Verhoeff’s Query: Is Vitamin D Effective Against Retinoblastoma?

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In 1966, Verhoeff suggested that retinoblastomas might be sensitive to vitamin D because they sometimes undergo calcification and spontaneous regression. In recent years, the antineoplastic effect of vitamin D has been established in vitro and in vivo. This study presents evidence that vitamin D inhibits the growth of the human retinoblastoma cell line (Y-79) grown in athymic mice. In mice treated with ergocalciferol, the subcutaneous retinoblastomas were smaller and showed increased tumor necrosis and calcification. Unfortunately, the vitamin D caused significant toxic reactions. Further studies that reduce the toxicity of vitamin D will be needed before its use in children with retinoblastomas can be advocated. To our knowledge, this is the first demonstration of the activity of ergocalciferol against a tumor in vivo and it suggests that ergocalciferol or one of its derivatives may be an effective chemotherapeutic agent against retinoblastomas in humans.

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Retinoblastoma, the most common intraocular tumor of childhood, has a high rate of spontaneous regression. Characteristically, calcium deposition is seen in retinoblastomas, especially when they undergo spontaneous or induced regression.

In recent years, the role of vitamin D as an antineoplastic agent has been established. Researchers have shown that vitamin D is capable of inducing inhibition and differentiation of several human cancers in vitro, including leukemia, breast cancer, malignant melanoma, and histiocytic lymphoma.

Recent reports have also shown that vitamin D can inhibit tumor growth in vivo. In a short report, Sato et al13 showed that 1-alpha-hydroxyvitamin D3 suppressed growth of murine sarcoma 180 and inhibited pulmonary metastases from murine Lewis lung carcinoma implanted subcutaneously in mice. Honma et al17 used 1-alpha-hydroxyvitamin D3 and 1,25-dihydroxycholecalciferol to prolong survival time of syngeneic mice and nude mice inoculated with murine myeloid leukemia cells. Moore15 also reported the inhibition of a myelomonocytic cell line treated with 1,25-dihydroxycholecalciferol in athymic mice. Eisman et al14 showed that 1,25-dihydroxycholecalciferol inhibits xenografts of human colon cancer and malignant melanoma in immunosuppressed mice. Furthermore, a 19-year prospective epidemiologic study has shown that dietary vitamin D is protective against colorectal cancer in men.

Evidence exists that the antineoplastic actions of vitamin D are mediated by the vitamin D receptor. Vitamin D binds its intracellular receptor and affects gene expression by a mechanism similar to other steroid hormones. There are several forms of vitamin D that bind to the receptor. The two predominant natural forms are vitamin D3, which comes strictly from diet, and vitamin D2, which comes from diet and can be synthesized in the skin in a photochemical reaction. As vitamins D3 and D2 are hydroxylated first in the liver and again in the kidney, they become more potent. The potency of the hydroxylated analogues of vitamin D3 and D2 is similar. We present evidence that vitamin D2 inhibits the growth of heterotransplanted Y-79 retinoblastoma cells in athymic mice.

MATERIALS AND METHODS

Animals

Forty-five congenitally athymic National Cancer Center male and female mice bred at this facility and maintained in a...
laminar air-flow room were used for this experiment. The mice, weighing between 16 and 24 g at the beginning of this study, were randomized by sex and weight into three groups of 15. They were fed autoclaved rodent chow (Wayne Research Labs) from which they received approximately 60 mg/d of calcium, 40 mg/d of phosphorus, and 45 ng/d of vitamin D. All manipulations involving the mice were performed under sterile conditions.

Tumors

The Y-79 retinoblastoma cell line that has been maintained in the athymic mice in this laboratory was harvested by aseptic technique three hours before tumor cell injection. The tumor was minced by mechanical disaggregation followed by light centrifugation to obtain a single cell suspension. Cell counts were performed with a hemacytometer using the trypan blue dye exclusion test to verify cell viability. The mice were anesthetized with 0.03 to 0.05 mL of a 50:50 by volume mixture of xylazine (Rompun), 20 mg/mL, and ketamine hydrochloride (Ketalar), 100 mg/mL, injected intramuscularly into the flank. Local anesthesia (proparacaine hydrochloride) was also applied topically to the right eye. Under sterile conditions, 1.0 × 10⁶ cells suspended in 0.06 mL of serum-free F-12 media (Gibco) were injected subcutaneously in the dorsal midscapular region of each mouse through a 22-gauge needle; 1.25 × 10⁶ cells suspended in 25 μL of serum-free F-12 were injected into the vitreous cavity of the right eye of each mouse through a 30-gauge needle. Injections were made immediately behind the pars plana under magnification. Gentamicin sulfate (Gentacidan), 3 mg/mL/d, was applied topically to the right eye for three days following the injection.

The mice were examined three times a week for tumor growth. Once visible, tumors were measured in three dimensions using calipers. The geometric mean diameter was computed by taking the cube root of the length × width × height. The mice were also weighed and assessed for skin changes, diarrhea, perioral lesions, and lethargy at least once a week.

**Ergocalciferol Treatment**

The vitamin D₃ was first dissolved in 95% alcohol (VWR, Omni-Solve) and then diluted in mineral oil with a final alcohol content of 6% to 10%. Because vitamin D₃ in solution can have a short shelf life, new stock solutions were prepared every ten days and stored in the dark under argon gas at −70°C.

Treatment was initiated five days before tumor cell injection. Mice received either 7.8 mg/kg of ergocalciferol, 2.8 mg/kg of ergocalciferol, or 0.1 mL of mineral oil vehicle injected subcutaneously through a 25-gauge needle. Treatments were given five days a week for five weeks.

**Serum Collection and Handling**

Retro-orbital blood samples for calcium determinations were withdrawn from four mice per group before treatment and 24 hours after the 9th, 17th, and 25th treatments. The bleeding was performed with microliter capillary tubes that were then centrifuged to separate the serum. The serum sample was stored in Eppendorf tubes (Eppendorf Instruments, Hamburg, West Germany) in the dark at −70°C. Hemolyzed and turbid serum samples were excluded from the study because these characteristics may affect the calcium content. The insoluble material may result from the interaction of calcium with proteins and fatty acids. Serum calcium concentrations were determined within 40 days of collection by a colorimetric assay (Sigma) using a Varian (Varian, Palo Alto, Calif) spectrophotometer.

**Pathology**

A necropsy was performed on all mice. Mice that survived to the completion of the study were killed 24 hours after their final treatment. Subcutaneous tumors were excised, measured, and weighed. A piece of each tumor was stored at −70°C and later assayed for calcium content. The right eye, kidney, liver, and a portion of the subcutaneous tumor were fixed in a 0.5% glutaraldehyde and 2% formaldehyde solution for histopathologic examination. Pieces of liver and kidney from each animal were also stored at −70°C and later assayed for calcium content. Six tumor specimens, two kidneys, and two livers from each group were submitted for percentage calcium determinations using the inductively coupled plasma technique.

Tissue sections of the tumors, eyes, livers, and kidneys were stained with either hematoxylin-eosin or the von Kossa stain for calcium for histopathologic examination. Two hematoxylin-eosin–stained sections per tumor and per eye were examined for presence of tumor and percentage of necrosis. One von Kossa–stained section of each tissue was examined for calcification. By dividing the estimated area of necrotic or calcified tissue on a section by the total cross-sectional area of the tissue, one of us (D.M.A.) estimated the percentages of tumor showing necrosis, calcification within necrotic areas of tumor, and calcification in the kidney and liver using coded slides without knowledge of the key. This analysis yielded a semiquantitative estimate of the percentages of calcification and necrosis. The eye, subcutaneous tumors, kidneys, and livers were also examined grossly and microscopically for either local or distant metastases.

**Statistics**

P values are computed using the Student’s t test. Error bars bars demarcate ± SEM.
Fig 3.—Histopathologic state of subcutaneous retinoblastoma. Top, 30-day-old control tumor composed of healthy malignant cells with many mitotic figures. Center, 30-day-old tumor from mouse that received 2.8 mg/kg of ergocalciferol five days a week for five weeks starting five days prior to tumor passage. Note areas of pale necrotic tumor cells and clusters of mineralized tumor cells (arrow). Bottom, 30-day-old tumor from mouse that received 7.8 mg/kg of ergocalciferol five days a week for five weeks. Majority of cells show degeneration. Foci of calcification are common (arrows) (hematoxylin-eosin, original magnification \( \times 645 \)).

RESULTS

Five treated and two control mice were excluded from the study because they either died under anesthesia or they were eaten by their littermates. Two anomalously high tumor calcium levels in treated mice were also excluded because they were two orders of magnitude different from all other values and this difference most likely reflected technical errors.

Tumor Inhibition

From the 12th day of the study until its completion, the average size of the subcutaneous tumors in the treated group was smaller than that in the control group (Fig 1). This finding was confirmed at necropsy. The average tumor weights from the high-dose and low-dose groups were 0.137 and 0.057 g, respectively, vs 0.685 g for the control group. Calcium assays of the subcutaneous tumors revealed a dose-dependent elevation of tumor calcium concentrations in treated vs control mice (Fig 2).

Histopathologic evaluation of the subcutaneous and intraocular tumors revealed a dose-dependent increase in estimated tumor necrosis in treated animals (Figs 3 and 4). Furthermore, the von Kossa stain for calcium revealed a dose-dependent increase in estimated calcification of the subcutaneous tumors (Fig 4). This finding correlates with the chemical assay of tumor calcium seen in Fig 2. The von Kossa calcium stain showed that treated and control intraocular tumors and livers had less than 5% estimated calcification. Treated kidneys, however, had more calcium staining than control kidneys.

Histopathologic examination of sections of the subcutaneous tumors and intraocular tumors revealed no local invasion. All of the subcutaneous tumors were encapsulated and the intraocular tumors did not extend beyond the orbit. Gross examination of the animals and histopathologic
examination of the kidney and liver did not reveal any metastatic disease. This result agrees with previous extensive necropsies of nude mice done in our laboratory that showed that the Y-79 retinoblastoma cells do not metastasize in nude mice when implanted subcutaneously or intracocularly.

Toxicity

Vitamin D toxicity was manifested by hypercalcemia, cachexia, and death. The serum calcium level in treated mice was elevated throughout the study. The average serum calcium levels ranged from 2.87 to 3.5 mmol/L (11.5 to 14.0 mg/dL) in treated mice vs 2.34 to 2.85 mmol/L (9.4 to 11.2 mg/dL) in control mice. Tissue calcium assays did not show a dose-dependent elevation of kidney calcium values in treated animals. In fact, liver calcium levels were higher in control than in treated animals (Fig 2). By the last week of the study, the mice treated with high doses had lost 30% of their original weight and the mice treated with low doses had lost 10%. Concurrently, control mice gained an additional 30% of their original weight. Finally, seven of 13 mice in the low-dose group and nine of 12 mice in the high-dose group died during the course of the study. No mice in the control group died (Fig 5).

COMMENT

This study shows that ergocalciferol inhibits the growth of retinoblastoma in athymic mice. This confirms the hypothesis that Verhoeoff presented more than 20 years ago. The mice in this experiment treated with ergocalciferol had smaller subcutaneous tumors than control mice and those tumors were more necrotic and calcified than tumors in control mice.

Vitamin D is fat soluble. It freely crosses the intact blood-retinal barrier. In this study, the inflammation and tissue damage that indicated a breakdown in the blood-retinal barrier made it even more penetrable to the vitamin D. Therefore, the ergocalciferol treatment should inhibit the growth of the intraocular tumors in the same manner that it inhibited the growth of the subcutaneous tumors. Variation in the sizes of intraocular tumors was difficult to appreciate, however, because the sclera presents a barrier to growth, and rapidly growing tumors soon fill the eye. Therefore, we had misgivings about the accuracy of measurements of intraocular tumor size. We deferred our evaluation of the effect of ergocalciferol on tumor size to the more easily measured subcutaneous tumors.

Verhoeoff suggested that the antineoplastic action of vitamin D might be secondary to its effect as a calcifying agent. This mechanism is unlikely because in vitro studies have shown that high levels of calcium inhibit the antineoplastic effect of vitamin D. In our study, mice treated with 2.8 mg/kg of ergocalciferol had lower tumor calcium levels than those treated with 7.8 mg/kg of ergocalciferol. Treated mice with lower tumor calcium levels had smaller tumors even though they received less ergocalciferol. The high tumor calcium levels in some of the treated mice may have inhibited the antiproliferative effect of vitamin D because high levels of intracellular calcium can stimulate proliferation of normal and cancer cells.

The antineoplastic effect of vitamin D is believed to be receptor mediated. The vitamin D receptor has one site that binds to vitamin D and one that binds DNA. By binding DNA, the vitamin D-vitamin D receptor complex affects gene expression. This action of vitamin D on DNA may precipitate its antineoplastic effect.

Although our study achieved tumor inhibition, necrosis, and calcification with ergocalciferol, it did so with significant systemic toxic reactions. Before its use in humans can be considered, methods of decreasing the toxic reactions of vitamin D must be studied. A recent in vivo study showed that toxic reactions can be decreased by avoiding hypercalcemia. Furthermore, maintaining low or normal serum calcium levels does not compromise the antineoplastic effect of vitamin D. In humans, hypercalcemia can cause nausea, vomiting, abdominal pain, lethargy, coma, dehydration, and renal failure. Should the treat-
ment of retinoblastomas with vitamin D become feasible, there are several available therapies for hypercalcemia that may be useful in ameliorating the toxic effects of vitamin D. Fluid and electrolyte replacement along with intravenous furosemide (Lasix) therapy causes a calcium diuresis by blocking tubular reabsorption of calcium. Oral phosphate inhibits bone resorption of calcium. Corticosteroids inhibit intestinal and bone resorption of calcium. Calcitonin inhibits bone resorption of calcium and promotes calcium diuresis. Calcitonin also has low toxicity. Finally, some antineoplastic agents—plemamicin (Mithramycin) and daclinomycin (actinomycin D)—have been shown to inhibit bone resorption and decrease serum calcium levels.

Eisman et al maintained normal serum calcium levels in mice treated with cholecalciferol by administering a low-calcium diet. This method of controlling vitamin D–induced hypercalcemia may also be effective in humans.

This study shows that ergocalciferol is effective in inhibiting growth of human retinoblastomas grown in athymic "nude" mice. This inhibition was achieved at doses that caused significant toxic reactions. Since the toxic reaction was most likely due to the hypercalcemia, a side effect of vitamin D that is not related to its principal antineoplastic effect, future studies with vitamin D may be more effective if treatment with vitamin D is combined with treatment for hypercalcemia. Further studies that reduce the toxic reaction of vitamin D are necessary before its use in children afflicted with retinoblastoma may be considered.

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References


