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Vitamin D deficiency promotes growth of MCF-7 human breast cancer in a rodent model of osteosclerotic bone metastasis

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ABSTRACT

Introduction: Breast cancer metastases to bone are common in advanced stage disease. We have recently demonstrated that vitamin D deficiency enhances breast cancer growth in an osteolytic mouse model of breast cancer metastasis. In this study, we examined the effects of vitamin D deficiency on tumor growth in an osteosclerotic model of intra-skeletal breast cancer in mice.

Methods: The effects of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] on proliferation and apoptosis of MCF-7 breast cancer cells, and changes in the expression of genes within the vitamin D metabolic pathway (VDR, 1α- and 24-hydroxylase) were examined *in vitro*. MCF-7 breast cancer cells were injected intra-tibially into vitamin D deficient and vitamin D sufficient mice co-treated with and without osteoprotegerin (OPG). The development of tumor-related lesions was monitored via serial X-ray analysis. Tumor burden and indices of proliferation and apoptosis were determined by histology along with markers of bone turnover and serum intact PTH levels.

Results: In vitro, MCF-7 cells expressed critical genes for vitamin D signalling and metabolism. Treatment with $1,25(OH)_2D_3$ inhibited cell growth and proliferation, and increased apoptosis. *In vivo*, osteosclerotic lesions developed faster and were larger at endpoint in the tibiae of vitamin D deficient mice compared to vitamin D sufficient mice (1.49 ± 0.08 mm^2 versus 1.68 ± 0.15 mm^2, $P<0.05$). Tumor area was increased by 55.8% in vitamin D deficient mice (0.81 ± 0.13 mm^2 versus 0.52 ± 0.11 mm^2 in vitamin D sufficient mice). OPG treatment inhibited bone turnover and caused an increase in PTH levels, while tumor burden was reduced by 90.4% in vitamin D sufficient mice and by 92.6% in vitamin D deficient mice. Tumor mitotic activity was increased in the tibiae of vitamin D deficient mice and apoptosis was decreased, consistent with faster growth.

Conclusion: Vitamin D deficiency enhances both the growth of tumors and the tumor-induced osteosclerotic changes in the tibiae of mice following intratibial implantation of MCF-7 cells. Enhancement of tumor growth appears dependent on increased bone resorption rather than increased bone formation induced by these tumors.

Introduction

Vitamin D deficiency in the general population is a health problem of enormous and increasing dimensions. The most important risk factors for developing vitamin D deficiency include low sunlight exposure [1–4], higher geographical latitude [1,5–7], skin pigmentation [7,8], heavy sunscreen use [9,10] as well as intake of diets containing inadequate levels of vitamin D [11–13]. The central role played by vitamin D in maintaining bone health and metabolism is reasonably well understood [10,14]. A healthy vitamin D status is critical for normal calcium and phosphate homeostasis, maintenance of optimal bone mass as well as for sustaining musculoskeletal function [15–17]. Severe vitamin D deficiency results in osteomalacia (adults) or rickets (children), a rare but highly symptomatic disease characterised by pain, bony deformities and fractures [13]. In contrast, mild to moderate vitamin D deficiency, as frequently seen in the general population, is asymptomatic. As vitamin D levels are not routinely screened, the clinical relevance of mild to moderate hypovitaminosis D is often underestimated.

In addition to the role of vitamin D in maintaining healthy bones, numerous studies have identified an association between vitamin D deficiency and increased cancer risk (e.g. in breast, prostate and colon

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cancers) [18–21]. Of particular interest in breast cancer, vitamin D deficiency has been associated with increased incidence, faster disease progression and poorer prognosis [18,22,23]. Moreover, the population of women at higher risk for developing breast cancer are also likely to simultaneously have poor vitamin D status. However, little research has been conducted to determine if the association between vitamin D deficiency and more aggressive breast cancer represents a causal relationship.

Secondary spread of breast cancer frequently involves metastasis to bone and can result in debilitating pain, immobility, fractures and spinal compression syndromes [24,25]. Breast cancer growth in bone most frequently produces osteolytic lesions and the pathophysiology underlying bone lysis has been extensively studied. However breast cancer cells induce osteosclerotic lesions in approximately 20% of all breast cancer cases with skeletal involvement [25]. The pathophysiological origins of these lesions are not well understood [26]. Clinical consequences of osteosclerotic metastases include bone pain and fractures similar to that seen in patients with osteolytic metastases. At least in part, this appears to be from the excessive formation of poorly organised and mineralized bone matrix contributing to weak mechanical qualities [25]. In addition, extensive osteosclerotic lesions may result in hypocalcemia (as opposed to hypercalcemia in osteolytic disease) due to skeletal accumulation of calcium in new woven bone [27].

We have previously demonstrated that in the relatively vitamin D insensitive MDA-MB-231 breast cancer cell line (which produces purely osteolytic lesions), early tumor growth in bone is enhanced by deficiencies of both calcium [28] and vitamin D [29]. It is unknown, however, whether vitamin D deficiency elicits a similar response pattern in tumors that generate predominantly osteosclerotic bone metastases. In this study, we therefore evaluated the effects of vitamin D deficiency on breast cancer tumor growth, both in the bone environment and in the mammary fat pad, of a cell line (MCF-7) which in our hands induces mixed osteolytic/osteosclerotic lesions when implanted in the tibiae of nude mice [26]. We utilized an established model of vitamin D deficiency in immuno-deficient mice, previously developed in our laboratory, that produces serum levels of 25(OH)D in the range 10–15 nmol/L, comparable to moderately severe vitamin D deficiency in humans [29]. In addition, these mice demonstrated evidence of secondary hyperparathyroidism and increased bone remodelling, as well reduced bone mass as a consequence of vitamin D deficiency.

Materials and methods

Breast cancer cell line

MCF-7 human breast cancer cell line was originally obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and was maintained in RPMI medium (GIBCO® Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS)(JRH Biosciences, Lenexa, KS, USA), 1% penicillin-streptomycin solution (GIBCO® Invitrogen, Carlsbad, CA, USA) and 0.02% human insulin (Humulin R, Eli Lilly& Co., Indianapolis, IN, USA).

Cell proliferation assay

MCF-7 cells were seeded in 12-well plates at a density of 2×10^4 cells/well. $1,25(OH)_2D_3$ (Sigma-Aldrich, St. Louis, MO, USA) at concentrations of 10^{-7} M, 10^{-8} M and 10^{-9} M or vehicle (ethanol) was added on day 1 with replacement every 24 h. Cells were counted daily for 6 days with trypan blue exclusion used to confirm cell viability. Cell proliferation and apoptosis were assessed by 5'-Bromodeoxyuridine (BrdU) incorporation (Amersham Life Science, Buckinghamshire, UK) and TUNEL labelling (Roche, Mannheim, Germany). All experiments were repeated three times.

Real-time RT PCR

Total RNA was isolated at 4, 8, 24 and 48 h after treatment with 10^{-8} M $1,25(OH)_2D_3$ using Nucleospin RNA II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) in accordance with the manufacturer's protocol. First strand cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) following oligo (dT) (Promega Corp., Madison, WI, USA) priming. Real time RT-PCR were performed using human-specific primers to VDR (GenBank accession no. NM_001017535), vitamin D 24-hydroxylase (CYP24, GenBank accession no. NM_000782) vitamin D 1α-hydroxylase (CYP27B1α, GenBank accession no. NM_000785), receptor activator of NFkB ligand (RANKL) (TNSF11) GeneBank accession no. NM_057149 and osteoprotegerin (TNFRSF11B) GenBank accession no. NM_002546 with iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on an iCycler iQ5 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). Human

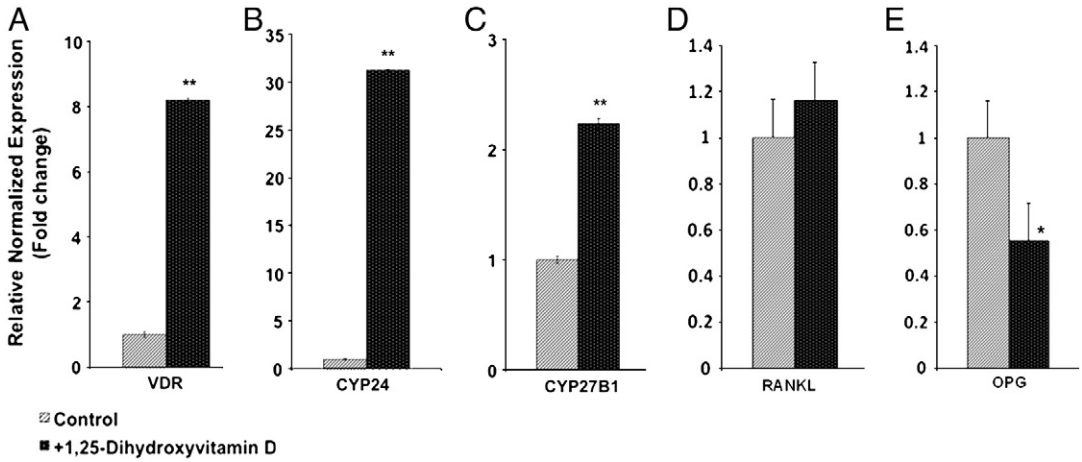

Fig. 1. mRNA expression of vitamin D-related genes, RANKL and OPG by 10^{-8} M $1,25(OH)_2D_3$ in cultivated MCF-7 breast cancer cells after 8 h treatment. (A) MCF-7 cells express VDR and are stimulated by $1,25(OH)_2D_3$ to increase VDR expression by approximately 8-fold. (B) The $1,25(OH)_2D_3$ inactivating enzyme, 24-hydroxylase (CYP24) is strongly induced (31-fold) by $1,25(OH)_2D_3$. (C) 1α-hydroxylase (CYP27B1) is up-regulated by about 2-fold when treated with $1,25(OH)_2D_3$. RANKL expression is not changed (D) and OPG expression is downregulated. (E) All gene expression results were obtained in single experiments performed in triplicates and are representative of three independent experiments. Different to control $^*P<0.05$, $^{**}P<0.001$.