Calcitriol Is a Potent Inhibitor of Retinal Neovascularization

Daniel M. Albert,[1] *Elizabeth A. Scheef,*[1] *Shoujian Wang,*[1] *Farideh Mehraein,*[1] *Soesiawati R. Darjatmoko,*[1] *Christine M. Sorenson,*[2] *and Nader Sheibani*[1,3]

Purpose. Vitamin D compounds inhibit the growth of a variety of tumors in preclinical and clinical studies. Among the mechanisms suggested for this inhibition is antiangiogenesis. Retinal angiogenesis is the basis for vision loss in several major blinding diseases. The purpose of this study was to evaluate the antiangiogenic activity of calcitriol (1,25-dihydroxyvitamin D_3) in vivo and its effect on retinal endothelial cell (EC) proliferation, migration, and capillary morphogenesis in vitro.

Methods. The mouse oxygen-induced ischemic retinopathy (OIR) model was used to assess the antiangiogenic activity of calcitriol. Ocular VEGF levels were determined by Western blot analysis of whole eye extracts from postnatal day (P) 15 mice during OIR. The effects of calcitriol on retinal EC proliferation, migration, and capillary morphogenesis were also assessed in vitro.

Results. Calcitriol-treated animals demonstrated a significant decrease in retinal neovascularization compared with control animals. This effect was dose dependent, and retinal neovascularization was significantly inhibited in calcitriol-treated mice. Although no deaths occurred, calcitriol administration was associated with increased serum calcium and a lack of increase in body weight in a dose-independent manner. The ocular level of VEGF was similar in control and calcitriol-treated animals. At a lower concentration of calcitriol, retinal EC capillary morphogenesis in solubilized basement membrane was inhibited without a significant inhibitory effect on EC proliferation and migration. The concentration of calcitriol required to inhibit retinal EC proliferation was significantly higher than that required to inhibit EC capillary morphogenesis.

Conclusions. These data suggest calcitriol is a potent inhibitor of retinal neovascularization and may be of benefit in the treatment of a variety of eye diseases with a neovascular component. (*Invest Ophthalmol Vis Sci.* 2007;48:2327–2334) DOI:10.1167/iovs.06-1210

From the Departments of [1]Ophthalmology and Visual Sciences, [2]Pediatrics, and [3]Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin.

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Corresponding author: Nader Sheibani, Department of Ophthalmology and Visual Sciences, University of Wisconsin Medical School, 600 Highland Avenue, K6/458 CSC, Madison, WI 53792-4673; nsheibanikar@wisc.edu.

Angiogenesis, the formation of new blood vessels from preexisting capillaries, is tightly regulated and normally does not occur except during developmental and repair processes. This strict regulation is manifested by a balanced production of positive and negative factors that keep angiogenesis in check.[1] However, the balance becomes abrogated under various pathologic conditions, such as cancer, diabetes, age-related macular degeneration, and retinopathy of prematurity (ROP), resulting in the growth of new abnormal blood vessels.

ROP is a potentially blinding eye disorder that affects premature infants.[2] Each year, approximately 1100 to 1500 infants in the United States develop ROP severe enough to require medical treatment. Unfortunately, 400 to 600 infants each year in the United States become legally blind from ROP.[3] ROP occurs when abnormal new blood vessels grow and spread throughout the retina. These newly formed blood vessels are fragile and leak, scarring the retina and resulting in retinal detachment, the main cause of visual impairment and blindness in ROP. ROP remains a serious problem despite striking advances in neonatology. Therefore, there is great interest in the development and identification of agents that can inhibit the growth of new blood vessels.

Oxygen-induced ischemic retinopathy (OIR) in the mouse is a highly reproducible model of angiogenesis in vivo and recapitulates the hyperoxia damage to the retinal vasculature and retinal neovascularization observed in humans with ROP.[4] The degree of neovascularization can be readily quantified by counting the number of vascular cell nuclei on the vitreous side. This model has been informative with regard to insight into the molecular and cellular mechanisms that contribute to ROP and the development and testing of agents with antiangiogenic activity.[5–8]

The antitumor activity of vitamin D compounds has been demonstrated in preclinical and clinical tests against a variety of cancers, including retinoblastomas.[9] However, the molecular and cellular mechanisms responsible for tumor growth inhibition require further delineation. The reduced vascularity observed in many tumors treated with vitamin D compounds suggests tumor vasculature may be a target. In vitro and in vivo studies have demonstrated that calcitriol can directly affect the activity of endothelial cells (ECs) and can impact their proliferation and sprouting.[10–12] However, the effects of calcitriol on retinal vascularization have not been previously addressed. In the present study, we demonstrate that calcitriol is a potent inhibitor of retinal neovascularization both in a mouse model of OIR in vivo and in retinal EC capillary morphogenesis in vitro, without a significant inhibitory effect on retinal EC proliferation and migration.

Materials and Methods

Calcitriol

Pure crystalline calcitriol (provided by Ilex Oncology Inc., San Antonio, TX) was prepared for injection as previously described.[9,13,14] This drug was diluted in mineral oil to concentrations of 0.025, 0.125, and 0.25 μg/mL. Each mouse in the treatment group received 0.0025,

0.0125, or 0.025 μg calcitriol (approximately 0.5, 2.5, or 5 μg/kg) per treatment. These doses were found, in previous toxicity and dose–response studies, to be effective with minimal toxicity, and they are well-accepted as therapeutic doses in many animal studies.[9,13,14] For in vitro studies, a stock solution of calcitriol in 100% ethanol (2 mM, 0.83 mg/mL) was prepared. Appropriate dilutions were prepared in growth medium. Control cells were incubated with ethanol concentrations similar to those of cells exposed to calcitriol, and ethanol levels were kept to less than 0.1%.

Mouse Model of Oxygen-Induced Ischemic Retinopathy

All experimental procedures involving animals were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. In the mouse OIR model, postnatal day (P)7 pups (8–10 pups) and their mother (C57BL/6J; Jackson Laboratories, Bar Harbor, ME) were placed in an airtight incubator and exposed to an atmosphere of 75% ± 0.5% oxygen (hyperoxia) for 5 days, as described previously.[4,15,16] Mice were then brought to room air for 5 days. To assess the antiangiogenic activity of calcitriol, half the pups were injected intraperitoneally with 0.0025, 0.0125, or 0.025 μg calcitriol in 0.1 mL mineral oil per day from P12 to P17, when maximum retinal neovascularization occurred. The other half, from the same litter, was injected with 0.1 mL mineral oil. One eye from each mouse was used for histochemical analysis and the other eye for histologic evaluation. These experiments were repeated at least three times for each dose of calcitriol.

Visualization and Quantification of Retinal Neovascularization

Vessel obliteration and the retinal vascular pattern on P17 were analyzed using retinal wholemounts stained with anti–collagen IV antibody, as previously described by us.[15,16] Retinas were viewed by fluorescence microscopy, and images were captured in digital format with the use of a microscope (Carl Zeiss, Chester, VA). Quantification of retinal neovascularization on P17 was performed, as previously described by us.[15,16]

Western Blot Analysis

VEGF protein levels were determined by blotting of whole eye extracts prepared from P15 mice during OIR (5 days of hyperoxia and 3 days of normoxia) when maximum levels of VEGF were expressed, as previously described.[15–17] Approximately 20 μg protein lysate was analyzed by SDS-PAGE (4%–20% Tris-Glycin gel; Invitrogen, Carlsbad, CA) and was blotted with a rabbit polyclonal anti–mouse VEGF antibody (1:2000 dilution; PeproTech, Rocky Hill, NJ). The same blot was also probed with a monoclonal antibody to β-catenin (1:3000; BD Transduction, Franklin Lakes, NJ) to verify equal protein loading in all lanes. For quantitative assessments, band intensities relative to loading controls were determined by scanning the blots (Molecular Dynamic Storm 860 Scanner and Image Quant Software; Amersham, Piscataway, NJ).

Effects of Calcitriol Treatment on Body Weight

In the OIR studies, all pups were of similar body weight before initiation of the experiment and after exposure to high oxygen. The side effects of calcitriol on the mouse body weights were determined during exposure to room air before and after treatment. Body weights from mice in each group were determined at P12 (before treatment) and P17 (after treatment). None of the experimental animals died during these experiments.

Determination of Serum Calcium Levels

Blood (0.2 mL) was collected from P17 mice treated with different doses of calcitriol or solvent control during OIR. The blood was allowed to clot at room temperature, and the collected serum was stored at −80°C until needed for analysis. Serum samples were sent to Marshfield Clinic (Marshfield, WI) for total serum calcium analysis. The serum calcium level is reported as milligram per deciliter.

Retinal EC Proliferation, Migration, and Capillary Morphogenesis

Mouse retinal ECs were prepared and maintained as described previously.[18] For cell proliferation assays, retinal ECs (10,000) were plated in triplicate in 96-well plates overnight. The next day, cells were fed with growth medium containing various concentrations of calcitriol or solvent control. Cells were allowed to grow for 3 to 9 days and were fed every 3 days with fresh medium containing appropriate concentrations of calcitriol. The degree of proliferation was assessed using the nonradioactive cell proliferation assay (CellTiter 96 AQ_{ueous}; Promega, Madison, WI), as recommended by the supplier. This is a colorimetric method for determining the number of viable cells in proliferation or chemosensitivity assays. The assay is dependent on the ability of viable cells to convert a novel tetrazolium compound, MTS, to a formazan product that is soluble in tissue culture medium. The quantity of formazan product, as measured by the amount of 490 nm absorbance, is directly proportional to the number of living cells in culture. Results are presented as percentage viability relative to solvent control cells.

Retinal EC migration was determined using both wound migration and transwell assays. Confluent monolayers of retinal ECs were wounded with a micropipette tip, rinsed with growth medium to remove detached cells, and incubated with growth medium containing calcitriol (10 μM) or ethanol (solvent control). Wound closure was monitored by phase microscopy, and digital images were obtained at 24 and 48 hours and were used for quantitative assessment of migration. For transwell migration, wells (8-μm pore size, 6.5-mm membrane; Costar, Lowell MA) were coated with solubilized basement membrane (200 μg/mL; Matrigel; BD Biosciences, San Jose, CA) or fibronectin (2 μg/mL) in PBS on the bottom at 4°C overnight. The next day, inserts were rinsed with PBS, blocked in PBS containing 2% BSA for 1 hour at room temperature, and washed with PBS. Cells were removed by trypsin-EDTA, counted, and resuspended at 1×10^6 cells/mL in serum-free medium. Inserts were placed in 24-well dishes (Costar) containing 0.5 mL serum-free medium, and 0.1 mL cell suspension was then added to the top of the insert. Cells were allowed to migrate through the filter for 3 hours in a tissue culture incubator. After incubation, the cells on top of the filter were scraped off using a cotton swab; the membrane was fixed in 4% paraformaldehyde and stained with hematoxylin and eosin. The inserts were mounted on a slide cell side up, and the number of cells that migrated to the bottom of the filter was determined by counting 10 high-power fields at 200× magnification.

Capillary morphogenesis assays in solubilized basement membrane (Matrigel; BD Biosciences) were performed as previously described by us.[18] Briefly, 0.5 mL solubilized basement membrane (Matrigel; BD Biosciences) was added to a cold 35-mm tissue culture plate and was incubated at 37°C for at least 30 minutes to allow the solubilized basement membrane (Matrigel; BD Biosciences) to harden. Retinal ECs were removed by trypsin-EDTA, resuspended at 1.5×10^5 cells/mL in the growth medium containing calcitriol (10 μM) or solvent control, and incubated on ice for 15 minutes After incubation, 2 mL cell suspension in the presence of calcitriol or solvent control was gently added to the solubilized basement membrane (Matrigel; BD Biosciences)–coated plates and incubated at 37°C. Cultures were monitored for 6 to 48 hours, and images were captured in digital format after 18 hours, when maximum organization was observed. Longer incubation did not result in further organization of ECs into a tubular network. The capillary network formed by control cells began to fall apart at 24 to 48 hours.

Statistical Analysis

Statistical differences between groups were evaluated with the Student unpaired *t*-test (two-tailed). Mean ± SD is shown. $P \leq 0.05$ was considered significant.

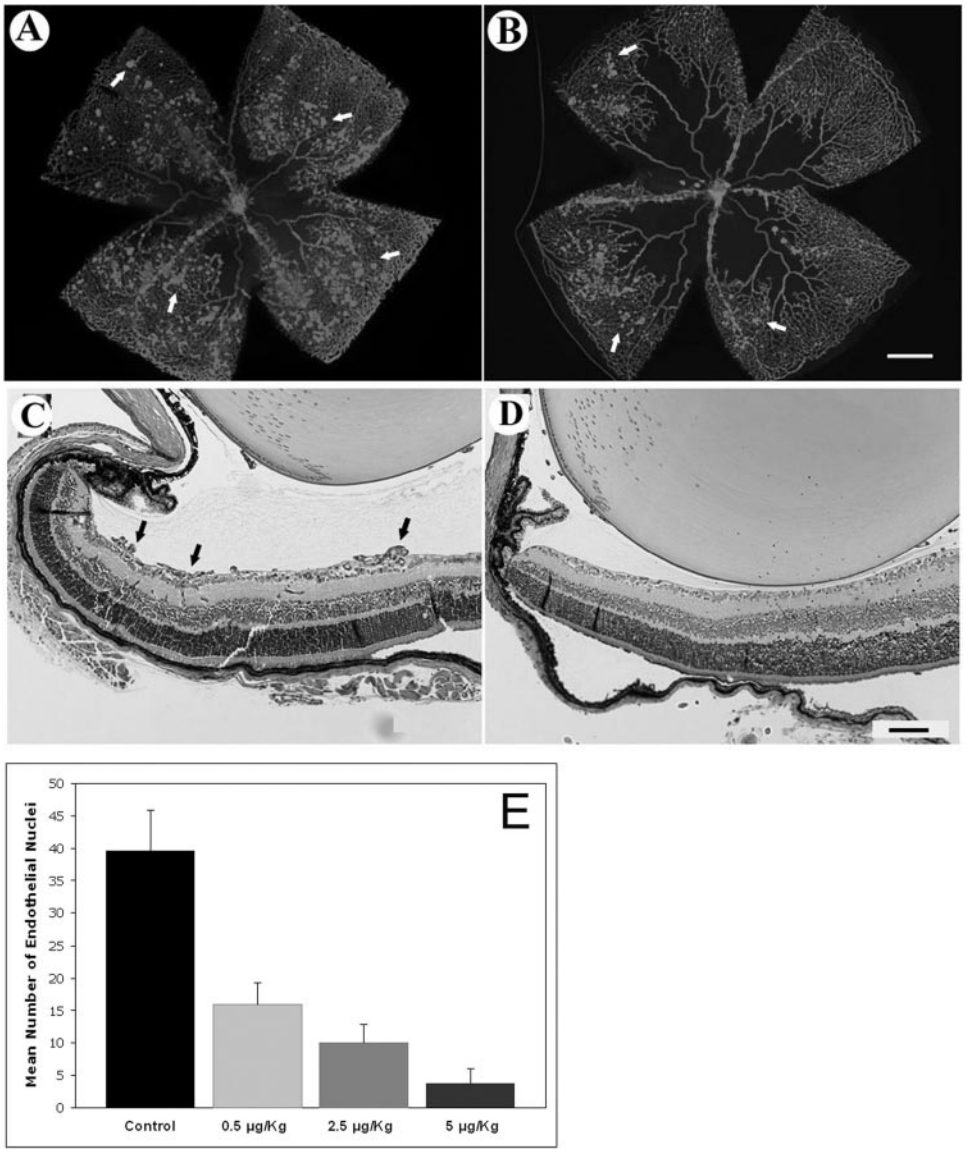

FIGURE 1. Assessment of retinal vasculature in control (**A**, **C**) and calcitriol-treated (**B**, **D**) mice during OIR. P7 mice were exposed to a cycle of hyperoxia and normoxia, and eyes were removed for appropriate analysis. (**A**, **B**) Wholemount collagen IV staining of retinal vasculature from P17 control and calcitriol-treated mice exposed to OIR, respectively. (**C**, **D**) Hematoxylin- and PAS-stained cross-sections prepared from P17 control and calcitriol-treated mice (5 μg/kg) exposed to OIR, respectively. *Arrows* show the new vessels growing into the vitreous. (**E**) Quantitative assessments of retinal neovascularization in eyes from P17 control and calcitriol-treated mice exposed to OIR. Data in each column are the mean ± SD values from four eyes of four mice. Note that there is a significant difference in the degree of neovascularization among control and calcitriol-treated mice ($P < 0.001$). These experiments were repeated three times with similar results. Scale bars: 500 μm (**A**, **B**); 50 μm (**C**, **D**).

RESULTS

Inhibition of Retinal Neovascularization by Calcitriol

In the mouse OIR model, P7 mice are exposed to a cycle of hyperoxia (75% oxygen) and normoxia (20% oxygen). Exposure to high oxygen results in the downregulation of proangiogenic factors, promoting the obliteration of existing vessels dependent on these factors. In addition, further development of retinal vasculature also becomes halted. However, when animals are returned to room air, the undervascularized retina becomes ischemic and promotes the production of proangiogenic factors such as VEGF, which drive the growth of abnormal new blood vessels. Maximum neovascularization occurs from P12 to P17.[4,15,16] Therefore, the effect of calcitriol on retinal neovascularization was evaluated by its administration from P12 to P17.

To visualize ischemia-induced retinal neovascularization, collagen IV staining of wholemount retinas was performed. P17 calcitriol-treated and control mice subjected to OIR demonstrated that peripapillary retinal capillaries dropped out, whereas the larger, well-developed radial retinal vessels extending from the optic disc persisted. Retinas from P17 control mice exposed to OIR contained many neovascular tufts extending from the surface of the retina at the junction between the perfused and nonperfused retina (Fig. 1A; arrows). In contrast, retinas from P17 mice treated with calcitriol demonstrated markedly reduced neovascularization (Fig. 1B; arrows).

Retinal neovascularization was also assessed histologically by counting vascular cell nuclei anterior to the inner limiting membrane (ILM), as previously described.[15,16] Briefly, serial sections obtained from the region around the optic nerve were stained with hematoxylin and periodic acid-Schiff (PAS) and were examined in a masked fashion for the presence of neovascular cell nuclei projecting into the vitreous from the retina. The neovascular cell nuclei score was defined as the mean number of neovascular nuclei per section found in eight sections (four on each side of the optic nerve) per eye. Retinas from P17 control mice subjected to OIR contained multiple neovascular tufts on their surfaces (Figs. 1A, 1C; arrows), with some extending into the vitreous. These tufts contained a significant number of neovascular nuclei anterior to the ILM (Fig. 1C; arrows). Retinas from mice treated with calcitriol showed significantly fewer preretinal neovascular tufts (Figs.

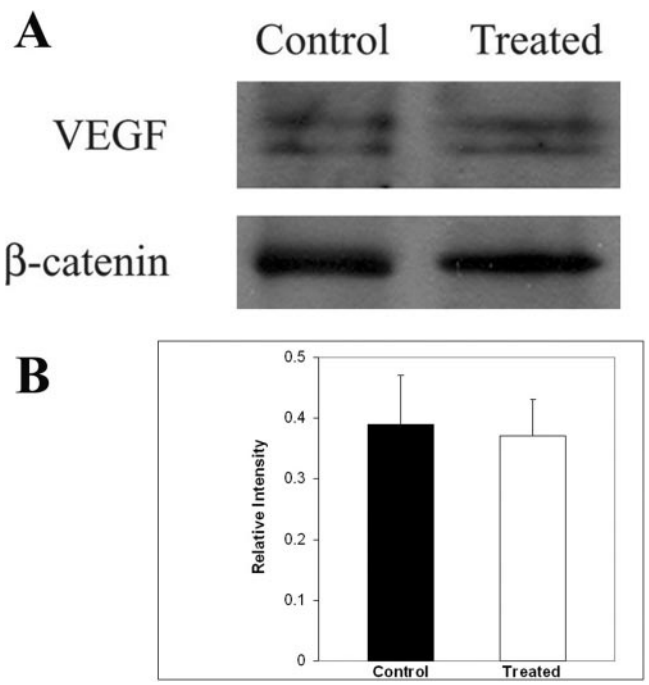

FIGURE 2. Assessment of VEGF levels in eyes from control and calcitriol-treated mice. Eye extracts prepared from control and calcitriol-treated (5 μg/kg) P15 mice (5 days of hyperoxia and 3 days of normoxia) were analyzed by SDS-PAGE and Western blotting. β-Catenin was used for loading control (**A**). Quantitative assessments of relative band intensities (**B**). Data in each column are the mean ± SD values of relative intensities of three experiments. Note that there is no significant difference in the relative amounts of VEGF expressed in control and calcitriol-treated eyes ($P < 0.5$).

1B, 1D; arrows). Retinal neovascularization in these mice was inhibited by greater than 90% at 5 μg/kg calcitriol (Fig. 1E; $P < 0.001$). A lower degree of inhibition was observed at lower doses of calcitriol. A 75% inhibition of neovascularization was observed at 2.5 μg/kg calcitriol, whereas 60% inhibition was observed at 0.5 μg/kg calcitriol. Therefore, the inhibition of angiogenesis by calcitriol occurred in a dose-dependent manner.

To determine whether the inability of retinas from calcitriol-treated mice to undergo neovascularization in response to ischemia was caused by lack of VEGF expression, we examined VEGF levels in eyes from P15 control and calcitriol-treated mice during OIR (5 days of hyperoxia and 3 days of normoxia). VEGF expression is maximally induced at P15 during OIR.[15,17] Figure 2A shows a blot of protein lysates prepared from whole eyes of control and calcitriol-treated P15 mice during OIR. VEGF expression levels in eyes from control and calcitriol-treated mice during OIR were not significantly different ($P < 0.56$; Fig. 2B).

Assessment of the Effects of Calcitriol on Body Weights and Serum Calcium Levels

Body weights of mice were determined at P12 and P17 after 5 days of injection with different doses of calcitriol or solvent control. Body weights of all the animals treated with calcitriol were similar before initiation of the experiment and after exposure to high oxygen. In control mice, body weights increased by approximately 30% from P12 to P17 (Fig. 3A; $P < 0.05$). In contrast, there was a lack of increase in body weight in mice that received calcitriol for 5 days (Fig. 3B). This effect

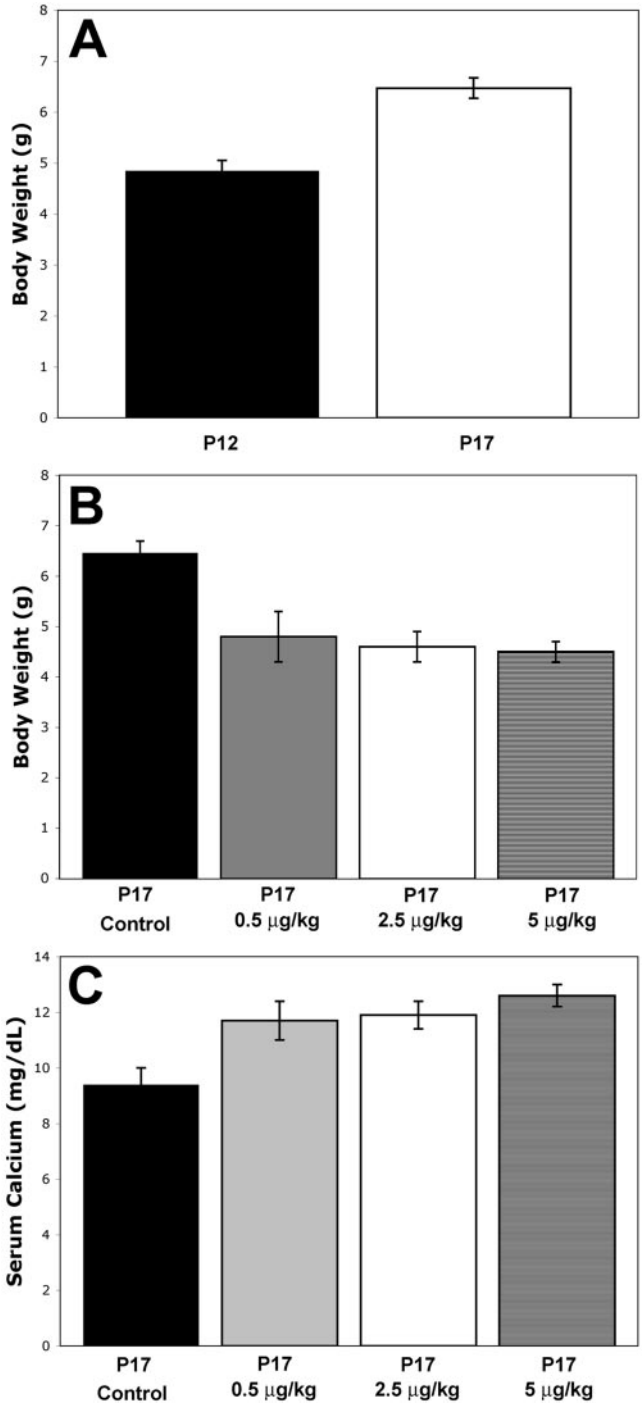

FIGURE 3. Effects of different doses of calcitriol treatment on body weights and serum calcium levels. Body weights of control (**A**) and calcitriol-treated (**B**) mice during oxygen-induced ischemic retinopathy were determined at P12 (before treatment) and at P17 (after treatment). Serum calcium levels were determined in P17 control and calcitriol-treated mice (**C**). Data in each column are the mean ± SD values of body weights or serum calcium levels of four mice from four experiments. Effects of calcitriol on body weights and serum calcium levels were dose independent. Note a significant increase in body weight of control mice from P12 to P17 ($P < 0.05$). The decrease in body weight and the increase in serum calcium levels of P17 mice treated with calcitriol compared with control treated mice were significant ($P < 0.05$).

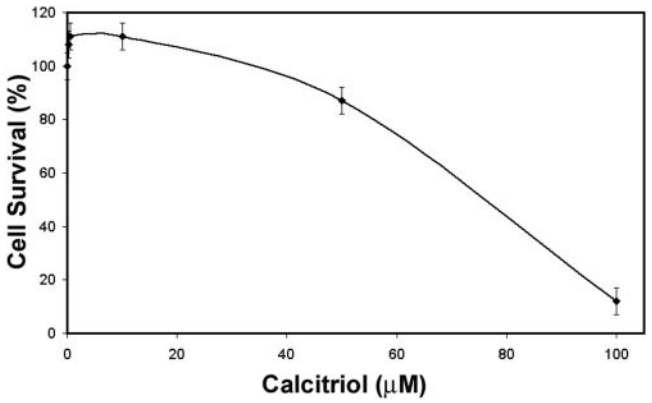

FIGURE 4. Effects of calcitriol on retinal EC proliferation. Retinal ECs were incubated with different concentrations of calcitriol for 3 days. The percentage of cell viability relative to the control treatment was determined using a nonradioactive cell viability assay. Note that calcitriol had no inhibitory effect on EC viability at concentrations below 10 μM and at 100 μM reduced cell viability by 90%. These experiments were repeated four times with similar results.

on body weight was independent of the doses of calcitriol used in these experiments. The decrease in body weight of P17 treated mice compared with P12 mice was not significant (Fig. 3B; $P < 0.5$) and was well within the range of variation in weights of animals in the same litter. However, the decrease in body weight of P17 treated mice compared with P17 control mice was significant (Figs. 3A, 3B; $P < 0.05$). Thus, calcitriol treatment was associated with a lack of increase in body weight compared with control mice. This side effect of calcitriol, and perhaps hypercalcemia, is commonly seen in many studies evaluating the therapeutic effects of calcitriol.[13,14,19] The total serum calcium levels were determined in P17 mice and were significantly increased in calcitriol-treated mice compared with control mice ($P < 0.05$). However, the differences in serum calcium levels among calcitriol-treated animals were not significant (Fig. 3C; $P < 0.5$). Thus, the changes in serum calcium levels were independent of the doses of calcitriol used here and were consistent with the lack of significant differences in the body weights of P17 mice treated with different doses of calcitriol (Figs. 3B, 3C).

Calcitriol Effects on Retinal EC Proliferation, Migration, and Capillary Morphogenesis

The effects of calcitriol on retinal EC proliferation, migration, and capillary morphogenesis have not been previously examined. Furthermore, the effects of calcitriol on the proliferation and migration of other types of ECs have been contradictory.[11] We next examined the effects of calcitriol on retinal EC viability, during both short-term (3 days) and long-term (9 days) incubation. Figure 4 shows the viability of retinal ECs incubated with different concentrations of calcitriol relative to cells incubated with solvent control for 3 days at 37°C. We observed minimal cytotoxicity at lower concentrations of calcitriol (0–10 μM). Significant cytotoxicity was only observed at 50-μM and higher concentrations of calcitriol. Calcitriol at 100 μM reduced retinal EC viability by approximately 90%. Incubation of retinal ECs with calcitriol (0–10 μM) for 9 days had minimal inhibitory effects on their viability, as was observed after 3 days of exposure (data not shown).

We also examined the effects of calcitriol on the migration of retinal ECs using both wound migration and transwell assays. We observed no significant effect on wound migration of retinal ECs incubated with 10 μM calcitriol compared with solvent control cells (Figs. 5A, 5B). When we used the transwell assay, calcitriol had no significant effect on the migration of retinal ECs through the filter coated with solubilized basement membrane (Matrigel; BD Biosciences; Fig. 5C). Although calcitriol appeared to enhance retinal EC migration through filters coated with fibronectin, this was not statistically significant when compared with solvent control (not shown). Therefore, calcitriol at 10 μM had minimal inhibitory effects on retinal EC migration in culture.

Retinal ECs, like many other types of ECs, rapidly organize into capillary networks when plated in solubilized basement membrane (Matrigel; BD Biosciences).[18] Wild-type retinal ECs incubated with the solvent control organized into a capillary network when plated in solubilized basement membrane (Matrigel; BD Biosciences) (Figs. 6A, 6C). However, incubation of retinal ECs with calcitriol (10 μM) completely blocked their ability to form capillary networks in solubilized basement membrane (Matrigel; BD Biosciences) (Figs. 6B, 6D). Calcitriol at lower concentrations was less effective (not shown), which was consistent with our in vivo dose-dependent inhibition of neovascularization by calcitriol.

DISCUSSION

Calcitriol (1α,25-dihydroxyvitamin D_3), the active hormonal form of vitamin D, has an inhibitory effect in a variety of cancers, including prostate, breast, colon, and retinoblastoma. These effects are mediated through the interaction of calcitriol with its receptor (vitamin D receptor [VDR]), which arrests the cancerous cell cycle at the G0-G1 transition through the up-regulation of cyclin-dependent kinase inhibitors P21 and P27.[20,21] Furthermore, growth-suppressing activity of calcitriol may occur through the induction of apoptosis. In retinoblastoma cells, this occurs through modulation of the expression of bcl-2 family members.[22] Recent studies demonstrated potential antiangiogenic activity for calcitriol, suggesting vitamin D compounds may inhibit tumor growth through the inhibition of angiogenesis.[11,23] In the present study, we showed that calcitriol is a potent inhibitor of retinal neovascularization in vivo and inhibits retinal EC capillary morphogenesis in vitro without a significant inhibitory effect on EC proliferation and migration.

How calcitriol affects angiogenesis has been unclear. Calcitriol has been reported to decrease[24] or to have no effect[25,26] on EC proliferation, to have no effect on capillary morphogenesis in vitro,[27] and to inhibit angiogenesis in vivo.[28] Mantel et al.[11] recently showed that calcitriol inhibits angiogenesis in vivo in a xenograft breast cancer model and EC proliferation and morphogenesis in vitro by inhibiting EC proliferation and induction of apoptosis. However, the effect of calcitriol on EC proliferation mediated by VEGF was small. The effects of calcitriol on retinal neovascularization, retinal EC proliferation, migration, and capillary morphogenesis have not been previously addressed. Calcitriol, at doses shown to be effective in inhibition of retinoblastoma with minimal toxicity, significantly blocked retinal neovascularization during OIR. The inhibition of angiogenesis by calcitriol was independent of changes in VEGF expression at P15, when maximum expression of VEGF occurred.

Given the potential role of calcitriol in the downregulation of bcl-2 expression in tumor cells,[22] it is reasonable to speculate that the downregulation of bcl-2 in ECs may contribute to a lack of neovascularization in mice treated with calcitriol. We have recently shown in this model that mice deficient in bcl-2 failed to neovascularize their retina despite sufficient expression of VEGF.[16] Furthermore, the up-regulation of P21 and P53

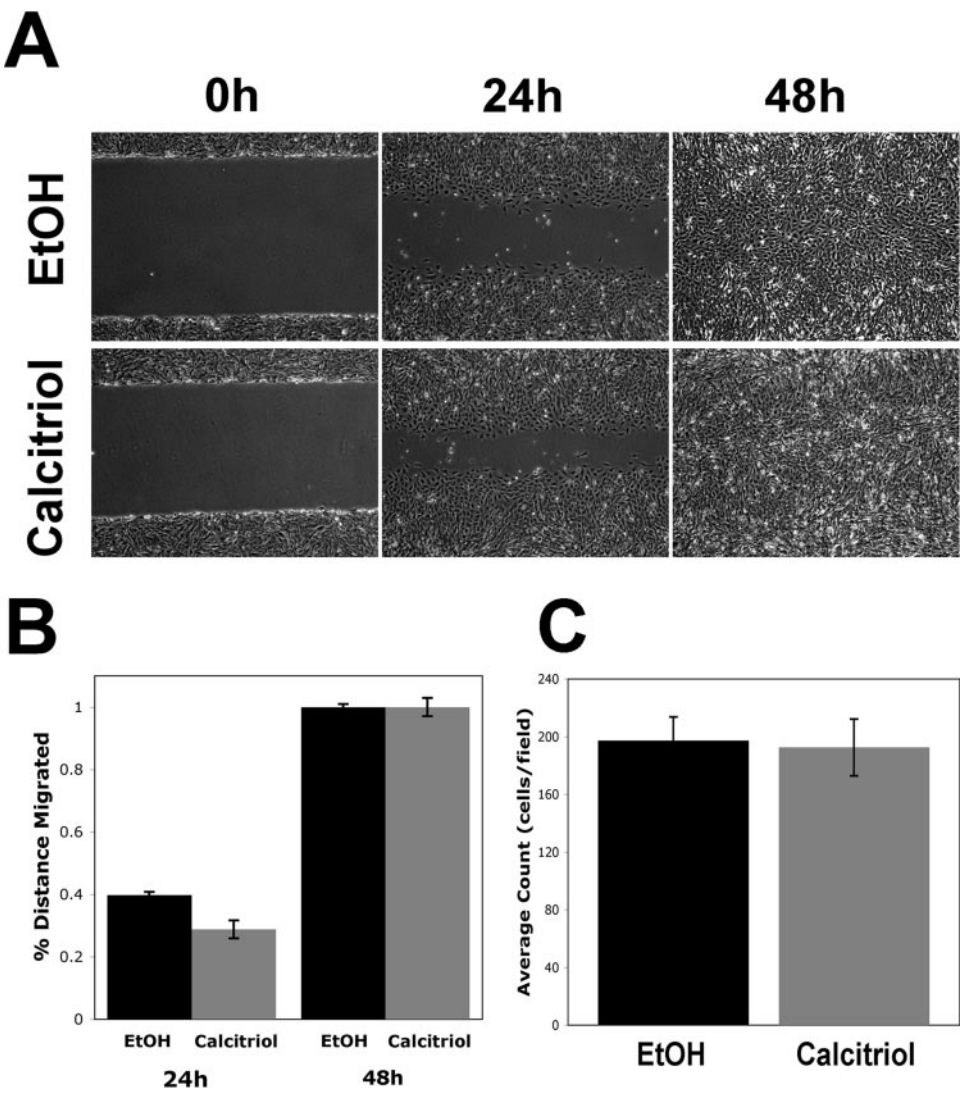

FIGURE 5. Effects of calcitriol on retinal EC migration. Retinal EC migration in the presence of ethanol (control) or calcitriol (10 μM) was determined using wound migration (**A**, **B**) and transwell (**C**) assays. Morphology of confluent monolayers of retinal EC wound closure was monitored by phase microscopy at different times after wounding (**A**). Quantitative assessment of wound migration (**B**). Data in each column are the mean values ± SD of percentage distance migrated of three separate experiments. Quantitative assessment of transwell migration (**C**). Data in each bar are the mean ± SD values of cells that migrated through the membrane in 10 high-power fields of three separate experiments. Note that there is no significant difference in the degree of migration among control and calcitriol-treated cells ($P < 0.5$).

by vitamin D reported to inhibit retinoblastoma tumor growth[29] may also promote EC death. The up-regulation of P53 could result in increased expression of thrombospondin−1 (TSP1) and other endogenous inhibitors of angiogenesis, in tumor cells and inhibition of angiogenesis.[30,31] We recently showed that Y-79 retinoblastoma cells express little or no TSP1, and expression of TSP1 in these cells has a dramatic effect on their proliferation in culture and tumor formation in nude mice.[32] Whether the incubation of Y-79 cells or ECs with calcitriol results in increased expression of TSP1, through the up-regulation of P53 expression, remains to be determined.

Our results with retinal ECs are consistent with previous reports that the effect of calcitriol at low concentrations on the inhibition of EC proliferation and migration is minimal. We did not observe a significant decrease in retinal EC viability or migration when calcitriol was used at concentrations up to 10 μM. A significant decrease in retinal EC viability was observed when calcitriol was used at concentrations of 50 μM and higher. In fact, calcitriol at 100 μM reduced retinal EC viability by 90%. These inhibitory concentrations are higher than those used in many studies that reported no or mild effects on EC proliferation. Therefore, inhibition of EC proliferation may require higher concentrations of calcitriol and may occur through VDR-independent mechanisms.[33]

Calcitriol at 10 μM completely abolished the ability of retinal ECs to undergo capillary morphogenesis. This concentration of calcitriol had minimal inhibitory effects on retinal EC proliferation, in short (3 days) or long (9 days) incubation, and on retinal EC migration. To gain further insight into the potential mechanism(s) involved in the inhibition of retinal EC capillary morphogenesis by calcitriol, we assessed retinal EC viability and cell cycle distribution during capillary morphogenesis in the presence or absence of calcitriol. We observed no significant effect of calcitriol on cell viability or cell cycle distribution during retinal EC capillary morphogenesis in solubilized basement membrane (Matrigel; BD Biosciences; not shown). Identifying genes whose expression is differentially affected by calcitriol during this process may provide additional insight into the cellular mechanisms involved and are the subject of current investigations in our laboratory. Thus, calcitriol is a potent inhibitor of retinal EC capillary morphogenesis despite its lack of inhibitory effects on retinal EC proliferation and migration in culture. This is consistent with our in vivo data demonstrating that retinal neovascularization was markedly inhibited in the presence of chemotherapeutic doses of calcitriol.

A toxic side effect of calcitriol treatment is decreased body weight, a consistent complication of hypercalcemia. The antineoplastic effect of calcitriol, however, is unrelated to either high serum calcium levels or calcium deposition in the tumors.[34,35] In fact, the clinical usefulness of vitamin D is limited by the toxic effects associated with hypercalcemia. We evalu-

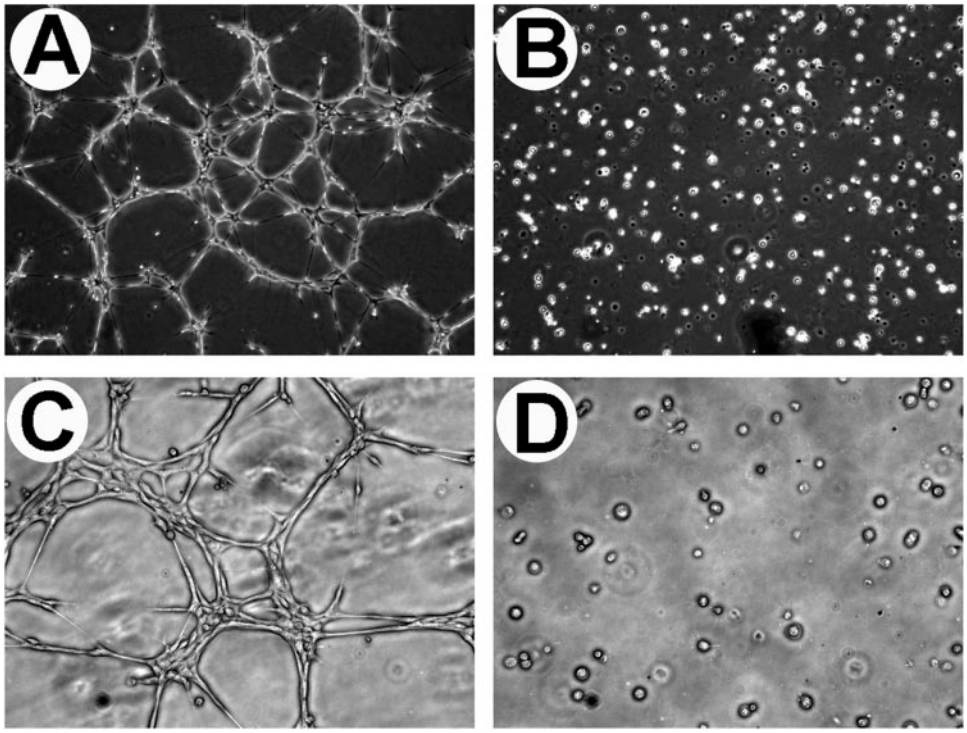

FIGURE 6. Effects of calcitriol on retinal EC capillary morphogenesis in solubilized basement membrane (Matrigel). The ability of retinal ECs to undergo capillary morphogenesis in the presence of solvent control (**A**, **C**) and calcitriol (10 μM; **B**, **D**) in solubilized basement membrane (Matrigel). Images were obtained after 18 hours. Calcitriol attenuated the ability of retinal ECs to undergo capillary morphogenesis. These experiments were repeated three times with similar results. The second two images (**C**, **D**) are higher magnifications ($\times$100) of the first two images (**A**, **B**; $\times$40).

ated the body weights of mice injected with solvent control or calcitriol during OIR. Body weights of mice injected with solvent control from P12 to P17 was increased by 30% (Fig. 3A), whereas body weights of mice injected with calcitriol did not significantly change (Fig. 3B). The lack of increase in body weight observed here is well within the expected range associated with the therapeutic use of calcitriol in the treatment of tumors and other conditions.[13,14,19,36] In cancer treatment studies, the doses of vitamin D used are approximately 5 to 10 times the doses used in renal failure or parathyroid disease.[37–39] The dose of calcitriol used here was based on data obtained in preclinical cancer studies with adult mice and may require further evaluation in younger mice. However, the impact of calcitriol on serum calcium levels in younger animals was identical with those we previously observed in older animals and was independent of the doses of calcitriol used here (Fig. 3C). Therefore, the inhibition of neovascularization observed here may be independent of hypercalcemia and changes in body weight and was directly proportional to the dose of calcitriol used (Fig. 1).

The dose-independent effects of calcitriol on serum calcium levels in our studies indicated that the changes in calcium level may be more sensitive to the levels of calcitriol. The lowest dose used here (0.5 μg/kg) may still be well above the threshold concentration of calcitriol for increased serum calcium levels. Therefore, careful evaluation of the effects of lower doses of calcitriol on serum calcium level and inhibition of angiogenesis may be essential. However, our results indicated a decrease in inhibition of neovascularization at lower doses of calcitriol despite similar levels of serum calcium. Thus, the use of lower doses of calcitriol may reduce increases in serum calcium level but will also compromise its antiangiogenic activity. Alternatively, serum calcium levels may be lowered by the use of pharmacologic agents such as bisphosphonates without compromising calcitriol antiangiogenic activity.

In summary, calcitriol is a hormone with multiple sites of action. Among its primary and secondary effects are hypercalcemia, impaired weight gain or induced weight loss (or both), and inhibition of angiogenesis. Although the purpose of the present study was to report the occurrence of angiogenesis inhibition in a dose-dependent manner after the administration of calcitriol, it is important that any respective roles that hypercalcemia and impaired weight gain play in the inhibition of angiogenesis be fully defined. Knowledge of such roles is critical in assessing the potential usefulness of calcitriol in newborns and in other clinical settings. The development of new vitamin D analogues that lack effects on serum calcium levels without compromising their antiangiogenic activity may have significant clinical value. Furthermore, these analogues may also allow evaluation of the relationship among hypercalcemia, impaired weight gain, and inhibition of angiogenesis.

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