TOXICITY AND DOSE-RESPONSE STUDIES OF 1α-HYDROXYVITAMIN D_2 IN LHβ-TAG TRANSGENIC MICE

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ABSTRACT

Purpose: The study objective is to determine the effectiveness of a vitamin D analogue, 1α-hydroxyvitamin D_2 (1α-OH-D_2), in inhibiting retinoblastoma in a transgenic retinoblastoma model (LHβ-Tag mouse) and to evaluate its toxicity. Previous studies of 1α-OH-D_2 in athymic mice with human retinoblastoma xenografts suggested efficacy in tumor suppression and suitability for human treatment.

Methods: LHβ-Tag mice (N = 142), 8 to 10 weeks old, were randomly assigned to treatment groups receiving either control (vehicle) or 0.1, 0.3, 0.5, or 1.0 μg/day of 1α-OH-D_2 via oral gavage five times a week for 5 weeks. Animals were then euthanized. The eyes were enucleated, processed histologically, and serially sectioned. Three sections of each eye were microscopically examined, and mean tumor area was measured using Optimus software. Toxicity was assessed by mortality, weight loss, serum calcium levels, and kidney calcification.

Results: The mean tumor size in each 1α-OH-D_2 group was smaller than in controls (*P* values <.02): control, 90,248 μm^2; 0.1 μg, 31,545 μm^2; 0.3 μg, 16,750 μm^2; 0.5 μg, 30,245 μm^2; and 1.0 μg, 16,049 μm^2. No dose-dependent response curve was evident. Mortality was higher in the groups receiving the 0.5 μg and 1.0 μg doses (*P* values <.01) than in the other treatment groups and the control group.

Conclusion: In the LHβ-Tag mouse, 1α-OH-D_2 inhibits retinoblastoma with no increased mortality at lower doses (0.1 to 0.3 μg). 1α-OH-D_2 has been approved by the Food and Drug Administration as an investigative drug for cancer treatment and has shown efficacy with low levels of toxicity in adult cancer trials. 1α-OH-D_2 meets the criteria for human clinical trials.

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INTRODUCTION

Retinoblastoma is the most common intraocular malignancy in childhood, occurring once in 20,000 live births worldwide.[1] Although current methods of treatment have achieved survival rates of better than 90%,[1] there is a need for improved treatment alternatives to provide better visual results and decrease the risk of secondary tumors in bilateral retinoblastoma.[2]

A candidate compound for clinical trials in human retinoblastoma must meet the following criteria in preclinical studies: (1) effectively inhibit the growth of retinoblastoma; (2) have low levels of toxicity; (3) be nonmutagenic; (4) have a defined mechanism of action; (5) be approved by the Food and Drug Administration (FDA) for investigative use in tumors; and (6) have been successfully tested in adult cancer. The vitamin D analogue 1α-hydroxyvitamin D_2 (1α-OH-D_2) is a nonmutagenic compound that has previously been shown to be effective with low levels of toxicity in dose-response studies using the athymic/human retinoblastoma model.[3] The antineoplastic mechanism of action of related vitamin D compounds has been identified.[4] 1α-OH-D_2 has been approved as an investigational drug in prostate cancer and has been successfully used in phase 1 and phase 2 studies (George Wilding, personal communication, July 30, 2001). The purpose of the present study is to determine the effectiveness of this compound in inhibiting retinoblastoma in a transgenic retinoblastoma model, the LHβ-Tag mouse,[5] and to evaluate its toxicity.

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MATERIALS AND METHODS

COMPOUND PREPARATION AND ADMINISTRATION

Pure crystalline 1α-OH-D_2 (graciously provided by BoneCare International, Inc, Madison, Wisconsin) was dissolved in 100% ethanol for a stock solution of 2.98 mg/mL. This solution was diluted in 0.1 mL of coconut oil to make drug concentrations of 0.1 μg, 0.3 μg, 0.5 μg, and 1.0 μg. Drug concentrations were confirmed by spectrophotometric analysis. The control group was given 0.1 mL of coconut oil. Stock solutions of drug were prepared fresh weekly and stored in amber glass bottles at -40°C to protect the compound from degradation due to temperature or UV light.

TOXICITY TRIAL

All experiments performed on animals conformed to the animal care and use polices defined by the Research Animal Resource Center at the University of Wisconsin and the ARVO statement on the Use of Animals in Ophthalmic and Vision Research.

A brief toxicity trial was done to determine the most efficacious doses of 1α-OH-D_2 for the dose-response study. Forty-two LHβ-Tag transgene-negative (as determined by polymerase chain reaction [PCR]) mice were divided into five treatment groups of seven animals each with a corresponding control group of seven animals. Drug doses were 0.3 μg, 0.9 μg, 1.8 μg, 3.0 μg, and 4.8 μg in 0.1 mL of coconut oil. The control group received 0.1 mL of coconut oil alone.

DOSE-RESPONSE STUDY

A total of 175 LHβ-Tag transgene-positive (as determined by PCR) 8- to 10-week-old mice were randomly assigned by sex and litter to one of five treatment groups. Doses were calculated from the preliminary toxicity study in transgene-negative mice (see "Results" section) and are as follows: vehicle (control) and 0.1 μg, 0.3 μg, 0.5 μg, and 1.0 μg of 1α-OH-D_2. Treatment was via oral gavage with a 1-inch 22-G steel gavage needle (1.25 mm in diameter) attached to a 1-mL syringe. Treatment was given five times per week for 5 weeks. Doses were skipped for up to 3 consecutive days in mice that lost weight (25% of baseline weight) and/or became lethargic and were continued when the affected animals regained weight and health. The mice were fed a vitamin D and calcium restricted diet to remove the effect of endogenous calcium on the treatment. Individual body weights were recorded twice per week during treatment and just prior to euthanization on the last treatment day.

TUMOR MEASUREMENT AND HISTOPATHOLOGIC STUDY

After euthanization, the eyes were enucleated and placed in 10% neutral buffered formalin for standard histopathologic sections. Four serially sectioned 5-μm-thick sections were cut from each of the superior, middle, and inferior areas of the globe and stained with hematoxylin-eosin. The four sections from each globe area were examined under a microscope, and the section with the largest tumor was used for measurement. The outline of the tumor in each section was traced from a microscopically digitized image and the area measured with Optimas software version 6.5 (MediaCybernetics, Silver Spring, Maryland). The three tumor areas from each representative portion of the globe were averaged together to obtain the mean tumor measurement of each eye expressed in micrometers squared (μm^2).

TOXICITY ASSESSMENT

Following euthanization, both kidneys were harvested from randomly selected mice in each treatment group (control group, 15 mice; 0.1 μg group, 14 mice; 0.3 μg group, 16 mice; 0.5 μg group, 17 mice; and 1.0 μg group, 17 mice). Kidneys were fixed in 10% neutral buffered formalin, processed histologically, and serially sectioned at 5 μm. Sections were stained by hematoxylin-eosin and von Kossa techniques. Two sections of each kidney were examined by a single masked reviewer and graded for degree of calcification according to the following scale: I, no calcium deposits; II, 1 to 7 foci of calcium deposits; III, 8 to 15 foci of calcium deposits; and IV, >15 foci of calcium deposits.

Serum samples from representative mice in each group (control group, 13 samples; 0.1 μg group, 13 samples; 0.3 μg group, 11 samples; 0.5 μg group, 9 samples; and 1.0 μg group, 11 samples) were obtained just prior to euthanization from the axillary vessels and analyzed for calcium levels by Marshfield Laboratories, Marshfield, Wisconsin.

Toxicity was assessed by percent of animals surviving the treatment schedule, changes in body weight, degree of kidney calcification, and serum calcium levels. Animals that died before the completion of the treatment were not assessed in the latter three categories.

STATISTICAL METHODS

The effect of dose on tumor area, change in animal weight, serum calcium levels, and kidney calcification was assessed using analysis of variance. The tumor area and serum calcium levels were log transformed prior to analysis to stabilize the variance. No transformation of the kidney calcification values produced approximately normally distributed data. These data were rank transformed (the rank of a data value is its numerical ordering) to obtain an approximate nonparametric analysis. The change in animal weight (from first to last measurement) was restricted

to animals that survived until the last measurement (week 5). The effect of dose on mortality was assessed by using a generalized linear model assuming binomial variability.

The effect of layer (animal batch) on response was accounted for in all analyses by including a blocking term in the model. All significant global tests for effect of dose were followed by pairwise analyses to assess differences between specific dose groups.

RESULTS

PRELIMINARY TOXICITY TRIAL TO DETERMINE DOSAGES

Forty-two LHβ-Tag transgene-negative (as determined by PCR) mice were divided into five treatment groups of 7 animals each with a corresponding control group. Toxicity was assessed by mortality. The majority of mice in the 1.8 μg (6 of 7 animals), 3.0 μg (6 of 7 animals), and 4.8 μg (7 of 7 animals) groups died within 2 weeks of treatment. One animal in each of the 1.8 μg and 3.0 μg groups survived the 5-week treatment schedule. Two of 7 animals in the 0.9 μg group died within 2 weeks of treatment initiation, and 3 animals in the same group died within 4 weeks of treatment. From these data, we calculated doses of 0.1 μg, 0.3 μg, 0.5 μg, and 1.0 μg of 1α-OH-D_2 for the dose-response study.

DOSE-RESPONSE EFFICACY STUDY

Results of the dose-response data are summarized in Table I. The number of animals surviving the treatment schedule is as follows: control group, 30 of 31 mice surviving (97%); 0.1 μg group, 32 of 35 animals surviving (91%); 0.3 μg group, 30 of 34 animals surviving (88%); 0.5 μg group, 26 of 37 animals surviving (70%); and 1.0 μg group, 24 of 38 animals surviving (63%) (Figure 1). Mean tumor area of each treatment group was as follows: control, 90,248 μm²; 0.1 μg; 31,545 μm²; 0.3 μg, 16,750 μm²; 0.5 μg, 30,245 μm²; and 1.0 μg, 16,049 μm² (Figure 2). Although no dose-dependent response curve is appreci-

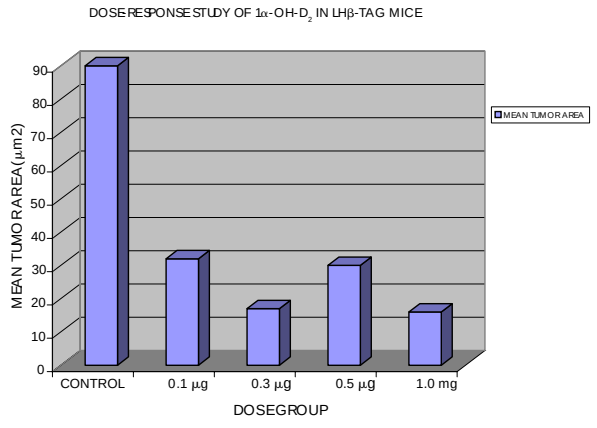

FIGURE 1

Survival curve of study animals. Survival rate was high in the 0.1 μg and 0.3 μg groups. However, mortality was significantly higher in the 0.5 μg and 1.0 μg groups when compared to control group and 0.1 and 0.3 μg groups (all *P* values <.01).

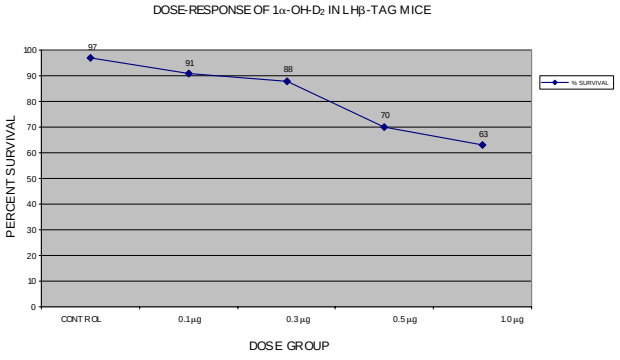

FIGURE 2

Mean tumor area of LHβ-Tag mice treated with 1α-OH-D_2, measured in micrometers squared (μm²). Reduction in tumor area is seen in all treated groups when compared to control group (all *P* values <.02); however, no dose-response curve is readily apparent.

TABLE I: SUMMARY OF DATA ANALYZED IN STUDY OF 1α-OH-D_2 IN LHβ-TAG MICE

DOSE GROUP	NO. OF MICE	% SURVIVAL	TUMOR AREA*	% TUMOR INHIBITION	SERUM CALCIUM (mg/dL)†	MEAN WT CHANGE (gm)‡
Control	31	96.77	90.25 ± 32.55	NA	7.9	0.605 ± 0.34
0.1 μg	35	91.43	31.55 ± 10.83	34.9	9.9	-1.27 ± 0.33
0.3 μg	34	88.24	16.75 ± 6.11	18.6	11.3	-3.02 ± 0.35
0.5 μg	37	70.27	30.24 ± 11.72	33.5	11.4	-3.13 ± 0.36
1.0 μg	38	63.16	16.05 ± 6.57	17.8	12.2	-3.4 ± 0.39

NA, not applicable.
*Tumor area was significant when compared to controls in all treated groups (all *P* values <.02).
†Serum calcium levels were higher in all treatment groups when compared to controls (all *P* values <.0001).
‡Body weight loss was significantly higher in the treatment groups when compared to controls (all *P* values <.0001).

ated, the average tumor measurement from each treatment group of 1α-OH-D_2 is statistically significant (all *P* values <.02) compared to controls.

Survival rate, change in body weight, and serum calcium data are summarized in Table I. Increases in mortality, weight loss, and serum calcium levels and a higher kidney calcification grade are seen as the dose of 1α-OH-D_2 increases. Mortality in the 0.5 μg and 1.0 μg treatment groups was higher than in all other treatment groups and the control group (all *P* values <.01). Weight loss (measured as change in weight from baseline measurement to final measurement) was statistically significant in all drug groups when compared to controls (all *P* values <.0001). Kidney calcification was significantly higher in the 0.3 μg, 0.5 μg, and 1.0 μg groups (all *P* values <.002). Serum calcification was significantly higher in all treatment groups when compared to controls (*P* values <.0001). Serum calcium was also significantly higher in the 0.3 μg and 1.0 μg groups when compared to the 0.1 μg group (*P* values <.009).

Some mice skipped treatment doses owing to weight loss or lethargy: control group, 0% doses skipped; 0.1 μg group, 4.4% doses skipped; 0.3 μg group, 6.1% doses skipped; 0.5 μg group, 7.7% doses skipped; and 1.0 μg group, 10.8% doses skipped. The effect of skipped dose was not statistically significant (*P* = .39).

DISCUSSION

In 1966, Frederick C. Verhoeff[6] proposed that retinoblastoma be treated with high doses of vitamin D. This hypothesis was based on his observations of the association of calcification of retinoblastoma with spontaneous and induced regression. To test this hypothesis, we studied the two standard forms of vitamin D, ergocalciferol (vitamin D_2) and 1,25-dihydroxy-D_3 (calcitriol) (Figure 3), in the athymic/Y79 retinoblastoma xenograft mouse model.[7,8] Subsequently, we studied the effect of calcitriol on the growth of retinoblastomas in the LHβ-Tag mouse model.[9] From these studies, we learned that both calcitriol and vitamin D_2 inhibited the growth of human retinoblastomas and transgenic mouse retinoblastomas, but the antineoplastic effect was related to either elevated serum calcium levels or calcification of the tumors.

We were encouraged that synthetic analogues of vitamin D_2 and calcitriol with retained or increased antineoplastic effect but decreased hypercalcemic activity might prove feasible for eventual human trials. This conclusion followed our experience with the synthetic analogue of calcitriol, 1,25-dihydroxy-16-ene-23-yne-vitamin D_3 (16,23-D_3). This compound was extensively studied in the athymic xenograft mouse model[10] and the LHβ-Tag transgenic mouse models.[11,12] These results have recently been reviewed in detail.[4]

Although 16,23-D_3 appears to be a promising drug for use in the treatment of retinoblastoma in children, it was not approved by the FDA until March 1999 for investigational use in human cancer patients. No adult data are currently available; consequently, we have carried out preclinical studies of another analogue of vitamin D_2, 1α-OH-D_2. As noted, 1α-OH-D_2 has been used successfully in our laboratory in the treatment of nude mice containing human retinoblastoma xenografts, has been approved by the FDA (in 1996) as an investigational drug in prostate cancer, and has been successfully used in phase 1 and phase 2 clinical studies of prostate cancer (George Wilding, personal communication, July 30, 2001). In the athymic/Y79 mouse model, tumor weight and volume in groups receiving doses of 0.2 or 0.3 μg/day of 1α-OH-D_2 were significantly lower than in controls and toxicity was less than with calcitriol and vitamin D_2.[3] In the studies now described, 1α-OH-D_2 inhibited retinoblastoma growth in the LHβ-Tag transgenic mouse model, with each treatment group (0.1 to 1.0 μg) showing a statistically significant effect as compared to the control group. The tumors in the treated animals ranged in size from 18% to 30% of the mean tumor area in the control group. There was not, however, an identifiable dose-dependent response curve. No statistically significant increase in mortality was seen between the 0.1 μg group, the 0.3 μg group, and the control group (Figure 1). However, statistically significant increases in mortality in the 0.5 μg and 1.0 μg groups were observed (both *P* values <.05).

Vitamin D receptor mRNAs are detectable in Y79 retinoblastoma cells, LHβ-Tag tumors, and human retinoblastoma specimens using reverse-transcriptase polymerase chain reaction (RT-PCR).[4] On the basis of experiments that were carried out on calcitriol and 16,23-D_3,[4] the mechanism of action appears to be related to increased p53-related gene expression resulting in increased apoptosis.

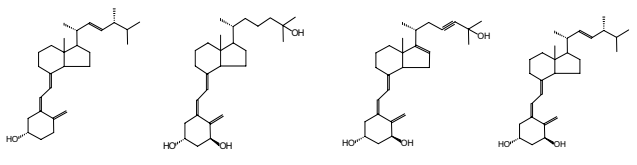

FIGURE 3
Vitamin D_2 (ergocalciferol) and its analogs, calcitriol (1,25-dihydroxy-D_3), 1,25-dihydroxy-16-ene-23-yne-vitamin D_3 (16,23-D_3), and 1α-hydroxyvitamin D_2 (1α-OH-D_2).

CONCLUSION

1α-OH-D_2 has been effective in inhibiting retinoblastoma growth in two animal models, the LHβ-Tag transgenic

mouse, as presently described, and the athymic/Y79 model, as previously reported.[3] This nonmutagenic compound has low toxicity. The mechanism of antineoplastic action has been demonstrated in related compounds, and studies are under way to show whether or not the mechanism of action is similar for 1α-OH-D_2. The drug has been approved by the FDA for investigational use in human tumors, and phase 1 and phase 2 data show the drug to have effectiveness and low toxicity in the treatment of human prostate cancer. We are now planning to move into phase 1 and phase 2 clinical trials of this drug in children.

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DISCUSSION

DR BARRETT G. HAIK. The treatment of patients with retinoblastoma has changed dramatically over the past 10 years. Until the mid-1990s, external beam irradiation was the primary modality employed in conservative therapy. As this therapy was refined over a half century, improved delivery systems reduced the amount of damage to adjacent ocular and orbital tissue caused by radiation.

However, in 1993, Eng and colleagues presented a statistically powerful study that demonstrated an extremely high incidence of radiation-associated tumors in long-term survivors of retinoblastoma. Although the association of radiogenic neoplasms in patients with retinoblastoma had been noted for many years, Eng's study provided the largest patient series with meticulous long-term follow-up.

At approximately the same time that Dr Eng was confirming the continued risks involved with radiation therapy, Judith Kingston and John Hungerford from St. Bartholomew's Hospital in London were reporting success in treating intraocular retinoblastoma with systemic chemotherapy. Subsequently, multiple ocular oncology centers throughout North America and Europe developed individual protocols based on their own experiences with chemotherapeutic agents for tumors of neural origin. Because no single chemotherapy protocol has been proven to be totally effective and all carry significant risk of side effects, none has been universally accepted.

Today, Dr Albert and his colleagues have presented their own extremely exciting and promising findings that a nonmutagenic synthetic analog of vitamin D_2, 1α-hydroxyvitamin D_2 (1α-OH-D_2), effectively inhibits growth of retinoblastoma in mouse models while offering low toxicity.

The prospect of taking Dr Albert's promising findings from the laboratory to the bedside is exciting. Nevertheless, established use of 1α-OH-D_2 will require proof of its efficacy as a single agent or as part of a multimodality regimen, and developing protocols for delivery of drugs and for monitoring side effects will take time and designing and implementing clinical trials will be complex because of the rarity of retinoblastoma. Many years of follow-up will also be required to assess the long-term impact of the incidence of second primary tumors.

Notwithstanding the time and efforts that will be required to test the use of this modality in the treatment of patients with retinoblastoma, Dr Albert's findings regarding 1α-OH-D_2 offer new hope to children afflicted with this sight- and life-threatening disease.