Vitamin D3 triggers antitumor activity through targeting hedgehog signaling in human renal cell carcinoma

Valérian Dormoy*, Claire Béraud, Véronique Lindner1, Catherine Coquard, Mariette Barthelmébs, David Brasse2, Didier Jacqmin3, Hervé Lang3 and Thierry Massfelder

INSPRM U682, Section of Kidney Cancer and Renal Physiopathology, University of Strasbourg, School of Medicine, Strasbourg 67085, France, 1 Department of Pathology, Mulhouse Hospital, Mulhouse 68000, France, JIPHC, CNRS UMR 7178, University of Strasbourg, Strasbourg 67037, France and 3 Department of Urology, New Civil Hospital of Strasbourg, Strasbourg 67091, France
* To whom correspondence should be addressed. Tel: +333–68-85-34-56; Fax: +333–68-85-34-59;
Email: valerian.dormoy@medecine.u-strasbg.fr

Human clear cell renal cell carcinoma (CCC) remains resistant to treatments despite the progress in targeted therapies. Several signaling pathways acting during renal development are reactivated during kidney tumorsogenesis; this is the case of the sonic hedgehog (SHH)-Gli. Interestingly, the precursor of active vitamin D3 (VD3), cholecalciferol, has been demonstrated to be a strong inhibitor of SHH-Gli signaling. Here, we show the preclinical efficacy of cholecalciferol in CCC both in vitro and in vivo. A panel of CCC cell lines, tumors and normal corresponding tissues from CCC patients were used to evaluate the expression of the VD3 receptor and metabolizing enzymes and the effects of cholecalciferol treatment. Subsequently, xenografted mice were treated with cholecalciferol in a prophylactic or therapeutic manner; their response and the adverse effects were evaluated on the basis of weekly monitoring, followed by blood collection procedures and X-ray micro-computed tomography. VD3 receptor and metabolizing enzymes are dramatically decreased in human cell lines and tumors. Cholecalciferol decreases cell proliferation and increases cell death by inhibition of the SHH-Gli pathway. Xenografted mice treated with cholecalciferol exhibit absence of tumor development or substantial growth inhibition. The treatment was shown to be safe; it did not induce calcification or calcium reabsorption. These findings establish that, although VD3 receptors and metabolizing enzymes are absent in CCC, cholecalciferol supplementation is a strong tool to block the reactivation of SHH-Gli pathway in this pathology, leading ultimately to tumor regression. Cholecalciferol may have highly therapeutic potential in CCC.

Introduction
Renal cell carcinoma (RCC) is the most lethal urologic tumor and the sixth leading cause of cancer deaths in Western countries. Each year, ~270 000 patients are diagnosed with this malignancy, resulting in ~110 000 deaths (1–3). RCC is represented by 80% of clear cell RCC (CCR) resistant to radio-, hormonal and chemotherapy, whereas immunotherapy is effective in only 15% of selected patients. Recent advances in understanding the molecular network of hypoxia-inducible factors have led to several novel targeted therapies. Drugs that modulate the downstream targets of the hypoxia-inducible factor pathway, including sunitinib, sorafenib, temsirolimus and bevacizumab, have proven beneficial in treating RCC (4). So far, the best known oncogenic signal in human CCC is constituted by the von Hippel-Lindau (VHL) tumor suppressor gene and hypoxia-inducible factors (5–8). Additional oncogenic events are required for CCC formation, and this concept has been clearly proven by the phosphatidylinositol-3-kinase (PI3K)/Akt, nuclear factor-kappaB (NF-xB) and mitogen-activated protein kinase pathways, which were constitutively activated and which promoted tumor growth in human CCC (9–11). It became clear that mechanistic similarities exist between tumorsogenesis and nephrogenesis and that tumor cells hijack developmental signaling molecular pathways for their own growth (12–15).

In this context, we have recently shown that the nephrogenic sonic hedgehog (SHH)-Gli signaling pathway and the transcription factor Lim1, required for normal kidney development, are reactivated in human CCC and are crucial for tumor growth (16,17). SHH-Gli signaling is characterized by the binding of one of the ligands (Hedgehog, Indian or Desert) on the Pcth1 receptor, releasing the inhibition on the second receptor of the pathway, Smo. As a result, the Gli transcription factors (Gli1, Gli2 and Gli3) are released from the membrane and they in turn regulate multiple targets involved more particularly in cell proliferation and differentiation (16). Thus, interfering with this pathway may allow the regulation of cell fate in pathologies where SHH-Gli signaling is reactivated or re-expressed.

Vitamin D3 (VD3) can take three different forms after conversion of the precursor 7-dehydrocholesterol (7-DHC, pro-vitamin D3): cholecalciferol, calcidiol and calcitriol (Supplementary Figure S1, available at Carcinogenesis Online). The vitamin D receptor (VDR) and all the VD3-metabolizing enzymes are expressed in the normal kidney, but their expression is lost during the malignant transformation to CCC and the implications of this loss remain unknown (18-19). We have more evidence linking the incidence of various cancers, including colon, breast and prostate cancers, to low serum VD3 levels (20–23). Apart from its systemic role in maintaining mineral homeostasis, calcitriol has local antiproliferative, apoptotic, differentiation-inducing and immunomodulatory effects in cancer and several pathologies as well (24–28). Interestingly, preliminary results concerning cholecalciferol and calcitriol reported that they appear to be two specific and effective natural inhibitors of the SHH-Gli pathway because they target one of the two receptors of the SHH-Gli system, i.e. Smo receptor in a VDR-independent manner (28,29). Thus, it has been demonstrated that Ptc1 directly inhibits the Smo receptor and induces the release of VD3 to inhibit Smo indirectly. Interfering with Smo receptor in a system where the factors of the SHH-Gli pathway are present may lead to the repression of the targets induced under normal activation. In addition, antitumor activity has been obtained with VD3, independently of the VDR, through the SHH-Gli pathway in a model of basal cell carcinoma (30). Because we have recently shown that the SHH-Gli pathway is re-expressed in human CCC and that its inhibition induced tumor regression in nude mice, we hypothesized that VD3, and particularly cholecalciferol, has the potential to influence human CCC growth, at least through the SHH-Gli pathway.

Materials and methods

Cell culture and reagents
Human CCC cell lines either deficient in VHL (786-O, A498) or expressing VHL (ACHN, Caki-1) were obtained from the American Type Culture Collection (Manassas, VA). Mesangial cells were obtained from glomeruli isolated from C57BL/6 mice. In brief, kidneys obtained from 8- to 9-week-old mice were subject to sequential sieving for extraction of glomeruli. Glomeruli were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) enriched with 30% foetal bovine serum and antibiotics (penicillin/streptomycin). MCs outgrew from the glomeruli in ~1 week. Cells were grown for 1 week in Dulbecco’s modified Eagle’s medium with high D-value content to avoid fibroblast growth. The cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal bovine serum (20% for the mesangial cells) and 1% antibiotics in 10% CO2 atmosphere.

Abbreviations: CCC, clear cell renal cell carcinoma; FITC, fluorescein isothiocyanate; RCC, renal cell carcinoma; SHH, sonic hedgehog; VD3, vitamin D3; VDR, vitamin D receptor; VHL, von Hippel-Lindau.

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Human tumor biopsies
Tumors and corresponding normal tissues were obtained from 21 patients in collaboration with the Department of Urology (Prof. D.Jaqmin and Prof. H.Lang) of the “Nouvel Hôpital Civil”, Strasbourg, France. The tumors were staged according to the tumor node metastasis (TNM) classification (31): six pT1, six pT2, six pT3 and three pT4. Informed consent was obtained from all patients and the study was approved by the French Human Ethics Committee. Tissues were freshly frozen and stored in liquid nitrogen immediately after surgical resection.

Western blot analysis
Protein extractions and membrane preparations were performed as described previously (32). Membranes were incubated with the appropriate dilution of the following primary antibodies: anti-Gli1 (1:2000; Millipore), anti-Gli2 (1:1000; Abcam), anti-Gli3 (1:1000; Abcam), anti-Lim1 (1:500; Millipore), anti-Smo (1:500; Santa Cruz), anti-VDR (1:500; Sigma–Aldrich) and anti-glyceraldehyde-3-phosphate-dehydrogenase (1:500; Millipore). The appropriate horseradish peroxidase-conjugated secondary antibody was used. Immuno-reactivity was visualized with the enhanced chemiluminescence immunoblotting detection kit (Millipore).

Proteome array analysis
We used the proteome Profiler™ Arrays (R&D Systems) specific for phosphokinases, apoptosis and angiogenesis to analyze cholecalciferol targets in human CCC; the experiments were set up according to the manufacturer’s protocol. In brief, 786-0 cells were seeded in six-well plates (100,000 cells/ml), grown for four days and then treated for 24 h with cholecalciferol (Sigma) or vehicle (Ctl). Cells were lysed and the lysates were added to the membrane array. Membranes were then incubated overnight with the detection antibody cocktail. Protein signals were detected as detailed previously (17). The BioID software (Vilber Lourmat) was used to quantify duplicate gene signals and results were expressed in average pixel density.

Real-time quantitative reverse transcription-polymerase chain reaction analysis
Total RNAs were extracted from CCC cells or tissues using the Trizol method (Invitrogen). The extracted RNA (5 µg) was reverse transcribed using the non-specific primer 15-mer oligonucleotide containing only deoxythymidine (dT) residues p(dT)15 (Roche Diagnostics), as described previously (17). Complementary DNAs (cDNAs) specific for the target genes were amplified using the “LightCycler-FastStart DNA Master SYBR Green” kit (Roche Diagnostics) with the primers depicted in Supplementary Table S1, available at Carcinogenesis Open. Each sample was analyzed thrice and quantified with the analysis software for LightCycler (Roche Diagnostics).

RNA interference
Tumor cells were seeded in 24-well plates (20,000 cells/ml), grown for 24 h and then were transiently transfected for 24 to 72 h with siGli2, a Gli2-targeting small interfering RNA (siRNA; Applied Biosystems) or control siRNA (scGli, Applied Biosystems), according to the manufacturer’s instructions.

cDNA overexpression
Human 786-O cells were seeded in 24-well plates (20,000 cells/ml), grown for 24 h and then treated for 6 to 24 h with various concentrations of cholecalciferol, calcitriol (Sigma) or vehicle (Ctl).

Bromodeoxyuridine incorporation
Human CCC cells were seeded in 96-well plates (20,000 cells/ml) and grown for 24 h. Cells were treated for 6 to 24 h with cholecalciferol, or the vehicle (Ctl). Bromodeoxyuridine was incorporated for 4 h before fixing the cells. Tests were then realized according to the protocol of the manufacturer (Calbiochem®, Merck KGaA), as described previously (32).

Fluorescence-activated cell sorting analysis
Human CCC cells were seeded in six-well plates (20,000 cells/ml), grown for 24 h and treated for 6 to 24 h with cholecalciferol or the corresponding volume of vehicle (Ctl). Cells were harvested and incubated in buffer containing Annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide. After centrifugation, the supernatant was withdrawn and cells were fixed in formol. Cells were then subjected to fluorescence-activated cell sorting (FACS). Side scatter and forward scatter detectors were used to gate the cells. Fluorescence detectors for propidium iodide and Annexin-V-FITC were used to distinguish between cell death and apoptosis of the gated cells. As we monitored fluorescence over time, Annexin-V-FITC-negative/propidium iodide-negative cells were considered viable cells. Annexin-V-FITC-positive/propidium iodide-negative cells were considered early apoptotic cells and Annexin-V-FITC-positive/propidium iodide-positive cells were considered end-stage apoptotic cells. The remaining cells (Annexin-V-FITC-negative/propidium iodide-positive cells) were considered non-apoptotic dead cells. Fluorescence analysis was performed using FACSort flow cytometer (Becton-Dickinson) and the fractions of viable cells, necrotic cells and apoptotic cells were determined using FCS express software (DeNovo Software, Los Angeles, CA).

Xenograft tumor model
All animal studies were in compliance with the French Animal Use Regulations and the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the French Bureau of Veterinarian Services. After a week of habituation in the facilities, 4-week-old athymic male mice (SWISS nu/nu-; Charles River Laboratories) were divided into six groups (n = 7 each): one received cholecalciferol from the diet directly after habituation (prophylactic diet group, PD); one received intraperitoneal i.p. injections of cholecalciferol after habituation (prophylactic injection group, PI); the third received cholecalciferol from the diet when tumors had grown to an overall volume of 100 mm³ (therapeutic injection group, TD); the fourth received cholecalciferol i.p. when tumors had grown to an overall volume of 100 mm³ (therapeutic injection group, TI); the fifth one received the diluent i.p. (control group, CD); and the sixth group received a normal diet (control diet, CT).

In the third week, 10 million 786-O cells were injected subcutaneously into the skin of the mice. Tumor volumes were measured as described previously (32). The protocol was as follows: cholecalciferol i.p. at 250 IU (international units)/mouse once every 2 weeks, or diet at 10 000 IU/kg (customized diet A04 Safe) for the treatment groups; the control groups received the vehicle alone or the normal diet (A04). Tumor growth was measured once a week and blood samples were collected at the first week (t0). At the end of the treatment, blood samples were collected, animals were killed and tumors were harvested. Then, paraffin was embedded into the tumors and they were cut in 4-µm-thick sections for subsequent immuno-histochemical analysis, described previously for the mitotic index, the apoptotic index and neovascularization (11).

Serum calcium and vitamin D3 metabolite analysis
Serum levels of 1,25(OH)2-VD3 and 25OH-VD3 were analyzed by enzyme immunoassays using commercial kits (Immunodiagnostic Systems). The intra-assay coefficient of variation for each assay was 10%, whereas the intra-assay coefficient of variation for each assay was 12% and 10%, respectively. The cross-reactivity of the 1,25(OH)2-VD3 assay for 25OH-VD3 was 0.01%. Serum total calcium was analyzed via a quantitative colorimetric assay using the Quantichrom Calcium assay kit (Gentaur).

X-ray microcomputed tomography
Animals were scanned with X-ray microtomography after killing to avoid any physical effects such as beam hardening because the observation of bone microstructure requires high-resolution acquisitions, leading to long X-ray exposure, incompatible with oncologic studies. The mice selected for X-ray analysis were taken after 20 weeks of treatment either with the diet or with the injections. Totally, 768 screenings were carried out for the region starting at the tail and ending at the neck of the animal. Images were reconstructed with a cone-beam reconstruction algorithm, producing real-time three-dimensional images with an isotropic voxel size of 0.055 mm. Structural indices were then calculated using a homemade software and GEHC Microview software (GE Healthcare, UK (33)). The bone analysis of all mice corresponds to the lumbar region. Determination of bone mineral density, bone mineral content and bone volume computed tomography images are a visual representation of the X-ray linear attenuation coefficients, which directly reflect the mineral content of bones. X-ray linear attenuation coefficients were converted to mineral density values using a dipotassium phosphate phantom containing seven solutions of K2HPO4 with known densities used to calculate a regression equation to convert linear attenuation coefficients, which directly reflect the mineral content of bones. X-ray linear attenuation coefficients were converted to mineral density values using a dipotassium phosphate phantom containing seven solutions of K2HPO4 with known densities used to calculate a regression equation to convert linear attenuation coefficients, which directly reflect the mineral content of bones. X-ray linear attenuation coefficients were converted to mineral density values using a dipotassium phosphate phantom containing seven solutions of K2HPO4 with known densities used to calculate a regression equation to convert linear attenuation coefficients, which directly reflect the mineral content of bones. X-ray linear attenuation coefficients were converted to mineral density values using a dipotassium phosphate phantom containing seven solutions of K2HPO4 with known densities used to calculate a regression equation to convert linear attenuation coefficients, which directly reflect the mineral content of bones.

Statistical analysis
All values are expressed as mean ± SEM. Values were compared using multifactorial analysis of variance followed by the Student-Newman-Keuls’s test for multiple comparisons. A value of P < 0.05 was considered significant.

Vitamin D3 in renal cell carcinoma

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Results

Vitamin D3 signaling pathway components are repressed in human CCC
It has been shown previously by immunohistochemistry and western blot that VDR and all the VD3-metabolizing enzymes are expressed in the normal adult kidney, but their expression is lost in patients with RCC (18). Here, we confirm these findings by RT–qPCR: in contrast to normal human kidney, the expression levels of VD3 receptor (VDR) and two enzymes required for the transformation of VD3, namely, CYP27B1 and CYP24A1, are dramatically reduced in a panel of human CCC cell lines expressing or not expressing VHL (Figure 1A) as well as in CCC tumor tissues at different stages (Figure 1B). In addition, we did not detect the VDR in total protein extracts from our panel of human CCC cell lines or in CCC tumor tissues at different stages (Figure 1C), showing the loss of VDR expression in human CCC.

SMO inactivation with cholecalciferol results in the downregulation of Gli2 in human CCC
We assessed afterward whether cholecalciferol affects SHH pathway expression. Using the reverse transcription quantitative PCR (RT–qPCR) approach, Gli3 expression was not affected by the treatments, but cholecalciferol induced a significant decrease of Gli2 expression from 6 to 24 h posttreatment, with a maximal level of messenger RNA 25 times less than that in the control at 24 h (Figure 2A). These observations were confirmed by the levels of the Gli proteins and Lim1, a Gli target in CCC (Figure 2B). Surprisingly, Gli1 messenger RNA was slightly overexpressed in response to cholecalciferol, but the protein level was significantly reduced over time (Figure 2A and 2B), indicating a possible instability of the RNA and a reduced stability of the Gli1 protein. Such effects were also observed in our panel of human CCC cell lines both expressing or not expressing the VHL gene (data not shown). No changes in the expression levels of the receptors of the SHH pathway (Smo and Ptc) were observed and the same PCR analysis for normal mesangial cells revealed no significant changes in the expression of the SHH pathway components (Supplementary Figure S2, available at Carcinogenesis Online). In addition, Gli2 inhibition by cholecalciferol was concentration dependent and was abolished in the absence of Smo, showing that cholecalciferol suppresses Gli2 through Smo targeting (Supplementary Figure S3A, available at Carcinogenesis Online).

Cholecalciferol, but not calcitriol, decreases cell density in human CCC cell lines but not in normal cells
The high specificity and efficiency of cholecalciferol in inhibiting the SHH pathway has been recently demonstrated (29). In CCC cell lines, cholecalciferol, at 50 nM, decreased cell density in a time- and concentration-dependent manner up to 90% at 24 h (Figure 3A). It is important to note that in normal renal cells such as mesangial cells, this form of VD3 started to have significant effects on cell density only at 50 nM of treatment (Figure 3A). In contrast to normal kidney cells, the decrease of CCC cell growth induced by cholecalciferol lasted at least 72 h (Supplementary Figure S3B, available at Carcinogenesis Online); and calcitriol, the active form of VD3, decreased cell growth at high concentrations, but to a lesser degree. Similar effects on cell density were obtained with Gli2 siRNA, and the expression of a plasmid-derived Gli2 in cholecalciferol-exposed cells rescued the effects induced by the treatment (Supplementary Figure S3C, available at Carcinogenesis Online).

Cholecalciferol decreases cell proliferation and increases apoptosis and non-apoptotic cell death of human CCC cell lines
The effect of cholecalciferol on cell growth was due, in part, to the inhibition of cell proliferation as assessed by bromodeoxyuridine-incorporation studies in our panel of human CCC cells, independently of VHL expression (Figure 3B and Supplementary Figure S4A, available at Carcinogenesis Online). Cholecalciferol treatments of 786-0 and Caki-1 cells induced loss
of cell viability in a time-dependent manner, as assessed by flow cytometry (Figure 3C and 3D; and Supplementary Figure S4B, available at Carcinogenesis Online). The analysis of the fluorescence led us to conclude that cholecalciferol induces non-apoptotic cell death with a maximum of 53% (±4.2%) at 24 h in 786-O cells and 51.2% (±3.9%) in Caki-1 cells (Figure 3D and Supplementary Figure S4B, available at Carcinogenesis Online). Cholecalciferol also induced cell apoptosis to a lesser degree, with a maximum of 9% (±0.8%) at 24 h in 786-O cells and 10.5% (±2.5%) in Caki-1 cells (Figure 3D and Supplementary Figure S4B, available at Carcinogenesis Online).

**Cholecalciferol acts through multiple signaling pathways and factors in human CCC**

We have shown recently that inhibiting the SHH-Gli pathway has an effect particularly on the main oncogenic pathways implicated in RCC (16), and cholecalciferol is a powerful inhibitor of this pathway. To get more insight into the mechanism accounting for the effect on tumor cell growth, we used proteome Profiler Arrays (coated with apoptotic, phosphoproteins or angiogenesis pathway markers; Figure 4A). Various signaling pathways and molecular factors were found to be regulated at the level of expression and/or activation, directly or indirectly, by cholecalciferol treatment (Figure 4B).
notable that many factors playing a role in the prevention of apoptosis, such as Akt, cIAP-1, cIAP-2, survivin or claspin, were repressed and the expression level of several proteins responsible for angiogenesis and metastasis processes, such as matrix metalloproteinases, growth factors and interleukins, were dramatically decreased (Figure 4C).

Supplementary Table S2 (available at Carcinogenesis Online) describes every evaluated target. Thus, cholecalciferol appears to be a powerful antiangiogenic molecule and an inhibitor of the expression and/or activation of various oncogenic pathways in human RCC (Figure 4D).

Human CCC-bearing mice treated with cholecalciferol show absence of tumor development or growth inhibition

We have next analyzed the effect of cholecalciferol treatment in vivo. To confirm the adequate targeting of the drug, the expression of Gli2 was analyzed in the tumors. There was a substantial decrease in Gli2.

Fig. 3. Cholecalciferol inhibits cell growth in human CCC, decreases proliferation and induces cell death. (A) Human CCC cell growth was assessed by counting adherent cells. Human 786-O cells, human Caki-1 cells and mesangial cells (MC) were seeded in 24-well plates (20,000 cells/ml), grown for 24 h and treated for 6 to 24 h with cholecalciferol at the concentrations depicted in the figure or with the diluent only (Ctl). Results are shown as mean ± SEM, n = 4; *P < 0.05 and **P < 0.01 from Ctl. (B) Human 786-O cells were treated for 6 to 24 h with cholecalciferol at the concentrations depicted in the figure or the diluent only (Ctl) and were processed for cell proliferation analysis with bromodeoxyuridine staining. Results are shown as mean ± SEM, n = 6; **P < 0.01 from Ctl. (C) Representative plots of the FACS analysis at 12 and 24 h after cholecalciferol treatment (30 nM); the untreated cells (Ctl) are shown at 24 h. Gate 1 contains the analyzed cells (SSC, side scatter; FSC, forward scatter) and the quadrants show the repartition of the cells in gate 1 with reference to their fluorescence (PI, propidium iodide). (D) Quantitative analysis of cell debris, non-apoptotic dead cells and apoptotic cells as a function of the time posttreatment in 786-O cells. Results are shown as mean ± SEM.
expression in tumors harvested from mice receiving injections and receiving cholecalciferol in the diet by ~90% and ~60%, respectively, compared with tumors from control-treated mice, showing adequate targeting of the drug (Figure 5A). In addition, we did not detect VDR in total protein extracts from tumors, thus dissociating the possible antitumor activity of VD3 on CCC through VDR (Figure 5A).

Tumor growth was almost completely abolished in the group treated with cholecalciferol injections from the first week of the study (PI group), as only four mice (57%) out of seven showed tumors at the end of the protocol. Starting the treatment after the development of a tumor of 100 mm3 (TI group) decreased tumor growth significantly, as the average level reached 180% of control at the last week, and it is notable that one mouse showed a complete tumor regression (Figure 5A and 5B).

In the two groups that received cholecalciferol in the diet, tumor growth decreased significantly by 45% for the prophylactic group (PD group) and by 25% for the therapeutic group (TD group). The lesser effect obtained in comparison with the groups treated with injections may be explained by the low bioavailability of the drug in the food. The effects of cholecalciferol were obtained by a decrease in tumor cell proliferation (Supplementary Figure S5A and B, available at Carcinogenesis Online) and to a lesser degree by an induction of apoptosis (Supplementary Figure SSC and D, available at Carcinogenesis Online), according to the results we obtained in vitro.

Cholecalciferol treatment of mice does not induce calcification or calcium reabsorption

The baselines for calcidiol (120 nmol/l) and calcitriol (115 pmol/l) levels, measured in the two control groups (control vehicle and control diet), are very similar to the ones monitored in humans (26). As a result of the elevation of cholecalciferol intake by the mice, either in the diet or through injections, there is an inhibition of the SHH-Gli pathway and an increase of the serum level of the cholecalciferol transformation products as well. Serum 25(OH)-VD3 increased by 220% from t0 in the diet groups and by 307% from t0 in the i.p. injection groups (Figure 6A). Serum 1,25(OH)2-VD3 increased by 165% from t0 in PD and TD groups and by 151% from t0 in PI and TI groups (Figure 6A).

As VD3 toxicity is associated with hypercalcemia and calcification, we measured serum calcium level and bone mineral content of...
the mice, to assess the eventual toxicity of cholecalciferol. There was no significant modification in the level of calcium in the mice supplemented with cholecalciferol, except for the therapeutic diet (TD) group, with a concentration of 6.8 mg/dl at the end of the study compared with 7.2 mg/dl at t₀ (Figure 6B). The calcification was evaluated by computed tomography of whole mice and the lumbar region of mice treated for 20 weeks was taken for the analysis (Figure 6C). The bone mineral content was not changed by cholecalciferol supplementation (Figure 6D).

Discussion

Numerous epidemiologic studies have shown the importance of VD3 in preventing various cancers and several pathologies (22,26,34,35). In addition, the therapeutic potential of 1,25(OH)₂-VD3, the biologically active metabolite of VD3, and its analogues in cancer are now relatively well documented (36). Previous studies have shown an association between levels of ultraviolet radiation, VD3 levels and kidney cancer incidence (19,37,38). However, the potential role of VD3 in the progression of human kidney cancer has not received much attention yet. Former studies have shown that calcitriol inhibited the growth of KU-2 RCC cell line (39). Later, Fujioka reported that VD3 had inhibitory effects in vitro and in vivo (40). Finally, a recent study reported that calcitriol has inhibitory effects on human CCC cell lines both in vitro and in vivo mediated through cyclin-A and induction of apoptosis (41). Because most of the effects of VD3 are mediated by its interaction with VDR, it is not surprising that the antitumor activity described in the literature with VD3 was achieved via the VDR. Nevertheless, in accordance with our results presented here, it has been shown that the VDR and all the VD3-metabolizing enzymes are not expressed in CCC (Figure 1) (18). This raised the question of the mechanism of VD3 antitumor activity in a context where the VDR is absent.

The SHH-Gli pathway is activated by binding the hedgehog ligand to the patched receptor (Pch1), releasing the smoothened receptor (Smo) and the downstream transcription factors Glis from inhibition (42). Glis transcription factors have numerous downstream target genes in human CCC, including vascular endothelial growth factor, transforming growth factor, cyclinD1, Gli itself and Lim1 (the last shown to be an oncogene in human CCC) (17). Recent reports provided evidence of Pch-dependent secretion of a VD3-related compound, which functions as an endogenous inhibitor of the SHH-Gli signaling pathway by repressing the activity of the Smo receptor (28,29). It has been reported that VD3 inhibits the SHH-Gli signaling pathway at the level of Smo in a VDR-independent manner, suggesting that VD3 controls the activity of two independent signaling pathways: the SHH-Gli and the VDR pathways, both highly relevant in tumorigenesis and tumor treatment. Furthermore, cholecalciferol is bound to Smo with high affinity, and when zebrafish embryos are treated with VD3, the resultant effects mimic the smo(-/-) phenotype, confirming the inhibitory action in vivo (29). Finally, it has been recently reported that in the skin of VDR-null mice, SHH-Gli pathway
components are overexpressed and are associated with epidermal tumor formation (43). Taken together, these data show clearly the link between VD3 and the SHH-Gli pathway, and this may be highly relevant in cancer research and treatments. Here, we found another clue concerning the mechanism of cholecalciferol effects by showing that re-establishment of Gli2 expression rescues cholecalciferol-mediated antiproliferative effects in the absence of VDR.

As presented earlier, one report has shown that the inhibitory effects of VD3 on human CCC are the result of the inhibition of Akt phosphorylation and caspase-9 activity (41), whereas in prostate and breast cancer cells, it has been demonstrated to involve upregulation of proapoptotic Bax and Bcl-xL (44). Here, we show that cholecalciferol inhibits Gli2 expression in VDR-negative cells. As a result, we observed Akt activation through its phosphorylation and upregulation of Bax expression, confirming the results of the herein-mentioned studies. Importantly, we also identified a broad range of target genes involved in cell proliferation and apoptosis, including cIAP and survivin (inhibition) and TRAIL-R2 and p21 (activation), and in cell mobility or angiogenesis, including members of the matrix metalloproteinase family, epidermal growth factor, hepatocyte growth factor, fibroblast growth factors and vascular endothelial growth factor, which clearly increase the potential spectrum of effects of VD3 on tumor development. In addition, these results substantially increase our understanding of the molecular mechanism of the inhibitory effects of cholecalciferol on human CCC growth and most probably invasion. It will be important in the future to decipher in detail the molecular network of this naturally occurring inhibitory pathway, which may lead to the design of efficient and safe anticancer therapies.

One of the most important findings herein is the direct demonstration that cholecalciferol treatment inhibited or completely blocked the growth of human CCC when incorporated in a therapeutic or prophylactic manner. Cholecalciferol increased serum levels of calcidiol and calcitriol and could be converted to calcitriol by tumor cells through the VDR, but we proposed that the effect of VD3 on tumor growth is VDR independent as xenografted tumors did not express VDR. These results clearly link cholecalciferol to Gli expression and antitumor effects. The antitumor activity may be attributed to inhibition of tumor cell proliferation and induction of tumor cell apoptosis as observed in cultured cells, but not to antiangiogenic effects. The absence of effects on tumor neovascularization might be quite surprising in the light of the results of cholecalciferol target genes that include vascular endothelial growth factor. However, this observation could be attributed to the expression of additional target genes having opposite effects on vascularization, which were not uncovered by our experimental approach. Reports dealing with the prognostic value of vascularization in human CCC have shown one of the following: no effect on patient survival, better survival, or worse prognosis (45–47). It is notable that the levels of VD3 metabolites are very similar in mice and humans. In addition, the physiological response to the intake of cholecalciferol compared with the levels of calcidiol and calcitriol is similar too. Vitamin D3 prescription showed benefits for the prevention and treatment of several cancers. We report here that
cholecalciferol may be particularly efficient and safe to prevent and treat human CCC, in particular because of its inhibition of the SHH-Gli pathway, which we have shown recently to be re-expressed in kidney cancer. Thus, cholecalciferol supplementation in patients may have at least two main effects: it elevates the levels ofVD₃ metabolites, thus preventing cell malignancies and other defects resulting from a lack of these hormones; and it inhibits the developmental and oncogenic SHH-Gli pathway to decrease tumor cell growth.

The therapeutic potential of calcitriol, the biologically active metabolite ofVD₃, and its analogues in cancer is well documented (36,48). Here, we show that cholecalciferol is safe; it did not show significant toxicity and that was not only reflected by the absence of effects on the body weights of the mice but also by the absence of effects on various bone parameters and serum calcium levels. Data from the present study support the crucial role played by hedgehog signaling in RCC and show the potential of cholecalciferol to prevent and treat renal cancer in a safe way. These results argue about the need for further pre-clinical and clinical evaluation of cholecalciferol in terms of inhibiting human CCC growth and any probable invasion that should preclude its use as a therapeutic agent for this refractory disease.

Supplementary material

Supplementary Figures S1–S5 and Tables S1 and S2 can be found at http://carcin.oxfordjournals.org/.

Funding

INSERM (Institut National de la Santé et de la Recherche Médicale), University of Strasbourg; the French Foundation for Medical Research (Fondation pour la Recherche Médicale en France - Comités d’Alsséac) to T.M.

Acknowledgements

We thank Dr A. Donai for helpful suggestions, Dr J. Barths for allowing us to perform FACS analysis and R. A. Hanna for text correction.

Conflict of Interest Statement: None declared.

References


Received March 9, 2012; revised July 2, 2012; accepted July 22, 2012