1α,25-Dihydroxyvitamin D₃ is a preventive factor in the metastasis of lung cancer

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Introduction

Lung metastatic neoplasms are the major cause of cancer mortality. Despite progress in the development of diagnostic techniques for the early detection of lung cancer and improvements in surgical procedures, the prognosis of patients with lung cancer is generally poor, even in the early stages of the disease, as compared with other malignant neoplasms (1). An estimated 90 000 new cases of lung cancer were diagnosed in 2003 in Japan, and lung cancer will remain the leading cause of cancer death. From a clinical viewpoint, metastasis to mediastinal lymph nodes from the primary lesion is a particular challenge to the treatment of lung cancer and if lung cancer cells enter the metastatic phase the chance of recovery for the patient drops remarkably.

A biologically active metabolite of vitamin D₃, 1α,25-dihydroxyvitamin D₃ [1α,25(OH)₂D₃], is involved in essential cell regulatory processes, such as proliferation, differentiation and apoptosis (2–5). It has been shown that this hormone promotes cellular differentiation and inhibits the proliferation and invasive potential of a number of different cancer cells in vitro (6,7). Studies in vivo demonstrated that 1α,25(OH)₂D₃ slows the progression of breast, prostate and other carcinomas (8–11). These findings, in conjunction with the fact that the vitamin D receptor (VDR) is present in normal and tumor cells, suggest that 1α,25(OH)₂D₃ acts primarily as an intrinsic and preventive factor to stop the growth and metastasis of cancer cells (12–14). These properties suggest the possible clinical use of 1α,25(OH)₂D₃ in the treatment of cancers. However, the potent hypercalcemic activity of 1α,25(OH)₂D₃ has precluded its application as a pharmacological agent and limited a number of approaches using animal models (15). For this reason, various 1α,25(OH)₂D₃ analogs with reduced calcemic activity that retain the antiproliferative activity have been synthesized (6,16). In addition, epidemiological studies suggest that vitamin D₃ is involved in the pathogenesis of some cancer types. Several lines of evidence indicate an inverse correlation between dietary vitamin D intake or sunlight exposure and the prevalence of colorectal cancer and an association of high vitamin D₁ intake with a low incidence of breast and prostate cancer, although similar studies on human lung cancer have yet to be conducted (17–19).

In the cascade of biological events that leads to the establishment of systemic metastasis, tumor cells must complete a sequence of interactions with the host homeostasis mechanisms and avoid destruction by the host defenses (20–22). Metastasis begins with an invasive process by which tumor cells penetrate the epithelial basement membrane, enter the underlying host stroma surrounding the primary neoplasm and subsequently reach and penetrate the vasculature. Because of the large number of capillary blood vessels in the lungs, tumor cells circulating in the blood are more likely to lodge in these organs, thereby leading to substantial metastatic growth there. Thus, metastasis of cancer to the lung is a particularly

Abbreviations: 1α,25(OH)₂D₃, 1α,25-dihydroxyvitamin D₃; DMBA, 7,12-dimethylbenz[a]anthracene; FBS, fetal bovine serum; GFP, green fluorescent protein; LLC, Lewis lung carcinoma; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; PTH, parathyroid hormone; VDR, vitamin D receptor; VDRE, vitamin D response element; VEGF, vascular endothelial growth factor.

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Materials and methods

In this study we have focused on the direct effects of 1\(\alpha,25(\text{OH})_2\text{D}_3\) on the critical process by which metastatic lung cancer cells in the bloodstream migrate to the potential target organ and attach and lodge in the microvasculature there in a model without the calcemic activity and host defense-related effects of 1\(\alpha,25(\text{OH})_2\text{D}_3\). We employed a direct metastasis model in which the entrapment of tumor cells and metastatic growth are quantified following the injection of Lewis lung carcinoma (LLC) cells expressing green fluorescent protein (GFP) (LLC-GFP cells) into the vein and used VDR knockout (VDR\(^{-/-}\)) mice. A major advantage of using LLC-GFP cells is that imaging requires no preparative procedures and, therefore, is uniquely suited for visualizing live LLC-GFP cells is that imaging requires no preparative procedures and, therefore, is uniquely suited for visualizing live tissue during tumor progression (23,24). In addition, GFP labeling is extremely effective for measuring the number and volume of metastasis nodules in target organs. VDR\(^{-/-}\) mice were generated from C57BL/6 mice by one of the co-authors and cloned. The medium used for culturing the tumor cells and for all assays continued for 3 days. The medium was changed 2 days thereafter.

Materials and methods

Cells

Cloned metastatic variants of LLC cells were kindly supplied by the Institute of Development, Aging and Cancer (Tohoku University). The LLC cells were stably transfected with the expression vector pEGFP-1 (Clontech, Palo Alto, CA) by electroporation. The transfected LLC cells (LLC-GFP cells) were cultured in a selective medium that contained 300-800 \(\mu\)g/ml G418 (Geneticin; Roche Diagnostics, Mannheim, Germany) for 6 days. Using flow cytometry, the brightest fluorescent cells above the 95th percentile were sorted and cloned. The medium used for culturing the tumor cells and for all assays was RPMI-1640 (Gibco BRL, Life Technologies, Grand Island, NY) supplemented with l-glutamine (0.29 mg/ml), kanamycin (0.06 mg/ml) and 10% heat-inactivated fetal bovine serum (FBS).

Animals

VDR\(^{-/-}\) mice were generated by homologous gene targeting as described previously (19). Null mutant mice were obtained by intercrossing a heterozygous VDR knockout female and a heterozygous male. Mice were weaned at 3 weeks of age and then fed ad libitum either a normal diet (1.2% calcium, 0.6% phosphorus and 108 IU vitamin D\(_3/100\) g), a high calcium diet (2% calcium, 1.25% phosphorus, 108 IU vitamin D\(_3/100\) g and 20% lactose), a vitamin D-deficient diet (0.6% calcium, 0.3% phosphorus and 0 IU vitamin D\(_3/100\) g) or a high calcium and vitamin D-deficient diet (2% calcium, 1.25% phosphorus, 0 IU vitamin D\(_3/100\) g and 20% lactose) (all from Clea Japan Inc., Tokyo Japan). The mice were maintained under pathogen-free conditions with a 12 h light/12 h dark cycle and were given free access to the assigned diet during the feeding period. Mice were injected i.v. with a single dose of 10\(^4\) LLC-GFP cells in a total volume of 0.2 ml in RPMI-1640 medium containing 10% FBS. The lungs were collected 1, 2 or 3 weeks or 18 days after the injection and the weights, metastatic nodule numbers and GFP+/actin mRNA ratio were measured. This study was conducted in accordance with the standards established by the Guidelines for the Care and Use of Laboratory Animals of Kobe Pharmaceutical University.

RT-PCR

Total RNA was prepared from LLC-GFP cells using ISOGEN (Nippon Gene, Tokyo Japan). Aliquots of 2 \(\mu\)g RNA were reverse transcribed with AMV reverse transcriptase (TaKaRa, Japan) and PCR amplified at 95°C for 40 s, 62°C for 40 s and 72°C for 1 min for 20-35 cycles, using primer sets specific for VDR (GenBank accession no. NM_0009504) (forward primer, 107-126; reverse primer, 286-305) and \(\beta\)-actin (GenBank accession no. X03672) (forward primer, 290-271; reverse primer, 305-326). Agarose gels (2%) were stained with ethidium bromide and visualized under UV lights. All assays were performed in triplicate.

Western blot analysis

Whole cell extracts were harvested in lysis buffer [1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, protease inhibitor mixture (Complete; Roche Molecular Biochemicals)] from LLC or LLC-GFP cells. Extracts containing 30 \(\mu\)g of each protein were then subjected to 7.5% SDS-PAGE. After separation by gel electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech). After blocking with Block Ace reagent (Dainippon Pharmaceuticals, Osaka, Japan) the membranes were incubated with the first antibody, a monoclonal anti-VDR antibody (9A7; Affinity Bioreagents Inc., Golden, CO). After incubation with the horseradish peroxidase-linked anti-IgG second antibody, the proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Cell cycle analysis

The effects of 1\(\alpha,25(\text{OH})_2\text{D}_3\) on the proliferation of LLC-GFP cells in vitro were assessed by cell counting and cell cycle analysis. Cells were seeded at a density of 2 \(\times\) 10\(^5\) cells/well in 6-well culture plates in RPMI-1640 medium containing 10% FBS for 24 h. After 24 h in serum-deprived RPMI-1640 medium, fresh medium containing 10% charcoal stripped FBS with or without 1\(\alpha,25(\text{OH})_2\text{D}_3\) (10\(^{-10} - 10^{-4}\) M) was added to cultured cells and incubation continued for 3 days. The medium was changed 2 days thereafter. 1\(\alpha,25(\text{OH})_2\text{D}_3\) was dissolved in ethanol and the final concentration of ethanol in all cultures did not exceed 0.1%. Cells were trypsinized at timed intervals and an aliquot was counted and analyzed. For analysis of the cell cycle, each group of cells was collected and washed once with phosphate-buffered saline (PBS) (calcium and magnesium free; –) and the cells were resuspended in PBS (–) containing 0.2% Triton X-100 and 100 \(\mu\)g/ml RNase and incubated at 37°C for 30 min. Cells were washed with PBS (–) and incubated with 0.5 ml of a DNA-staining solution containing propidium iodide (50 \(\mu\)g/ml) at 4°C for 20 min. The cells were analyzed with a flow cytometer equipped with an argon laser (488 nm) (Becton Dickinson FACScan) and the cell cycle distribution was analyzed using ModFit LT (Verity).

In vitro invasion assay using matrigel

The capacity of tumor cells to traverse a basement membrane matrix-coated filter has been shown to be representative of their invasiveness. Therefore, the capacity of tumor cells to migrate through matrigel-coated membranes was measured (26,27). Nucleopore filters (8 \(\mu\)m pore size) were coated with a 1:20 dilution of matrigel (Becton Dickinson, Bedford, MA). Representative filters were stained with crystal violet. Medium and 1\(\alpha,25(\text{OH})_2\text{D}_3\) were added to the upper and lower compartment of each blind well chemotactic chamber. A dried, coated filter was placed over the lower compartment. After reconstituting the matrigel, 5 \(\times\) 10\(^5\) cells were added to the upper compartment together with the same additives as were in the medium in the lower compartment of each chamber. Chambers were incubated for 24 h, after which the filters were removed, wiped clean on the upper surface and fixed in 10% formalin. The number of cells on the lower surface of the filter was counted under a fluorescence microscope. Data are presented as the number of cells per low power field enumerated from triplicate chambers.
Real-time quantitative PCR
LLC-GFP cells or lungs were homogenized in ISOGEN and total RNA was isolated. Aliquots of 2 μg RNA was reverse transcribed with AMV reverse transcriptase (TaKaRa, Japan) and PCR amplified at 95°C for 40 s, 62°C for 40 s and 72°C for 1 min for 35 cycles using primer sets specific for VDR, vascular endothelial growth factor (VEGF) (for primers see ref. 28), matrix metalloproteinase-2 (MMP-2) (GenBank accession no. NM_008610) (forward primer, 424–443; reverse primer, 558–576), MMP-9 (GenBank accession no. NM_013599) (forward primer, 149–167; reverse primer, 242–263), GFP (GenBank accession no. AF323988) (forward primer, 417–438; reverse primer, 490–511) and β-actin. A quantitative analysis of gene expression was performed using a GeneAmp 5700 Sequence Detection System (PE Biosystems, Foster City, CA) and a SYBR Green core reagent kit (PE Biosystems) as described (29).

Imaging and tumor scoring
A Leica fluorescence stereo microscope model MZ FL III (Leica Microsystems Inc., Tokyo, Japan) was used. Selective excitation of GFP was produced through a D480/40 band-pass filter and 510 DCXR dichroic mirror. Emitted fluorescence was collected through a DC300F long-pass filter and digital camera system. The numbers of metastatic tumors on the surface of the lungs were counted under the fluorescence stereo microscope. To quantify lung metastasis we counted GFP-expressing spots on the lungs.

Histological analysis
Tissues were fixed in 10% paraformaldehyde and embedded in paraffin. Sections (5 μm thick) were deparaffinized and then stained with hematoxylin and eosin according to standard procedures.

Assay for serum calcium and 1α,25(OH)2D3
Serum calcium and 1α,25(OH)2D3 levels were determined using a microcolorimetric assay (Wako, Japan) and radio receptor assay using radiolabeled 1α,25(OH)2D3, respectively.

Enzyme immunoassay for mouse VEGF
The mouse VEGF concentration in mouse serum was determined using a VEGF EIA kit (Quantikine M; R&D Systems, Minneapolis, MN, USA), based on a sandwich enzyme immunoassay, according to the manufacturer’s instructions. The concentration of VEGF in 100 μl of sample was estimated from a standard curve obtained using standard mouse VEGF serially diluted with a suitable buffer.

Intravenous injection of LLC-GFP cells into VDR+/+ and VDR−/− mice fed the high calcium, vitamin D-deficient diet and administered 1α,25(OH)2D3
Mice were injected i.v. with a single dose of 10⁶ LLC-GFP cells. Cells were first harvested by trypsinization and washed twice with PBS (−) and then injected in a total volume of 0.2 ml RPMI-1640 medium containing 10% FBS. A preventative protocol was designed in which 1α,25(OH)2D3 was administered continuously using an osmotic minipump (model 2 ML4 Alzet; Alza Corp., Palo Alto, CA) implanted s.c. on the same day as inoculation of LLC-GFP cells. An infusion rate of 1 μl of sample was estimated for 72 h (Figure 1D). The invasiveness of LLC-GFP cells was examined with an in vitro invasion assay using matrigel. LLC-GFP cells showed highly invasive activity and inhibition of cell invasion was highly sensitive to 1α,25(OH)2D3 treatment in a dose-dependent manner (Figure 1E).

Inhibition of the metastatic activity and angiogenesis-inducing activity of LLC-GFP cells by 1α,25(OH)2D3
LLC cells are known to highly express mRNAs of VEGF164 and VEGF120, involved in tumor-induced angiogenesis (32). LLC cells also express the mRNAs of MMP-2 and MMP-9, involved in invasion by tumor cells (33). To further investigate the mechanism of 1α,25(OH)2D3 inhibition of metastasis we examined the effects of treatment with 1α,25(OH)2D3 on the down-regulation of these genes. As shown in Figure 2A–D, all of these genes were strongly suppressed by treatment with 1α,25(OH)2D3 (10⁻⁹–10⁻⁷ M) for 24 h. As shown in Figure 2E, expression of VDR mRNA was unaffected by 1α,25(OH)2D3 treatment in LLC-GFP cells.

From these in vitro findings it seems that LLC-GFP cells have metastatic potential and responsiveness to 1α,25(OH)2D3. Furthermore, these results demonstrate that 1α,25(OH)2D3 inhibits the metastatic activity and angiogenesis-inducing activity of LLC-GFP cells by inducing down-regulation of VEGF164, VEGF120, MMP-2 and MMP-9 expression.

Tumorigenesis in VDR+/+ mice injected with an LLC-GFP cell suspension (LLC-GFP CSI VDR+/+ mice)
We i.v. injected VDR+/+ C57BL/6 mice (8 weeks of age) with a single dose of 10⁶ LLC-GFP cells and collected the lungs 18 days after injection. The LLC-GFP cells metastasized to the lungs from the circulation and generated numerous metastatic nodules, as shown by bright field stereo microscopy (Figure 3A) and fluorescence stereo microscopy (Figure 3B). Metastatic tumors expressing GFP were only detectable in the lungs, demonstrating organ-specific metastasis and stable expression of GFP by the cells. In order to investigate the metastatic potential of LLC-GFP cells, we i.v. injected VDR+/+ mice (8 weeks of age) with a single dose of 10⁶ cells and collected the lungs 1, 2 and 3 weeks after injection. Weights, metastatic nodule numbers and GFP/β-actin mRNA ratios of the lungs were measured. All of the animals (15/15 in each group) had highly fluorescent lung tumors and all values for the metastatic parameters increased with time (Figure 3C), demonstrating the appropriateness of using LLC-GFP cells for the evaluation of metastatic growth in VDR+/+ mice. All the animals injected with LLC-GFP cells survived for 2 weeks and only 3 of 15 had died by 3 weeks post-injection. Based on these in vivo observations, it seemed that lung metastatic tumors on day 18 after the injection provided the most appropriate
material for evaluating the anti-metastatic activity of 1α,25(OH)2D3.

Tumorigenesis in LLC-GFP CSI VDR+/+ and LLC-GFP CSI VDR−/− mice

First, we confirmed that VDR−/− mice showed an extreme hyper-vitaminosis D (18.54 ± 2.11 and 10 452.41 ± 849.81 pg/ml in VDR+/+ and VDR−/− mice, respectively) along with a severe hypocalcemia (9.54 ± 0.08 and 6.18 ± 0.13 mg/dl in VDR+/+ and VDR−/− mice, respectively) and a moderate hypophosphatemia (9.36 ± 0.45 and 7.03 ± 0.33 mg/dl in VDR+/+ and VDR−/− mice, respectively) when compared with VDR+/+ mice. Serum levels of 1α,25(OH)2D3 in VDR−/− mice were ~500-fold higher than those in VDR+/+ mice and nearly comparable with levels in mice i.v. injected with a single dose of 6.25 μg/kg 1α,25(OH)2D3 (34). To verify the hypothesis that 1α,25(OH)2D3 is an intrinsic factor that inhibits the metastatic growth of lung cancer cells, we i.v. injected VDR+/+ and VDR−/− mice (8-10 weeks of age) with a single dose of 106 LLC-GFP cells and collected the lungs on day 18 after injection. Numerous metastatic nodules (Figure 4A and E) and tumors expressing GFP (Figure 4C) were observed in the lungs of VDR+/+ mice, whereas only a small number of metastatic nodules (Figure 4B and F) and tumors expressing GFP (Figure 4D) were observed in the lungs of VDR−/− mice. The metastatic activities of LLC-GFP cells were remarkably reduced in VDR−/− mice when compared with VDR+/+ mice (Figure 4G). These findings clearly indicate that metastatic growth of LLC-GFP cells is strongly inhibited by serum 1α,25(OH)2D3, calcium and/or parathyroid hormone (PTH) that is enhanced or suppressed by a defect in VDR function. It has been reported that VDR−/− mice have extremely high serum levels of 1α,25(OH)2D3 and PTH resulting from extremely low serum levels of calcium and phosphate (25).

Tumorigenesis in the LLC-GFP CSI VDR+/+ and LLC-GFP CSI VDR−/− mice fed either a normal calcium diet, a high calcium diet, a vitamin D-deficient diet or a high calcium and vitamin D-deficient diet

Kato et al. reported that serum levels of PTH and calcium can be normalized in VDR−/− mice by providing a rescue diet containing high concentrations of calcium and lactose, however, the serum level of 1α,25(OH)2D3 cannot (35,36). It is generally accepted that expression of the PTH gene in the parathyroid gland is predominantly negatively regulated by serum calcium.
and that expression of the renal 1α-hydroxylase gene is positively and negatively regulated by PTH and 1α,25(OH)2D3, respectively (37,38). The reason why overexpression of the renal 1α-hydroxylase gene in VDR
/C0/C0 mice is not normalized by the rescue diet, irrespective of reduced expression of the PTH gene, has not been fully clarified and it has been postulated that negative regulation of renal 1α-hydroxylase gene expression by a product, 1α,25(OH)2D3 itself, is more important than positive regulation by PTH, and this negative feedback control is inoperative in VDR
/C0/C0 mice, resulting in overexpression of the 1α-hydroxylase gene (35). These previous observations raise the possibility that other intrinsic factors in addition to 1α,25(OH)2D3 may be involved in the reduced metastatic growth of LLC-GFP cells in VDR
/C0/C0 mice.

To exclude the above possibility and show that 1α,25(OH)2D3 plays an essential role in inhibition of the metastatic growth of LLC-GFP cells, we fed VDR
/+ and VDR
/- mice (3 weeks of age) either a normal diet, a high calcium diet, a vitamin D-deficient diet or a high calcium and vitamin D-deficient diet for 7 weeks. Then, we injected the mice with LLC-GFP cells and continued to feed the assigned diets before collecting the lungs.

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Fig. 2. Effect of 1α,25(OH)2D3 on VEGF and MMP mRNA expression in LLC-GFP cells. (A) VEGF164, (B) VEGF120, (C) MMP-2, (D) MMP-9 and (E) VDR mRNA expression in LLC-GFP cells treated with 1α,25(OH)2D3 (10⁻⁹-10⁻⁷ M) or vehicle (ethanol) for 24 h. Each bar represents the mean ± SE (n = 6). *P < 0.05; **P < 0.01; ***P < 0.001.
on day 18 after injection. In the VDR\textsuperscript{++} and VDR\textsuperscript{--} mice fed the normal diet injection of LLC-GFP cells did not affect the serum calcium levels (Figure 5A) and induced a small, but insignificant, decline in serum 1\(\alpha\),25(OH)\textsubscript{2}D\textsubscript{3} levels of both groups of mice (Figure 5B and C). In the VDR\textsuperscript{++} and VDR\textsuperscript{--} mice fed the high calcium diet the serum calcium levels of both groups of mice were within the normal range (Figure 5A).

The high calcium diet induced a significant decline in the serum 1\(\alpha\),25(OH)\textsubscript{2}D\textsubscript{3} levels of VDR\textsuperscript{++} mice, leading to a moderate hypovitaminosis D, and induced a small, but insignificant, decline in the serum 1\(\alpha\),25(OH)\textsubscript{2}D\textsubscript{3} levels of VDR\textsuperscript{--} mice, although the mice still had extremely severe hypervitaminosis D (Figure 5B and C). These findings clearly indicate that in VDR\textsuperscript{--} mice, irrespective of tumor burden, a high calcium diet is able to normalize the serum calcium level but fails to normalize the serum 1\(\alpha\),25(OH)\textsubscript{2}D\textsubscript{3} level, as reported previously (36). Feeding of the vitamin D-deficient and the high calcium and vitamin D-deficient diets resulted in complete elimination of 1\(\alpha\),25(OH)\textsubscript{2}D\textsubscript{3} from the serum of both VDR\textsuperscript{++} and VDR\textsuperscript{--} mice (Figure 5B and C). Intriguingly, metastatic growth of LLC-GFP cells was remarkably reduced in VDR\textsuperscript{--} mice fed either the normal diet or the high calcium diet, while it was not reduced or even enhanced in VDR\textsuperscript{--} mice fed the vitamin D-deficient and the high calcium and vitamin D-deficient diets (Figure 6A--C). Moreover, VDR\textsuperscript{++} mice fed the manipulated diets displayed an apparent inverse relationship between serum levels of 1\(\alpha\),25(OH)\textsubscript{2}D\textsubscript{3} and GFP mRNA expression (Figures 5C and 6C). These results indicate that physiological levels of 1\(\alpha\),25(OH)\textsubscript{2}D\textsubscript{3} have a preventive effect against tumorigenesis.

The vitamin D-deficient diet appeared to increase serum calcium levels in VDR\textsuperscript{++} and VDR\textsuperscript{--} mice with tumors.
This is probably due to LLC-GFP cells inducing hypercalcemia during the process of tumorigenesis (39,40). These results clearly indicate that serum 1α,25(OH)2D3 levels were negatively correlated with metastasis of LLC-GFP cells. Furthermore, we found about 3-fold higher serum levels of VEGF in vitamin D-deficient VDR+/+ and VDR−/− mice than VDR+/+ and VDR−/− mice fed the normal or high calcium diets (Figure 7). Moreover, our results indicate that VDR+/+ mice fed the above diets displayed an apparent inverse relationship between serum levels of VEGF and 1α,25(OH)2D3 (Figures 5B

**Fig. 5.** Serum concentrations of calcium and 1α,25(OH)2D3 in LLC-GFP CSI VDR+/+ and LLC-GFP CSI VDR−/− mice fed either a normal diet, a high calcium diet, a vitamin D-deficient diet or a high calcium and vitamin D-deficient diet. (A) Serum concentrations of calcium in VDR+/+ and VDR−/− mice. (B) Serum concentrations of 1α,25(OH)2D3 in VDR+/+ and VDR−/− mice. (C) An enlarged graph of serum 1α,25(OH)2D3 concentrations in VDR+/+ mice. N.D. is below the detection limit (1 pg/ml). Serum samples were collected on day 18 after injection. Each bar represents the mean ± SE (n = 15). *P < 0.05; **P < 0.01; ***P < 0.001. a–g in each individual column indicate a significant difference between the data with the same letter. Student’s t-test.
Tumor cells, including LLC cells, are able to produce several angiogenic factors, including basic fibroblast-like growth factor and VEGF. VEGF is the major inducer of tumor-induced angiogenesis and serves as an indicator of tumorigenesis in mice (41). In our in vitro study we found that VEGF mRNA expression in LLC-GFP cells was significantly suppressed by 1α,25(OH)₂D₃ treatment. Also, secretion of VEGF from LLC-GFP cells was decreased by 1α,25(OH)₂D₃ 24 h after treatment (data not shown). These findings support the hypothesis that 1α,25(OH)₂D₃ may play a critical role in the secretion of VEGF from LLC-GFP cells in vivo and VEGF gene expression may be regulated by
1α,25(OH)2D3. Our study clearly indicates that 1α,25(OH)2D3 is an intrinsic and preventive factor in the metastasis and angiogenesis of lung cancer cells, suppressing the production of metastatic and angiogenic factors by cancer cells.

**Effects of continuous treatment with 1α,25(OH)2D3 on the development of lung metastases in LLC-GFP CSI VDR+/+ and LLC-GFP CSI VDR−/− mice fed a high calcium and vitamin D-deficient diet**

Feeding of a high calcium and vitamin D-deficient diet resulted in complete elimination of 1α,25(OH)2D3 from and correction of calcium in the serum of both VDR+/+ and VDR−/− mice (Figure 8A). After 1α,25(OH)2D3 administration for 18 days using an osmotic minipump serum 1α,25(OH)2D3 levels in both VDR+/+ and VDR−/− mice fed a high calcium and vitamin D-deficient diet were significantly increased from 0–20 to 700–800 pg/ml. LLC-GFP CSI VDR+/+ mice receiving 1α,25(OH)2D3 show hypercalcemia, but LLC-GFP CSI VDR−/− mice receiving 1α,25(OH)2D3 did not show any significant change in plasma calcium levels when compared with the vehicle-treated control group (Figure 8A).

The number of mice that developed lung metastases was analyzed by measuring lung weight, nodule count and GFP mRNA expression in the lung by quantitative RT-PCR. These parameters were significantly lower in the 1α,25(OH)2D3-treated group as compared with the untreated group in LLC-GFP CSI VDR+/+ and LLC-GFP CSI VDR−/− mice fed a high calcium and vitamin D-deficient diet (Figure 8C). These results suggest that our experimental model might be useful for studying the pathophysiology of metastatic tumors in the lung microcirculation without the calcemic activity and host defenses triggered by 1α,25(OH)2D3 or its analogs.

**Discussion**

The main finding of this study was the ability of 1α,25(OH)2D3 to inhibit the metastasis and angiogenesis of tumor cells in vivo without an association with its calcium regulatory functions and regulation of the host defenses by 1α,25(OH)2D3 through the VDR. Given that 1α,25(OH)2D3 is primarily operative in vivo as a factor preventing the metastasis and angiogenesis of cancer cells even under physiological conditions, 1α,25(OH)2D3 could be very important in the prevention of cancers. It would be useful to perform an experiment like ours after generating primary tumors in different tissues in animal models. Unfortunately, despite an extensive literature search, we were unable to find such experiments using animals bearing transplantable cancers with a variety of serum 1α,25(OH)2D3 levels. Also, in previous studies an association between the inhibitory effect of 1α,25(OH)2D3 and its calcemic activity and/or regulation of the host defenses by 1α,25(OH)2D3 through the VDR could not be excluded. Several investigators have reported that calcium and vitamin D play important roles in the inhibition of cell proliferation and colon and prostate cancer development (41–44). However, whether the calcemic activity of and regulation of the host defenses by 1α,25(OH)2D3 contribute to the effects on cancer cells has not been elucidated.

VDR−/− mice are an animal model lacking vitamin D activity, which is involved in not only calcium metabolism in the intestine, kidney and bone, but also a broad range of essential cell regulatory processes, including cellular proliferation, differentiation and apoptosis. Also, VDR−/− mice display important defects in macrophage function and cellular immunity in vitro and in vivo (45). However, irrespective of these important functions of 1α,25(OH)2D3, almost all the other abnormalities observed in VDR−/− mice except for high serum levels of 1α,25(OH)2D3 can be reversed by feeding a high calcium diet (35,45–47). Through dietary manipulation this animal model has helped us to more precisely define the direct effects of 1α,25(OH)2D3 on cancer cells and tumor growth and metastasis under conditions without the calcemic activity and other actions of 1α,25(OH)2D3 in the host.

In the present study, to fully visualize and quantify metastasis in vivo, we developed GFP-expressing Lewis lung carcinoma cells as a far more powerful model with which to study the mechanisms of tumor progression, including regional and distant metastasis representative of lung cancer. LLC-GFP cells express the VDR and their proliferative and invasive activities are significantly inhibited by 1α,25(OH)2D3 treatment. The mechanism of this inhibition in response to
1α,25(OH)₂D₃ has not been made clear. However, our observations in vitro suggest that the mRNA levels of MMP-2, MMP-9 and VEGF, which are the most important factors for tumor invasion and tumor-induced angiogenesis, were decreased by 1α,25(OH)₂D₃ treatment in a dose-dependent manner (Figure 2). Also, we observed secretion of MMP-2, MMP-9 and VEGF into the culture medium by gelatin zymography and ELISA. All of these secretions were inhibited by MMP-9 and VEGF into an autocrine inhibitory loop. (Figure 2). Also, we observed secretion of MMP-2, MMP-9 and VEGF into the culture medium by gelatin zymography and ELISA. All of these secretions were inhibited by MMP-9 and VEGF into an autocrine inhibitory loop.

Here we have focused on the direct effects of 1α,25(OH)₂D₃ on the direct effects of 1α,25(OH)₂D₃ on the critical processes of metastatic lung cancer cells without an association with its calcemic activity and regulation of the host defenses. Therefore, we employed VDR⁻/⁻ mice and a direct metastasis model, involving the entrapment of VDR-positive and 1α,25(OH)₂D₃-responsive LLC-GFP cells in a vein. When VDR⁻/⁻ mice were fed the normal and high calcium diets they displayed a high serum level of 1α,25(OH)₂D₃ and the metastatic growth of LLC-GFP cells was remarkably decreased. However, no such effect was observed in VDR⁻/⁻ mice fed a vitamin D-deficient diet. These results suggest that serum 1α,25(OH)₂D₃ directly inhibits the metastatic and angiogenesis-inducing activity of LLC-GFP cells irrespective of the serum concentration of calcium. Furthermore, administration of 1α,25(OH)₂D₃ prevented tumorigenesis in VDR⁺/⁺ and VDR⁻/⁻ mice. The hypercalcemia observed when high doses of 1α,25(OH)₂D₃ are administered appears to be mediated through the nuclear receptor, because VDR⁻/⁻ mice could be treated with extremely high doses of 1α,25(OH)₂D₃ without any side-effects, whereas VDR⁺/⁺ mice showed lethal hypercalcemia on administration through other means. One possibility may be 1α,25(OH)₂D₃-dependent transcriptional repression through a negative regulatory VDRE, as proposed for the vitamin D 1α-hydroxylase gene by Murayama et al. (38). Another possibility is that 1α,25(OH)₂D₃ mediates its inhibitory action on MMP and VEGF secretion via an autocrine inhibitory loop.

Fig. 8. Effects of continuous treatment with 1α,25(OH)₂D₃ on the development of lung metastases in LLC-GFP CSI VDR⁺/⁺ and LLC-GFP CSI VDR⁻/⁻ mice fed a high calcium and vitamin D-deficient diet. 1α,25(OH)₂D₃ (1 µg/kg/day) or vehicle was infused continuously by an osmotic minipump implanted s.c. on the same day as the LLC-GFP cell injection. Serum and lungs were collected on day 18 after the cell injection. (A) Serum calcium. (B) Serum 1α,25(OH)₂D₃ concentration. (C) Lung weight, lung nodule counts under a fluorescence stereo microscope and GFP mRNA expression of the lung of LLC-GFP CSI VDR⁺/⁺ and LLC-GFP CSI VDR⁻/⁻ mice. Each bar represents the mean ± SE (n = 10). *P < 0.05; **P < 0.01; ***P < 0.001. Significant difference from vehicle-treated animals. Student’s t-test.
of 1 μg/kg/day 1α,25(OH)2D₃. These results clearly suggest that the inhibitory effect of 1α,25(OH)2D₃ on the metastasis of lung cancer cells is not related to the calcemic activity and other actions of 1α,25(OH)2D₃ in the host. The beneficial effects of 1α,25(OH)2D₃ in cancer treatment have been supported by many studies with 1α,25(OH)2D₃ and synthetic analogs. However, despite a few encouraging results, clinical applications have been limited due to an extremely narrow therapeutic window, i.e., effective doses cannot be administered without inducing hypercalcemia (15). Therefore, whether the calcemic activity of and regulation of the host defenses by 1α,25(OH)2D₃ contribute to the effects on cancer cells has not been elucidated. In this paper we report for the first time that 1α,25(OH)2D₃ inhibits the metastasis and angiogenesis of lung cancer cells without an association with the calcemic activity and other actions of 1α,25(OH)2D₃ in the host.

With regard to VDR ablation and immune defects and tumorigenesis, Mathieu et al. reported that VDR−/− mice display important defects in macrophage function and cellular immunity in vitro and in vivo. However, these defects are an indirect consequence of disruption of the VDR because they can be restored through normalization of calcium levels (45). Thus, in our experimental model VDR−/− mice with corrected serum calcium levels had normal immune functions. Zinser et al. have investigated the roles of the VDR in host defense activity and 7,12-dimethylbenz[a]anthracene (DMBA)-induced skin carcinogenesis in vivo using VDR−/− mice (54). In their experiments mice were repeatedly exposed to DMBA to induce the formation of skin tumors. Chemically induced carcinomatous growth and the progression of tumorigenesis occurred more rapidly in VDR−/− than VDR+/+ mice. The authors suggested that VDR ablation is associated with enhanced sensitivity to tumor formation. The development of skin tumors in response to the chemical carcinogen DMBA in VDR−/−, but not VDR+/+, mice suggests that the VDR acts as a tumor suppressor in the epidermis. Also, Kallay et al. suggested that loss of the VDR may provide a link to the complex process of multi-step carcinogenesis by causing direct DNA damage when 1α,25(OH)2D₃-mediated growth control is diminished (55). Their report indicated that the anti-tumorigenic effects of 1α,25(OH)2D₃ are mediated through the VDR.

From our study it is now clear that the metastatic growth of lung cancer cells is remarkably reduced in response to increased serum levels of 1α,25(OH)2D₃, but not serum calcium levels and the host defenses. Furthermore, we found that metastatic growth of LLC-GFP cells was increased in VDR−/− mice fed a vitamin D-deficient diet or a high calcium and vitamin D-deficient diet compared with VDR+/+ mice fed a normal diet (Figure 6A-C). VDR+/+ mice fed the manipulated diets displayed an apparent inverse relationship between the serum levels of 1α,25(OH)2D₃ and GFP mRNA expression (Figures 5C and 6C). Thus, these results in VDR+/+ mice support the hypothesis that physiological levels of 1α,25(OH)2D₃ have a preventive effect against tumorigenesis. Renal and extra-renal production of 1α,25(OH)2D₃ may also be a part of important mechanisms in the anti-tumor effects of dietary vitamin D or circulating 1α,25(OH)2D₃. However, serum 1α,25(OH)2D₃ levels in VDR−/− mice are not physiological or feasible for use in human patients. In this study we would like to emphasize that VDR−/− mice with corrected hypocalcemia and hypervitaminosis D can be used as an experimental model for screening the anti-cancer effects of new vitamin D analogs in vivo.

In conclusion, we have shown that 1α,25(OH)2D₃ acts on the metastatic growth of lung cancer cells directly and is an intrinsic factor for the prevention or treatment of cancer. Moreover, we suggest that serum levels of 1α,25(OH)2D₃ vary inversely with tumorigenesis and that 1α,25(OH)2D₃ may work as an intrinsic factor for the prevention of metastasis in intact animals. These findings should encourage the further development of nutritionally based models for the chemoprevention of metastatic cancer using vitamin D. Current research is aimed at exploring the potential use of 1α,25(OH)2D₃ as a preventive factor and anti-proliferative agent against cancer.

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