Differential Antiproliferative Effects of Calcitriol on Tumor-Derived and Matrigel-Derived Endothelial Cells

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**Abstract**

The most active metabolite of vitamin D, calcitriol, is growth inhibitory for various tumor types *in vitro* and *in vivo* and inhibits the growth of endothelial cells freshly isolated from tumors [tumor-derived endothelial cells (TDEC)]. We compared the effects of calcitriol on Matrigel-derived endothelial cells (MDEC) and TDEC isolated from Matrigel plugs and squamous cell carcinoma tumors, respectively. TDEC and MDEC expressed vitamin D receptor (VDR) and responded to calcitriol by increasing VDR protein expression. Although no mutations were found in VDR from either cell type, Scatchard plot analysis revealed a higher ligand-binding affinity in TDEC ($K_d$, 0.26 nmol/L) than MDEC ($K_d$, 0.65 nmol/L). The VDR signaling axis in both cells was intact as shown using nuclear translocation and 24-hydroxylase promoter-luciferase reporter assays. However, unlike TDEC, MDEC were resistant to calcitriol-induced growth inhibition. Calcitriol (10 nmol/L) resulted in a 12.3% growth inhibition of MDEC compared with 47% in TDEC. In TDEC, calcitriol resulted in induction of apoptosis were seen in calcitriol-treated MDEC. These results show that TDEC were more sensitive than MDEC to the antiproliferative effects of calcitriol despite apparently normal VDR content and structure of signaling axis in both cell types.

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**Introduction**

The most active metabolite of vitamin D, 1,25-dihydroxycholecalciferol (calcitriol), is a key regulator of bone and mineral metabolism (1) and can modulate the growth and differentiation of several normal and malignant cell types (2). Calcitriol, in nanomolar concentrations, has a direct antiproliferative effects in prostate (3), breast (4), and colon cancer (5), squamous cell carcinoma (6), and leukemia (7) models; calcitriol promotes differentiation, cell cycle arrest, and apoptosis (6, 8, 9). In addition to the direct effects on tumor cells, calcitriol also inhibits the proliferation of endothelial cells and angiogenesis in tumors (10, 11). Angiogenesis is required for growth of tumors larger than a few millimeters and agents that selectively inhibit endothelial cells can inhibit and prevent tumor growth (12). Neovascularization and endothelial cell proliferation are attractive therapeutic targets, because data indicate a decreased risk of acquired resistance (13).

Vitamin D receptor (VDR) is expressed in several endothelial cell types, including bovine aortic endothelial cells, mouse endothelial cells, and human capillaries (10, 11). Calcitriol and its analogues inhibit embryonic angiogenesis in chick chorioallantoic membranes (14) and vascular endothelial growth factor (VEGF)–induced endothelial cell sprouting and elongation in *in vitro* rat and mouse model systems (15, 16). *In vivo*, calcitriol treatment decreases vessel density and VEGF expression (15, 16). Calcitriol inhibits Kaposi sarcoma cell growth *in vitro* and reduces the production of interleukin-6 and interleukin-8, autocrine growth factors for this highly vascular tumor (17). Expression of mRNA and protein for 25(OH)D$_3$-1α-hydroxylase (CYP27B), the rate-limiting enzyme for synthesis of calcitriol from 25(OH)D$_3$, is present in human renal arteries and postcapillary venules from lymphoid tissue (18). These studies suggest a potential role of calcitriol in the biology of endothelial and tumor cell microenvironment.

Blood vessels in tumors are different from those in normal tissues. Tumor vessels have abnormal branching and defects in the endothelial monolayer and often have an incomplete basement membrane and an abnormal pericyte coat (19, 20). These abnormalities observed in tumor vessels could be due to imbalance between proangiogenic and antiangiogenic factors in the tumor microenvironment, which could trigger the extensive and uncontrolled proliferation of tumor vasculature (21). Bovine capillary endothelial cells isolated from normal tissues grow slowly and eventually die when cultured in standard medium, but they grow rapidly and survive in tumor-conditioned medium (22). These differences in phenotype suggest that endothelial cells differ when grown in dissimilar microenvironments (tumor or normal).

We developed a method to freshly isolate endothelial cells from tumor and normal tissues (23). The endothelial cells that migrate into tumors [tumor-derived endothelial cells (TDEC)] are significantly growth inhibited by calcitriol, but endothelial cells isolated from normal tissues are not (10). These mature endothelial cells isolated from normal tissues are not representative of the neo-vascular endothelial cells that migrate into the tumor microenvironment. In C3H/HeJ mice, we directly compare the effects of calcitriol on microvascular endothelial cells isolated freshly from tumor (TDEC) and Matrigel plugs [Matrigel-derived endothelial cells (MDEC)].

**Materials and Methods**

Chemicals and reagents. Calcitriol (Hoffmann-LaRoche, Nutley, NJ) was reconstituted in 100% ethanol and stored, protected from light, under a layer of nitrogen gas at −70°C. All handling of calcitriol was done with indirect lighting. Immediately before use, calcitriol was diluted to the final
concentrations in tissue culture medium or sterile saline. For most application, calcitriol was used at 10 nmol/L because this concentration consistently shows antiproliferative effects in multiple assays in a variety of tumor cell types.

Cells and model systems. TDEC were isolated from the murine squamous cell carcinoma VII/SF tumor model in C3H/Hej mice as described previously (24). TDEC were cultured routinely on 1% gelatin-coated flasks in DMEM/10% (v/v) fetal bovine serum/1% penicillin and streptomycin. For all experiments, TDEC used were at low passages (<8 passages).

MDEC were isolated from Matrigel plugs implanted in C3H/Hej mice based on an adaptation of the method of Passaniti et al. (25). Matrigel (0.5 mL) containing 50 ng/mL human recombinant basic fibroblast growth factor (FGF; Promega, Madison, WI) and 10 units/mL heparin (Sigma, St. Louis, MO) were inoculated s.c. into the opposite flank of the mice. After 2 weeks, Matrigel plugs were carefully dissected out without any other tissue contamination, washed in PBS, and cultured on 1% gelatin-coated cell culture dish. The Matrigel plugs were stretched and loosen to facilitate cell migration. Within 2 weeks, cells from the Matrigel plugs migrated out to the cell culture dish and were initially cultured in DMEM enriched with 20% sarcoma 180 conditioned medium, 10% fetal bovine serum, 5 units/mL heparin, basal medium Eagle’s vitamin solution (Invitrogen, Carlsbad, CA), HEPES (Invitrogen), 50 μg/mL endothelial cell growth supplement (Fisher, Hampton, NH), and 1% penicillin/streptomycin; these cells were designated as cells at passage 0. Endothelial cells used in subsequent in vitro experiments were not beyond passage 8 and were cultured in DMEM/10% (v/v) fetal bovine serum/1% penicillin streptomycin. We used indirect staining of anti-mouse platelet endothelial cell adhesion molecule 1 (PECAM-1; CD31) antibody (PharMingen, BD Biosciences, San Jose, CA) and fluoroscein-conjugated anti-rat IgG antibody (PharMingen) to identify the isolated cells using flow cytometry (BD FACScan, BD Biosciences). Matrigel plugs were processed for H&E and anti-mouse PECAM-1 antibody staining. The Institutional Animal Care and Use Committee of Roswell Park Cancer Institute approved all of the procedures involving animals.

Reverse transcription-PCR. Total RNA was isolated by RNeasy Mini kit (Qiagen, Valencia, CA). Reverse transcription of VDR and glyceraldehyde-3-phosphate dehydrogenase was done using 2 μg total RNA and the SuperScript III transcriptase (Invitrogen) and Tag DNA polymerase (Invitrogen). Specific primers used to amplify the whole coding region of mouse VDR (NM_009504) were 5’-ATGGGAGCAATGGCACGC-3’ and 5’-TGCAAGAGATCTCATTGGC-3’. PCR products were TA subcloned into pCR2.1 vector (Invitrogen). Plasmids containing the insert from at least 15 colonies were isolated and sequenced using ABI PRISM 3130XL sequencer (Applied Biosystems, Foster City, CA). Primers used for the PCR products were 5’-CAAGGT- GACCAGCTCTATGAA-3’ and 5’-TGGTGACGACCCACCTGAT-5’ (518 bp). FGF receptor 2 (5’-AAAGGTGACAAACAGCTGGAG-3’ and 5’-CAGAGT-GAAAGATATCCCGATAG-3’; 625 bp). Sequences for primer pairs were as follows: factor VIII (5’-TGGAGGAATCTCATTGCCG-3’ and 5’-AAAAACATAGCCATT-5’; 786 bp), neuropilins 1 and 2, and tumor endothelium markers 1, 5, and 6 (518 bp). Sequences for primer pairs for VEGF receptors 1 and 2, neuropilins 1 and 2, and tumor endothelium markers 1, 5, and 6 were described previously (26). The PCR products were resolved on 1.5% agarose gels.

Hormone-binding assay. Cells were plated in 12-well plates for 24 hours before treatment with varying concentrations of 1α,25-(26,27,28,29)-dihydroxyvitamin D3 (Perkin-Elmer, Boston, MA) with or without 100-fold excess of unlabeled 1,25-dihydroxyvitamin D3 for 4 hours in 37°C. They were then washed with ice-cold PBS, lysed with 100% ethanol, and transferred to scintillation vials for counting the radioactivity. Specific binding was then calculated by subtracting nonspecific binding from total binding. Scatchard plots were generated using equation: B / F = 1 / Kd + B / Rm, where B and F are the bound and free ligands, respectively, Kd is the ligand dissociation constant, and Rm is the maximal binding of ligand.

Nuclear translocation. Cells were plated on chamber slides 24 hours before treatment with 10 nmol/L calcitriol for 15 minutes. They were then fixed in 2% paraformaldehyde in 5 minutes at room temperature, washed in PBS for 10 minutes, permeabilized with 0.05% Triton X-100, and washed with PBS for 5 minutes. The chamber slides were incubated with primary antibodies either anti-VDR (Affinity Bioreagents, Golden, CO) or anti-retinoid X receptor α (RXRα; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies for 1 hour at room temperature. After washing with PBS, the slides were incubated with Cy2-conjugated AffiniPure goat anti-rat IgG (Jackson Immunoresearch, West Grove, PA) or rhodamine (TRITC)–conjugated AffiniPure donkey anti-rabbit IgG (Jackson Immunoresearch) for 30 minutes at room temperature. The cells were washed again in PBS for 10 minutes before mounted in Vectashield with 4’,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) followed by analysis with a confocal microscopy (Leica Microsystems, Bannockburn, IL).

24-Hydroxylase promoter-luciferase reporter assay. Cells were plated on 48-well plates for 24 hours in 5% charcoal-stripped medium before 3-hour infection with a constant dose of adenovirus containing β-galactosidase and either varying doses of adenovirus containing 24-hydroxylase promoter-luciferase construct or adenoviral empty vector control in serum-free medium. Then, the cells were treated with varying doses of calcitriol for 48 hours in 5% charcoal-stripped medium. Cells were harvested by removing the medium and washed twice with PBS. Cell lysates were obtained using three times of freeze-thaw method in 50 μL of 1× Reporter Lysis Buffer (Promega) and gentle vortex. The cell lysates were then subjected to Luciferase Assay Reagent (Promega) and the light produced was measured with a luminometer (Veritas Microplate Luminometer, Turner Biosystems, Sunnyvale, CA). The activity of β-galactosidase was obtained using β-Galactosidase Enzyme Assay System (Promega) and the absorbance was measured with an ELISA reader (Spectra Max 340PC) at 520 nm. The relative luciferase units were obtained by normalizing the luciferase unit with the activity of β-galactosidase and total protein from each lystate.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. TDEC and MDEC were plated at the range of 1 × 10⁴ to 2 × 10⁵ per well into 96-well tissue culture plates (Corning Glass, Inc., Corning, NY) and incubated at 37°C in a humidified atmosphere containing 5% CO₂. Twenty-four hours after plating, cells were treated for 48 hours with either vehicle or varying doses of calcitriol. Cells were harvested by adding 20 μL of 0.5% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) for 3 hours at 37°C. The medium was removed and the cells were solubilized with 10% SDS/10 mM HCl for overnight at 37°C. The absorbance was read with an ELISA reader (Spectra Max 340PC) at 490 nm.

Cell cycle analysis. Endothelial cells were plated and treated at a final concentration 10 nmol/L calcitriol. Cells were harvested 48 hours after treatment by trypsinization, washed using PBS, and fixed with 70% ethanol. The cells were stained with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) or rhodamine (TRITC)–conjugated anti-rat IgG antibody (PharMingen) and samples were analyzed with a confocal microscope (Leica Microsystems, Bannockburn, IL).

Bromodeoxyuridine incorporation assay. Cells were plated in 96-well plates 24 hours prior treatment as described above in cell cycle analysis. After 48 hours, bromodeoxyuridine labeling solution (Roche, Indianapolis, IN) was added into each well for 4 hours before fixing. Antibody staining and addition of substrate procedures were done as described (27). The absorbance was read with an ELISA reader (Spectra Max 340PC) at 370 nm.

Annexin V-phycocerythrin/7-amino-actinomycin D staining. Annexin V-phycocerythrin/7-amino-actinomycin D labeling was done according to the manufacturer’s instructions (PharMingen) and samples were analyzed by flow cytometry. Briefly, the cells were treated for 48 hours with either vehicle or 10 nmol/L calcitriol. Cells were trypsinized and washed with PBS before resuspending in assay binding buffer. Annexin V-phycocerythrin/7-amino-actinomycin D labeling was done at room temperature for 15 minutes before analysis by flow cytometry (BD FACScan).

Western blot analysis. Twenty-four hours after plating, the cells were treated with vehicle or 10 nmol/L calcitriol. Antibodies used include

5-HT2C receptor antagonist, and 5-HT2C receptor agonist.
PECAM-1 (Emfert, Würzburg, Germany), VDR (Santa Cruz Biotechnology), p21 (Santa Cruz Biotechnology), p27 (Santa Cruz Biotechnology), Bel-2 (Santa Cruz Biotechnology), Akt (Cell Signaling, Danvers, MA), p-Akt (Ser473; Cell Signaling), total Erk (p44/42 mitogen-activated protein kinase; Cell Signaling), p-Erk (Santa Cruz Biotechnology), poly(ADP-ribose) polymerase (PharMingen), and caspase-3 (Cell Signaling). Anti-rabbit and anti-mouse horseradish peroxidase–conjugated secondary antibodies were purchased from Amersham Life Sciences (Arlington Heights, IL) and actin was detected using the actin kit from Oncogene Research Products (Boston, MA).

In vitro alkaline phosphatase reactions. Alkaline phosphatase reactions were done on nuclear extracts of TDEC and MDEC treated with 10 nmol/L calcitriol or vehicle for 24 hours. Nuclear protein extract was prepared as described previously (28). The nuclear extract (50 μg) was incubated with 5 μl of 10× phosphatase buffer and 30 units calf intestinal alkaline phosphatase (Promega) in a 50 μl reaction volume and incubated for 1 hour at 37°C. The mock reactions did not contain enzyme. The lysis buffer used contain only protease inhibitor cocktail. Phosphatase reactions were stopped by addition of sample buffer used in Western blotting and subjected to electrophoresis as described above.

Statistical analysis. The statistical of differences were assessed by use of the two-way ANOVA (two-sided). All data were expressed as the mean (SD) of at least three determinants unless stated otherwise.

Results
Isolation and identification of MDEC. Endothelial cells were isolated from Matrigel plugs that were implanted s.c. into C3H/HeJ mice. An intact lumen containing RBC stained in H&E (Fig. 1A, left) and anti-mouse PECAM-1 antibody by flow cytometry (Fig. 1A, left) and anti-mouse PECAM-1 antibody (Fig. 1A, right) documented endothelial cells migrated into the Matrigel plugs. Migrating cells were cultured in 1% gelatin-coated cell culture dishes. When compared with TDEC (Fig. 1B, right), MDEC (Fig. 1B, left) showed distinct morphologic differences. Although they were isolated from the same strain of mice (C3H/HeJ), MDEC have long, branching, and cytoplasmic projections. Using indirect staining of anti-mouse PECAM-1 antibody by flow cytometry (Fig. 1C, left) and Western blot analysis of PECAM-1 protein expression (Fig. 1C, right) both MDEC and TDEC (23) were identified as an enriched population of endothelial cells. Further studies using reverse transcription-PCR showed that both TDEC and MDEC expressed other endothelial cell markers, such as factor VIII, FGF receptor 2, neuropilin receptors, vascular cell adhesion molecule 1, and tumor endothelium markers (Fig. 1D). However, TDEC expressed little VEGF receptors mRNA when compared with MDEC. The doubling time of TDEC and MDEC were 24 to 28 and 32 to 36 hours, respectively. These two populations of endothelial cells (TDEC and MDEC) were then compared with respect to calcitriol response.

Comparison of VDR between TDEC and MDEC. Using Western blot analysis, both TDEC and MDEC expressed similar basal levels of VDR protein and responded to 10 nmol/L calcitriol by increasing VDR protein expression in a time-dependent manner (Fig. 2A). Although MDEC had higher basal VDR expression compared with TDEC, treatment with calcitriol resulted in increase of VDR protein expression in both cell types across time. An additional middle band was observed in both cell types following calcitriol treatment. These doublet or triplet bands may represent different phosphorylated forms of VDR as indicated in a dephosphorylation assay using alkaline phosphatase (Fig. 2B). The more slowly migrating forms of VDR were either reduced or converted to a faster migrating form on treatment with alkaline phosphatase, indicating that phosphorylation of VDR is a post-translational process.

Figure 1. Isolation and identification of MDEC. A, H&E (left) and PECAM-1 (right) staining. Matrigel plugs harvested from the animals were fixed in either formalin or zinc fixative before paraffin embedding processing. The specimens were then processed for regular H&E and PECAM-1 (brown) stainings. Arrows, intact lumen of blood vessel. Magnification, ×400 (H&E) and ×100 (PECAM-1). B, phase-contrast picture of isolated MDEC (left) and TDEC (right) cultured in a cell culture dish. Magnification, ×200. C, expression of PECAM-1 in MDEC. Left, representative flow cytometry immunofluorescence profiles for expression of PECAM-1 in MDEC. Gray peak, PECAM-1; black peak, control for nonspecific immunofluorescence. Right, Western blotting of PECAM-1. Protein levels of PECAM-1 were detected by Western blot analysis. TDEC were positive for PECAM-1 as described previously (10). D, expression of endothelial cell positive markers using reverse transcription PCR. TDEC was used as positive control for endothelial cell. VEGFR1, VEGF receptor; FGFR2; FGFR receptor 2; NRP1, neuropilin; VCAM1, vascular cell adhesion molecule 1; TEM, tumor endothelium marker; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Representative of at least three independent experiments.
To determine whether there is any difference in the VDR mRNA level between the two cells, we amplified the whole coding region of VDR sequence using reverse transcription-PCR and subjected the PCR product for sequencing. The PCR products from both cells appeared similar in size and quantity (Fig. 2C). There were no mutations found in either sequence when compared with the VDR from the mouse database (NM_009504; data not shown). However, dose-response saturation plot (Fig. 2D, left) and Scatchard plot analysis (Fig. 2D, right) suggested that the kinetics of receptor-ligand binding were different in these cells. The dose-response saturation plot, which represents only specific binding, approached saturation at 31 ± 0.59 and 24 ± 3.5 fmol/mg (P = 0.001) of [3H]-1,25-dihydroxyvitamin D3 for TDEC and MDEC, respectively. These results indicate that the VDR level (as measured by maximum 1,25-dihydroxyvitamin D3 binding capacity/mg protein) is higher in TDEC than in MDEC. As shown in Scatchard plot, linear regression analysis showed a single class of specific, high-affinity receptors with lower equilibrium binding dissociation constant (Kd) in TDEC (0.26 ± 0.001 nmol/L) than in MDEC (0.65 ± 0.000005 nmol/L; P = 0.0016).

Activity of VDR-RXR heterodimer in the nucleus was similar between TDEC and MDEC. Subcellular localization of VDR and RXRα (a heterodimer partner of VDR) in both cell types was the same. VDR in both TDEC and MDEC accumulated in the nucleus after treatment with calcitriol (Fig. 3A). Using 24-hydroxylase promoter-luciferase reporter assay, the VDR-RXR heterodimer complex in TDEC and MDEC translocated into the nucleus and transactivated the reporter gene in a dose-dependent manner (Fig. 3B). With increasing dose of the reporter gene, the endogenous VDR from both cells transactivated the exogenous DNA in a similar rate when treated with 10 nmol/L calcitriol. However, at a constant dose of the reporter gene and varying concentrations of
calcitriol, VDR in TDEC transactivated the DNA at a higher rate than in MDEC (Fig. 3C). Taken together, these findings indicate that the VDR signaling was intact and functional in both TDEC and MDEC, but more efficient ligand binding and transactivation occurred in TDEC than MDEC.

**Effect of calcitriol on cell proliferation.** To study the effects of calcitriol on the cells growth, TDEC and MDEC were treated with 0 to 500 nmol/L calcitriol for 48 hours and cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyloxazole tetrazolium bromide assay. Calcitriol, at nanomolar concentrations, inhibited cell growth of TDEC. However, the growth of the MDEC was only minimally affected by the treatment with calcitriol (Fig. 4). TDEC responded to calcitriol in a dose-dependent manner, and at 500 nmol/L, calcitriol resulted in >60% inhibition of cell growth. At 10 nmol/L concentration of calcitriol, ~47 ± 2.7% growth inhibition was seen in TDEC compared with 12.3 ± 0.36% (P = 0.00017) growth inhibition in MDEC. At higher concentrations of calcitriol, growth inhibition was never higher than 12% in calcitriol-treated MDEC.

**Effect of calcitriol on cell cycle distribution and DNA synthesis.** The effects of calcitriol on cell cycle distribution in TDEC and MDEC were assessed by propidium iodide staining. Following 48-hour treatment with 10 nmol/L calcitriol in TDEC, there was a 10.6 ± 1.64% increase in G0/G1-phase cell population (P = 0.058) and a 6.8 ± 1.2% decrease (P = 0.04) in S-phase cell population (Fig. 5A). In contrast, calcitriol did not induce G0/G1

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**Figure 3.** Nuclear translocation and transactivation activity of VDR in TDEC and MDEC. A, confocal microscopy showed subcellular localization of VDR (green) and RXRxalpha (red) in cells treated with 10 nmol/L calcitriol for 15 minutes. Nuclei were stained with 4,6-diamidino-2-phenylindole (blue). Arrows, colocalization of VDR and RXRxalpha in the nuclei (white). B and C, calcitriol-induced transactivation activity of VDR-RXRxalpha heterodimer in both TDEC (B) and MDEC (C). Cells were infected with adenoviral vector varying doses of adenovirus carrying a 24-hydroxylase promoter-luciferase reporter and a constant dose of adenoviral β-galactosidase expression vector for 3 hours before treatment of calcitriol for 48 hours (C). Dotted lines, normalized luciferase activity from vehicle-treated cells; solid lines, normalized luciferase activity from calcitriol-treated cells. Luminescence data were normalized with β-galactosidase activities, and the empty adenoviral control vector showed minimal luciferase activity (data not shown). RLU, relative luciferase unit; Ad-p24lac, 24-hydroxylase promoter luciferase adenoviral construct.
cell cycle arrest in MDEC (Fig. 5B). The effect of calcitriol on the expression of the cell cycle cyclin-dependent kinases p21 and p27 was examined in both endothelial cell populations. p27 protein expression was induced and p21 protein expression was decreased in calcitriol-treated TDEC but not in MDEC (Fig. 5C).

To investigate the effect of calcitriol on DNA synthesis, TDEC and MDEC were examined for bromodeoxyuridine incorporation. DNA synthesis was inhibited by treatment of calcitriol only in TDEC (Fig. 5D). Compared with vehicle, calcitriol caused ~38 ± 4.7% (P = 8.32 × 10⁻⁵) reduction of bromodeoxyuridine incorporation in TDEC; there was no change in bromodeoxyuridine incorporation in MDEC.

**Effect of calcitriol on apoptotic signaling pathways.** Using Annexin V/7-amino-actinomycin D staining, the extent of apoptosis induced by calcitriol was examined in TDEC and MDEC. Treatment with calcitriol resulted in a significant increase in total cell death (38.6 ± 1.03%; P = 0.0087) after 48 hours in TDEC (Fig. 6A) but not in MDEC (4.1 ± 0.59%; P = 0.012; Fig. 6B). Using Western blot analysis, treatment of calcitriol caused a significant reduction of p-Erk and p-Akt protein in TDEC and no change in total Erk and total Akt; no changes in these proteins were seen in MDEC (Fig. 6C). Furthermore, a reduction in Bcl-2 and full-length poly(ADP-ribose) polymerase and an increase in cleavage of caspase-3, all markers of apoptosis, were observed in TDEC treated with calcitriol, but no such effects was seen on MDEC.

**Discussion**

We compared the effect of calcitriol on two types of endothelial cells that differ in origin: TDEC from syngeneic squamous cell carcinoma tumors (23) and MDEC from Matrigel plugs implanted into the flanks of C3H/HeJ mice. MDEC were selected because the Matrigel microenvironment contains a variety of extracellular matrix proteins and an angiogenic stimulus, basic FGF. This matrix may replicate at least in part in the environment the tumor cells create in vivo. Endothelial cells migrate into the plug in a similar fashion to the manner in which migration into the tumor occurs (29–31). Numerous studies have used the implanted Matrigel plugs to assess the efficacy of angiogenesis inhibitors in vivo (32–34), supporting the applicability of this model for tumor-associated angiogenesis. Because the Matrigel plugs were harvested after 2 weeks, the endothelial cells appear to be "neovascular" when compared with those isolated from adult organs and thus are reflective of angiogenesis in normal tissue.

Using this methodology, we isolated and cultured an enriched population of endothelial cells for in vitro studies. Although TDEC and MDEC have different morphologies in culture, both cells express PECAM-1 (CD31), a surface marker characteristic of endothelial cell lineage. Both cell types express RNA of several endothelial cell markers, including factor VIII, FGF receptor, neuropilins, and tumor endothelium markers. In contrast, VEGF receptor 2 expression is observed in MDEC and not in TDEC. This suggests that the growth of TDEC, under standard in vitro culture conditions (5% CO₂ and 20% O₂), may be more dependent on the FGF rather than the VEGF signaling pathway. The cells in Matrigel plugs, which are PECAM-1 negative, may be pericytes or stromal cells that were recruited during neovascularization. Pericytes stabilize vessel walls and may have a role in endothelial cells proliferation, survival, permeability, migration, and maturation (35). These nonendothelial cells did not overgrow the in vitro cultures as MDEC were stained with PECAM-1 antibody using indirect fluorescence-activated cell sorting staining.

Freshly isolated TDEC and MDEC have a similar growth profile with a doubling time of 24 to 36 hours and were used before 8 to 10 passages. At later passage number, cell morphology was changed and the expression of endothelial cell markers and cytokine induction of inducible nitric oxide synthase were lost (23, 36).

Treatment with calcitriol resulted in growth inhibition of TDEC in a dose-dependent manner. However, MDEC were relatively resistant to the antiproliferative effects of calcitriol. This finding is intriguing because both TDEC and MDEC expressed substantial level of VDR and responded in a time-dependent manner to treatment of calcitriol by inducing VDR protein expression. It is well established that the effect of calcitriol is largely mediated by VDR, which possess transcription factor activity on ligand activation. VDR seems to be essential for the antiproliferative effect of calcitriol. In VDR knockout mice, carcinogen-induced tumors are unresponsive to calcitriol, whereas similarly induced tumors in wild-type mice do respond to calcitriol (37). The fact that the mRNA sequence of VDR from TDEC and MDEC was similar to the database (NM_009504) excludes the possibility of mutation in VDR in both cells.

VDR in TDEC has a higher binding affinity for calcitriol when compared with MDEC. The VDR is a high-affinity, low-capacity receptor with a dissociation constant of ~0.1 nmol/L. We showed previously that VDR present in different tissues have slightly different binding affinity to calcitriol and tumors from animals generally have lower binding capacity when compared with other vitamin D target tissues, such as kidney, intestinal mucosa, skin, and bone (38). The higher binding affinity of VDR in TDEC than in MDEC could be due auxiliary factors in TDEC that are not present in MDEC that might facilitate and stabilize the binding of calcitriol to its receptor. This might explain the observation of more efficient transactivation activity in TDEC than
in MDEC. Transactivation in TDEC reached saturation at 25 nmol/L, whereas saturation occurred at 100 nmol/L in MDEC, suggesting that high doses of calcitriol can transactivate in MDEC without exhibiting growth inhibitory effects. In addition, the role of intracellular vitamin D–binding proteins to promote the delivery of ligand to the VDR has been implicated in New World primate cells (39).

The finding that calcitriol resulted in an induction of VDR in MDEC without an antiproliferative effect suggests that there could be modifications in the VDR-mediated signaling pathway in these endothelial cells. Immunofluorescent staining showed that the VDR-RXR complex in both cells colocalized to the nucleus after treatment with calcitriol. VDR heterodimerizes with the RXR for high-affinity binding to target genes (40). VDR and RXR distribute equally between the cytoplasm and the nucleus and can heterodimerize in the absence of calcitriol (41). The addition of calcitriol stabilizes the heterodimer and promotes trafficking into the nucleus and binding to the vitamin D response elements (42). CYP24, the major calcitriol-catabolizing enzyme, contains two vitamin D response elements at the promoter regions (43). Using a CYP24 promoter-luciferase reporter construct, the VDR-RXR complex transactivated the exogenous promoter in both cells; in TDEC, the construct was activated at a higher rate. High expression of CYP24 can shorten the half-life of calcitriol and can prevent sufficient accumulation of calcitriol to exert its biological effects (44). In several human prostate carcinoma cell lines, growth inhibition by calcitriol is in inverse proportion to the CYP24 activity of each cell line (45). Pertinent to these studies, TDEC and MDEC differ in their expression of CYP24. In MDEC, CYP24 is overexpressed compared with TDEC and the mechanism of this differential expression may be explained by hypermethylation of the CYP24 promoter in TDEC.

Another explanation for the differential response to calcitriol is the inherent expression of coregulators in each cell type. Upon binding of ligand, the activated VDR initiates a cascade of protein-protein interaction at the DNA to modify the chromatin and to recruit the transcriptional apparatus. Recruitment of the WINAC, SRC-1/p160, and DRIP complexes by VDR to the DNA has been shown using chromatin immunoprecipitation assays (46,47). SRC-1/p160 and DRIP complexes contain intrinsic histone acetyltransferase activity and interact with the basal transcription machinery (48). Activated VDR could also repress transcription by recruiting nuclear receptor corepressor and mSin3 complexes, which possess histone deacetyltransferase activity (49). Overexpression or underexpression of any of these complexes could directly affect the function of the VDR in regulating gene transcription. The concept of a tissue or cell type selectivity for a nuclear receptor, such as VDR, is not unique; estrogen receptor exerts variable response following estradiol, tamoxifen, and raloxifene depending on tissue type (50).

**Figure 5.** Calcitriol caused cells to arrest at G0/G1 phase and reduced S-phase activity in TDEC but not in MDEC. A and B, propidium iodide staining using flow cytometry was done with 48-hour treated cells. Representative flow cytometry profile of TDEC (A) with vehicle (left) and 10 nmol/L calcitriol (right) and MDEC (B) with vehicle (left) and 10 nmol/L calcitriol (right). Western blot analysis of p21 and p27 indicates correlation of cell cycle arrest with modulation of cyclin-dependent kinase inhibitor protein expression. Representative of at least three independent experiments. D, calcitriol reduced S-phase activity in TDEC but not in MDEC. Cells were plated on 96-well plates before 48-hour treatment of either vehicle (black columns) or 10 nmol/L calcitriol (gray columns). Cells were then processed as described (27). *, P = 8.32 × 10−5, significantly different from vehicle control (two-sided ANOVA). Columns, mean percentage from three independent experiments; experiments, bars, SD.

These studies show that calcitriol has a selective effect on endothelial cells derived from a tumor microenvironment compared with endothelial cells that migrate into Matrigel plugs. Although there are studies suggesting that the tumorendothelium is different from endothelium found in normal tissues (20), a comparison of endothelial cell types in these different microenvironments has not been examined extensively with regards to effects of calcitriol. Our model system for isolation of these two populations of endothelial cells allows for a comparison between tumor and nontumor endothelium. Further studies are necessary to more precisely define the differences between the two. These studies also suggest the utility of calcitriol in cancer therapy as a novel therapeutic agent that targets both tumor and tumor endothelium without adversely effecting normal vasculature. Information obtained from this study will allow a better understanding toward the development of an efficacious calcitriol therapeutic approach for future clinical trials of human malignancies.

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Figure 6. Calcitriol caused increased binding of Annexin V in TDEC but not in MDEC. A and B, Annexin V and 7-amino-actinomycin D (7-AAD) staining using flow cytometry was done with 48-hour treated cells. Treatment of TDEC (A) with vehicle (left) and 10 nmol/L calcitriol (right) and MDEC (B) with vehicle (left) and 10 nmol/L calcitriol (right). C, Western blot analysis of various proteins in survival and apoptotic signaling correlates to induction of apoptosis in TDEC. Representative of three independent experiments.

References


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