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Metastatic growth of lung cancer cells is extremely reduced in Vitamin D receptor knockout mice☆

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Abstract

Lung metastatic neoplasms are the major cause of cancer mortality. Despite the progress of diagnostic techniques and improvements in surgical procedures, the prognosis of patients with lung cancer is generally poor, even in the early stages of cancer [Cancer: Principles and Practice of Oncology, vol. 1, fifth ed., Lippincott-Raven, New York, 1997, p. 849]. Epidemiological studies indicate a positive correlation with the prevalence of cancers and low serum levels of Vitamin D metabolites [Am. J. Clin. Nutr. 54 (1991) 193s; Cancer Epidemiol. Biomark. Prev. 9 (2000) 1059]. 1α,25-Dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] is a potent inhibitor of cancer cell proliferation in vitro [Proc. Natl. Acad. Sci. U.S.A. 78 (1981) 4990; Endocrinol. 139 (1998) 1046; Mol. Endocr. 15 (2001) 1127]. There is, however, no report demonstrating that 1α,25$(OH)_2D_3$ is operative in vivo to inhibit metastatic growth of cancer cells. To verify this possibility, we generated a stable transfectant of the Lewis lung carcinoma (LLC) cell expressing green fluorescent protein (GFP) and examined its metastatic activity in wild-type mice and Vitamin D receptor (VDR) knockout mice that exhibit no Vitamin D-dependent calcemic activity and extremely high serum levels of 1α,25$(OH)_2D_3$ due to the overexpression of the 1α-hydroxylase gene [Nat. Genet. 16 (1997) 391; Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 9831]. Here, we show that 1α,25$(OH)_2D_3$ inhibits metastatic growth of lung cancer cells in the defined animal model and may work as an intrinsic factor for prevention of metastasis in intact animals. These findings establish a critical role for 1α,25$(OH)_2D_3$ in lung metastatic neoplasms and provide a new model for metastasis of malignant cells.

Keywords: VDR knockout mice; Vitamin D; Lung cancer; Tumor; Metastasis

1. Introduction

1α,25-Dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] inhibits proliferation of a variety of cultured cells from different tissues, including prostate, colon, pancreatic, breast and lung cancers. It is also shown that, 1α,25$(OH)_2D_3$ modulates in vivo tumor growth in several types of animal tumor models. Vitamin D receptor (VDR) VDR knockout mice ($VDR^{-/-}$) exhibit no Vitamin D-dependent calcemic activity and extremely high serum levels of 1α,25$(OH)_2D_3$ due to the overexpression of the 1α-hydroxylase gene [1]. From a clinical viewpoint, they display a phenotype that closely resembles human Vitamin D-dependent rickets type II including 1α,25$(OH)_2D_3$ resistance, hypocalcaemia, secondary hyperparathyroidism, osteomalacia and alopecia. This animal model ca be used for the evaluation of the potential of 1α,25$(OH)_2D_3$ and its analogs to inhibit tumor growth without inducing hypercalcemia. We examined the effect of 1α,25$(OH)_2D_3$ on tumor growth and metastasis of lung cancer cells using $VDR^{-/-}$.

2. Materials and methods

2.1. Cell culture

Cloned metastatic variant LLC cells were kindly supplied by the Institute of Development, Aging and Cancer, Tohoku University. The LLC cells were stably transfected the jellyfish *Aequorea victoria* green fluorescent protein (GFP) with an expression vector by the electroporation. The transfected LLC cells (LLC–GFP cells) were cultured in a selective medium that contained 300 μg/ml of G418 (Geneticin, Roche, Diagnostics, Mannheim, Germany) for 6 days. Using flow cytometry, the brightest fluorescent

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cells above the 95 percentile were sorted and cloned. The medium used for culturing the tumor cells and for all assays was RPMI-1640 medium (Life Technologies, Grand Island, NY) supplemented with L-glutamine (0.29 mg/ml), kanamycin (0.06 mg/ml) and 10% heat-inactive fetal bovine serum (FBS).

2.2. Animals

$VDR^{-/-}$ mice were generated by homologous gene targeting as described previously [1]. Null mutant mice were obtained by intercrossing the heterozygous VDR knockout female and male mouse. Mice were weaned at 3 weeks of age and were then fed ad libitum either a normal diet (Clea Japan Inc., Tokyo, Japan; ingredients: 1.2% calcium, 0.6% phosphorus and 1.08 IU/g Vitamin D_3), a high calcium diet (Clea Japan Inc.; ingredients: 2% calcium, 1.25% phosphorus, 1.08 IU/g Vitamin D_3, and 20% lactose) or a Vitamin D-deficient diet (Clea Japan Inc.; ingredients: 0.6% calcium, 0.3% phosphorus and 0 IU/g Vitamin D_3). Mice were injected intravenously with a single dose of 10^6 of LLC–GFP cells in a total volume of 0.2 ml in RPMI-1640 medium containing 10% FBS. The lungs were collected 18 days after the injection and the weights, metastatic nodule numbers, and GFP/β-actin mRNA ratio were measured.

2.3. Real-time quantitative PCR for GFP and β-actin

The lung was homogenized in ISOGEN (Nippon Gene, Japan) and total RNA was isolated. The purified RNA was reverse-transcribed. Quantitative analysis of gene expression was performed using the GeneAmp 5700 Sequence Detection System (PE Biosystems, Foster City, CA) and the SYBR Green core reagent kit (PE Biosystems, Foster City, CA) as described.

2.4. Assay for serum calcium and 1α,25(OH)$_2$D$_3$

Serum calcium and 1α,25$(OH)_2D_3$ levels were determined by micro colorimetric assay (Wako, Japan) and radio receptor assay, respectively.

2.5. Statistical analyses

Data are presented as mean ± S.E.M. The Student's *t*-test was used for group analysis. *P*-values <0.05 were considered significant.

3. Results

As the first step to verify our hypothesis, we checked responsiveness to 1α,25$(OH)_2D_3$ and the metastatic potential of LLC–GFP cells. LLC–GFP cells showed highly invasive activity and the inhibition of the cell invasion was highly sensitive to 1α,25$(OH)_2D_3$ treatment. To examine the metastatic potential of LLC–GFP cells, we injected wild-type C57BL/6 mice (WT mice), 8 weeks of age, intravenously with a single dose of 10^6 of LLC–GFP cells. The tumor formation was recognized and it seemed that the lung metastatic tumors of LLC–GFP cells on day 18 after the injection provide the most appropriate views for evaluating anti-metastatic activity of 1α,25$(OH)_2D_3$. We injected WT ($VDR^{+/+}$) and $VDR^{-/-}$ mice, 8–10 weeks of age, intravenously with a single dose of 10^6 of LLC–GFP cells and collected the lungs on day 18 after the injection. Numerous metastatic nodules and tumors expressing GFP were observed in the lungs of $VDR^{+/+}$ mice, whereas only a small number of metastatic nodules and tumors expressing GFP were observed in the lungs of $VDR^{-/-}$ mice. The metastatic activities of LLC–GFP cells were remarkably reduced in the $VDR^{-/-}$ mice when compared to the $VDR^{+/+}$ mice (Fig. 1). This result indicate that extremely

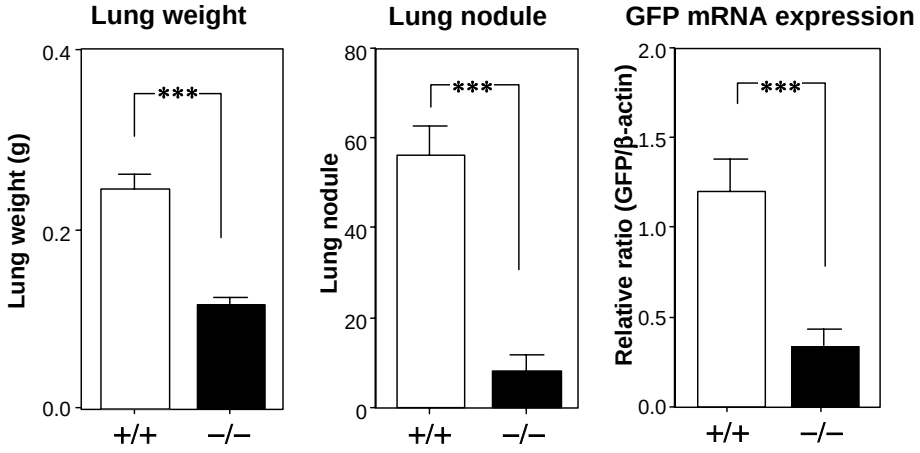

Fig. 1. Metastatic tumors of $VDR^{+/+}$ and $VDR^{-/-}$ mice were detected by the lung weight, the lung nodule counts under fluorescence stereo microscope and GFP mRNA expression of the lung analyzed by real-time quantitative RT-PCR. Each bar represents the mean ± S.E.M. $^{***}P < 0.001$ ($n = 15$).

Table 1
Metastatic growth of the LLC–GFP cells and serum calcium and 1α,25(OH)$_2$D$_3$ levels of the VDR$^{+/+}$ and VDR$^{-/-}$ mice fed either a normal diet, a high calcium diet or a Vitamin D-deficient diet

	GFP mRNA expression in lung (arbitrary unit)	Serum 1α,25(OH)$_2$D$_3$ (pg/mL)	Serum Ca (mg/dL)
Normal diet			
VDR$^{+/+}$ ($n = 14$)	1.2 ± 0.2** a	15.7 ± 2.4*** a	9.5 ± 0.2** a
VDR$^{-/-}$ ($n = 13$)	0.3 ± 0.1** b	8104.0 ± 561.0*** b	5.9 + 0.1** b
High Ca diet			
VDR$^{+/+}$ ($n = 20$)	1.4 ± 0.2**	7.5 ± 1.0***,## a,c	9.9 ± 0.1 c
VDR$^{-/-}$ ($n = 15$)	0.4 ± 0.1** c	7164.8 ± 469.6*** d	9.2 ± 0.1## b,d
Vitamin D deficient diet			
VDR$^{+/+}$ ($n = 18$)	1.9 ± 0.2## a	0.0 ± 0.0***,### a,c	11.5 ± 0.3## a,c
VDR$^{-/-}$ ($n = 15$)	1.9 ± 0.2### b,c	39.5 ± 14.6***,### b,d	10.4 ± 0.2## b,d

Values are means ± S.E.M. (n). a, b, c, d in parameter indicate the significant difference between the data of the same letter.
** $P < 0.01$.
*** $P < 0.001$.
$P < 0.01$.
$P < 0.001$.

high serum levels of 1α,25(OH)$_2$D$_3$ in the VDR$^{-/-}$ mice play an essential role in the inhibition of the metastatic growth of LLC–GFP cells. However, other intrinsic factors in addition to 1α,25(OH)$_2$D$_3$ may be involved in the reduced metastatic tumor growth. To clarify this possibility, we conducted the same experiment using the VDR$^{+/+}$ and VDR$^{-/-}$ mice, 3 weeks of age, fed either a normal diet, a high calcium diet or a Vitamin D-deficient diet for 7 weeks. The results clearly indicated that the metastatic growth of LLC–GFP cells was remarkably reduced in the VDR$^{-/-}$ mice fed the normal or high calcium diet, while no reduction of the tumor growth was observed in the VDR$^{-/-}$ mice fed the Vitamin D-deficient diet (Table 1).

4. Discussion

These findings clearly indicate that the metastatic growth of LLC–GFP cells is strongly inhibited by a factor(s) that is enhanced or suppressed by a defect in VDR function. It has been reported that VDR$^{-/-}$ mice exhibit extremely high serum levels of 1α,25(OH)$_2$D$_3$ and parathyroid hormone (PTH) resulting from extremely low serum levels of calcium and phosphate [1,2]. In the VDR$^{-/-}$ mice, the abnormality of serum levels of PTH and calcium can be normalized by a rescue diet containing high concentrations of calcium and lactose, however, the abnormality in the serum level of 1α,25(OH)$_2$D$_3$ cannot be normalized by the rescue diet [3,4]. The reason why the overexpression of the renal 1α-hydroxylase gene in VDR$^{-/-}$ mice is not normalized by the rescue diet, irrespective of the reduced expression of the PTH gene, has not been fully clarified. It has been postulated that the negative regulation of the 1α-hydroxylase gene by a product, 1α,25(OH)$_2$D$_3$ itself, is more important than the positive regulation by PTH. Therefore, this negative feedback control is not operative in VDR$^{-/-}$ mice resulting in the overexpression of the 1α-hydroxylase gene. These previous observations raise the possibility that other intrinsic factors in addition to 1α,25(OH)$_2$D$_3$ may be involved in the reduced metastatic growth of LLC–GFP cells. In the present study, the metastatic growth of LLC–GFP cells was remarkably reduced in the VDR$^{-/-}$ mice fed the normal or high calcium diet, while tumor growth was not reduced in the VDR$^{-/-}$ mice fed the Vitamin D-deficient diet. These results demonstrate that 1α,25(OH)$_2$D$_3$ inhibits metastatic growth of lung cancer cells in the defined animal model and may work as an intrinsic factor for prevention of metastasis in intact animals. These findings establish a critical role for 1α,25(OH)$_2$D$_3$ in lung metastatic neoplasms and provide a new model for metastasis of malignant cells.

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