Vitamin D Analogues Increase p53, p21, and Apoptosis in a Xenograft Model of Human Retinoblastoma

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PURPOSE. To study the antineoplastic effect of vitamin D analogues in a xenograft model of human retinoblastoma.

METHODS. Athymic mice were injected subcutaneously with Y79 cells and treated 5 days a week with either mineral oil (control group) or the vitamin D analogues calcitriol or 1,25-dihydroxy-16-ene-23-yne vitamin D_3 (16,23-D_3). BrdU was injected 1 hour before death. Animals were killed after 1, 2, 3, or 5 weeks. Paraffin-embedded sections of the tumors were studied for cell proliferation by monitoring for BrdU incorporation and cell death by terminal transferase dUTP-nick end labeling (TUNEL), 3′-overhang ligation, and histology. Sections of the tumors were immunostained for p53 and p21.

RESULTS. There was no significant difference in incorporation of BrdU among the three groups, suggesting that cell proliferation is unaffected by vitamin D analogues. TUNEL was increased in tumors treated with vitamin D analogues compared with the control group. This increase was statistically significant for calcitriol in the time frame examined, but not statistically significant for 16,23-D_3. Alternatively, the ratio of proliferation to cell death was significantly different for both calcitriol and 16,23-D_3 compared with control tumors after 3 weeks of treatment. Dying cells contained DNA strand breaks with overhanging nucleotides and nuclear changes characteristic of apoptosis. There was an increase in staining for p53 and p21 in areas associated with cell death in specimens treated with vitamin D analogues.

CONCLUSIONS. Vitamin D analogues appear to attenuate retinoblastoma tumor growth in athymic mice by increasing apoptosis. Cell death is associated with the upregulation of both p53 and p21. (*Invest Ophthalmol Vis Sci.* 2003;44:4192–4199) DOI:10.1167/iovs.02-1198

Retinoblastoma is the most common ocular tumor in children, occurring in 1/14,000 to 1/20,000 births.[1] Current treatments for retinoblastoma, which include surgery, radiation, and chemotherapy, achieve a survival rate of 90%.[2] Unfortunately, these treatments are mutagenic, increasing the risk of secondary tumors in adulthood.[3] This increased risk has stimulated interest in evaluating new treatments. In 1966, Frederic Verhoeff reported that calcium deposits were often associated with regions of dying cells in retinoblastoma. He proposed that vitamin D could have an inhibitory effect on retinoblastoma because of its calcifying activity.[4] This line of therapy was not initially explored, however, because of the marked toxicity of vitamin D, due to its potent hypercalcemic effect and the absence of an experimental model of retinoblastoma. The establishment of human retinoblastoma cell lines and the creation of transgenic mouse models of retinoblastoma have provided useful tools to study this hypothesis. Vitamin D receptors were found in the human retinoblastoma cell line Y79, and inhibition of the growth of those cells in culture was demonstrated after treatment with the hydroxylated form of vitamin D_3 (calcitriol) and vitamin D_2.[5] New vitamin D analogues that have a reduced effect on phosphocalcic metabolism have also been developed, and studies have shown that they attenuate retinoblastoma tumor growth in both an athymic (nude) mouse model[6,7] and a transgenic mouse model.[8] The mechanisms underlying this antitumor effect, however, are not well characterized.

Vitamin D, especially its active physiological metabolite, 1α,25-dihydroxy-vitamin D_3 (1,25(OH)$_2$D$_3$), plays a major role in calcium and phosphate homeostasis. During the past decade, emphasis has been placed on its role in cellular differentiation and proliferation and it has been shown to have broad potential as an anticancer agent against different malignant cell lines derived from melanoma,[9] myeloid leukemia,[10,11] breast cancer,[12–14] prostate cancer,[15,16] colon cancer,[17] and retinoblastoma.[5] The 1,25(OH)$_2$D$_3$ analogue binds with high affinity to a specific nuclear receptor (Vitamin D receptor, or VDR).[18] This binding induces a conformational change that activates the VDR and the dimerization with another nuclear receptor, the retinoic X receptor (RXR).[19] The heterodimer then binds to specific DNA motifs called Vitamin D response elements (VDREs) in the promoters of target genes and activates their transcription.[20,21] Target genes can be involved in phosphocalcic metabolism, or in regulating cell division and cell death (for review see Ref. 22). Vitamin D treatment induces an arrest in the G_1 phase of the cell cycle in numerous cancer cell lines,[23–29] which is probably caused by the upregulation of one or both of the cyclin-dependent kinase inhibitors (CDKIs) p21$^{WAF1/cip1}$ or p27^{kip1}. A VDRE has been found in the promoter of the p21 gene, indicating that vitamin D may directly activate its transcription.[30] No VDRE has been identified in the p27 gene, and its upregulation seems to be more cell-type dependent.[31]

Vitamin D treatment also can induce apoptotic death of tumor cells.[32–36] The mechanisms involved in this induction are not well understood and appear to vary with the cell type. Vitamin D–induced apoptosis seems to be mediated through a p53 pathway in certain cancer cell lines such as glioma,[34] whereas cell death induced by the treatment of some breast cancer cell lines with vitamin D analogues appears to be mediated by a p53-independent pathway. In these cells, the BCL-2 family of apoptotic regulatory proteins seems to play a major

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A. Vitamin D$_2$ B. Calcitriol   C. 16,23-D$_3$
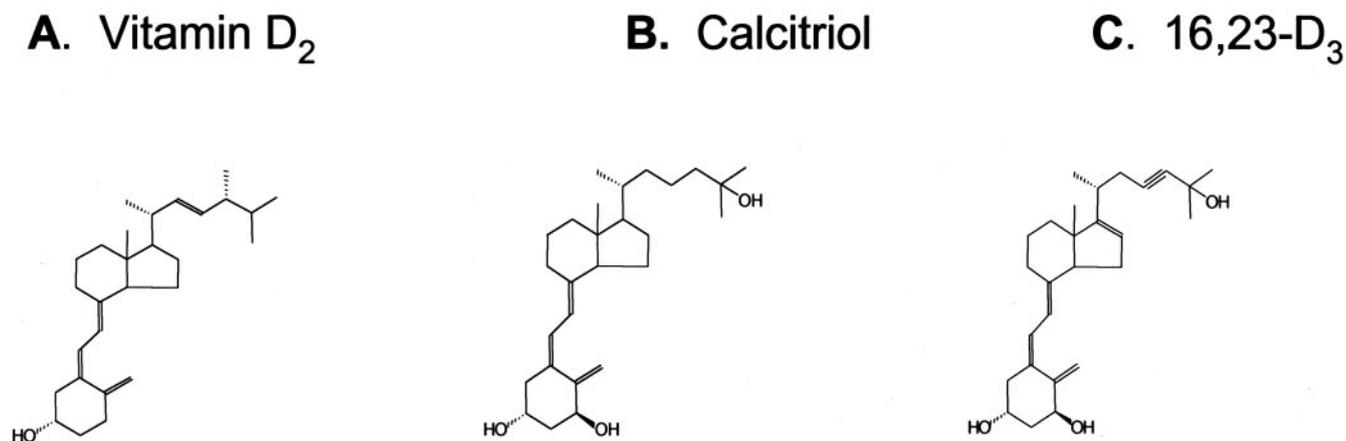
FIGURE 1. Chemical structure of vitamin D$_3$, and the two analogues calcitriol and 16,23-D$_3$. Calcitriol is the hydroxylated form of vitamin D$_3$. A second analogue 16,23-D$_3$ has been modified by the creation of a double bond at position C16 in the D-ring, and the creation of a triple bond at position C23.[37] Calcitriol has been shown to have significant effects on phosphocalcic metabolism, whereas the modifications in 16,23-D$_3$ have greatly reduced these effects.[38]

role in induction of apoptosis, with downregulation of anti-apoptotic protein such as BCL-2 and upregulation of proapoptotic molecules such as BAX.[36]

The purpose of this study was to investigate the antineoplastic action of vitamin D analogues in retinoblastoma. We used subcutaneous xenografts of the human retinoblastoma cell line Y79 in athymic mice and studied the effects of the hydroxylated form of vitamin D$_3$, 1,25-dihydroxycholecalciferol (calcitriol), and one of its analogues, 1,25-dihydroxy-16-ene-23-yne vitamin D$_3$ (16,23-D$_3$) (Fig. 1).[37] Unlike calcitriol, 16,23-D$_3$ has fewer side effects on phosphocalcic metabolism.[38] In this study, we report that the vitamin D–mediated attenuation of tumor growth is associated with an increase in apoptotic cell death and not a reduction in tumor cell proliferation. Increased cell death also correlates with an increase in both p53 and p21, suggesting that vitamin D analogues mediate apoptosis by activating a p53-dependent pathway.

MATERIALS AND METHODS

Animal Model

The use of animals in this study was approved by the Research Animal Resources Center at the University of Wisconsin and conformed to the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Sixty athymic nude (*nu*) female mice (Harlan Sprague-Dawley, Indianapolis, IN), aged from 8 to 10 weeks, were each given a dorsal subcutaneous injection of 1×10^7 Y79 human retinoblastoma cells suspended in 500 μL of 1:1 Iscove medium containing 20% fetal bovine serum and basement membrane matrix suspension (Matrigel; BD Bioscience, Bedford, MA). Culture methods for Y79 cells have been described elsewhere.[5] Tumors were allowed to grow for 5 days before the randomization of the animals and the beginning of the treatment. Two weeks before the treatment and during the whole study, the animals were fed a calcium- and vitamin D–deficient diet (Purina Mills, Inc., St. Louis, MO) to reduce hypercalcemia induced by the drugs.

Treatment Protocol and Tumor Size Assessment

Two vitamin D analogues were included in our study: the hydroxylated form of vitamin D$_3$, calcitriol (Sigma-Aldrich, St. Louis, MO) and one of its analogues 16,23-D$_3$ (Ilex Oncology, San Antonio, TX; Fig. 1).[37] The stock solution of each drug was prepared by dissolving the pure crystalline form in 100% ethanol to a solution concentration of 2.98 mg/mL. These stock solutions were kept in the dark at −40°C to prevent degradation, and their concentration was determined weekly by spectrophotometric analysis. Each analogue (0.05 μg calcitriol and 0.5 μg 16,23-D$_3$) was diluted further in 250 μL of mineral oil (MO) to make an injectable emulsion. Mineral oil alone was injected in the control group.

Drugs were administered by intraperitoneal injection five times a week for 5 weeks. The mice were randomized into three equal groups: one control group receiving injections of MO, one group receiving injections of calcitriol, and one group receiving injections of 16,23-D$_3$. Tumor size was measured externally three times a week with a caliper. The mice were also weighed twice a week and monitored daily to detect signs of secondary hypercalcemia such as lethargy or dehydration.

To study the kinetics of the antineoplastic effect, we killed five mice from each group after 1, 2, 3, or 5 weeks of treatment. One hour before death, animals were injected intraperitoneally with 0.02 mg/μL 5-bromo-2′-deoxyuridine (BrdU, 100 μg/kg; Calbiochem, San Diego, CA) and 0.01 mg/μL 5-fluoro-2′-deoxyuridine (FrdU, 6.7 μg/kg; Calbiochem) to monitor cell proliferation as a function of DNA synthesis. Animals were killed by cervical dislocation under isofluorane anesthesia. After death, subcutaneous tumors were harvested, measured, and weighed.

Histochemistry of Tumors

Each tumor was fixed in 4% paraformaldehyde in phosphate buffer (100 mM, pH 7.4) overnight. The tumors were then embedded in paraffin. Five-micrometer serial sections were obtained from each tumor and were affixed on slides (Vectabon; Vector Laboratories Inc., Burlingame, CA). Adjacent sections for each tumor were stained with hematoxylin and eosin (H&E), anti-BrdU antibodies, terminal transferase biotin-dUTP nick end labeling (TUNEL), 3′-overhang ligation, anti-p53 antibodies and anti-p21 antibodies.

Anti-BrdU Immunostaining

Tumor cells that had incorporated BrdU were detected with an anti-BrdU antibody using a BrdU staining kit (Calbiochem). Briefly, the sections were deparaffinized. Endogenous peroxidase was neutralized by pretreatment with 0.5% hydrogen peroxide in methanol and rehydrated. Tumor sections were digested with trypsin 0.05% for 9 minutes at 37°C and rinsed with water. A denaturing solution was applied for 30 minutes, after which the sections were washed with phosphate-buffered saline (PBS; pH 7.6) and immersed in a blocking solution for 10 minutes. The sections were then incubated overnight at 4°C with an anti-BrdU monoclonal antibody. A biotinylated goat-anti-mouse anti-

FIGURE 2. Vitamin D analogues slow tumor growth. Y79 xenograft tumors were harvested from mice killed after 1, 2, 3, or 5 weeks of treatment with MO, calcitriol, or 16,23-D$_3$. Each tumor was measured with a caliper to determine its volume. The mean tumor volume ($\pm$ SEM) for each treatment group after different periods of treatment is shown. Significantly different comparisons are marked with *brackets* and probabilities. The results show a modest but significant decrease in tumor volume at 1 week for both calcitriol and 16,23-D$_3$ compared with the treatment with MO. At 3 weeks, only calcitriol exhibited a significant effect, but after 5 weeks both analogues significantly attenuated tumor growth.
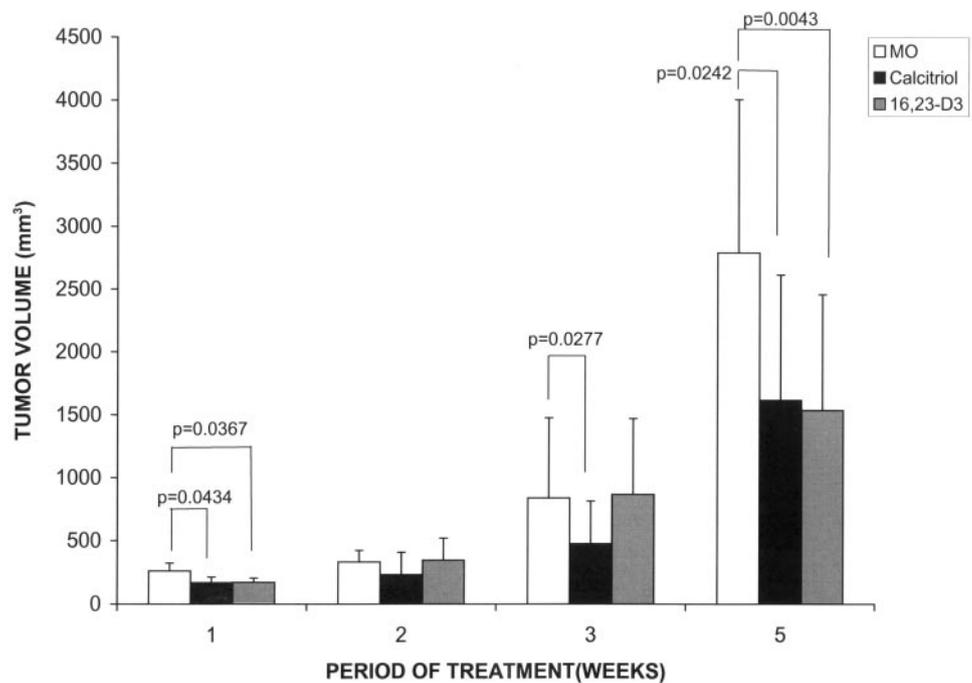
body was used for the secondary antibody. Labeled cells were identified with an avidin peroxidase kit (ABC; Vector Laboratories), with diaminobenzidine used as a substrate.

TUNEL Assay

Histologic analysis of DNA fragmentation was used to identify dying cells in paraffin sections of the xenograft tumors. TUNEL was performed as described by Gavrieli et al.[39] Briefly, the 3′ ends of nicked DNA were labeled by addition of biotin-16-dUTP (Roche Diagnostics, Indianapolis, IN), using terminal deoxynucleotide transferase (Promega, Madison, WI). Labeled DNA was then identified with the avidin peroxidase kit (ABC; Vector Laboratories), with diaminobenzidine used as a substrate.

3′-Overhang Ligation

Because TUNEL labels degraded DNA in both apoptotic and necrotic cells, we stained sections using the 3′-overhang ligation technique described by Didenko and Hornsby.[40] This method specifically labels double-stranded DNA breaks with 3′-overhanging ends, which are characteristic of fragments in apoptotic cells. Briefly, a 200-bp DNA fragment, corresponding to the multiple cloning site of a plasmid vector (pBK-CMV; Stratagene, La Jolla, CA) was generated by polymerase chain reaction using *Taq* DNA polymerase (Roche Diagnostics), plasmid-specific primers, and 125 μM cold dNTPs spiked with 100 μM biotin-16-dUTP (Roche Diagnostics). The labeled DNA was then applied over tissue sections and ligated to the nicked DNA using T4 DNA ligase enzyme (Promega). After sections were washed, labeled nuclei were viewed by first incubating them with streptavidin-Texas red (Jackson ImmunoResearch, West Grove, PA) followed by fluorescence microscopy (Axiophot; Carl Zeiss Meditec, Thornwood, NY).

p53 and p21 Immunostaining

Paraffin-embedded sections were labeled with antibodies against human p53 and human p21 (monoclonal DO-1 and monoclonal Ab1, respectively; Oncogene Research Products, Boston, MA), according to the protocol described by Nork et al.[41] Briefly, sections were deparaffinized, and endogenous peroxidase was neutralized by pretreatment with 0.5% hydrogen peroxide in methanol. Sections were subjected to antigen retrieval by incubating them in 100 mM Tris buffer (pH 9.5) at 90°C for 30 minutes (p53) and 10 minutes (p21) followed by 60

minutes at room temperature. The sections were rinsed in water and PBS and blocked with PBS containing 4% (wt/vol) bovine serum albumin (PBS-BSA) for 30 minutes. The sections were then incubated overnight at 4°C with a primary antibody against human p53 (0.8 μg/mL in PBS-BSA) or against human p21 (0.5 μg/mL in PBS-BSA). After they were washed with PBS, the sections were incubated with a biotinylated goat anti-mouse antibody (Vector Laboratories) for 30 minutes. Labeled cells were identified by using the avidin peroxidase kit (ABC; Vector Laboratories) with diaminobenzidine used as a substrate.

Quantification of Cell Proliferation and Cell Death

To quantify tumor cell proliferation and tumor cell death, 6 to10 random photomicrographs of each section stained either for BrdU or for TUNEL (two sections for each tumor) were taken with Nomarski interference optics at 400× magnification. For each photograph, we recorded the number of positively stained cells counted in at least 200 cells.
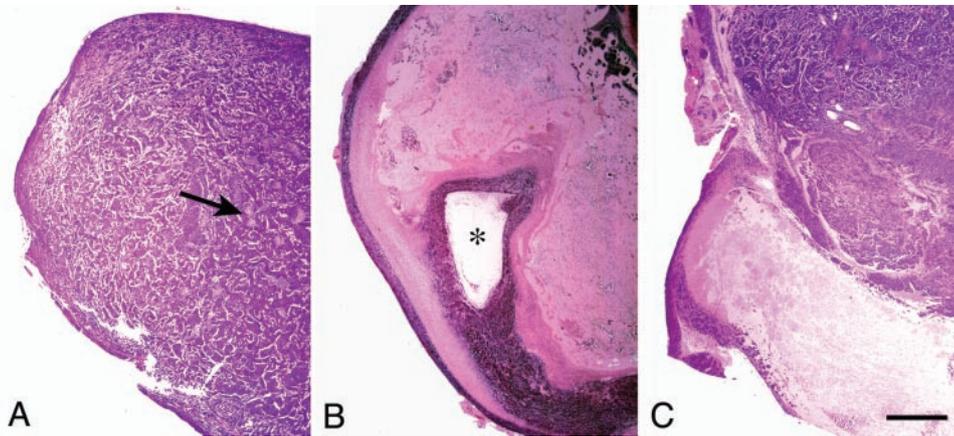
Statistical Analysis

The effect of treatment and the length of treatment on tumor size, TUNEL, and BrdU staining and on the ratio of BrdU over TUNEL were each assessed by analysis of variance. Tumor size and BrdU/TUNEL ratios were transformed to the log scale and the TUNEL values to the square root scale to stabilize the variance. 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 pair-wise analyses to assess differences between specific treatment groups.

RESULTS

Effect of Vitamin D Analogues on Tumor Growth

Consistent with previous studies,[6] both calcitriol and 16,23-D$_3$ were able to retard tumor growth in the Y79 xenograft model of retinoblastoma (Fig. 2). The results showed a significant decrease in tumor volume at 1 week with both calcitriol and 16,23-D$_3$, compared with the treatment with MO ($P = 0.0434$

FIGURE 3. Gross morphologic features of MO- and Vitamin D–treated xenograft tumors. H&E-stained sections were obtained from each tumor. Sections obtained from tumors after 3 weeks of treatment are shown. (**A**) A representative region of a tumor from a mouse treated with MO. Although these tumors clearly showed evidence of regions of cell death (not shown) they also typically exhibited large solid masses of cells that contained structures resembling Homer-Wright Rosettes (*arrow*). (**B**) A region of a tumor treated with calcitriol. Large regions of these tumors were acellular, with layers of viable cells at the periphery and surrounding a penetrating blood vessel (✱). (**C**) A region of a tumor treated with 16,23-D$_3$ showing an acellular region adjacent to a solid mass of cells. Similar to the other two specimens shown, the periphery of the acellular region was surrounded by layers of viable cells, whereas the solid mass contained organized rosette structures. Scale bar, 1500 μm.

and $P = 0.0367$, respectively), although tumor growth in all groups was minimal at this time. No significant difference was found at 2 weeks, and only the tumors treated with calcitriol were significantly smaller ($P = 0.0277$ for calcitriol versus MO; $P = 0.0277$ for calcitriol versus 16,23-D$_3$) after week 3. After 5 weeks, both analogues had significantly limited tumor growth compared with the control group ($P = 0.0242$ for calcitriol versus MO; $P = 0.0043$ for 16,23-D$_3$ versus MO).

Tumors from each treatment group were also analyzed by histology for morphologic differences. Representative H&E-stained sections are shown in Figure 3. All tumors contained acellular regions surrounded by layers of viable-appearing cells. Acellular regions appeared to be more prevalent in vitamin D-treated tumors. All tumors also contained solid masses of cells, but these regions appeared to be larger in tumors from MO-treated mice. The solid tumor masses exhibited evidence of cellular organization into structures that resembled Homer-Wright rosettes (Fig. 3A). Some tumors also exhibited viable cells surrounding blood vessels that had penetrated the tumor mass (Fig. 3B).

Effect of Vitamin D Analogues on Tumor Cell Proliferation and Cell Death

To determine whether the decrease in tumor growth induced by vitamin D analogues was due to a reduction in cell proliferation, an increase in cell death, or both, we first studied cell proliferation by monitoring BrdU incorporation into tumor cells. We found no significant differences in BrdU incorporation between the control and either of the treated groups (Fig. 4A) at any time point ($P = 0.6294$). To evaluate tumor cell death, we monitored TUNEL on adjacent sections of the same samples. At 5 weeks, cells positive for TUNEL were detected in control tumors and in each treatment group, indicating that cell death is a naturally occurring process in these tumors as they grow (Fig. 4B). The rate of cell death, however, in groups treated with vitamin D analogues and calcitriol was significantly greater than in the group treated with MO ($P = 0.0154$). Tumors treated with calcitriol showed an elevated level of TUNEL over the entire course of the treatment. Tumors treated with 16,23-D$_3$ also showed an increase in cell death relative to the ones treated with MO, but this effect was not statistically significant. In addition, the effect was delayed compared with calcitriol (Fig. 4B), first becoming evident at 3 weeks of treatment.

We also investigated the relationship between the rate of proliferation and cell death in each tumor by calculating the ratio of the percentage of BrdU incorporation to the percentage of TUNEL-positive cells in each tumor (Table 1). The ratio was generally higher in MO-treated tumors during the first 3 weeks of treatment. At 5 weeks, the ratio dropped significantly ($P = 0.0035$, comparing 3 and 5 weeks of treatment), suggesting an overall decrease in tumor growth in this model after extended periods, regardless of treatment. Exposure to vitamin D analogues typically produced lower ratios than MO treatment. This decrease was significant in tumors treated for 3 weeks with both calcitriol ($P = 0.0210$) and 16,23-D$_3$ ($P = 0.0453$). Similar to the MO treatment group, vitamin D-treated tumors exhibited a decrease in growth after 5 weeks relative to earlier time points. It is likely that the decrease in the ratio of cell proliferation to cell death is primarily a consequence of an increase in cell death, because neither analogue had a significant effect on proliferation when assessed as a single variable (Fig. 4A).

Cause of Cell Death in Xenograft Tumors

Because TUNEL identifies fragmented DNA in both apoptotic and necrotic nuclei, we characterized further the mechanism of cell death induced by vitamin D analogues. Examination of H&E-stained sections showed that dying cells exhibited either pyknotic nuclei or nuclear fragmentation characteristic of cell death by apoptosis (Fig. 5A). In addition, fragmented DNA in the nuclei of dying cells could be labeled by the Didenko and Hornsby ligation technique,[40] indicating the presence of 3'-overhanging nucleotides typical of DNA fragments in apoptotic cells (Fig. 5B). These characteristics of apoptosis were evident in all three treatment groups, but were more prevalent in tumors treated with vitamin D analogues, consistent with the TUNEL results.

Correlation of p53 and p21 Immunostaining with Regions of Cell Death

To understand further the mechanism underlying tumor growth inhibition induced by vitamin D analogues, we studied the possible roles of p53 and p21 in this antitumor effect. Serial sections taken from each tumor were stained sequentially for BrdU incorporation, TUNEL, p53, and p21. Figure 6 shows a comparison of the four different stains after 3 weeks of treatment. All tumors contained an outer region of cell proliferation juxtaposed to an inner zone of cell death. These regions encircled a central acellular core. Immunostaining for p53 was evident in the region of dying cells in all treatment groups, but

A

B

FIGURE 4. Effect of vitamin D treatment on tumor cell proliferation and cell death. (A) Vitamin D analogues did not affect tumor cell proliferation. Mice bearing Y79 xenograft tumors were treated for 1, 2, 3, or 5 weeks with MO, calcitriol, or 16,23-D_3. BrdU incorporation was used to monitor tumor cell proliferation. Shown is the percentage of cells incorporating BrdU for each group after different periods of treatment (mean $\pm$ SEM, $n = 5$ at each time point). No difference was found among the different treatment groups at the different time points, suggesting that vitamin D analogues do not affect tumor cell proliferation. (B) Vitamin D analogues increased cell death. Sections from the same samples shown in (A) were examined by TUNEL. Shown is the percentage of cells positive for TUNEL for each group after different periods of treatment (mean $\pm$ SEM, $n = 5$ at each time point). Cells positive for TUNEL were found in each treatment group, including the control. The number of TUNEL-positive cells increased with time in each group, but was greater in tumors treated with vitamin D analogues and was significantly enhanced in animals treated with calcitriol ($P = 0.0154$ versus MO). Tumors treated with calcitriol also showed nearly homogeneous TUNEL over the course of the treatment. Tumors treated with 16,23-D_3 showed an increase in cell death relative to MO, but this effect was delayed compared with calcitriol. The difference in the kinetics of cell death may reflect different mechanisms of action for each analog.
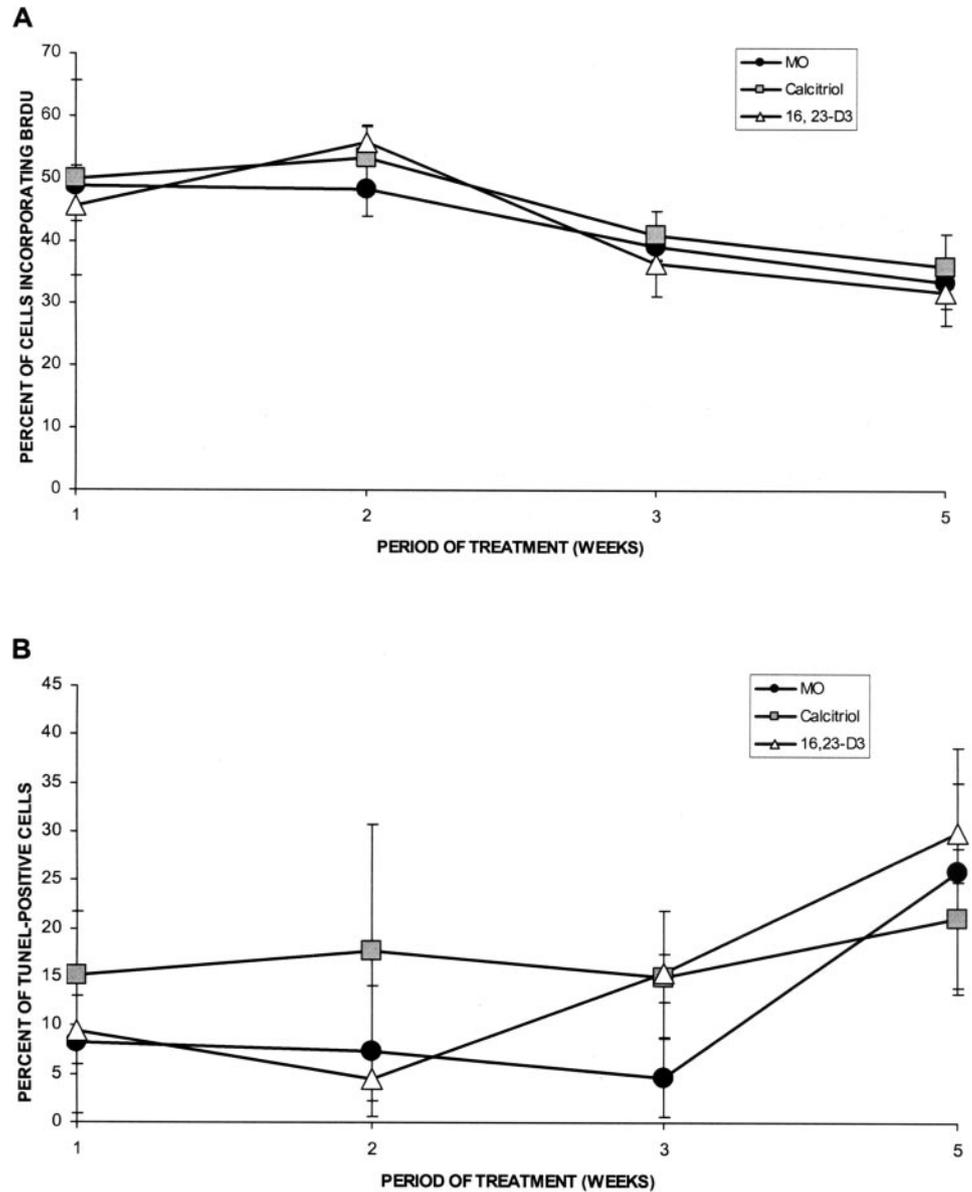
was more pronounced in tumors treated with vitamin D analogues. Immunostaining for p21 showed a pattern similar to p53, but only in tumors treated with vitamin D analogues.

Some staining for p21 was evident in MO-treated tumors, but this pattern was nonnuclear and therefore likely to be nonspecific.

TABLE 1. Effect of Vitamin D Treatment on the Ratio of Tumor Cell Proliferation and Death

Week	Mineral Oil Ratio	Calcitriol		16,23-D_3	
		Ratio	*P* versus MO	Ratio	*P* versus MO
1	7.877	2.946	0.1203	4.287	0.2342
2	9.747	3.850	0.1150	15.098	0.4216
3	8.253	2.681	0.0210	2.587	0.0453
5	1.598	1.850	0.6264	1.064	0.1698

Shown is the mean ratio of cell proliferation to cell death in tumors harvested after different times of treatment in each group of mice. Data for calcitriol and 1,25-dihydroxy-16-ene-23-yne vitamin D_3 (16,23-D_3) were tested against the mineral oil (MO) control group at each time point. The statistical probability for each treatment is shown in the adjacent column. These data indicate that tumor growth was retarded in each group by 5 weeks of treatment. In the first 3 weeks, vitamin D analogues typically yielded lower ratios than MO treatment, suggesting a reduction in tumor growth during this period. The most prominent effects of vitamin D treatment occurred at the 3-week time point, when both analogues exhibited a significant difference relative to the MO group.
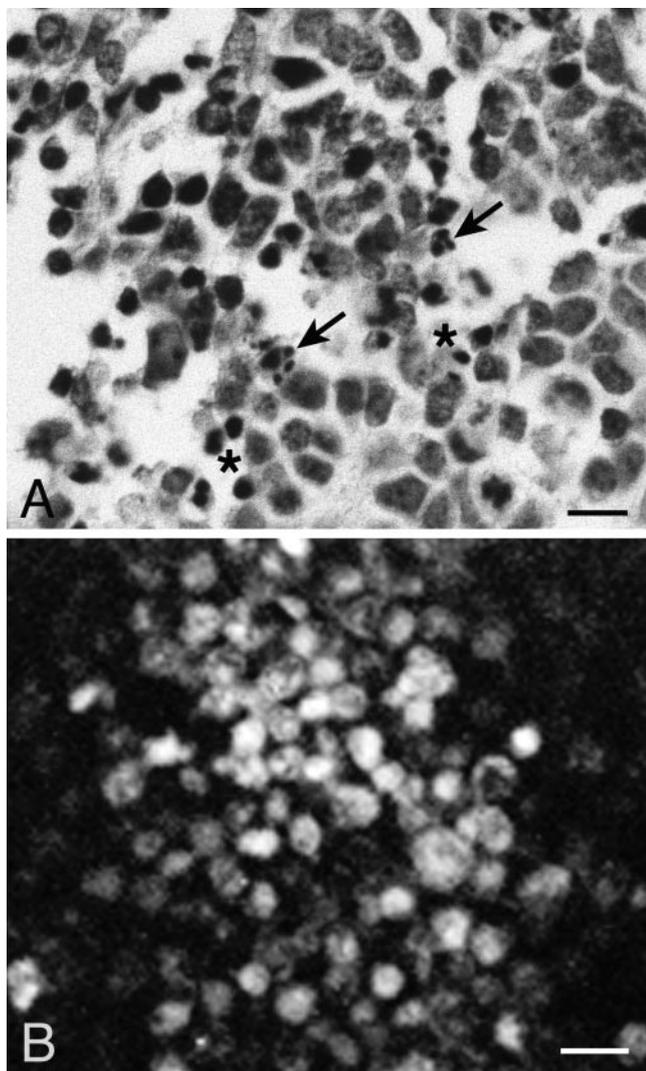
FIGURE 5. Cell death occurred by apoptosis in xenograft tumors. Two methods were used to determine whether apoptosis was the mechanism of cell death in Y79 xenograft tumors. Sections were taken from tumors after treatment with MO, calcitriol, or 16,23-D$_3$, and stained with either H&E to examine nuclear morphology or the 3′-overhang ligation technique to assess the nature of the DNA fragmentation found in dying cells. (**A**) H&E-stained section from a tumor treated for 5 weeks with 16,23-D$_3$. The nuclei in dying cells were either pyknotic (✶) or in various stages of fragmentation (*arrows*). (**B**) Fluorescence micrograph of dying cells in tumor treated with 16,23-D$_3$ for 5 weeks, labeled by 3′-overhang ligation.[40] This technique ligates a biotin-tagged DNA PCR fragment with 3′-overhanging nucleotides to fragmented DNA in nuclei with similar 3′-overhanging ends. Dying cells in the xenograft tumors were strongly positive. In this experiment, the proportion of cells that stained with the ligation technique was similar to the proportion of cells that stained by TUNEL in adjacent sections. Scale bars, 20 μm.

DISCUSSION

In a review of spontaneously regressed retinoblastoma, Frederic Verhoeff [4] made the observation that calcium deposits were frequently associated with areas of necrosis. He proposed that a calcifying agent, such as vitamin D, could kill tumor cells or inhibit their proliferation in retinoblastoma and strongly suggested its use as a treatment. Verhoeff's theory was not tested experimentally at that time, because vitamin D is toxic in high doses and there were no suitable models of retinoblas-

toma. Since then, less toxic analogues of vitamin D have been synthesized, and both in vitro and in vivo models of retinoblastoma have been developed. VDRs have subsequently been identified in retinoblastoma cell lines[5] and less toxic analogues have shown an effect on slowing tumor cell growth in retinoblastoma cell culture,[5] Y79 human retinoblastoma xenograft tumors in nude mice,[6,7] and a transgenic mouse model of retinoblastoma.[8] The mechanisms involved in this effect are not well understood and could be due to a decrease in tumor cell proliferation, an increase in tumor cell death, or both. To elucidate this question, we chose to study the mechanism of action of vitamin D analogues on Y79 retinoblastoma tumors grown in athymic mice.

Our results indicate that the vitamin D analogues, 16,23-D$_3$ and calcitriol, have no significant effect on cell proliferation, but induce increased apoptotic cell death in Y79 xenograft tumors. Similar results have been reported in a preliminary study involving a third vitamin D analogue, 1α hydroxy vitamin D$_2$.[37] This supports Verhoeff's initial suggestion on the potential action of vitamin D as an agent to kill tumor cells in retinoblastoma. It should be noted that Y79 cell xenograft tumors may have different molecular characteristics not typically found in native human retinoblastoma tumors. Although the xenograft tumor model has characteristics of aggressive retinoblastomas, possibly as a consequence of Y79 cells' having an immortalized phenotype, most tumors are composed of a variety of cell types in various states of differentiation,[42] and they may not respond equally to vitamin D analogues.

The absence of effect of vitamin D analogues on retinoblastoma cell proliferation may be surprising, considering their effects on other cancer cell lines. In some breast cancer, prostate cancer, myeloma, and pancreatic cancer cell lines, vitamin D analogues cause a cell cycle arrest in the G$_1$ phase of the cell cycle.[23–31] Vitamin D–mediated cell cycle arrest is thought to be due to the upregulation of CDKIs, such as p21, which play a major role in cell cycle control. In G$_0$ and early G$_1$, the retinoblastoma protein (pRb) is hypophosphorylated and binds to and inactivates transcription factors from the E2F/DP family. Mitogenic signals result in the successive phosphorylation of pRb by cyclin-dependent kinases (cdks), causing the release of E2F/DP heterodimers that initiate the transcription necessary for cell cycle progression toward the S phase.[43] CDKIs inhibit the activity of cdks, maintaining pRb in its hypophosphorylated state and causing an arrest of the cell cycle. The upregulation of p21 by vitamin D treatment in certain cancer cell lines explains the cell cycle arrest induced by this treatment. However, in retinoblastoma, cell cycle control is disrupted, because the rate-limiting step for this tumor to develop is the inactivation of both alleles of the retinoblastoma susceptibility gene (RB1).[44] Thus, the upregulation of p21 cannot induce cell cycle arrest by the predicted mechanism.

Alternatively, our results show that treatment with vitamin D analogues primarily caused an increase in apoptosis. It has been shown that apoptosis occurs naturally in human retinoblastoma,[40] but this event is exacerbated after vitamin D treatment. The ability of vitamin D to trigger apoptosis in retinoblastoma is similar to results obtained in other cancer cells such as in breast cancer,[32] glioma,[33–34] and colon cancer.[35] Vitamin D affects gene expression by to binding and activating its receptor (VDR). The activated VDR then translocates to the nucleus where it interacts with VDREs present in the promoter of target genes. Numerous target genes are sensitive to vitamin D$_2$ and some of them are involved in apoptosis. The p21 gene, for example, possesses a VDRE[30] and induces apoptosis in human malignant glioma cell lines[45] and in WERI-RB1 and Y79 retinoblastoma cell lines[46] in cell transfection and transduction studies. In addition, p53, one of the first tumor-suppressor genes linked to apoptosis, plays a major role in regulating
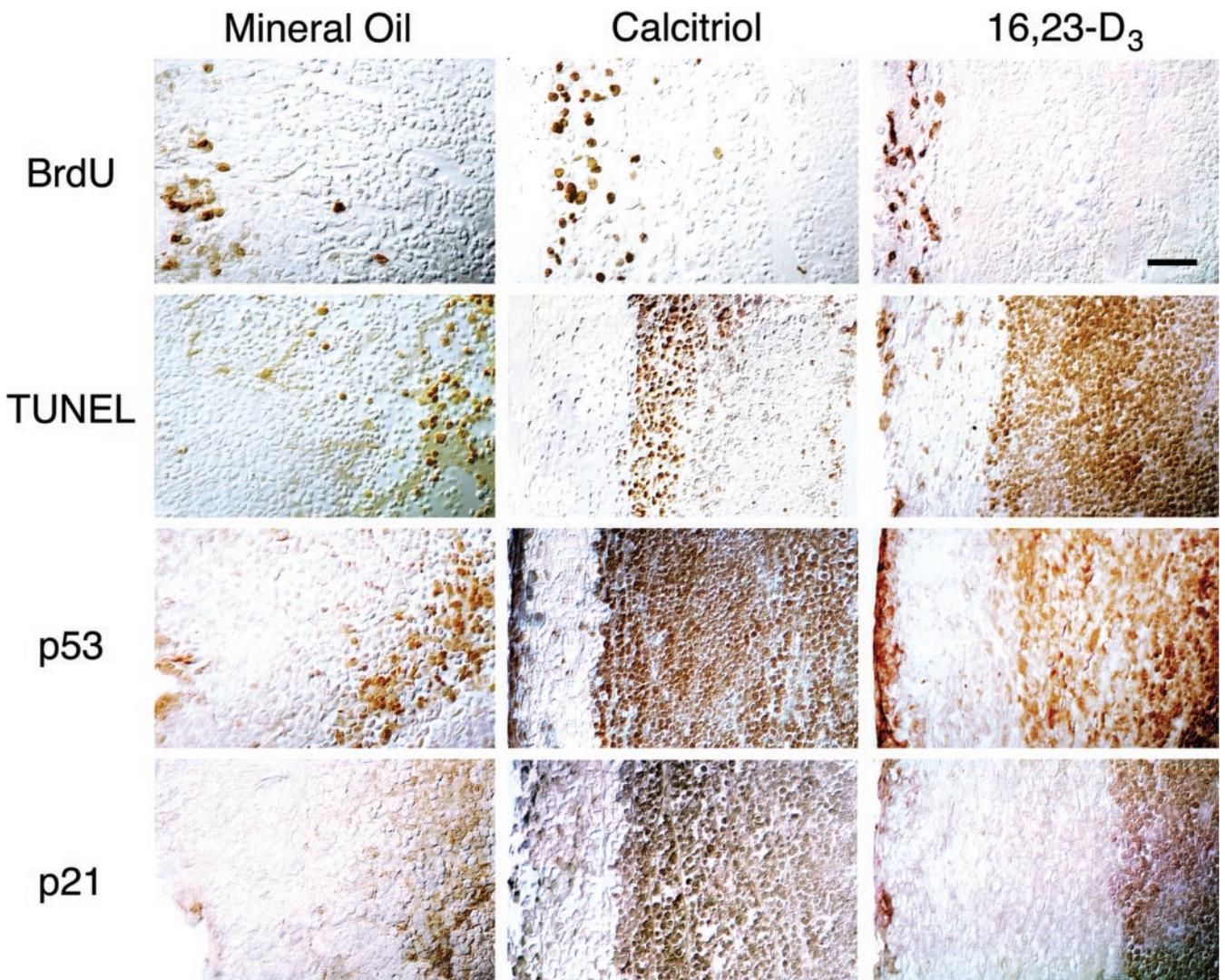
FIGURE 6. Cell death correlated with p53 and p21 immunostaining. Histochemical labeling of Y79 xenograft tumor sections after 3 weeks of treatment with MO, calcitriol, or 16,23-D₃. Serial sections were stained sequentially for BrdU incorporation, TUNEL, and antibodies against human p53 or p21. BrdU incorporation showed areas of actively dividing cells on the periphery of the tumor mass. Regions of cell death were adjacent to this zone of proliferation. Cell death was more prevalent in sections from tumors treated with vitamin D analogues. Immunostaining for p53 and p21 showed typical nuclear localization for these proteins and correlates with the regions of dying cells. Scale bar, 50 μm.

apoptotic cell death in retinoblastoma.[41,47] It too has been shown to be upregulated after treatment with vitamin D in glioma cell lines[34] although, because this gene does not contain a VDRE, the mechanism of its upregulation is not clear. The roles that p21 and p53 play in the vitamin D–mediated cell death in retinoblastoma is unknown. Convention dictates that p53 activates p21 expression.[47] However, because vitamin D is able to increase p21 expression directly, it is possible that the toxic effects of p21 in retinoblastoma cells subsequently stimulate the activation of p53. Apoptosis then ensues by p53-mediated upregulation of cell death regulatory genes such as BAX.[48] The exact sequence of the molecular events that occurs in retinoblastoma cells treated with vitamin D is the subject of ongoing studies in our laboratory.

Both vitamin D analogues stimulated an increase in cell death compared with MO, although kinetic analysis suggests that each analogue acts differently (Fig. 4B). Calcitriol caused immediate and sustained cell death, whereas 16,23-D₃ exerted a more gradual effect. This difference may reflect the higher toxicity of calcitriol. In addition to affecting gene transcription, calcitriol could independently cause an increase in the concen-

tration of intracellular calcium and trigger a variety of biochemical events leading to cell death.[49]

In summary, in the present study vitamin D analogues increased the rate of apoptosis in a mouse xenograft model of human retinoblastoma. Because vitamin D analogues have a low mutagenic potential relative to other treatments for retinoblastoma, further understanding of its mechanism of action may someday lead to its routine use in treating these tumors, consistent with the initial suggestion proposed by Frederic Verhoeff nearly 40 years ago.

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