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Vitamin D3 Administration Induces Nuclear p27 Accumulation, Restores Differentiation, and Reduces Tumor Burden in a Mouse Model of Metastatic Follicular Thyroid Cancer

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We have previously demonstrated *in vitro* that 1α,25-dihydroxyvitamin D3 (calcitriol) treatment increases p27 expression and decreases cell proliferation in cultured thyroid carcinoma cell lines. We hypothesized that *in vivo* treatment with calcitriol would have a beneficial effect on thyroid carcinoma growth and progression. Five × 10^6 WRO (human thyroid follicular carcinoma derived) cells were implanted in the neck in 4- to 5-wk-old female SCID mice in an orthotopic xenograft model. Animals (n = 15) were treated ip three times a week for 21 d with 0.75 μg/kg calcitriol or vehicle. Mice were killed 21 d after tumor implantation, tumor volume was measured, and excised tumor tissue was examined by light microscopy and immunohistochemistry for p27 and thyroglobulin reactivity. Average tumor volume in control mice after 21 d of vehicle treatment was 2002 ± 207 mm^3 compared with a mean tumor volume of 1241 ± 115 mm^3 in animals receiving calcitriol, reflecting a 38% reduction in tumor volume size ($P < 0.003$). Tumors from vehicle-treated animals demonstrated morphological features of epithelial malignancies with characteristics of insular carcinoma and multiple metastases to the lungs. Tumors excised from calcitriol-treated animals demonstrated signs of differentiation with restoration of thyroglobulin staining. This was associated with a marked accumulation of p27 immunoreactivity in the nuclear compartment. These studies demonstrate that *in vivo* calcitriol administration can effectively restore p27 accumulation in thyroid carcinoma cells, an effect associated with appreciably enhanced cellular differentiation, reduction in tumor burden, and prevention of metastatic growth. (*Endocrinology* 145: 5840–5846, 2004)

THYROID CARCINOMAS EXHIBIT a wide spectrum of differentiation from some of the most indolent carcinomas (micropapillary carcinoma) to the most rapidly lethal of human malignancies (anaplastic carcinoma) (1, 2). Thyroid cancers also exhibit several markers of differentiation status including expression of thyroglobulin. Thus, these cells provide a robust model to examine the effects of targeted therapies affecting cellular differentiation.

Epidemiological studies have associated vitamin D attained through nutrition and sun exposure with reduced cancer risk (3). In addition, the hormonally active form of vitamin D, 1α,25-dihydroxyvitamin D_3 (vitamin D3 or calcitriol), has been shown to have a role in the modulation of the proliferation and differentiation of several malignant cell types including pancreatic cancer, squamous cell cancer, breast cancer, and colon cancer (4–7). Mechanisms of vitamin D effect include inhibition of proliferation associated with cell cycle arrest and, in some models, induction of differentiation, reduction in invasiveness and angiogenesis, and enhanced apoptosis (8). Furthermore, prevention of metastasis in experimental models of breast cancer and prostate cancer has been demonstrated (9, 10).

Recent studies from our laboratory using an *in vitro* model demonstrated a dose-dependent antiproliferative effect of calcitriol on thyroid cancer cell growth as determined by [H^3]thymidine incorporation and MIB-1 immunolabeling (11). Calcitriol did not induce apoptosis in these thyroid cancer cell lines; rather, the mechanism of this effect was due to G_1-cell phase arrest and increased nuclear protein expression of the cyclin-dependent kinase inhibitor $p27^{kip1}$ (p27). It is well known that p27 is not primarily regulated by transcriptional or translation control and, in this model, accumulation of p27 was the result of diminished phosphorylation, targeting to the proteasome, and consequent reduction in ubiquitin-mediated degradation (11). The role of p27 in the biological behavior of thyroid cancer has been shown to be important, as down-regulation of p27 correlates with metastatic spread (12, 13).

The purpose of the present study was to develop an *in vivo* model of thyroid cancer growth and metastasis and to evaluate the therapeutic effect and mechanism of calcitriol treatment in this model. Fidler (14) has demonstrated that human colon carcinomas are heterogeneous for a variety of biological properties that include invasion and metastasis and has found that, for colon cancer xenografts, regardless of their malignant potential in the patient, the tumor did not metastasize unless it was implanted orthotopically. Only when tumor cells were injected into the cecum or spleen of nude mice was hepatic metastasis evident. The invasive phenotype was also influenced by the organ environment, given that

Abbreviation: p27, $p27^{kip1}$.

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cells grafted sc did not produce degradative enzymes and the cells did not metastasize. In contrast, cells in the cecum did both, suggesting that the orthotopic implantation of cells yielded metastatic subpopulations of cells suitable for the study of metastasis (14). Based on this rationale, we sought to develop an orthotopic model of thyroid cancer cell growth.

Materials and Methods

Cells and chemicals

The human thyroid follicular carcinoma cell line WRO was a generous gift of Dr. J. Fagin (University of Cincinnati, Cincinnati, OH). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1× nonessential amino acids, and antibiotics (Sigma-Aldrich Co. Ltd., Irvine, UK) until confluent.

1α,25-Dihydroxyvitamin D_3 (vitamin D3 or calcitriol) was kindly provided by LEO Pharmaceutical Products (Ballerup, Denmark) and was dissolved in 80% propylene glycol in PBS as previously reported (9, 10), which also served as vehicle.

Development of an orthotopic mouse model of thyroid cancer

The human thyroid follicular carcinoma-derived cell line WRO was cultured as previously described (11). Cells were trypsinized, trypsin activity was neutralized with normal serum, and then cells were washed twice in serum-free medium. In preliminary studies, varying numbers of cells were injected into a sc site on the back or, to develop an orthotopic model of thyroid carcinoma, into the neck in the region of the thyroid bed. In these preliminary studies, we determined that 5×10^6 cells provided the optimal number of cells to form a palpable tumor at either site within 3 d. In parallel studies, the orthotopic model grew faster and developed lung metastases after 2 wk; the sc model did not develop lung metastases.

For the analysis of calcitriol action *in vivo*, 5×10^6 cells were orthotopically implanted in the neck of 4- to 5-wk-old female SCID mice (n = 15) and monitored for the development of tumor growth. Toxicity was monitored by assessing body weight, and overall health status was monitored every other day. Serum calcium was measured at the start of the experiments and at the time of death.

Calcitriol therapy

In preliminary studies, animals were treated with 0.50 to 1.0 μg/kg calcitriol dissolved in 80% propylene glycol in PBS as previously reported (9, 10). The effectiveness of this treatment on tumor growth was not different between doses of 0.70 and 1.0 μg/kg; therefore, the dose used for subsequent studies was 0.75 μg/kg. Animals were given calcitriol or vehicle alone ip three times per week as described previously (9) for 21 d. Treatments commenced 4 d after tumor cells were implanted. Animal handling and treatment protocols were approved by the Ontario Cancer Institute Animal Care Committee.

Analysis of tumor growth

Tumor growth and volume were measured using calipers every 3 d (tumor volume in mm^3 = tumor width × tumor width × tumor length/2). Mice were killed 21 d after tumor cell implantation, and the tumors were excised, weighed, and measured. Excised tumor tissue was fixed in formalin and embedded in paraffin and was examined by light microscopy and immunohistochemistry for p27 and thyroglobulin staining. Complete necropsies were performed with survey of all tissues for metastatic disease grossly and by light microscopy. Metastatic foci were counted and measured.

Immunocytochemistry

Harvested tumors were sectioned at 4-μm intervals. For cell differentiation, thyroglobulin content was determined with a polyclonal antibody (Dako Corp., Carpinteria, CA) applied at 1:8000 dilution for 30 min after pepsin pretreatment. For localization of p27, a monoclonal antibody that recognized both phosphorylated and nonphosphorylated p27 (Transduction Laboratories, Newington, NH) was used at 1:1000 for 60 min after microwave antigen retrieval. The immunological reactions were visualized with the UltraStreptavidin detection system level 2 (Signet, Dedham, MA) and 3,3-diaminobenzidine tetrahydrochloride as the chromogen. As a negative control, the primary antibody was replaced with normal mouse ascites.

A semiquantitative analysis was performed to evaluate the distribution and staining intensity of p27 and thyroglobulin positivity. For these studies, distribution was evaluated as follows: grade 1, staining in less than 5% of cells; grade 2, staining in 5–25% of cells; grade 3, staining in 25–50% of cells; grade 4, staining in 50–75% of cells; and grade 5, staining in more than 75% of cells. Intensity was graded in three grades: grade 1, weak intensity of positivity, identified as less intense than positive control tissue on the same slide; grade 2, moderate intensity, equivalent to that of positive control tissue on the slide; and grade 3, more intense staining than positive control tissue on the slide.

Statistical analysis

Data are presented as mean ± SE. Differences were assessed by the unpaired, two-sided t test. $P < 0.05$ was considered as statistically significant.

Results

In vivo model of thyroid cancer

An *in vivo* model of human thyroid cancer was established with the growth of WRO cell line tumor xenografts in the

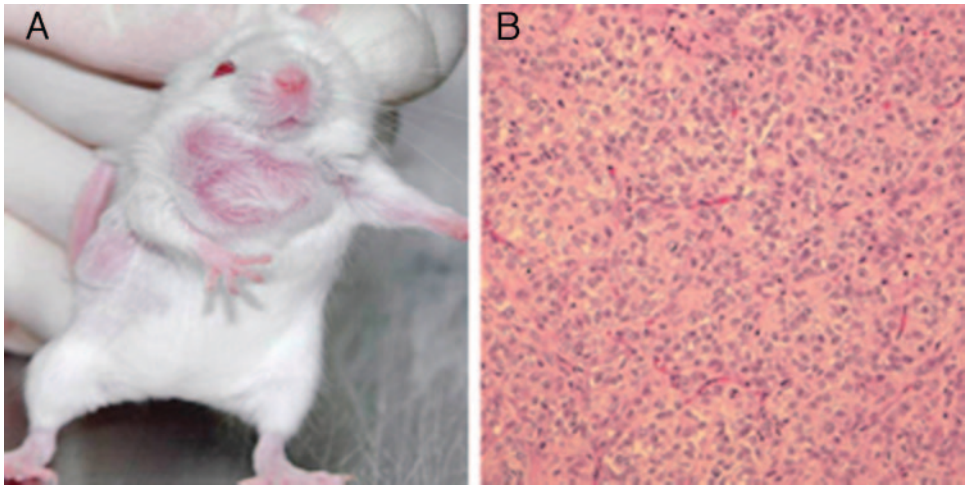

FIG. 1. An orthotopic model of follicular thyroid carcinoma. A, Growth of the human thyroid cancer cell line WRO orthotopic xenografts in SCID mice. WRO cells (5×10^6) were injected in the neck of 6-wk-old mice as indicated. Representative tumorous growth in control, untreated animals after 3 wk of injection is shown. B, The tumors are composed of epithelial cells with solid growth patterns but no evidence of thyroid follicle formation. Original magnification, ×125.

orthotopic cervical location in SCID mice (Fig. 1A). These tumors demonstrated morphological features of epithelial malignancies with characteristics of poorly differentiated thyroid carcinoma of follicular cell derivation (Fig. 1B). More significantly, lungs harvested at 21 d demonstrated metastatic disease secondary to the growth of the orthotopic cervical xenografts reminiscent of the human disease. Interestingly, this metastatic behavior was not seen when the same cells were implanted sc in the back.

Effect of calcitriol on WRO thyroid cell line xenograft growth

After 21 d, mean tumor volume in vehicle-treated mice reached 2002 ± 207 mm^3. This was significantly ($P < 0.003$) inhibited to 1241 ± 115 mm^3 in calcitriol-treated animals, reflecting an approximately 38% reduction in tumor volume (Fig. 2A). This treatment was not associated with any significant toxicity as evidenced by unchanged serum calcium

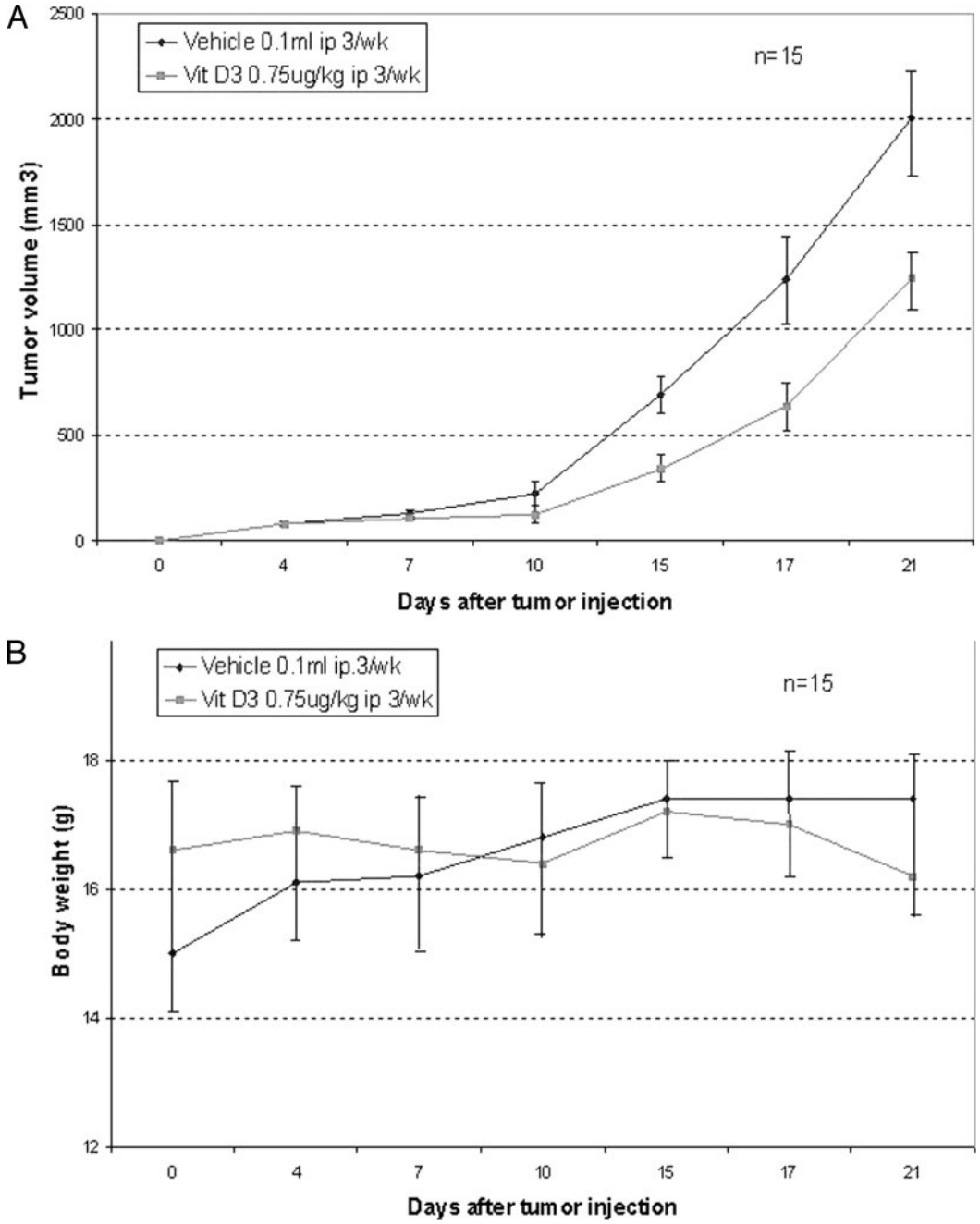

FIG. 2. Effect of calcitriol (vitamin D3) on WRO follicular thyroid carcinoma cell xenograft tumors. A, WRO cells (5×10^6) were injected in the neck of 6-wk-old female SCID mice. Treatment consisted of intraperitoneal injection of calcitriol (three times per week) for 3 wk at a dose of 0.75 μg/kg. Control animals received vehicle alone. The indicated tumor volumes represent the mean ± SEM of three independent experiments each, with five animals in each treatment group. Statistical analysis confirmed $P < 0.003$ *vs.* vehicle-treated control was reached after 10 d, and the differences in tumor size remained significant for the 3-wk duration of the experiments. B, There was no difference in the weights of animals in the treatment group compared with controls.

levels (2.35 ± 0.2 mmol/liter at baseline *vs.* 2.55 ± 0.5 mmol/liter at time of death; normal range, 2.1–2.55 mmol/liter) and no significant difference in the weights of control or treated animals monitored every 2–4 d (Fig. 2B). We subsequently examined whether the mechanisms of action of calcitriol we had previously observed *in vitro* (11) were translatable to this *in vivo* model.

p27 Immunohistochemistry

Excised tumor specimens from calcitriol- and vehicle-treated animals were examined by immunohistochemistry for p27 accumulation (Fig. 3). Tumors excised from calcitriol-treated animals displayed prominent accumulation of p27 immunoreactivity within the nuclear compartment (Fig. 3). This is consistent with our *in vitro* observations, where calcitriol increased p27 expression in WRO cells as demonstrated by Western blotting analysis (11). Semiquantitative analysis revealed grade 3 distribution (25–50% of cells positive) and grade 1 (weak) intensity in all control tumors compared with grade 5 distribution (>75% of cells positive) and grade 3 (strong) intensity in all treated tumors.

Thyroglobulin immunohistochemistry

Tumors excised from calcitriol-treated animals demonstrated signs of differentiation with focal follicle formation and some restoration of thyroglobulin staining (Fig. 4). Indeed, focal thyroglobulin positivity was identified only in calcitriol-treated animals (distribution grade 2, 5–25% of cells). The intensity of staining was weak to moderate in all foci. In comparison, no detectable thyroglobulin was identified in any (n = 15) of the vehicle-treated control animals.

Lung metastasis

Because the lungs represent a common site for human follicular thyroid carcinoma metastasis, we examined the *in vivo* effects of calcitriol treatment on the development of metastatic disease to the lung in this mouse model. As noted above, mice develop lung metastases secondary to growth of the orthotopic thyroid cancer xenografts. Representative autopsy photographs showing lungs from control and calcitriol-treated animals are shown in Fig. 5A. Analyses of calcitriol-treated animals showed no gross evidence of pulmonary metastases compared with the grossly evident

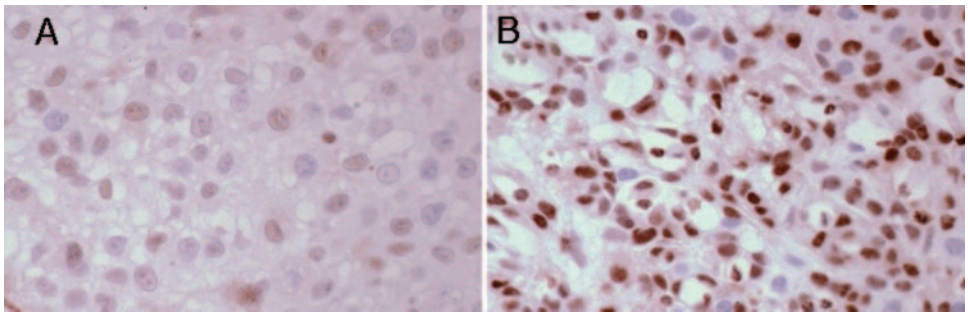

FIG. 3. Calcitriol (vitamin D3) enhances p27 accumulation in thyroid follicular carcinoma cells *in vivo*. WRO cells (5×10^6) cells were injected in the neck of 6-wk-old SCID mice and treated with calcitriol or vehicle control. Shown are immunohistochemical studies of p27 reactivity in excised orthotopic tumor xenografts in control vehicle-treated (A) and calcitriol-treated (B) animals. Note the intense and diffuse positivity in the calcitriol-treated tumor (B) compared with the scant staining in the control tumor (A). Original magnification, ×265.

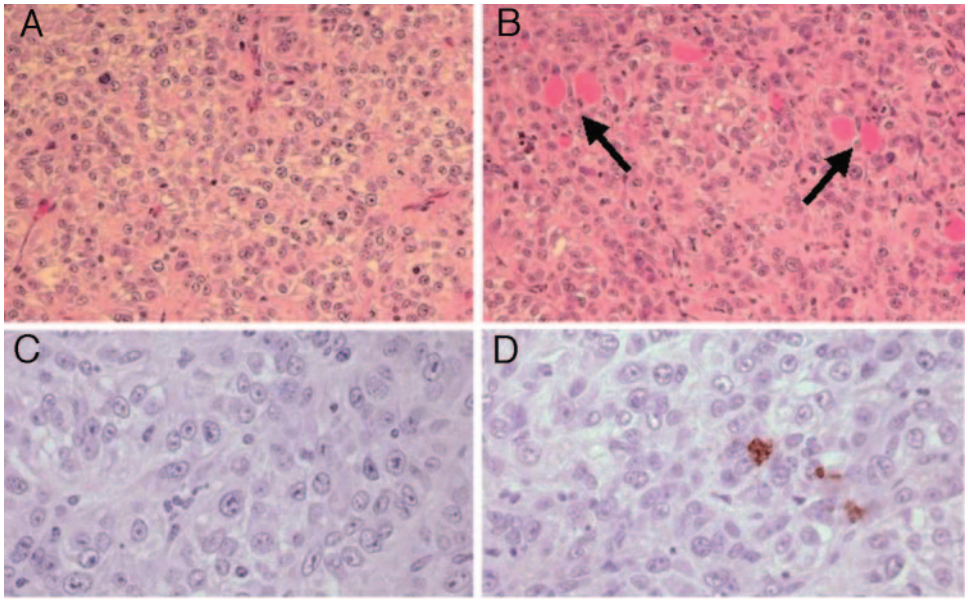

FIG. 4. Calcitriol (vitamin D3) restores differentiation and thyroglobulin expression in thyroid follicular carcinoma cells *in vivo*. Representative hematoxylin and eosin-stained slides (A and B) and immunohistochemical stains of the differentiation marker thyroglobulin (C and D) in excised orthotopic tumor xenografts in control vehicle-treated (A and C) and calcitriol-treated (B and D) animals are shown. In animals treated with calcitriol, there is focal evidence of follicle formation (B, *arrows*) as well as thyroglobulin positivity (D). Original magnifications: A and B, ×160; C and D, ×265.

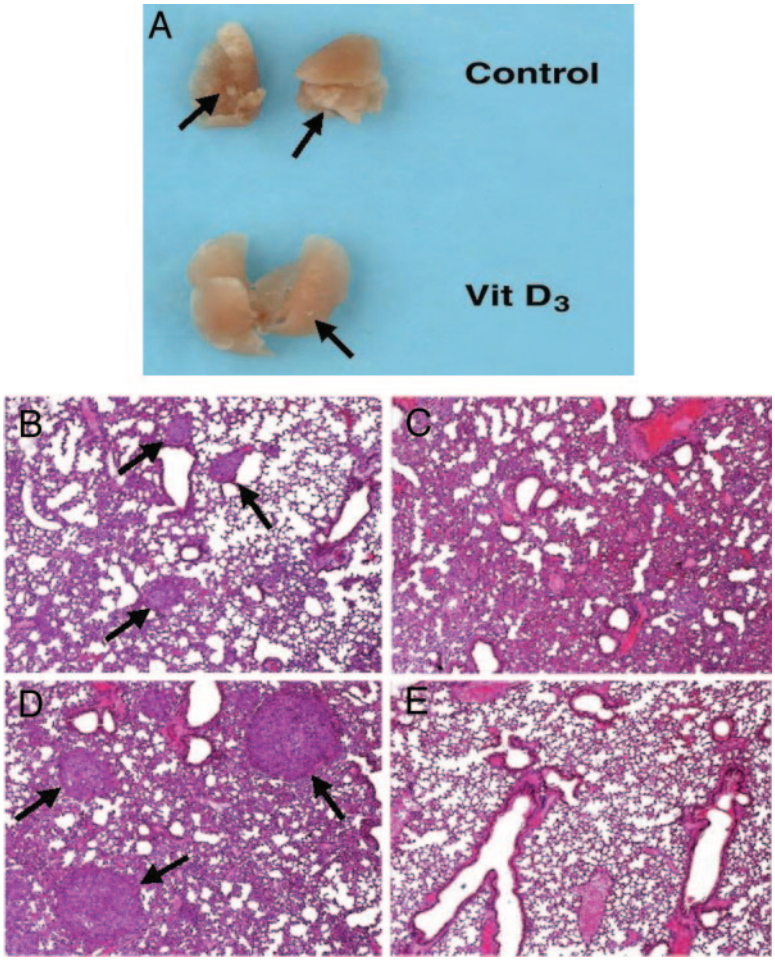

FIG. 5. Calcitriol (vitamin D3) reduces thyroid follicular cancer lung metastases. A, On gross examination, the lung of a vehicle-treated mouse contains multiple metastatic deposits (*arrows*), whereas a calcitriol-treated mouse has only a single small lesion; most mice treated with calcitriol had no detectable lung pathology. Corresponding light microscopic examinations at low magnification (B and C) and high magnification (D and E) illustrate multiple metastatic deposits in the control mouse (B and D), whereas none are identified in a corresponding calcitriol-treated animal (C and E). Original magnifications: A and B, ×25; C and D, ×75.

disease seen in vehicle-treated animals. This effect was confirmed histologically, as numerous large lung metastases were confirmed in vehicle-treated mice. In contrast, lungs from 12 of 15 calcitriol-treated mice showed no lung metastases. The remaining three calcitriol-treated animals demonstrated small metastatic foci that were detectable only by microscopic examination, each focus measuring less than 3 mm in maximum dimension (Fig. 5, B and C). In contrast, 14 of the 15 vehicle-treated control animals showed metastatic lung disease with tumor foci averaging 8 mm in diameter (Fig. 5, A–C). Immunohistochemical analysis for p27 was carried out on the lung metastases; in control animals, metastatic tumor deposits exhibited grade 3 distribution of p27 and grade 1 positivity. The three calcitriol-treated animals with metastatic foci exhibited more abundant and intense p27 reactivity in the small foci of tumor, staining that was graded as grade 5 distribution and grade 3 positivity. No thyroglobulin positivity was identified in the lung metastases.

Discussion

The potential for vitamin D in cancer prevention and treatment has been recognized for decades, as early observations suggested an inverse association between cancer rates and sun exposure (3). A recent study has concluded that vitamin D derived from sun exposure may improve the prognosis of breast, colon, and prostate cancer (15), and a significant variation in prognosis by season of diagnosis was observed. Diagnoses during summer and fall, the seasons with the highest level of vitamin D, revealed the lowest risk of cancer death, suggesting that a high level of vitamin D at the time of diagnosis and, thus, during cancer treatment may improve prognosis of the three cancer types studied. The strongest epidemiological evidence for a protective role for vitamin D comes from prospective studies of dietary vitamin D intake and colorectal cancer development. A clear inverse association is evident for vitamin D intake and colon or colorectal cancer (16–23). 1,25-Dihydroxyvitamin D3 (calcitriol), the active metabolite of vitamin D, has been demonstrated to have significant antineoplastic activity for which several mechanisms have been proposed (8). These include inhibition of proliferation associated with cell cycle arrest, differentiation, reduction in invasiveness and angiogenesis, induction of apoptosis, and altered activation of growth factor signaling (24–28). Mechanisms differ between tumor models and experimental conditions, and no singular unifying hypothesis about the mechanism of antineoplastic activity has thus far emerged (8).

A role for vitamin D in the modulation of proliferation and differentiation in a variety of cell types has been observed including squamous cell cancer, breast cancer, prostate cancer, and colorectal cancer (4–7, 24–28). In thy-

roid cancer, previous studies from our laboratory investigated the effects of calcitriol and its noncalciomimetic analog EB1089 on the growth *in vitro* of a number of thyroid carcinoma cell lines (11). Calcitriol and EB1089 exhibited antiproliferative effects in a dose-dependent manner as determined by thymidine incorporation and MIB-1 immunolabeling (11). Both agents resulted in similar G_1-phase arrest; however, neither apoptosis nor differentiation was observed (11). This effect in follicular WRO cells is consistent with the presence of functional vitamin D_3 receptors that we have also documented by RT-PCR analysis (data not shown). Increased nuclear protein expression of the cyclin-dependent kinase inhibitor, p27 in cells treated with calcitriol or EB1089 was mediated by the phosphatase phosphatase and tensin homolog and its downstream target phosphorylated activated kinase/protein kinase B in WRO cells (11). We thus sought to develop an *in vivo* model of thyroid cancer cell growth to determine whether this *in vitro* effect of calcitriol was translatable to the murine model.

In the current study, thyroid cancer xenografts grew orthotopically in the neck of immunodeficient mice with subsequent development of lung metastases. Both the primary and metastatic tumors were responsive to calcitriol treatment, evidenced by decreased tumor volume as well as number of lesions and extent of metastatic growth.

A beneficial role for vitamin D in the prevention of breast and prostate cancer metastasis has also been observed in other experimental model systems (9, 10). In the present study, the mechanism of the inhibitory effect of calcitriol in the *in vivo* treatment of thyroid cancer confirmed our previous *in vitro* observations noted above (11). This was reflected by an increased accumulation of the cyclin-dependent kinase inhibitor p27 in tumors in calcitriol-treated animals compared with control/vehicle-treated animals and enhanced cellular differentiation supported by focal increases in tumor thyroglobulin positivity in calcitriol-treated animals.

Recent studies have noted a potentially beneficial effect of vitamin D in another endocrine tumor model, specifically insulinoma cells, by yet another mechanism (29). Calcitriol-induced apoptosis in this system was dependent on the function of p53 and was associated with an increase in protein levels of transcription factor nuclear factor-$\kappa\beta$. Calcitriol-induced cell death was regulated by members of the Bcl-2 family of apoptosis regulatory proteins, as demonstrated by calcitriol-induced up-regulation of proapoptotic Bax and Bak and the lack of cytotoxicity in Bcl-2-overexpressing insulinoma cells. Furthermore, these studies revealed that calcitriol-mediated arrest of insulinoma cells in G_1 phase was associated with the abnormal expression of p21 and G_2/M-specific cyclin B2 genes and involved the DNA damage-inducible factor GADD45.

It is evident that calcitriol exerts its antiproliferative effects by numerous mechanisms depending on the cell type and tumor model system. Clinically, recent phase I trials in prostate cancer have demonstrated that intermittent dosing allows substantial dose escalation and has produced potentially therapeutic peak calcitriol concentrations. A phase II study reported encouraging levels of activity for the combination of high-dose calcitriol and docetaxel administered on a weekly schedule in patients with androgen-independent prostate cancer (8).

Additional work is needed to elucidate the molecular mechanisms of vitamin D's antineoplastic properties and the optimal clinical applications. Nevertheless, this novel mouse model of metastatic follicular thyroid cancer indicates that calcitriol and its noncalciomimetic analogs are worthy of future investigation in thyroid cancer. In particular, our data support the consideration of vitamin D analogs in clinical trials as adjuvant treatment of inoperable and radioactive iodine-resistant forms of thyroid follicular-derived carcinomas.

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