significantly larger in vitamin D–deficient than in vitamin D–sufficient mice after 2 weeks (radiographic osteolysis: +121.5%; histologic tumor area: +314%; P < 0.05). Although OPG treatment reduced the size of radiographic osteolytes and tumor area in both groups, tumors remained larger in OPG-treated vitamin D–deficient compared with OPG-treated vitamin D–sufficient mice (0.53 ± 0.05 mm² versus 0.19 ± 0.05 mm²; P < 0.05). We conclude that vitamin D deficiency promotes the growth of human breast cancer cells in the bones of nude mice. These effects are partly mediated through secondary changes in the bone microenvironment, along with direct effects of vitamin D on tumor growth.

Cancer Res; 70(5); 1835–44. ©2010 AACR.

Introduction

Epidemiologic studies in the United States and worldwide (1, 2) have shown an association between the increasing incidence of breast cancer with decreasing sunlight exposure. Vitamin D is produced endogenously in the skin via a sunlight-dependent mechanism, and low annual sunlight exposure (due to seasonal changes or geographic latitude) is a known cause of vitamin D deficiency (2–4), making vitamin D a candidate for modulating the associations between sunlight exposure and cancer. Consistent with this hypothesis, several epidemiologic studies found a strong correlation between vitamin D deficiency and increased incidence of breast, prostate, and colon cancers (1–5).

Vitamin D insufficiency and deficiency are widespread among postmenopausal women who are also at increased risk of developing breast cancer (6). Vitamin D deficiency is associated with increased breast cancer incidence and to poorer prognosis in women with early-stage disease (7). Therefore, the hypothesis that vitamin D deficiency promotes breast cancer growth is plausible. However, a causal link between vitamin D status and breast cancer growth has not been established in vivo.

In addition to classic hormonal actions of bioactive 1,25(OH)2D in calcium homeostasis, paracrine actions of vitamin D have been reported, highlighting its significance in tissues other than kidney, bowel, parathyroid, and bone. 1,25(OH)2D is an antiproliferative, prodifferentiation, and proapoptotic factor in normal tissue [e.g., skin (8), mammary gland (9, 10), and colon (11)] and malignant tissues [e.g., breast (9, 10, 12), prostate (13), and colon (11, 14) cancer]. However, apart from direct inhibitory effects on cancer cell proliferation, vitamin D may also suppress breast cancer growth in bone indirectly via modulation of the bone microenvironment. We previously reported that increasing bone remodeling by dietary calcium insufficiency promotes breast cancer growth, whereas inhibition of bone resorption inhibits breast cancer growth in bone (15, 16). Thus, vitamin D deficiency may also promote breast cancer growth in the bone environment by altering the osteoclast-mediated release of growth factors from the bone matrix (15–18) and/or through its direct effects on cancer cell behavior.
Vitamin D deficiency results in elevated parathyroid hormone (PTH) secretion (secondary hyperparathyroidism; refs. 19, 20). Elevated PTH levels stimulate calcium mobilization from skeletal stores (21, 22). At the same time, PTH promotes the renal conversion of 25-hydroxyvitamin D [25(OH)D] to 1,25-dihydroxyvitamin D [1,25(OH)2D] to increase intestinal calcium absorption. PTH stimulates the osteoblastic PTH receptor (17) to increase expression of receptor activator of NF-κB ligand, a potent activator of osteoclast recruitment and bone resorption. An abnormal increase in bone remodeling ensues, with a bias toward bone resorption, causing bone loss. In the presence of cancer, tumor-secreted PTH-related protein (PTHrP) mimics PTH actions, leading to the formation of osteolytic lesions. Subsequently, growth factors released from the bone matrix, such as transforming growth factor-β, insulin-like growth factors, and cytokines, complete a positive feedback loop, resulting in further tumor growth (17, 18, 23, 24).

Given the known effects of vitamin D deficiency on bone remodeling (25, 26), and the evidence for the inhibitory effects of 1,25(OH)2D on breast cancer cell proliferation (9, 10, 12), we hypothesized that vitamin D deficiency would promote breast cancer tumor growth in mice either directly or by effects on bone remodeling. In this study, we determined the effects of vitamin D deficiency on the growth of breast cancer implanted within bone and in the mammary fat pad. To identify effects mediated by bone resorption, we also blocked bone resorption via concurrent treatment with osteoprotegerin (OPG; ref. 27).

Materials and Methods

Breast cancer cell line. The MDA-MB-231-TxSA variant of MDA-MB-231 human breast cancer cells (28) was maintained in DMEM (Life Technologies Invitrogen) supplemented with 10% FCS (JRH Biosciences) and 1% penicillin-streptomycin solution (Life Technologies Invitrogen).

Cell proliferation assay. MDA-MB-231-TxSA cells were seeded in 12-well plates at a density of 1 × 104 per well. 1,25(OH)2D3 (Sigma-Aldrich) at concentrations of 10−7, 10−8, and 10−9 mol/L or vehicle (ethanol) was added on day 1 with replacement every 24 h. Cells were counted daily for 6 d with trypan blue exclusion used to confirm cell viability. Cell proliferation and apoptosis were assessed by 5′-bromodeoxyuridine incorporation (Amersham Life Science) and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) labeling (Roche). All experiments were repeated thrice.

Real-time reverse transcription-PCR. Total RNA was isolated at 4, 8, 24, and 48 h after treatment with 10−8 mol/L 1,25 (OH)2D3 using Nucleospin RNA II kit (Macherey-Nagel GmbH & Co. KG). First-strand cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen) after oligo(dT) (Promega Corp.) priming. Real-time reverse transcription-PCR was performed using human-specific primers to VDR (GenBank accession no. NM_001017535), vitamin D 24-hydroxylase (CYP27α; GenBank accession no. NM_000782), and vitamin D 1α-hydroxylase (CYP27B1; GenBank accession no. NM_000785) with iQ SYBR Green Supermix (Bio-Rad Laboratories) on an iCycler IQ5 real-time PCR detection system (Bio-Rad Laboratories). Human glyceraldehyde-3-phosphate dehydrogenase gene expression was used for cDNA normalization.

Generation of vitamin D–deficient mice. Animal studies were conducted in accordance with a protocol approved by our Institutional Animal Ethics Committee. An initial study was conducted to confirm the establishment of sustained vitamin D deficiency in nontumor-bearing nude mice. Three-week-old female BALB/c nu/nu mice (ARC) were kept for 10 wk under specific pathogen-free, temperature-controlled conditions with 12-h day/night cycles. Mice were given ad libitum either normal mouse chow (vitamin D–sufficient diet) containing 0.79% calcium and 2,000 IU/kg cholecalciferol (vitamin D3) or a vitamin D–free diet, identical except for absence of added cholecalciferol (Specialty Feeds). Mice were housed in incandescent light conditions to minimize endogenous production of vitamin D. Every 2 wk, mice were anaesthetized with ketamine/xylazine (75/10 mg/kg) i.p. and 100 μL blood was collected retro-orbitally. After completion of this study, mice were euthanized and the tibiae were removed for analysis by micro–computed tomography (CT). Sustained vitamin D deficiency was established after 6 wk on a vitamin D–free diet (see Results), and this period was used to generate vitamin D–deficient mice for tumor implantation studies.

Intratibial xenograft model of breast cancer metastasis. Mice after 6 wk on vitamin D–sufficient and vitamin D–free diets were randomized into two treatment groups—vitamin D sufficient (n = 7) and vitamin D deficient (n = 7)—and anaesthetized. As previously described (15), 5 × 104 cells/10 μL of cell suspension were slowly injected through the anterior tuberosity of the proximal tibia in both limbs. The analgesic carprofen (Rimadyl; 5 mg/kg s.c.) was given at the time of inoculation for pain control. MDA-MB-231-TxSA breast cancer cells produce lytic bone lesions in this model (15, 16), which were followed by X-rays taken 10 d after the procedure and again after the end point at 14 d. The identical experiment was repeated and extended to 28 d (n = 7 per group), with X-rays and blood collected at biweekly intervals. To investigate osteoclast-independent effects of vitamin D on tumor growth in bone, a third experiment was performed using the extended end-point (28 d) model and treatment with recombinant OPG (OPG-Fc). Recombinant OPG (OPG-Fc; Amgen, Inc.), a fusion protein consisting of OPG (amino acids 22–194) and the Fc domain of human IgG (27), was used in this study. Vitamin D–sufficient (n = 7) and vitamin D–deficient (n = 7) mice were concurrently treated with 1 mg/kg/d of OPG-Fc s.c. beginning 3 d before intratibial inoculation of breast cancer cells as previously described (15). Subsequently, mice were administered 3 mg/kg of OPG-Fc every third day throughout the study. After euthanasia, the tibiae and femora were removed for micro-CT and histologic analyses.

Biochemical assays. Blood sampling was conducted either retro-orbitally after 4, 6, and 8 wk or via cardiac puncture after 10 wk. Plasma intact PTH (iPTH; Mouse Intact PTH ELISA kit, Immutopics, Inc.), 25(OH)D, tartrate-resistant acid phosphatase 5b (TRAcP5b), rat/mouse procollagen type
In N-terminal propeptide (PINP; all from Immunodiagnostic Systems Ltd.), and serum calcium (QuantiChrom Calcium Assay kit, BioAssay Systems) were assessed.

Radiographic quantification of osteolytic lesions. At days 10, 14, 21, and 28 after intratibial inoculation, mice were anaesthetized and digital radiographs of their hind limbs were recorded. Image analysis software (ImageJ, NIH) was used to quantify areas of osteolytic destruction.

Micro-CT analysis. Micro-CT images were obtained using a SkyScan 1172 scanner (SkyScan) at 100 kV, 100 μA with a 1-mm aluminum filter. One thousand and eight hundred cross-sectional projections were collated at a resolution of 6.93 μm/pixel. Sections were reconstructed using a modified Feldkamp cone-beam algorithm, with beam hardening correction at 50%. Three-dimensional modeling was achieved using the VGStudio MAX v1.2 software (Volume Graphics GmbH).

Histology. Tibiae harvested at days 14 and 28 after intratibial injection were fixed in phosphate-buffered 4% paraformaldehyde (pH 7.4) for 24 h. Samples were decalcified in 10% EDTA (pH 7.5) at 4°C for 2 wk and paraffin embedded. Five-micrometer longitudinal serial sections from three representative levels were cut from each specimen and stained with H&E for routine examination. Three separate sections located ∼200 μm apart were assessed using BIOQUANT OSTEO image analysis software to determine total tumor area. Osteoclasts were identified by histochemical staining for TRAcP, a marker for osteoclasts, as previously described (15). Tumor sections were analyzed for mitotic activity (29, 30) and stained with ApopTag TUNEL kit for apoptosis.

Figure 1. MDA-MB-231-TxSA breast cancer cells respond to 10⁻⁸ mol/L 1,25(OH)₂D₃. A, the 1,25(OH)₂D₃ inactivating enzyme CYP24 is modestly upregulated by 1,25(OH)₂D₃. B, MDA-MB-231-TxSA cells express VDR and respond to 1,25(OH)₂D₃ stimulus by increasing VDR expression by ∼4-fold. **, P < 0.001. C, CYP27B1, activating enzyme of 25(OH)D₃, is expressed in these cells and downregulated. *, P < 0.05. D, MDA-MB-231-TxSA growth is inhibited by 1,25(OH)₂D₃ with maximal effects at 10⁻⁸ and 10⁻⁷ mol/L. Cell proliferation is significantly reduced by 10⁻⁸ and 10⁻⁷ mol/L 1,25(OH)₂D₃. *, P < 0.05. Cell apoptosis is not altered by 1,25(OH)₂D₃, BrdUrd, 5′-bromodeoxyuridine.
The ratio of positive cells was calculated from 10 random fields of nonnecrotic tumor measured at ×400 magnification.

**Bone histomorphometry.** Serial sections of nontumor-bound distal femora were analyzed for bone volume and bone turnover. In TRAcP-stained sections, the number of osteoclasts (identified as TRAcP positive, multinucleated cells adjacent to bone surfaces) per millimeter bone surface, as well as percentage osteoclast surface per bone surface, was calculated (three representative levels, ×200 magnification). Additionally, osteoblast surface per bone surface was assessed in H&E-stained sections (×400 magnification).

**Orthotopic implantation of MDA-MB-231-Tx cells.** Cells were prepared as above and suspended in cold 50% Matrigel/PBS at a concentration of 2 × 10^7 cells/mL. Suspension (100 μL) was implanted s.c. in the mammary fat pad (n = 15). Tumor size was measured in three dimensions by calipers to determine tumor volume (31) every second day and by tumor weight at euthanasia on day 20.

**Statistical analysis.** All data are presented as mean ± SE. Statistics were performed using Student’s t test for simple comparison of two means. One-way ANOVA followed by Tukey’s post hoc test was used for multiple comparisons. Differences were considered statistically significant when P < 0.05.

**Results**

1,25(OH)₂D directly inhibits breast cancer cell proliferation.** The MDA-MB-231-TxSA cell line expressed the VDR and responded to 1,25(OH)₂D₃. When treated with 10⁻⁸ mol/L 1,25(OH)₂D₃, CYP24 expression was enhanced, peaking at 4 hours (by 1.8-fold; Fig. 1A), whereas VDR peaked at 8 hours into treatment (by 4-fold), consistent with the activation of the VDR and of transcription of the VDR-responsive gene encoding the 1,25(OH)₂D₃-inactivating enzyme, CYP24 (Fig. 1B). Conversely, 1,25(OH)₂D₃ downregulated the 25(OH)D₃-activating enzyme, CYP27B1 by ~20% (Fig. 1C).

The maximal inhibitory effects on cell proliferation were found at 10⁻⁸ mol/L 1,25(OH)₂D₃ (Fig. 1D). After 6 days of culture, a 47.6% reduction in cell number was observed in cells treated with 10⁻⁸ mol/L 1,25(OH)₂D₃. Treatment with 10⁻⁹ mol/L 1,25(OH)₂D₃ had no effect on cell proliferation, whereas at a higher concentration 10⁻⁷ mol/L 1,25(OH)₂D₃ had no additional inhibitory effect on cell proliferation. 1,25(OH)₂D₃ did not alter proportions of apoptotic cells.
Dietary vitamin D restriction induces vitamin D deficiency. Sustained vitamin D deficiency was established in mice after 4 weeks on a vitamin D–free diet and maintained through the remaining 6 weeks of the study. Vitamin D–deficient mice had plasma 25(OH)D levels of ≤15 nmol/L (11.5 ± 0.5 nmol/L), whereas their vitamin D–sufficient counterparts exhibited levels of ≥100 nmol/L (119.1 ± 7.3 nmol/L; Fig. 2A). These 25(OH)D levels in mice are comparable with humans (26), where vitamin D levels ≤15 nmol/L indicate severe vitamin D deficiency and levels ≥100 nmol/L indicate vitamin D repletion (32).

At the time of cancer cell inoculation (week 6), vitamin D–deficient mice showed a trend toward increased serum PTH levels (Fig. 2A). By 10 weeks, serum PTH levels were significantly raised in vitamin D–deficient mice, consistent with hypoparathyroidism secondary to vitamin D deficiency. Serum calcium levels, however, were not significantly different in vitamin D–sufficient and vitamin D–deficient mice at 6 weeks (2.39 ± 0.01 mmol/L versus 2.32 ± 0.07 mmol/L, respectively; \( P = 0.20 \)), and these levels did not change significantly throughout the study (data not shown). More importantly, plasma TRACP5b and P1NP levels were significantly increased in vitamin D–deficient compared with vitamin D–sufficient mice at each time point (Fig. 2A).

Vitamin D–sufficient and vitamin D–deficient mice had similar weights throughout the study period (Fig. 2C). Vitamin D deficiency reduced bone density as revealed by micro-CT analysis of the proximal tibiae. In particular, statistically significant reductions in trabecular bone volume (33% less), trabecular thickness (16% less), and trabecular number (23% less) were observed (Fig. 2B).

Vitamin D deficiency enhances breast cancer–induced osteolysis in the tibial metaphysis. Having established stable vitamin D deficiency in mice, we evaluated the effect of vitamin D deficiency on breast cancer growth in bone. When monitored by radiographic imaging, osteolytic lesions appeared earlier in vitamin D–deficient than in vitamin D–sufficient mice (Fig. 3B). Furthermore, radiographic lesions increased

Figure 3. Progression of osteolytic lesions as monitored by X-ray imaging (n = 7). A, vitamin D–deficient mice developed lesions faster and more extensively than vitamin D–sufficient mice. Arrows, lytic lesion. Lytic lesion areas formed in vitamin D–deficient mice are significantly larger than those in vitamin D–sufficient mice until day 21. B, vitamin D–deficient mice showed a decreased time to first lesion compared with vitamin D–sufficient mice. C, histomorphometric analysis showed that tumor area was larger in vitamin D–deficient compared with vitamin D–sufficient mice at day 14 but not at day 28. *, \( P < 0.05 \) versus vitamin D sufficient.
in size more rapidly in vitamin D–deficient mice (Fig. 3A). Specifically, lesion areas in vitamin D–deficient mice were increased by 105.5% (day 10), 121.5% (day 14), and 26.9% (day 21) compared with vitamin D–sufficient mice. By day 28, there was no statistically significant difference in the sizes of lytic lesions in these two groups, which were typically >1 mm².

Consistent with the radiographic lesion areas shown in Fig. 3A, histologic analysis of tumor area revealed significantly larger tumors in vitamin D–deficient mice terminated 14 days after intratibial injection of cancer cells (Fig. 3C). In contrast, histologic analysis 28 days after cancer cell inoculation did not show any differences between the two groups.

To confirm that the effects of vitamin D deficiency on bone remodeling paralleled those seen in nontumor-bearing mice, nontumor-involved distal femoral metaphyses were assessed histologically for bone remodeling activity. In vitamin D–deficient mice, we observed a 37.5% reduction in trabecular bone volume (% bone volume 34.8 ± 1.54% versus 55.6 ± 0.6% in vitamin D–sufficient mice; P < 0.001), a 121% increase in osteoclast surface (% osteoclast surface 15.3 ± 0.8% versus 6.9 ± 0.3% in vitamin D–sufficient mice; P < 0.001), and a 63% increase in osteoblast surface (% osteoblast surface 22.8 ± 1.0% versus 14.0 ± 0.5% in vitamin D–sufficient mice). Taken together, the results indicate that breast cancer growth in bone is enhanced in states of vitamin D deficiency in association with increased bone turnover.

**Vitamin D deficiency increases breast cancer growth in bone in the absence of endosteal bone resorption.** To investigate the interaction between tumor cell growth and the bone microenvironment independent of bone resorption, experiments were repeated with OPG treatment to silence bone resorption (27). Radiographic analysis showed that development of lytic lesions was profoundly inhibited by OPG treatment in both vitamin D–deficient and vitamin D–sufficient mice. Histologic analysis revealed that treatment with OPG entirely suppressed endosteal osteoclasts in the bones of both vitamin D–sufficient and vitamin D–deficient mice (Fig. 4), along with a marked increase in trabecular bone volume associated with growth-related retention of primary spongiosa as has been previously reported for OPG and bisphosphonate treatments (16, 27). In addition, profound suppression of plasma TRAcP5b levels (Fig. 5A) was observed in OPG-treated mice, despite elevated iPTH levels (Fig. 5B). However, TRAcP5b levels remained higher in vitamin D–deficient OPG-treated versus vitamin D–sufficient OPG-treated mice. Whereas no endosteal osteoclasts were present in OPG-treated mice, occasional periosteal osteoclasts were observed, which may have contributed to the measured plasma TRAcP5b levels (data not shown). Periosteal osteoclastic bone resorption has previously been reported to be more resistant to OPG inhibition than endosteal bone resorption (27).

Tumor burden at study completion was greatly reduced by OPG treatment in both vitamin D–deficient and vitamin D–sufficient mice, consistent with previous findings that tumor growth in bone is dependent on bone resorption. Intriguingly, in the presence of OPG, a 184.4% increase in tumor burden was observed in vitamin D–deficient mice (Fig. 5C).

**Vitamin D deficiency enhances tumor cell proliferation.** Tumors from vitamin D–deficient mice had higher mitotic

![Figure 4. Representative TRAcP-stained sections from each treatment group at end point (28 d). Magnification, ×200. Arrows, osteoclasts. T, tumor; B, bone. There were no detectable osteoclasts in OPG-treated mice.](image-url)
activity compared with vitamin D–sufficient mice irrespective of OPG treatment. OPG treatment significantly reduced the proportion of actively dividing cells in both diet groups (Fig. 6A). Rates of cell apoptosis were not altered by vitamin D deficiency or by OPG treatments (Fig. 6B).

Vitamin D deficiency does not alter tumor growth in the mammary fat pad. Tumor weights were similar in both vitamin D–sufficient and vitamin D–deficient mice (0.11 ± 0.02 g in vitamin D–deficient versus 0.10 ± 0.02 g in vitamin D–sufficient mice; P = not significant). Growth rates of tumor size as measured progressively by calipers were also similar at all time points (see Supplementary Fig. S1).

Discussion

Vitamin D deficiency promotes tumor growth in a murine model of bone metastasis, resulting in larger osteolytic lesions. Our results indicate that epidemiologic evidence of inverse associations between vitamin D status and breast cancer progression may have causal significance (1).

We present a model of vitamin D deficiency in nude mice, which is simple to generate (as it does not require treatment of pregnant dams), stable, and highly reproducible. We successfully induced profound vitamin D deficiency in female nude mice characterized by low plasma levels of 25(OH)D, elevated PTH levels, bone loss, and increased bone remodeling. Plasma 25(OH)D concentrations <15 nmol/L and marked secondary hyperparathyroidism are changes similar to those reported in humans with vitamin D deficiency (25, 33). Of note, serum calcium levels were not altered, indicating that calcium homeostatic mechanisms were not overwhelmed and that calcium levels were maintained through higher levels of PTH and at the expense of accelerated bone remodeling and skeletal calcium mobilization. Maintenance of normal calcium levels is also seen in humans with vitamin D deficiency (34).

Plasma TRAcP5b and P1NP were elevated in vitamin D deficiency, consistent with the effect of hyperparathyroidism on bone turnover. The gradual decline in absolute TRAcP5b and P1NP levels in both groups of mice is expected due to slowing growth rates associated with normal aging. Besides detection of these systemic changes, we confirmed increased numbers of osteoclasts and osteoblasts in the proximal tibial metaphyses by histomorphometry. Thus, we have established a model of severe vitamin D deficiency in which to investigate effects of vitamin D deficiency on breast cancer growth in bone.

The mechanisms by which vitamin D deficiency promotes tumor growth in bone in this study are currently uncertain. However, several mechanisms are conceivable. First, as shown above, vitamin D deficiency induces hyperparathyroidism (34, 35) and leads to an increase in bone resorption.
Increased bone resorption is known to enhance intraskeletal tumor growth (15, 23, 24). Hence, our findings create an extension to the widely accepted concept of the vicious cycle (23, 24), with the participation of vitamin D deficiency augmenting the process. At later time points (day 28), in the absence of OPG treatment, the effects of vitamin D deficiency on tumor size are lost. It is possible that enlarging tumors in the bone cavity progressively control the local environment by increasing bone resorption through release of PTHrP and other proresorptive factors, thereby escaping the constraints of the normal bone microenvironment. This is supported by our finding that vitamin D deficiency effects on growth are maintained at 28 days when OPG treatment is provided to block both bone resorption and escape from the bone environment.

Inhibition of bone turnover by OPG interrupts the vicious cycle. Inhibition of osteoclast activity reduced tumor growth significantly in both groups of mice, consistent with our previous results studying effects of antiresorptive treatments on tibial tumor growth with this cell line (16). Based on these results, it is apparent that growth of breast cancer cells in the bone is largely dependent on the ability of the tumor cell to manipulate bone turnover. However, even when OPG treatment was used to silence bone resorption, tumor areas were larger in bones of vitamin D-deficient mice, indicating a contribution of vitamin D status independent of bone environment. Radiological evidence of inhibition of bone resorption and histologic demonstration of an absence of endosteal osteoclasts confirmed that bone resorption in these mice was profoundly abrogated.

Therefore, there seems to be a contribution from vitamin D deficiency in aggravating tumor growth in bone that is independent of bone resorption. The data suggest that vitamin D deficiency may enhance tumor growth in the bone environment by two means: first, via increasing bone resorption, and second, by an action that is independent of the bone effects of vitamin D deficiency. However, TRAcP5b levels were relatively higher in these vitamin D-deficient compared with vitamin D-sufficient mice, indicating that a difference in osteoclast activity may be maintained despite OPG treatments. Although OPG treatment caused complete disappearance of osteoclasts within the medullary cavity of the tibia (Fig. 4) including bone surfaces adjacent to tumors, osteoclasts were observed on periosteal bone surfaces remote from tumor, which may have contributed to the increased TRAcP5b levels and induced a systemic enhancement of tumor growth.

In addition to its effects on the bone microenvironment, vitamin D deficiency may remove vitamin D–dependent inhibition of tumor cell proliferation. 1,25(OH)2D seems to have autocrine and/or paracrine actions. For example, in normal mammary epithelia and mammary neoplasms, it is reported to have antiproliferative, prodifferentiation, and proapoptotic effects (8, 11, 36). Our results indicate that 1,25(OH)2D3 has direct antiproliferative effects on MDA-MB-231-TxSA cells, which is consistent with observations of others (8, 11, 36) showing 1,25(OH)2D3 dose-dependent inhibition of cancer cell proliferation in vitro (breast, colon, thyroid, skin, and head and neck). In contrast to previous studies showing parental MDA-MB-231 cells as a VDR− mouse cell line (37), we showed that the MDA-MB-231-TxSA variant used in our studies expresses the VDR and that 1,25(OH)2D3 upregulated VDR and CYP24 expression. This cell line also expresses CYP27B1 and thus could locally convert 25(OH)D to biologically active vitamin D, 1,25(OH)2D. In the presence of exogenous 1,25(OH)2D3, a modest downregulation of CYP27B1 was noted, suggesting the operation of negative feedback regulation of endogenous 1,25(OH)2D production. Alternative mechanisms for the effects of vitamin D deficiency in vivo could include immune modulation (22), loss of direct effects of 25(OH)D on cells, or some other alteration of the tumor environment. The lack of significant effects of vitamin D deficiency on the growth of MDA-MB-231-TxSA cells implanted in the mammary fat pad, and the loss of effects at later time points in the tibia, suggests that the effects of vitamin D deficiency on tumor growth may be site and/or growth stage specific.

The results of this study, with the currently published epidemiologic data, highlight the potential importance of correcting vitamin D deficiency in women with breast cancer (5, 38). Humans acquire vitamin D primarily through biosynthesis in the skin (2, 34), although dietary sources of vitamin D are important contributors to maintain healthy levels of vitamin D (33, 39). It is reported that diets enriched in vitamin D and
calcium may prevent tumorigenicity in mammary tissue (39). Serum 25(OH)D levels required to optimally support healthy bones lie above 75 nmol/L (35). At intermediate 25(OH)D levels between 20 and 75 nmol/L, negative effects on bone, including increased bone turnover and secondary hyperparathyroidism, have been reported (26, 40). Although these intermediate effects have not been addressed in the current study, they may also affect the progression of breast cancer growth in bone. However, we acknowledge that the effects of vitamin D deficiency on cancer growth observed in this study may be limited to this murine model and are not necessarily directly translatable to human disease.

In summary, our work provides evidence that maintenance of adequate vitamin D levels may reduce the ability of breast cancer cells to grow in the bone environment. These results indicate a need for clinical trials in humans to investigate the value of correcting vitamin D deficiency in limiting the progression of breast cancer.

References

30. Ladekarl M. Choice of methodology for quantifying cancer structures in tissue sections. A comparison of 2- and 3-dimensional estimators

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Mambdouh Khalil and his staff for excellent animal care, the staff at the NANO Major National Research Facility at the Electron Microscope Unit (University of Sydney) for technical assistance, Professor Toshiyuki Yoneda for the provision of the TsA cell line, and Amgen, Inc. for OPG.

Grant Support

National Health and Medical Research Council of Australia grant 352332 and The University of Sydney Cancer Research Fund.

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.

Received 08/26/2009; revised 12/13/2009; accepted 12/14/2009; published OnlineFirst 02/16/2010.


33. Vieth R, Chan PCR, MacFarlane GD. Efficacy and safety of vitamin D3 intake exceeding the lowest observed adverse effect level. Am J Clin Nutr 2001;73:288–94.


Vitamin D Deficiency Promotes Human Breast Cancer Growth in a Murine Model of Bone Metastasis

Li Laine Ooi, Hong Zhou, Robert Kalak, et al.

Cancer Res 2010;70:1835-1844. Published OnlineFirst February 16, 2010.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-09-3194

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/02/15/0008-5472.CAN-09-3194.DC1

Cited articles
This article cites 39 articles, 6 of which you can access for free at:
http://cancerres.aacrjournals.org/content/70/5/1835.full#ref-list-1

Citing articles
This article has been cited by 8 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/70/5/1835.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.