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Epigenetic silencing of CYP24 in the tumor microenvironment☆

Candace S. Johnson[a,*], Ivy Chung[b,1], Donald L. Trump[b]

[a] Department of Pharmacology & Therapeutics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY, USA
[b] Department of Medicine, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, USA

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ABSTRACT

Calcitriol (1,25 dihydroxycholecalciferol) has significant anti-tumor activity *in vitro* and *in vivo* in a number of tumor model systems. We developed a system for isolation of fresh endothelial cells from tumors and Matrigel environments which demonstrate that CYP24, the catabolic enzyme involved in vitamin D signaling, is epigenetically silenced selectively in tumor-derived endothelial cells (TDEC). TDEC maintain phenotypic characteristics which are distinct from endothelial cells isolated from normal tissues and from Matrigel plugs (MDEC). In TDEC, calcitriol induces G_0/G_1 arrest, modulates p27 and p21, and induces apoptotic cell death and decreases P-Erk and P-Akt. In contrast, endothelial cells isolated from normal tissues and MDEC are unresponsive to calcitriol-mediated anti-proliferative effects despite intact signaling through the vitamin D receptor (VDR). In TDEC, which are sensitive to calcitriol, the CYP24 promoter is hypermethylated in two CpG island regions located at the 5′end; this hypermethylation may contribute to gene silencing of CYP24. The extent of methylation in these two regions is significantly less in MDEC. Lastly, treatment of TDEC with a DNA methyltransferase inhibitor restores calcitriol-mediated induction of CYP24 and resistance to calcitriol. These data suggest that epigenetic silencing of CYP24 modulates cellular responses to calcitriol.

1. Introduction

Studies in our laboratory demonstrate that calcitriol (vitamin D or 1,25 dihydroxycholecalciferol), a central factor in bone and mineral metabolism [1,2], has significant anti-tumor activity *in vitro* and *in vivo* in a number of murine syngeneic and human xenograft tumor model systems [3–6]. Calcitriol induces cell cycle arrest and related kinases, decreases survival signaling molecules and induces modulators of apoptosis in various tumor types [4–10]. Clinically in phase I studies, we have determined the MTD of calcitriol alone and in combination with a number of cytotoxic agents and/or glucocorticoids, where there is synergy [11–20] and we have completed a phase II trial on androgen-independent prostate cancer where the combination of dexamethasone and calcitriol resulted in an enhanced anti-tumor effect. Induction of CYP24, the enzyme primarily responsible for calcitriol catabolism [21], may be a factor in bioavailability, calcitriol exposure and the anti-proliferative activity pre-clinically and clinically. In addition, the endothelial cells in tumors are sensitive to calcitriol and uniquely modulate CYP24 expression through epigenetic changes [22].

Epigenetic events affect gene expression without alteration in DNA gene sequence and lead to transcriptional gene silencing and inactivation of tumor suppressor genes in human cancer [23]. While many studies document epigenetic changes in tumor cells, only limited data support a role for epigenetic changes in the "normal" cells found in the tumor microenvironment. Epigenetic changes are found in the stromal fibroblasts from normal human breast tissue and breast carcinoma [24,25] and in the tumor stroma and endothelium of localized human prostate cancer [26,27]. Calcitriol also inhibits proliferation of endothelial cells and can inhibit angiogenesis in a number of tumor model systems [28–30]. We have established a method for the isolation of fresh, tumor-derived endothelial cells (TDEC) that maintain phenotypic characteristics which are distinct from endothelial cells isolated from normal tissues [31] and from Matrigel plugs (MDEC) [32]. These endothelial cell populations differ in their response to calcitriol and in their expression of CYP24. We discuss here the epigenetic silencing of CYP24 in these cells and the potential role on the tumor microenvironment in these activities. These unique differences in the epigenetic silencing of calcitriol-induced CYP24 gene expression in endothelial cells from the tumor microenvironment directly impact on calcitriol-mediated signaling pathways and ultimately on therapeutic application.

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* Corresponding author. Tel.: +1 716 845 8300; fax: +1 716 845 1258.
E-mail address: Candace.Johnson@RoswellPark.org (C.S. Johnson).
[1] Current address: Vascular Biology Program Children's Hospital, Harvard Medical School, Boston, MA 02115, USA.

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2. Results/discussion

2.1. Isolation of TDEC and MDEC

Using an antibody to PECAM or platelet cell adhesion molecule, a positive marker for endothelial cells [31,32], tumor cells are harvested, dissociated, labeled and sorted for PECAM-positive cells. In a dissociated SCC or PC-3 tumor, the sorted population is >95% positive for PECAM and yields $1–1.5 \times 10^6$ cells from $2–4 \times 10^7$ dissociated tumor cells. Cells highly fluorescent for PECAM (positive sort) represent 4–7% of the total population of cells from the tumor and have a doubling time of 50 h as compared to 12–20 h for most tumor cell lines. These PECAM-positive cells are also positive for the uptake of acetylated low-density lipoprotein (Dil-ac-LDL), factor VIII and demonstrate endothelial-like characteristics by electron microscopy (presence of Weibel–Palade bodies). In addition, cell survival throughout this entire procedure is >90% and PECAM-positive endothelial cells are available for analysis within 6–8 h of tumor harvest. Cells are utilized fresh or after only 1–2 passages. Cultures maintain a number of phenotype markers through 10 passages or 5–6 weeks in culture. For the appropriate "normal" endothelial cell control, microvascular endothelial cells were isolated from Matrigel plugs that were implanted subcutaneously into mice [32]. In selecting a "normal" EC, it was important to create a situation similar to a tumor without the tumor present to isolate neovascular endothelial cells that were recruited toward an antigenic stimulus. We developed a model to create a "normal" microenvironment where endothelial cells would be recruited, but where the tumor cells would not be present [22,31–34]. Endothelial cells were cultured and propagated in cell culture dishes coated with 1% gelatin. When compared to TDEC, MDEC showed distinct morphologic differences of long, branching and cytoplasmic projections. The doubling times of TDEC and MDEC were 24–28 h and 32–36 h, respectively.

2.2. 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 nM calcitriol by increasing VDR protein expression in a time dependent manner [32]. Although MDEC had higher basal VDR expression compared to 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 λ phosphatase. The more slowly migrating forms of VDR were either reduced or converted to a faster migrating form upon treatment with λ phosphatase, indicating that phosphorylation of VDR is a post-translational process.

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 semi-quantitative RT-PCR and subjected the PCR product for sequencing. The PCR products from both TDEC and MDEC appeared similar in size and quantity. There were no mutations found in either sequence when compared to the VDR from the mouse database. However, dose–response saturation plot and Scatchard plot analysis 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 fmol/mg and 24 ± 3.5 fmol/mg ($P = 0.001$), of [^{3}H]1,25-D_3 for TDEC and MDEC, respectively. These results indicate that the vitamin D receptor level (as measured by maximum 1,25-D_3 binding capacity per milligram of protein) is higher in TDEC than in MDEC. By Scatchard plot analysis, linear regression analysis demonstrated a single class of specific, high affinity receptors with lower equilibrium binding disassociation constant (*Kd*) in TDEC (0.26 ± 0.001 nM) than in MDEC (0.65 ± 0.000005 nM) ($P = 0.0016$). 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 [32]. 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 nM 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. 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. Studies using other types of endothelial cells also demonstrate VDR expression upon treatment with calcitriol, indicating possible role of VDR in the biology of these cells [33,34,35].

2.3. Anti-proliferative effects of calcitriol on TDEC/MDEC

We studied the effects of calcitriol on the growth of MDEC and TDEC [32,36]. Cells were treated with varying doses of calcitriol for 48 h and cell viability was determined using the MTT 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. The resistance to calcitriol's effect was also observed in other types of normal endothelial cells, including those isolated from mouse brain, lung and yolk sac. The effects of calcitriol on cell cycle distribution in TDEC and MDEC were assessed by PI staining where calcitriol-induced cell cycle arrest in TDEC and not in MDEC. The protein expression of p27 was induced and p21 was decreased in calcitriol-treated TDEC but not in MDEC. Further effects on DNA synthesis were examined and found a reduction of BrdU incorporation in TDEC and no change in MDEC. Using annexin V/7-AAD staining, treatment with calcitriol resulted in a significant increase in total cell death in TDEC and not MDEC. 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. Furthermore, a reduction in Bcl-2 and full length PARP 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.

To evaluate whether calcitriol treatment *in vivo* has an impact on tumor vasculature, we quantified the mean vessel density in SCC tumor and Matrigel plugs removed from animals treated daily 3× with calcitriol (0.625 μg/mouse), a dose that resulted in a significant anti-tumor effect [6–8]. Three areas were evaluated from each tissue and mean vessel density calculated based on the number of CD31 stained microvessels in each field. As shown in Fig. 1A, a significant reduction was observed in the number of vessels after calcitriol treatment in the tumor with little effect observed in the Matrigel. To quantitate apoptosis, we utilized CD31 and activated caspase-3 double staining to identify endothelial cells undergoing apoptosis. After treatment with calcitriol, an increase was observed in the number of apoptotic cells in the tumor and not in the Matrigel plug (Fig. 1B). Additional studies demonstrated that treatment with calcitriol resulted in an increase of vessel permeability at 8 h post-injection and a decrease in blood volume compared to control tumors as measured by MR imaging. These studies suggest that calcitriol targets the vasculature and results in significant endothelial cell death and ultimately collapse of the vasculature in the tumor.

2.4. Calcitriol-induced CYP24 expression in TDEC and MDEC

CYP24 or 25-hydroxyvitamin D-24-hydroxylase is the key enzyme responsible for initiating the vitamin D degradation path-

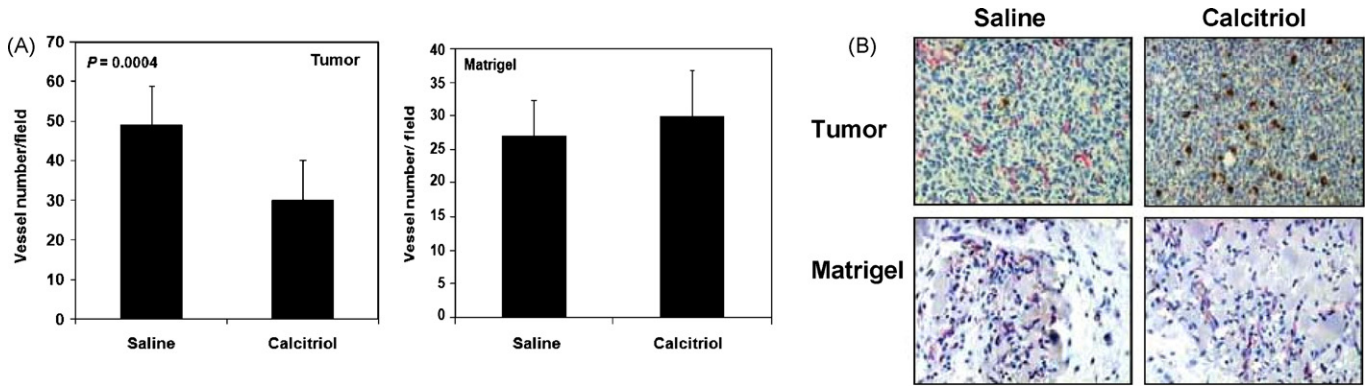

Fig. 1. Calcitriol effect on the vasculature *in vivo*. (A) The effects of calcitriol (daily 3× 0.625 μg/mouse) on mean vessel density (MVD) in both SCC tumors and Matrigel plugs where 15 fields and 3 animals in each group were counted and (B) apoptotic endothelial cells (dark staining) in SCC tumors and Matrigel plugs as determined by double staining of CD31 (endothelial cells) and caspase-3 (apoptosis) on frozen sections from 5 animals/group treated with calcitriol as described above.

way by directing the side chain metabolism of 25-hydroxylated vitamin D metabolites, including calcitriol [37]. This enzyme is expressed in kidney and most vitamin D target tissues [38]. CYP24 can be induced by calcitriol via positive transcriptional regulation because of the presence of two VDREs in the CYP24 promoter region. This mechanism provides negative feedback regulation for attenuation of excessive vitamin D effects within the tissues.

Both TDEC and MDEC activate VDR signaling pathway upon treatment of calcitriol [22,32]. However, treatment of 10 nM calcitriol at various time points induced CYP24 mRNA and protein expression in MDEC but not in TDEC. The induction of CYP24 mRNA in MDEC was observed as early as 3 h and was sustained for over 48 h post-treatment. Similarly, using antibody specific to CYP24 (a gift from Cytochroma, Inc.), we observed that CYP24 protein was expressed in a time dependent manner in MDEC while no CYP24 protein expression was observed in TDEC. Briefly, [^{3}H]-25-D_3 oxidative metabolites were extracted by liquid/liquid partition using tetrahydrofuran and ethyl acetate, dried and analyzed by high performance liquid chromatography (HPLC) on Zorbax SIL column (Agilent Technologies Inc., Palo Alto, CA) using hexane/isopropanol (94:6, v/v) as the mobile phase [22]. We further confirmed the enzymatic activity of CYP24 protein induced by calcitriol in both cell types using high performance liquid chromatography (HPLC). In MDEC, treatment of 10 nM calcitriol for 24 h and 48 h resulted in approximately 4- and 7-fold increase of CYP24 enzymatic activity, respectively when compared to the vehicle ($P < 0.005$); no significant induction of CYP24 activity was observed in calcitriol-treated TDEC [22].

Differential induction of CYP24 by calcitriol in MDEC and TDEC could be a mechanism accounting for the lack of response in MDEC to calcitriol-mediated growth inhibition. To test this hypothesis, we used a siRNA-specific for CYP24 to treat MDEC concurrent with calcitriol. Treatment of 10 nM calcitriol for 48 h resulted in a maximum of 15% cell kill of MDEC. MDEC were transfected with siRNA-CYP24, siRNA-NS or untransfected (mock) for 24 h before addition of 10 nM calcitriol for 48 h. When MDEC was treated with siRNA-CYP24, there was a down-regulation of protein expression and enzymatic activity and there was a 43% growth inhibition in MDEC with CYP24 siRNA as compared to 15% in the cells treated with siRNA-NS or mock control, indicating that CYP24 protects MDEC from the growth inhibitory effects of calcitriol.

2.5. Transcription gene silencing of CYP24 in TDEC

Since both TDEC and MDEC were isolated from the same strain background of mice (C3H/HeJ) but different microenvironments (tumor vs non-tumor), the observation that calcitriol failed to induce CYP24 in TDEC was intriguing. CYP24 is a well known vitamin D target gene, which is ubiquitously expressed to regulate calcitriol homeostasis in the body [21]. The cytochrome P450 (CYP) family of enzymes is involved in numerous cellular functions including metabolism of eicosanoids, biosynthesis of cholesterol, synthesis and degradation of biogenic amines, hydroxylation of retinoic acid as well as the metabolism of vitamin D [39]. Using microarray (Fig. 2A), we compared the overall expression of CYPs in TDEC and MDEC and found suppression across a number of CYPs, except CYP2C44, a CYP that metabolizes arachidonic acid [40]. These results were confirmed using qRT-PCR. Because CYP24 promoter hypermethylation in osteoblastic cells has been indicated in a previous report [41], we examined the promoter region of CYP24 gene in TDEC for CpG island hypermethylation and epigenetic gene silencing as a possible molecular mechanism fro these effects. We characterized the methylation status of two proximal CpG islands in the 5′ region of CYP24, region 1 (−1131 to −241) and region 2 −214 to +800) in TDEC and MDEC (Fig. 2B). Methylation-specific PCR analysis showed hypermethylation of region 1 in TDEC but not in MDEC. However, region 2 in both cells was found to be hypermethylated. Bisulfite sequencing from both cells revealed that the CpG dinucleotides found in the amplified sequence of regions 1 and 2 were significantly hypermethylated in TDEC when compared to MDEC and these findings were also confirmed by MassArray, a quantitative method of DNA methylation analysis (Fig. 2C). Whether VDREs at the distal promoter region are also heavily methylated in order for the CYP24 gene to be transcriptionally silenced, is yet to be determined.

Tumor-derived endothelial cells (TDEC) are in close contact with the tumor cells within the tumor microenvironment. Therefore, the extent of methylation for CpG islands in regions 1 and 2 was also determined for SCC (squamous cell carcinoma), RIF (radiation-induced fibrosarcoma) and TRAMPC2 (prostate) tumor cell lines and then compared to TDEC isolated from the same tumor type [22]. Both SCC and TDEC/SCC were equally hypermethylated (83/84% vs 79/87%, regions 1/2, respectively), however, RIF tumor cells were not as hypermethylated as TDEC/RIF (48/65% vs 74/82%, respectively) and TRAMP was not hypermethylated when compared to TDEC/TRAMP (26/28% vs 69/77%, respectively). Therefore, despite the consistent result that the CYP24 promoter was hypermethylated in TDEC regardless of the tumor model system examined, tumor cells from these same models differed in their extent of hypermethylated. It should be noted that these models differ in their sensitivity to the anti-proliferative effects of calcitriol *in vivo* with SCC the most sensitive, followed by RIF and TRAMP [4–6,42].

Based on the above data, we next tested whether 5-aza-dC, a classical DNA methyltransferase (DNMT) inhibitor, could reactivate calcitriol-induced CYP24 transcription in TDEC [22]. Treatment of TDEC with 0.25 μM 5-aza-dC for 96 h followed by 10 nM calcitriol

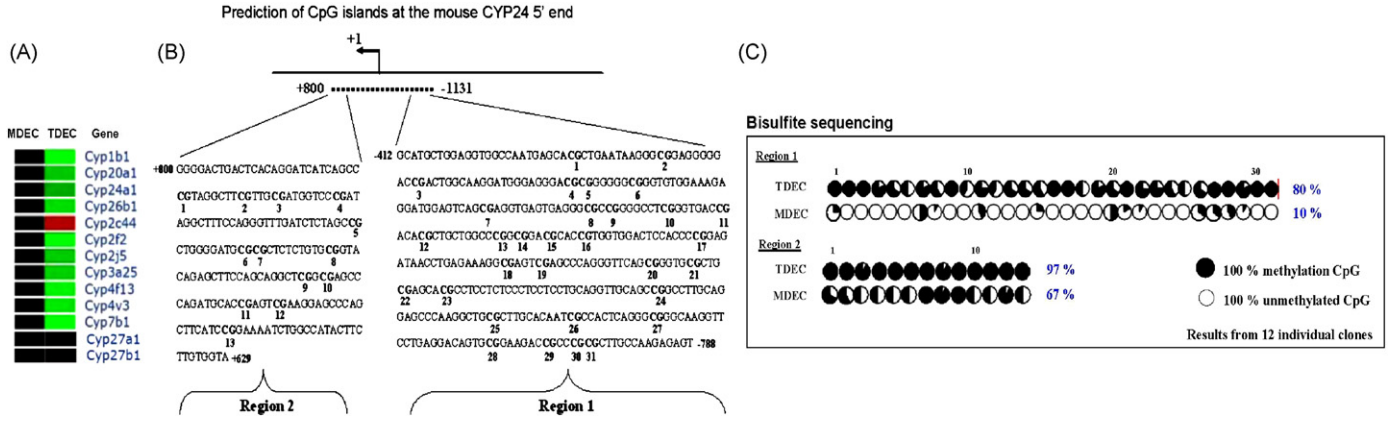

Fig. 2. CYP24 enzymes in TDEC and MDEC. (A) Microarray screen of CYP enzymes in TDEC and MDEC, (B) schematic diagram showing the position of the two CpG islands at the promoter region of CYP24, (C) methylation analysis of CpG sites located at the CYP24 promoter using bisulfite sequencing. Each circle represents one CpG site.

resulted in a time dependent induction of CYP24 mRNA ($P < 0.005$). By Western blot analysis, TDEC had a higher expression of the DNA methyltransferase enzymes, DNMT1 and DMNT3b when compared to MDEC.

3. Conclusion

While calcitriol is anti-proliferative to a wide variety of tumor cells *in vitro* and *in vivo*, we demonstrate that calcitriol has significant anti-tumor effects on TDEC and these effects are not observed on MDEC or endothelial cells isolated from normal tissues. These differences in calcitriol sensitivity of TDEC and MDEC may be due to the epigenetic silencing of the CYP24 promoter that is hypermethylated in two CpG island regions of TDEC. In addition, when MDEC are incubated with conditioned media from tumor cells, these cells become sensitive to calcitriol concurrent with hypermethylation of the CYP24 promoter. These studies demonstrate epigenetic differences in "normal" cells that only differ in their microenvironment: tumor vs normal. Only recently has evidence suggested a role for epigenetic changes for normal cells that are in the tumor microenvironment. Future studies will examine the mechanisms of epigenetic silencing of CYP24 in endothelial cells in the normal and tumor microenvironments and to determine effects on calcitriol-mediated signaling pathways.

Calcitriol has anti-proliferative effects in pre-clinical prostate models and studies suggest that the endothelial cells are epigenetically silenced at the CYP24 promoter. The silencing of the CYP24 promoter has impact on calcitriol-mediated therapies. While clinical studies have demonstrated limited anti-tumor effects of calcitriol (alone or in combination with steroids/docetaxel) in the treatment of prostate cancer, these studies have been seriously hampered: (1) there has been limited availability of a drug formulation which allows consistent pharmacologic administration of high dose calcitriol; (2) the optimal biologic dose or maximum tolerated dose of calcitriol has not be delineated; and (3) studies have been limited to phase I and II trials and to small and/or poorly designed phase II trials. Studies that overcome these limitations are underway and we are optimistic that an appropriate dose and formulation of calcitriol will available and able to be tested for its anti-angiogenic effects. In addition, if there is a defect in CYP24 signaling in tumor-derived endothelial cells that facilitates the growth of tumor-derived endothelial cells, ways to modulate CYP24 expression or activity in the tumor microenvironment could lead to a more effective treatment plan. Therefore, the information gained through these studies will permit the design of more effective therapeutic approaches for cancer.

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