Antineoplastic Effect of 1,25-Dihydroxy-16-ene-23-yne-Vitamin D₃ Analogue in Transgenic Mice With Retinoblastoma

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Objectives: To evaluate the in vivo efficacy and clinical toxic effects of the 1,25-dihydroxy-16-ene-23-yne-vitamin D₃ analogue in β-luteinizing hormone-Tag (LHβ-Tag) transgenic mice with heritable retinoblastoma.

Methods: Forty-two mice (8-10 weeks old), randomly assigned to experimental (n = 21) or control (n = 21) groups, received intraperitoneal injections of 0.05 μg of 1,25-dihydroxy-16-ene-23-yne-D₃ in 0.5-mL mineral oil vehicle (experimental group) or 0.5 mL of mineral oil vehicle (control group) for 5 weeks. One experimental and 3 control animals died of injection-related trauma. Eyes were enucleated 1 week after treatment and were examined histologically in a masked fashion.

Results: All experimental and control animals showed evidence of tumor. The tumors in the experimental mice showed a significantly smaller cross-sectional area (0.88 ± 0.08 mm²) compared with that in the control mice (1.12 ± 0.12 mm²) (P = .02). All mice completed the treatment and showed no clinical evidence of toxic effects.

Conclusions: Tumors in transgenic mice with retinoblastoma treated with 1,25(OH)₂-16-ene-23-yne-D₃ showed a 21% smaller cross-sectional area compared with that in the control mice, without producing clinically apparent toxic effects. This compound may be useful as adjuvantive therapy in the treatment of retinoblastoma.

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RETINOBLASTOMA is the most common primary intraocular malignancy in children, with an incidence of 1 case in 20,000 live births worldwide. In 1966, Verhoeff, who gave retinoblastoma its name and who had studied the tumor for more than 50 years, stressed the observation that, in cases of regressed retinoblastoma, the tumors were heavily calcified. He proposed using calcifying agents, particularly vitamin D, to kill or inhibit the proliferation of retinoblastoma. Subsequently, high-affinity vitamin D receptors have been identified in the Y-79 human retinoblastoma cell line. Vitamin D has been shown to inhibit the growth of retinoblastoma in tissue culture, in Y-79 human retinoblastoma cell line grown in athymic “nude” mice, and in β-luteinizing hormone-Tag (LHβ-Tag) transgenic mice, a model for hereditary retinoblastoma. Marked hypercalcemic toxic effects resulted from the large doses required to achieve a therapeutic effect, but the mechanism by which vitamin D inhibits neoplastic proliferation seems to be separate from its effect on calcium metabolism.

Over the past decade, numerous vitamin D analogues have been developed that seem more potent than the active metabolite of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), in inducing differentiation and inhibiting proliferation of neoplastic cells. Notably, they are less potent in inducing intestinal calcium absorption and bone calcium mobilization. Herein, we examined the in vivo efficacy and clinical evidence of toxicity of the 1,25-dihydroxy-16-ene-23-yne-vitamin D₃ analogue (1,25(OH)₂-16-ene-23-yne-D₃) in LHβ-Tag mice with heritable retinoblastoma. This model has been previously well described in the English-written articles and provides an opportunity to evaluate the benefits of various

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MATERIALS AND METHODS

ANIMALS

Forty-two LHβ-Tag transgenic mice were randomized into 2 groups of 21 animals in each. The mice were 8 to 10 weeks old at the start of treatment and were weighed by group daily. The details of animal care concerning mice treated with 1,25(OH)2D3 have been previously reported. Four mice (3 control and 1 experimental) died of injection-associated trauma before conclusion of the study and were excluded from the analysis. Twenty experimental and 18 control animals completed the treatment.

COMPOUND PREPARATION

Pure crystalline 1,25(OH)2-16-ene-23-yne-D3 (provided by Milan Uskokovic, PhD, Hoffmann-LaRoche, Nutley, NJ) was dissolved in 100% ethanol and stored in amber bottles under nitrogen gas at -40°C. Stock solutions were prepared at a concentration of 1 mg/mL in absolute ethanol or ethyl alcohol at the onset of the study and then every 7 days due to the short half-life of 1,25(OH)2-16-ene-23-yne-D3. Before injection, the compound was diluted in mineral oil and the concentration was confirmed spectrophotometrically. The experimental mice received 0.05 µg of 1,25(OH)2-16-ene-23-yne-D3 in a mineral oil vehicle. The control mice received 0.5 mL of mineral oil. The treatment was administered by intraperitoneal injection with a 25-gauge needle on a daily basis, 5 days a week for 5 weeks. Toxic effect was assessed by survival, daily weights, and clinical manifestations of hypercalcemia, such as poor fur grooming and lethargy. Blood calcium data were obtained in a toxicity trial (Table), and, consequently, these values were not obtained from the LHβ-Tag mice during the treatment trial.

PRELIMINARY TOXICITY TRIAL

To assess the relative toxicity of 1,25(OH)2-16-ene-23-yne-D3, a preliminary toxicity trial was done. Thirty-two ICR (nontransgenic) mice (Harlan Sprague Dawley) were randomly divided into 2 groups of 16 animals (group A and group B) and were treated for 5 weeks. Group A received 0.5-mL intraperitoneal injections with 0.05 µg of 1,25(OH)2D3 in a mineral oil vehicle, and group B received 0.05 µg of 1,25(OH)2-16-ene-23-yne-D3 in mineral oil vehicle. Blood calcium levels were measured several times throughout the study (Table). As in previous experiments, toxicity was assessed by survival and daily weights.

ANALYSIS OF TUMOR SIZE

Mice were killed 1 week after completion of treatment, and their eyes were enucleated, fixed in 10% neutral buffered formalin, and submitted for histologic processing. Paraffin-embedded eyes were sectioned at 5 µm and stained with hematoxylin-eosin. The technician was masked during slide processing.

Because tumor size from a given eye can vary from slide to slide, only sections through the pupil-optic-nerve-tumor plane were studied. Because the mean diameter of the optic nerve in the LHβ-Tag model is 225 µm, a total of 45 5-µm sections can be cut. By limiting sections to those cut through the anatomic center of the globe, equivalence between experimental and control animals for biases was achieved.

Three pupil-optic nerve sections from each eye were prepared and grossly inspected. The technically superior (eg, free of folds, knife marks or other cutting artifacts) section was chosen for digitization by a masked observer. If the sections were of equal quality, a section was randomly selected by a masked observer. Slides were coded, and investigators remained masked. The sections were digitized using a color scanner (UMAX, UMAX Data System, Taiwan, Republic of China) and a commercially available software package (Adobe Photoshop, Adobe Systems Inc, Mountain View, Calif). Each tumor was traced from the digitized image. The area was calculated in pixels by a software package (Image-Pro Plus, Media Cybernetics, Silver Spring, Md) and converted into square millimeters. Additional parameters, such as degree of retinal involvement, the involvement of other ocular structures, and tumor morphology, were evaluated independently in a masked fashion, as previously described. Degree of retinal involvement was scored as the presence of a single focus of tumor, as multiple foci, or as confluent involvement. It was also estimated by percentages of uninvolved retina, retina with tumor limited to the inner nuclear layer, or full-thickness replacement of the retina. Involvement of ocular structures was scored, including vitreous, lens, ciliary body, iris, angle, anterior chamber, cornea, choroid, retinal pigment epithelium, optic nerve, and extraocular extension. Tumor morphology was quantified by percentage of tumor calcification, percentage of tumor necrosis, proportion of undifferentiated tumor, Homer Wright rosettes, Flexner-Wintersteiner rosettes, and the number of mitoses in 6 high-power fields. The degree and character of inflammation were also scored.

Autopsies were not performed, and the effects of treatment on other tissues besides the eye were not studied. Because of the relatively short time after completion of treatment during which the mice were killed (1 week), conclusions about the long-term effects on tumor growth cannot be made.

STATISTICAL ANALYSIS

Tumor area was evaluated statistically by averaging each pair of eyes and conducting a 2-sample t test on these averages. The t test was done on the logarithmic scale to induce normality and to account for outliers. A nonparametric analysis, using the Wilcoxon rank sum test for the same data, was also performed and the results were concordant with those of the t test. Differences were considered significant at the .05 level. All averages given in the text are on the raw scale.

To evaluate the other parameters, individual mice were again treated as independent units and mean values were taken for each mouse. The medians of these averages in the 2 groups were compared using the Wilcoxon rank sum test.

chemotherapeutic protocols. In the transgenic mice, bilateral tumors arising from the inner nuclear layer of the retina develop; in 10% to 25% of them, midbrain tumors develop. The antigenic profile of mice tumors resembles that of human retinoblastoma, but it differs in rosette morphology and antigen distribution.
Toxicity Trial Results*

<table>
<thead>
<tr>
<th>No. of Mice</th>
<th>Mean Weight, g</th>
<th>Weight Change, %</th>
<th>Blood Calcium, mg/dl.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 33</td>
<td>Day 1</td>
</tr>
<tr>
<td>Group A: 1,25(OH)2D3</td>
<td>16</td>
<td>4</td>
<td>30.38</td>
</tr>
<tr>
<td>Group B: 1,25(OH)2-16-ene-23-yne-D3</td>
<td>16</td>
<td>16†</td>
<td>30.66</td>
</tr>
</tbody>
</table>

*1,25(OH)2D3 indicates vitamin D metabolite, 1,25-dihydroxyvitamin D3. 1,25 (OH)2-16-ene-23-yne-D3, vitamin D3 analogue.
†One mouse died of injection-related trauma.

CLINICAL EVIDENCE OF TOXIC EFFECTS

All animals survived the 5-week treatment without clinical evidence of toxic effects. All of the mice that received 1,25(OH)2-16-ene-23-yne-D3 gained weight. In the experimental mice, the average weight of female mice increased from 19.35 to 31.23 g (61.40%); that of male mice increased from 22.64 to 30.66 g (35.42%). In the control mice, average weight of female mice increased from 19.43 to 28.83 g (48.34%); that of male mice increased from 24.46 to 31.56 g (29.03%). Overall, at the end of the study, average weight did not differ across treatment groups or by sex.

TUMOR MORPHOLOGY

Examination of tumors in the experimental group revealed less calcification (48%±14% vs 89%±11%, P=.012) and less necrosis (40%±11% vs 78%±10%, P=.026) than in the control group. The tumors in the experimental group tended to have fewer mitotic figures than those in the control group (37.6±7.1 vs 57.1±8.4, P=.052). No significant difference was found in degree of retinal involvement or involvement of other ocular structures.

COMMENT

Vitamin D has manifold actions, modulating calcium and phosphate metabolism, and regulating cellular differentiation and proliferation. It induces differentiation of both murine and human leukemic cells and increases differentiation of lymphocytes, monocytes, fibroblasts, melanocytes, and osteoblasts. Vitamin D inhibits growth of several human cancer lines, including breast, prostate, and colon cancer cells. The therapeutic potential of vitamin D has been limited by the hypercalcemic toxic effects resulting from the large doses required to demonstrate a therapeutic effect. Many different vitamin D analogues have been developed, which are less calcemic but which retain their therapeutic properties. The analogue used in our study, 1,25(OH)2-16-ene-23-yne-D3, has been found to be effective in suppressing growth of several human tumor lines, while being 33 times less effective than 1,25(OH)2D3 in stimulating intestinal calcium absorption and 50 times less active in bone calcium mobilization when administered to vitamin D-deficient chicks. This vitamin D analogue was 10 to 25 times less active than...
1,25(OH)$_2$D$_3$ in causing hypercalcemia when administered to leukemic mice.\textsuperscript{23}

The mode of action of vitamin D is similar to that of some corticosteroid hormones. It noncovalently binds to an intracellular receptor. The corticosteroid-receptor complex then associates with the nuclear DNA of the target cell to initiate protein synthesis or to selectively repress gene transcription.\textsuperscript{19} The mechanism by which vitamin D analogues selectively induce differentiation, while suppressing parathyroid hormone synthesis and secretion, is unclear, but several possibilities have been proposed.\textsuperscript{15,24-26} Analogues with low calcemic activity have a low affinity for serum vitamin D binding protein. This results in a higher proportion of the biologically active, unbound form of the compound in circulation and in a shorter half-life caused by more rapid clearance.\textsuperscript{23} Pharmacokinetic differences between vitamin D and its analogues may result in selectivity of these compounds.\textsuperscript{25,26} Evidence also suggests that vitamin D analogues act differentially at the vitamin D receptor site and that specific analogues may act on distinct receptor functions.\textsuperscript{24}

The mode of action by which 1,25(OH)$_2$-16-ene-23-yne-D$_3$ inhibits tumor growth has not been clearly established. It may be related to its ability to inhibit angiogenesis. The active metabolite of vitamin D$_3$, 1,25(OH)$_2$D$_3$, and a synthetic analogue, 22-oxa-1$\alpha$,25-dihydroxyvitamin D$_3$, have been shown to inhibit angiogenesis using the chick chorioallantoic membrane assay in a dose-dependent manner.\textsuperscript{27} The metabolite 1,25(OH)$_2$D$_3$ also inhibits angiogenesis when administered to immuno-suppressed mice that had received intradermal injections of human tumor cells.\textsuperscript{28} As with other solid tumors, retinoblastoma requires blood vessels for tumor growth to progress. To enlarge, the tumor must find a way to obtain oxygen and nutrients and to dispose of waste products.\textsuperscript{29} Folkman\textsuperscript{30} has demonstrated that tumor growth is angiogenesis dependent. Without proliferation of new vessels, most tumors remain quiescent at a size of only several millimeters.\textsuperscript{31}

The relationship between angiogenesis and retinoblastoma growth kinetics has not yet been fully elucidated in the transgenic mouse model. We have observed that transgenic LHB-Tag murine retinoblastomas are relatively small, localized, and without obvious vascularization during the first 3 to 4 months after birth of mice, but they subsequently show exponential growth concomitant with neovascularization.\textsuperscript{9,32} A variety of human retinoblastoma cell lines have been shown to stimulate angiogenesis, using the endothelial cell migration and chick chorioallantoic membrane assays,\textsuperscript{33} and to produce products very similar to, and possibly the same as, basic fibroblast growth factor, an angiogenic substance.\textsuperscript{34} Clinically, rubeosis iridis with secondary neovascular glaucoma is a common finding in cases of advanced retinoblastoma.\textsuperscript{35} Transgenic mice with retinoblastoma demonstrated a smaller mean vessel count in eyes treated with intraperitoneal injections of 1,25(OH)$_2$D$_3$.\textsuperscript{32} A high degree of tumor neovascularization correlates with development of metastasis and a poor prognosis in mammary, prostatic, head and neck, and early non–small cell lung cancer.\textsuperscript{36-39} The prognostic significance of density of new blood vessels in retinoblastoma is unclear.

Vitamin D and its analogues may inhibit retinoblastoma growth by several mechanisms. These compounds are known to influence cell differentiation and
proliferation through complex mechanisms that have not been fully elucidated. Oncogenes may be in part responsible for induction of tumor angiogenesis,22 and vitamin D may interact with them to reduce this effect. The c-myc mRNA concentration decreases after exposure to 1,25(OH)₂D₃. This is due to both decreased transcription and decreased stability of the c-myc mRNA.41,42 Exposure to 1,25(OH)₂D₃ also results in decreased phosphorylation of the retinoblastoma gene product in many cell types, which may be the reason for retardation of G₀/G₁ transition into the S phase of the cell cycle.43 The effect may also be partially due to local changes in prostaglandin or growth factor levels.41,43

Although chemotherapy did not play a major role in the treatment of retinoblastoma in the past,44 chemoreduction of retinoblastoma is now an area of active research. The effectiveness of chemotherapy in decreasing metastatic spread and improving survival is unknown.44 In February 1996, the Retinoblastoma Clinical Trial Consortium met at the National Eye Institute to plan a national clinical trial for chemoreduction of retinoblastoma and proposed regimens that include carboplatin-vincristine-etoposide with and without cyclosporin A with focal therapy for consolidation of response (see editorial by Ferris and Chew in this issue of the ARCHIVES). Inhibition of cell proliferation, induction of cell differentiation, and inhibition of angiogenesis are some of the mechanisms that are considered important in suppressing malignant growth.28 Vitamin D₃ and its analogues have demonstrated these antitumor effects and may be helpful as adjuvant therapy in helping to control tumor progression. Retinoids, cytokines, and vitamin D₃ and its analogues act synergistically to enhance differentiation in several human tumor lines in vitro.46 All of these compounds have demonstrated antiangiogenic properties.25,47 The combination of antiangiogenic agents with cytotoxic drugs has been found to improve the effect of the latter.48,49 Vitamin D analogues may potentiate conventional therapy, possibly allowing the use of lower dosages of the cytotoxic drugs that have many negative side effects.

Children with heritable disease will develop an average of 4 to 5 retinoblastomas, and individuals with bilateral retinoblastoma have a 200-fold increased risk of developing secondary nonocular neoplasms in early adulthood2 (particularly osteosarcoma20,31). Radiotherapy further increases the total incidence of secondary tumors,52 and chemotherapy, especially the alkylating agents, may also contribute to an increased risk of secondary neoplasms.49,53 Therapies with drugs of low toxicity, which may be used in conjunction with other modes of treatment to achieve chemoreduction, are needed.

Our study presents data supporting the antineoplastic, nontoxic effects of 1,25(OH)₂-16-ene-23-yne-D₃ in transgenic mice with retinoblastoma. Further research is necessary to establish long-term effects of therapy on tumor growth and development and further define its potential usefulness as an adjuvant therapy for retinoblastoma.

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REFERENCES
