Vitamin D Inhibits Angiogenesis in Transgenic Murine Retinoblastoma

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Purpose. Vitamin D compounds have been shown to inhibit tumor growth in a transgenic retinoblastoma murine model. The mechanism of action has not been defined clearly, although an antiangiogenic action has been proposed.

Methods. Transgenic retinoblastoma mice received high (0.05 μg) and low (0.025 μg) doses of vitamin D3 by intraperitoneal injection 5 times per week for 5 weeks. Control animals were injected with mineral oil vehicle alone. At 5 months of age, the animals were killed and eyes were enucleated and processed for light microscopy. Paraffin-embedded sections were stained with an immunoperoxidase stain (GS-1) specific for mammalian vascular endodielium. Sections were graded by a single masked reviewer, and intraobserver reliability was assessed. Mean vessel counts were made for each group.

Results. The high-dose group had the lowest mean vessel count (8.5), followed by the low-dose group (10.1). The control group had the highest mean vessel count (14.1). Vitamin D-treated animals (high- and low-dose groups combined) had significantly fewer vessels ($P = 0.001$) than untreated controls.

Conclusions. These results support the hypothesis that inhibition of angiogenesis is a mechanism of action for vitamin D in the transgenic retinoblastoma mouse model. Invest Ophthalmol Vis Sci. 1995; 36:83-87.

Human retinoblastoma requires the presence of blood vessels to support tumor growth. Retinoblastoma cells grow in a uniform, collar-like pattern around blood vessels with necrosis at a constant radius around blood vessels. It is not known whether the angiogenic stimulus is derived from tumor cells themselves or from ischemic retina. Cultured retinoblastoma cell lines, however, have been shown to produce tumor angiogenesis factors. The natural progression of transgenic murine retinoblastoma indicates that tumors are relatively avascular and are localized in the first 3 to 4 months after birth. Exponential growth is observed with the onset of vascularization at 4 to 5 months of age. Thus, both human and murine retinoblastoma seem to be highly dependent on angiogenesis.

Vitamin D3 and its synthetic analogs have been shown to inhibit angiogenesis using the chick chorioallantoic membrane assay in a dose-dependent manner. Vitamin D and its metabolites have been shown to affect proliferation and differentiation of many tissues, as recently reviewed by Reichel and associates. In cell culture studies, 1,25 (OH)2D3 (calcitriol) suppressed growth of cutaneous melanoma, breast carcinoma, renal cell carcinoma, histiocytic lymphoma, and leukemia. In immunosuppressed mice, it inhibited the growth of xenografts of human melanoma and colon cancer.

Vitamin D compounds are also effective in retarding the growth of human retinoblastoma (Y79) in nude mouse xenografts and in cell culture. We have recently reported the antineoplastic effect of vita-
min D compounds in the transgenic retinoblastoma mouse model. In the latter study, retinal tumors in calcitriol-treated transgenic retinoblastoma mice were absent or smaller and were less invasive than tumors in untreated animals.

*Griffonia simplicifolia* (GS-I; formerly called BS-I, or *Bauuna simplicifolia*) is a plant lectin. In a survey of 10 species of mammal, Alroy et al found that GS-I bound unequivocally to the vascular endothelium of every species tested, except humans. The major sugar to which it binds is α-D-galactose. The immunohistochemical reaction is through avidin-biotin-peroxidase, after exposure to the lectin probe. To determine if calcitriol's antineoplastic effect was related to its antiangiogenic properties, we used GS-I staining in eyes of transgenic retinoblastoma mice treated with calcitriol and untreated control animals using GS-I staining.

**MATERIALS AND METHODS**

This study was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was undertaken with the approval of the institutional review board.

Additional 5- to 6-μm thick sections were cut from paraffin blocks of formalin-fixed eyes of transgenic retinoblastoma mice treated with two doses of 1,25(OH)2D3 (calcitriol; Hoffmann-LaRoche, Nutley, NJ) from an experiment previously reported. Calcitriol-treated animals received 0.05 μg (high dose) or 0.025 μg (low dose) of the drug in 0.05 ml mineral oil 5 times per week for 5 weeks beginning at age 2 months. The control group received 0.05 ml mineral oil with the same injection schedule. Animals were sacrificed at 5 months of age.

The slides were deparaffinized and stained with GS-I (Vector Laboratories, Burlingame, CA) with specific and nonspecific controls as described by Alroy et al. In brief, the sections were exposed to 2% hydrogen peroxide to neutralize native peroxidase. They were then placed in a solution of 100 μg/ml mouse liver powder (Capellel, Cochraneville, PA) in phosphate-buffered saline (Gibco, Grand Island, NY) for 10 minutes. The slides were blotted dry, and sections were incubated with GS-I lectin (Vector Labs, Burlingame, CA) for 30 minutes, then washed three times in phosphate-buffered saline. They were then incubated with horseradish peroxidase in phosphate-buffered saline with diaminobenzidine and hydrogen peroxidase added for 8 to 10 minutes. After washing for 5 minutes in tap water, sections were counterstained with methyl green, dehydrated, and coverslipped. The vasculature in tissues, such as the retina and extracellular structures, provided an internal positive control for GS-I staining.

Each slide was evaluated by a masked reviewer (MTS). Any sharply demarcated brown vessel that was clearly separated from adjacent tissue, regardless of shape (circular or strand), was counted as a vessel. Vessel lumens and red blood cells were not necessary for the identification of vessels, and fields with multiple but discontinuous vascular formations were considered to represent multiple positive vessels. Fields with heavy background staining, hemorrhage, and necrosis were not included.

Microvessels in tumors were counted on a 400X field (40X objective and 10X ocular). Ten fields were counted on each slide, and the results were recorded. The average number of stained vessels was determined. For tumors with areas smaller than ten 400X fields, the entire tumor was counted. For intrarater reliability, each slide was counted twice by the reviewer.

Because interclass correlation exists between right and left eyes of individual mice, the average vessel count of each animal was determined. Analysis of variance was used to test whether differences in mean...
pared to the control group, the number of vessels was significantly lower in the treated animals (9.4 versus 14.1; \( P = 0.011 \)), although there was considerable overlap in the distribution of vessel counts across treatment groups.

**DISCUSSION**

Angiogenesis, or new blood vessel formation, occurs only in a few physiological and nonmalignant pathologic conditions, such as embryonic development,\(^\text{21}\) ovulation,\(^\text{22,23}\) wound healing,\(^\text{24,25}\) and inflammation.\(^\text{26}\) In these conditions, angiogenesis is self-limiting and eventually subsides. In contrast, tumor angiogenesis does not remit in malignant conditions.\(^\text{27}\) Although hypervascularization in solid tumors had long been noted,\(^\text{28}\) Algire and coworkers\(^\text{29}\) were the first to describe neovascularization as a factor in tumor growth. Folkman et al have demonstrated that tumor growth is angiogenesis dependent.\(^\text{30-32}\)

Angiogenesis has been suggested as an indicator of neoplastic transformation.\(^\text{33-35}\) Human solid tumors

**RESULTS**

Pearson correlation analyses indicated that tumor vessels were counted with a high degree of intraobserver reproducibility \((r = 0.88; P < 0.00001)\). They also indicated that the treatment affected right and left eyes equally \((P = 0.864)\). Retinoblastoma was present in 38 of 43 mice surviving the treatment protocol. Five of 27 (19%) calcitriol-treated mice and none of the 16 control mice failed to develop tumors.

Mean vessel counts for the 38 tumor-bearing animals were compared (Table 1). The high-dose group (Fig. 1) demonstrated the least amount of vascularization \((mean = 8.5 vessels)\); the low-dose group showed a slightly higher count \((Fig. 2) \ (mean = 10.1)\), whereas the untreated control mice had the highest vessel count \((mean = 14.1) \ (Fig. 3)\). When the vessel counts of both calcitriol-treated groups were com-

**FIGURE 2.** (A) Low-dose group. Hematoxylin and eosin-stained section of retinoblastoma, original magnification \(\times 400\). (B) GS-I staining of the same eye shows mild vascularity. Count for this field = 15. GS-I/methylene green, original magnification \(\times 400\).

vessel counts between treatment groups were statistically significant. Eyes without tumor were not included in this determination \(\) (six eyes from the high-dose group and four eyes from the low-dose group).

**FIGURE 3.** (A) Control eye with a highly undifferentiated tumor with numerous mitotic figures. Hematoxylin and eosin, original magnification \(\times 400\). (B) High degree of vascularity seen in the same eye when stained with GS-I. Count for this field = 30. GS-I/methylene green, original magnification \(\times 400\).
display an avascular and vascular growth phase. In the early prevascular state, tumors grow slowly and may remain small for long periods of time. In the transition from avascular to vascular states, angiogenesis is induced. As new capillary sprouts reach the tumor, growth becomes exponential with local invasion and distant metastasis. Inhibition of angiogenesis has, therefore, been proposed as a therapeutic approach for cancer by Folkman.

Calcitriol might inhibit tumor growth by employing its various antiangiogenic properties, such as cellular differentiation, growth inhibition, and antiangiogenic effects. Oikawa has demonstrated vitamin D3 and a synthetic analog, 22-oxa-1,25 dihydroxy vitamin D3, inhibits angiogenesis using the chick allantoic membrane assay. The present study suggests that calcitriol inhibits angiogenesis in transgenic murine retinoblastoma. This property may play a major role in the antineoplastic effect of vitamin D3. The antiangiogenic effect may be dose dependent, although these data indicate no significant difference between treatment groups. Vitamin D3 mediates its action through a steroid-receptor complex that affects nuclear DNA and may regulate synthesis of various RNA encoding proteins, transcription factors, and even oncogenes. Although this study suggests a strong role of the antiangiogenic properties of vitamin D3 in tumor inhibition, the complete absence of tumor formation in the eyes of five animals, together with inhibition of growth of human retinoblastoma cell lines, may indicate that additional mechanisms exist. It is possible that the effect of vitamin D on RNA coding proteins, transcription factors, and oncogenes may be its primary anti-neoplastic effect, with inhibition of angiogenesis a secondary effect. Further studies are needed to determine the exact mechanism of tumor control by vitamin D.

**Key Words**

retinoblastoma, vitamin D3 compounds, transgenic mice, angiogenesis

**References**

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