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Pancreatic cancer cells express 25-hydroxyvitamin D-1α-hydroxylase and their proliferation is inhibited by the prohormone 25-hydroxyvitamin D_3

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The steroid hormone 1,25-dihydroxyvitamin D_3, [1,25$(OH)_2D_3$, calcitriol], the active metabolite of vitamin D, exerts pleiotropic antitumor effects against several malignancies. However, the clinical use of this hormone is limited by hypercalcemia. 25-Hydroxyvitamin D_3, the prohormone of 1,25$(OH)_2D_3$, is hydroxylated to the active hormone by the enzyme 25-hydroxyvitamin-1-α-hydroxylase [1α(OH)ase]. 1α(OH)ase is found primarily in the kidney, but also is expressed in the prostate, colon and other tissues. Using immunohistochemistry, we report that 1α(OH)ase is highly expressed in both normal and malignant pancreatic tissue. Expression of this enzyme and enzymatic activity was also detected in four pancreatic tumor cell lines. 25(OH)D_3 inhibited the growth of three of four pancreatic cell lines in a manner that correlated with the level of induction of the cyclin-dependent kinase inhibitors p21 and p27 and with the induction of cell cycle arrest at the G_1/S checkpoint. The growth of a cell line stably transfected with a mutant *Ki-ras* allele and of a second cell line with an endogenous *Ki-ras* activating mutation was also inhibited by 25(OH)D_3, indicating that activating Ki-Ras mutations, which occur in almost 90% of pancreatic adenocarcinomas, do not interfere with the growth-inhibitory effects of 25(OH)D_3. The expression of 1α(OH)ase in normal and malignant pancreatic tissue and the antiproliferative effects of the prohormone in these cells, suggest that 25(OH)D_3 may offer possible therapeutic and chemopreventive options for pancreatic cancer.

Introduction

Pancreatic cancer accounts for ~30 000 new cancers per year in the US and 30 000 deaths (1,2). It is the most rapidly fatal of all cancers; the average survival after diagnosis is <6 months (3). The high case-fatality rate of pancreatic cancer is due to the absence of specific symptoms, which results in its detection at an advanced and incurable stage, and to the resistance of pancreatic tumors to standard chemotherapies (4,5).

Differentiation therapy, alone or in combination with existing therapies, is a rapidly developing field of clinical and experimental oncology (6,7). During the last decade, it has become apparent that pro-differentiation agents, such as vitamin D_3 (8–10), retinoic acid (11–13) and their derivatives exhibit antiproliferative effects against many tumor cell types. In addition to inducing terminal differentiation of transformed cells, these agents also exhibit potent pro-apoptotic properties (10,14). Moreover, vitamin D_3, retinoic acid and their analogs can act synergistically with standard cancer therapies such as chemotherapy and ionizing radiation (15–21). These properties of pro-differentiation agents, coupled with their relatively low toxicity, have pushed them to the forefront of new chemopreventive/chemotherapeutic approaches for cancer.

The steroid hormone 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$, calcitriol], the active metabolite of vitamin D, is being studied extensively for the treatment of several malignancies including prostate, breast and pancreatic cancer (reviewed in refs 9 and 10). Because the principal drawback of the systemic administration of 1,25$(OH)_2D_3$ is hypercalcemia [10], analogs of 1,25$(OH)_2D_3$ that exhibit similar growth-inhibitory and anti-metastatic properties but with reduced calcemic effects have been developed as anticancer drugs (22). For example, the 1,25$(OH)_2D_3$ analog EB1089 (Seocalcitol), is currently being studied in clinical trials for gastrointestinal malignancies (23) including a Phase-II trial for the treatment of pancreatic adenocarcinoma (24).

Previous studies have reported the expression of both the receptor for 1,25$(OH)_2D_3$ (VDR) and the retinoid receptor (RXR) in several pancreatic cancer cell lines, and *in vitro* and *in vivo* data have supported the use of analogs of 1,25$(OH)_2D_3$ and retinoic acid against pancreatic cancer. For example, Kawa *et al*. demonstrated that 22-oxa-1,25-dihydroxyvitamin D_3 inhibited the growth of three of nine pancreatic cancer cell lines and also inhibited the growth of the cell line BxPC-3 when it was grown as tumor xenografts in nude mice (25,26). Colston *et al*. reported that EB1089 was a potent growth inhibitor of the GER cell line *in vitro* and *in vivo* (27). More recently, Petterson *et al*. demonstrated that EB1089 was more potent than 9-*cis*-retinoic acid as an inhibitor of three pancreatic cell lines *in vitro* (28). These results suggest that 1,25$(OH)_2D_3$ analogs may be useful therapeutic agents in some pancreatic tumors.

In addition to the use of synthetic analogs of 1,25$(OH)_2D_3$, another strategy to limit the problem of hypercalcemia is to administer the inactive prohormone 25-hydroxyvitamin D_3 [25(OH)D_3]. In 1998, Schwartz *et al*. demonstrated that several prostate cancer cell lines and human prostate cancer cells in primary culture possess 25-hydroxyvitamin D-1α-hydroxylase [1α(OH)ase], the enzyme that converts the pro-hormonal form of vitamin D, 25(OH)D_3, to 1,25$(OH)_2D_3$ (29). In

Abbreviations: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 1α(OH)ase, 25-hydroxyvitamin-1-α-hydroxylase; PBS, phosphate-buffered saline; VDR, 1,25$(OH)_2D_3$ receptor; VDRE, VDR response element.

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prostate primary cultures, 25(OH)D_3 was found to have inhibitory effects comparable to those of 1,25$(OH)_2D_3$ (29,30). Because the conversion from prohormone to active hormone occurs within the cell, the problem of systemic hypercalcemia is greatly minimized. Because 25(OH)D_3 has been approved by the FDA for human use (e.g. for treating vitamin D deficiency), this drug would also be an attractive candidate for human clinical trials in cancers that express 1α(OH)ase. In this study, we used a polyclonal antibody against human 1α(OH)ase to study the expression pattern of the enzyme in various normal and cancerous tissues, including the pancreas. We also investigated the antiproliferative effects of 25(OH)D_3 in a panel of pancreatic tumor cell lines that were shown previously to exhibit differential responses to 1,25$(OH)_2D_3$.

Materials and methods

Cell lines

The BxPC-3, Hs700T, Hs766T and AsPC-1 cell lines were purchased from the American Type Tissue Collection (ATCC). AsPC-1 cells harbor a K-*ras* mutation (GGT to GAT transition at codon 12), that is observed in 70–80% of pancreatic cancers (31). Hs700T.*ras* cells were obtained by transfecting Hs700T cells with a plasmid expressing a codon 12 mutant Ki-Ras under the control of the human cytomegalovirus (CMV) promoter (a gift from Dr Peter Howley, Harvard University School of Medicine). Cells were selected with puromycin (2 μg/ml) for 7 days and individual clones were isolated using pyrex cloning rings. Cells were cultured in Dulbecco's modified essential medium supplemented with 10% fetal calf serum and antibiotics (penicillin-streptomycin). Hs700T.*ras* cells were cultured in the presence of 0.5 μg/ml puromycin.

Chemicals

25(OH)D_3 and 1α,25$(OH)_2D_3$ were purchased from Biomol (Plymouth Meeting, PA). They were dissolved in 100% ethanol and stock solutions were kept in −80°C, and were protected from light until used. The final ethanol concentration in all treatments was 0.1% (v/v). This concentration had no effect on the rate of growth of pancreatic cells (data not shown).

Antibody against 1α(OH)ase

The antiserum was produced under contract by Sigma-Aldrich (St Louis, MO). Briefly, an 8-branched multi-antigenic peptide consisting of amino acids 266–289 (sequence RHVERREAEAAMRNGGQPEKDLES) was used as an immunogen. The sequence of the synthetic peptide was confirmed by sequencing and the peptide was injected into two rabbits. Serum was collected and the polyclonal antibodies were affinity-purified using the purified peptide.

Cell-transfections immunocytochemistry

Transfection of human 1α(OH)ase cDNA cloned into the mammalian expression vector pcDNA 3.1 was carried out using the Lipofectamine transfection reagent according to manufacturer's instructions (Gibco BRL, Gaithersburg, MD). Non-transfected COS cells served as a negative control. Twenty-four hours after transfection the cells were fixed in 10% buffered formalin, permeabilized with acetone and labeled with anti-human 1α(OH)ase antibody. Rhodamine-labeled goat anti-rabbit antibody was used for secondary detection. Cells were visualized using fluorescence microscopy. LNCaP cells stably expressing exogenously-transfected 1α(OH)ase have been described previously [35]. Immunocytochemistry for mock-transfected and 1α(OH)ase-transfected LNCaP cells was performed by fixing the cells in 1:1 methanol: acetone mixture for 5 min followed by incubation with primary anti-1α(OH)ase antibodies (1:50 dilution) and with a FITC-labeled anti-rabbit secondary antibody (1:1000 dilution; Sigma-Aldrich) for 20 min. Cells were counterstained with Hoechst 33342 for visualization of nuclei.

Immunohistochemistry

For frozen tissues, cryostat sections were prepared and mounted on glass slides by thaw-mounting, followed by fixation in 3.7% formaldehyde, or by 100% acetone, following by incubation in blocking solution containing 1% bovine serum albumin in phosphate-buffered saline (PBS). After washing in PBS, the sections were incubated in primary rabbit affinity-purified anti-1α(OH)ase (25 μg/ml) in PBS-BSA, followed by goat anti-rabbit IgG conjugated to horseradish peroxidase. The peroxidase labeling was then detected using diaminobenzidine-peroxide substrate, followed by counterstaining in hematoxylin. For paraffin sections, a similar procedure was used although the first antibody step was preceded by an antigen retrieval step using either acid citrate solution boiled in a microwave oven for 10 min, or 3 M urea in a water bath at 95°C for 30–45 min. No peroxide-blocking step was used as this could interfere with the reactivity of some antigens, and endogenous peroxidase is not a major concern for the tissues examined.

Cell proliferation assay (MTT assay)

MTT assays were performed using a kit from Roche Applied Science (Indianapolis, IN). Briefly, 5000 cells were plated onto 24 well-plates and treated with 25(OH)D_3 or with 1,25$(OH)_2D_3$. After 7 days, cell viability was assessed according to the manufacturer's protocol. 200 μl aliquots from each reaction were transferred into a 96-well plate and absorbance values were measured at 560 nm with an automated plate reader (Molecular Devices, Sunnyvale, CA). Each experiment was performed in triplicate. Values were normalized against a blank (cells treated with ethanol alone) and were reported as percent inhibition along with SE values.

Gel electrophoresis, western transfer and immunoblotting

For analysis of 1α(OH)ase expression levels, an equal number of cells (~1 × 10^6) were directly lysed in 1.5× SDS buffer and 10 μl were used for immunoblot analysis. For p21 and p27 protein analysis, cells were washed three times with ice-cold PBS and resuspended in PBS with 1% NP-40 containing 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/ml aprotinin, 2 μg/ml antipain, 1 mM phenylmethyl sulphonyl fluoride, 1 mM $NaVO_4$, 1 mM NaF, 1 μM microcystin L and 2 mM EDTA. Cells were lysed on ice for 15 min and centrifuged for 10 min to separate cell debris. The protein concentration of each sample was determined by the modified Lowry assay (Bio-Rad, Hercules, CA). A total 40–50 μg of whole cell protein extract was resolved on a 10–12% SDS–polyacrylamide gel and transferred onto Hybond ECL nitrocellulose membrane (Amersham, Arlington Heights, IL). Membranes were stained with 0.15% Ponceau red (Sigma Chemical, St Louis, MO) to ensure equal loading and transfer, and then blocked with 5% (w/v) dried non-fat milk in Tris–borate saline (TBS) buffer (Tris base 20 mM, NaCl 137 mM, pH 7.6). Immunoblotting for 1α(OH)ase was performed with the rabbit polyclonal antibody at 1:1000 dilution. Immunoblotting for p21 and p27 was performed using affinity-purified rabbit polyclonal antibodies (BD-Pharmingen, San Diego, CA). Blotting with a mouse monoclonal antibody raised against human β-actin (Sigma Chemical) was used to control for loading and blotting errors. Following incubation with the primary antibody, the membranes were washed in TBST, incubated with an anti-rabbit secondary antibody and immunoreactive bands were visualized using an Enhanced Chemiluminescence (ECL-Plus) reagent kit according to the manufacturer's recommendations (Amersham). Films were exposed to the membranes for varying periods of time, and scanned with a personal scanner (Microtec, San Jose, CA). Optical densities of the immunoreactive bands were measured using the NIH Image Analysis Program.

Quantitative, real-time PCR (RT–PCR)

For RT–PCR, total RNA was isolated from each pancreatic tumor cell line (~1 × 10^6 cells), using Tri-Reagent (Sigma-Aldrich). Following spectrophotometric quantification, 5 μg of RNA was first treated with RNase-free DNase and then used as substrate for first-strand cDNA synthesis, using a reverse-transcriptase kit (Invitrogen, Carlsbad, CA), according to manufacturer's protocol. Successful first-strand synthesis was confirmed by running a 5 μl aliquot of each reaction on a 0.8% (w/v) agarose gel. Five microliters from each reaction were used as template for PCR. RT–PCR was carried out using the Platinum Quantitative PCR SuperMix-UDG kit (Invitrogen) according to the manufacturer's instructions. In order to quantify the amount of 1α mRNA, 500 ng of total cDNA was combined with Platinum Quantitative PCR SuperMix-UDG, ROX reference dye and primers at a concentration of 10 μM each, in a total reaction volume of 25 μl. The sequences of the forward and reverse primers were 5′-CACTTGCTGCCTGGAGGCTCAAGTG-3′ and 5′-ACAGCGTGGACACAAACACC-3′, respectively. The forward primer was labeled with JOE dye. Plasmid DNA, in amounts ranging from 10^4 to 10^{-4} pg, containing a 1α(OH)ase encoding region was used to construct a standard curve. Reaction conditions for quantification of unknowns and standards were 50°C for 2 min, 95°C for 2 min, followed by 55 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. All reactions were carried out in a 96-well plate in an icycler® RT–PCR Detection System (Bio-Rad). β-Actin expression levels were used to normalize 1α(OH)ase levels. In order to quantify the amount of β-actin, identical reaction conditions as for 1α(OH)ase quantification were used with minor changes. 250 ng total cDNA was used along with forward and reverse primers with the sequences 5′-GATGTGGATCAGCAAGCAGGA-3′ and 5′-CACCGGCCTAGAAGCATT-TGCGGTG-3′ respectively. The reverse primer was labeled with the JOE dye. A standard curve was constructed using plasmid DNA encoding β-actin (a gift from Dr Suzy Torti, Wake Forest University School of Medicine) in quantities ranging from 10^5 to 10^{-2} pg.

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Measurement of 1α(OH)ase enzyme activity

When cultures reached ~80% confluence, the media was removed and was replaced with basal medium plus 50 nM of $25(OH)D_3$ containing 0.1 μCi $[^3H]25(OH)D_3$ and 10 μM DPPD and incubated for 2 h for 1α(OH)ase enzyme activity analysis. The 1α(OH)ase enzyme activity was determined by high performance liquid chromatography using methylene chloride/isopropanol (19:1) as the mobile phase to prevent 10-oxo-19-nor-$25(OH)D_3$ contamination as described (32). DPPD, an antioxidant, was added during the incubation to prevent the free radical, non-enzymatic auto-oxidation of $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$.

Cell cycle analysis

Exponentially growing cells were plated in 100-mm Petri dishes and treated with 2 μM $25(OH)D_3$. After 24 or 48 h, the cells were harvested, fixed with 70% ethanol at a cell density of 1×10^6 cells/ml, and cell cycle distribution was analyzed by flow cytometry using FACS analysis. For FACS analysis, cells were washed twice in IFA buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 4% FCS, 0.1% sodium azide) and then resuspended in 0.5 ml IFA containing RNase A (5 U/ml) and propidium iodide (50 μg/ml). Following a 15-min incubation at 37°C, the cells were subjected to flow cytometry using a BD LSR instrument (Becton Dickinson, San Jose, CA) equipped with a 488-nm (blue) argon and a 32-nm (UV) helium–cadmium laser. Data acquisition was performed using CellQuest software and data analysis with ModFit LT (2.0) software (Variety Software House, Topsham, ME).

Results

1α(OH)ase is expressed in both normal and malignant pancreatic tissue

A synthetic peptide comprised of amino acids 266–289 of the human 1α(OH)ase protein, which shares a 70% homology with the mouse protein (Figure 1A), was used to inject rabbits and the antibody was purified using immobilized peptide. The activity and specificity of the antibody was tested in monkey COS cells. Untransfected cells exhibited no immunoreactivity (Figure 1B, bottom right panel). However, transfection of a plasmid expressing the human 1α(OH)ase gene under the control of a CMV promoter followed by immunocytochemical staining with the polyclonal antibody resulted in strong staining activity in a punctate pattern consistent with a mitochondrial localization of 1α(OH)ase (Figure 1B, top and bottom left panels). To further test the specificity of this antibody, we performed western transfer and immunoblot analysis on extracts of human renal tubule cells, BxPC-3 pancreatic tumor cells, LNCaP cells and LNCaP cells transfected with 1α(OH)ase. As shown in Figure 1C, a band of approximate molecular weight of 55 kDa exhibited strong immunoreactivity with this antibody. Notably, LNCaP cells, which have demonstrated very low 1α(OH)ase enzymatic activity (33,43), had the lowest levels of 1α(OH)ase immunoreactivity which was strongly enhanced by transfection of the 1α(OH)ase expression plasmid. Immunocytochemical staining of untransfected LNCaP cells resulted in little or no staining, whereas the transfected cells exhibited strong cytoplasmic staining with the polyclonal antibody (Figure 1D). Taken together, these results indicate that the polyclonal antibody raised against the 23 amino acid peptide demonstrates specific activity against human 1α(OH)ase in several cell lines.

Using this antibody, we examined the expression pattern of 1α(OH)ase in pancreatic tissue. Both cryostat sections from frozen specimens and paraffin-embedded tissue sections were used. As shown in Figure 2, 1α(OH)ase expression was detected in centroacinar cells and ducts (Figure 2A, left and right panels), as well as in islets of Langerhans (Figure 2A, left panel). Similar to frozen sections, expression was detected in the ducts and the islets in paraffin-embedded tissue (Figure 2B). In sections from which the primary antibody was omitted from the immunohistochemical staining procedure, no expression was detected (Figure 2B, right bottom panel).

The expression of 1α(OH)ase in normal pancreas prompted us to investigate whether it is also expressed in adenocarcinoma of the pancreas, as well as in other malignant tissues. Two samples obtained from infiltrating adenocarcinoma of the pancreas displayed robust and extensive expression of 1α(OH)ase in (Figure 2C, left two panels). High expression levels were also found in ductal breast carcinoma (Figure 2C, top right) and in a section of pediatric renal cell carcinoma (Figure 2C, bottom right). Furthermore, we found positive staining for 1α(OH)ase in the autonomic ganglia in colon and in the bile ducts of the liver (data no shown). A summary of our results in comparison with the results reported previously by Zehnder *et al.* (41) is shown in Table I. Our results confirm previous findings of expression of 1α(OH)ase in normal pancreas. Moreover, they demonstrate for the first time that 1α(OH)ase is also expressed in pancreatic adenocarcinoma.

1α(OH)ase mRNA is expressed in four different pancreatic cell lines

The finding that 1α(OH)ase protein is expressed in pancreatic tumor tissue raised the possibility that pancreatic tumor cells could be growth-inhibited by $25(OH)D_3$. To investigate this, we first examined whether the enzyme is expressed in pancreatic tumor cell lines. We used three different cell lines that have been shown previously to exhibit differential sensitivity to $1,25(OH)_2D_3$ (26,27). The BxPC-3 and Hs700T show high to moderate sensitivity to $1,25(OH)_2D_3$, whereas the Hs766T cell line is insensitive (32,33). We also used the AsPC-1 cell line, which harbors a Ki-Ras mutation and has been shown previously to be growth inhibited by $1,25(OH)_2D_3$ (30). Quantitative, RT–PCR was performed on total RNA isolated from each cell line, using primers to the human 1α(OH)ase mRNA and β-actin mRNA as a control. As shown in Figure 3, all four cell lines express the mRNA for 1α(OH)ase, although at different levels. To further investigate whether the 1α(OH)ase that is expressed in these cells is enzymatically active, we measured 1α(OH)ase activity as described (33). As shown in Figure 3C, all four cell lines showed some conversion activity to $1,25(OH)_2D_3$ with no significant differences among them. Interestingly, there was no significant correlation between the levels of 1α(OH)ase mRNA and activity in these cell lines. Thus, all four pancreatic cell lines express 1α(OH)ase and are able to convert $25(OH)D_3$ to $1,25(OH)_2D_3$ *in vitro*.

$25(OH)D_3$ exerts antiproliferative effects on three out of four pancreatic cell lines

We next examined the antiproliferative effects of $25(OH)D_3$ and $1,25(OH)_2D_3$ on the four pancreatic cell lines. Because of the high mobility of these cells when plated in low density and of their propensity to aggregate, our attempts to perform clonogenic survival assays with these cells were not successful. Therefore, we analyzed their response to these two agents using the MTT assay. As shown in Figure 4A, treatments with increasing concentrations of $25(OH)D_3$ induced a significant dose-dependent inhibition of proliferation of three of the four cell lines. For $1,25(OH)_2D_3$, the order of sensitivity of the cell lines was BxPC-3 > AsPC-1 > Hs700T ≫ Hs766T. These results are similar to those reported by Kawa *et al.* (26) in which the order of sensitivity of the cell lines to

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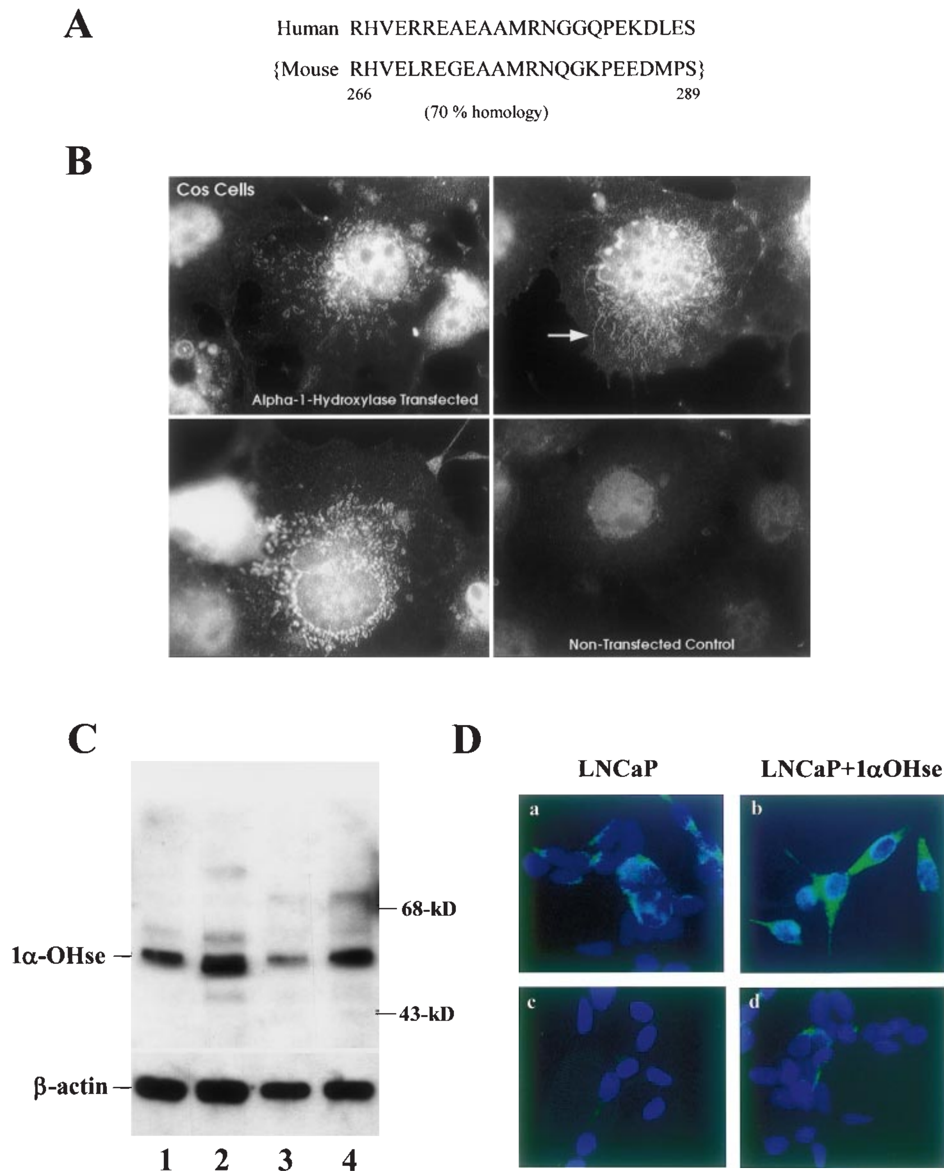

Fig. 1. Characterization of rabbit polyclonal antibody (Ab) raised against human 1α(OH)ase. (**A**) Amino acid sequence of the peptide used to raise the antibodies in rabbits which corresponds to amino acids 266–289 of the human 1α(OH)ase. The mouse sequence in the same region shares 70% homology and is shown below. (**B**) The polyclonal anti-1α(OH)ase antibody recognizes the protein when the 1α(OH)ase is transfected into COS monkey cells. The two top and bottom left panels show three different transfected cells. Arrow points to 'fiber-like' structures consistent with a mitochondrial localization of 1α(OH)ase. Non-transfected control cells (bottom right), fail to exhibit any immunoreactivity against this antibody. (**C**) Immunoblot of extracts from renal tubule epithelial cells (lane 1), BxPC-3 pancreatic tumor cells (lane 2), LNCaP prostate tumor cells (lane 3) and LNCaP cells transfected with 1α(OH)ase expression plasmid (lane 4). The membrane was incubated with the rabbit polyclonal anti-1α(OH)ase antibody (top panel), and then was stripped and re-probed with an anti-β-actin monoclonal antibody as a loading control. (**D**) Immunocytochemical detection of transfected 1α(OH)ase in LNCaP cells. Cells were either mock-transfected (**a** and **c**) or transfected with human 1α(OH)ase expression plasmid (**b** and **d**) and incubated with primary anti-1α(OH)ase antibody followed by FITC-labeled anti-rabbit secondary antibody (**a** and **b**), or only with secondary antibody (**c** and **d**). (Magnifications **b** = ×600; **d** = ×200).

$1,25(OH)_2D_3$ was BxPC-3 > Hs700T ≫ Hs766T. A similar order of sensitiviy was observed for $25(OH)D_3$. However, $25(OH)D_3$ was less potent than $1,25(OH)_2D_3$, since a ~30–50-fold higher dose of $25(OH)D_3$ was required to achieve 50% inhibition of the most sensitive cell lines BxPC-3 and AsPC-1 compared with $1,25(OH)_2D_3$ (IC_{50} values of 10–18 nM for $1,25(OH)_2D_3$ and IC_{50} 800–900 nM for $25(OH)D_3$ (Figure 4B). Hs700T cells were less sensitive to $1,25(OH)_2D_3$ and to $25(OH)D_3$ whereas the Hs766T cell line was not substantially inhibited by either treatment. The differential expression of the enzyme did not correlate with sensitivity to $25(OH)D_3$ since the Hs700T cell line, which is moderately sensitive to $25(OH)D_3$, had lower levels of the enzyme than the Hs766T cell line, whose growth was not inhibited by $25(OH)D_3$.

Lack of growth inhibition of the Hs766T cell line in response to $1,25(OH)_2D_3$ and to $25(OH)D_3$ is not due to lack of activation of VDR binding to the VDR response element (VDRE)

The dramatic differences in the response to $1,25(OH)_2D_3$ and $25(OH)D_3$ between the cell lines raised the possibility that these differences might be due to differential activity of the VDR (expression and/or activation). To investigate this

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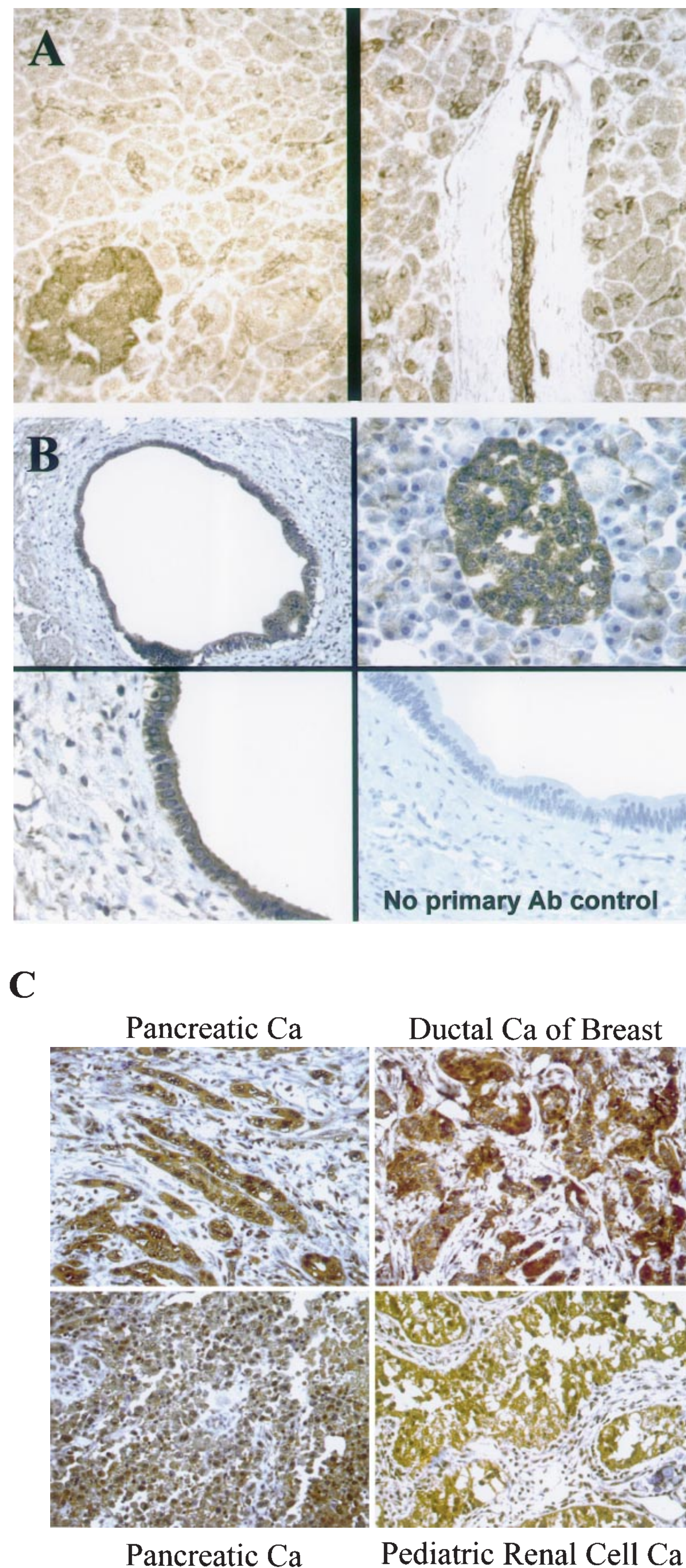

Fig. 2. (**A**) Cryostat sections showing presence of 1α(OH)ase in centroacinar cells and ducts of the pancreas, as well as in islets of Langerhans. (**B**) Paraffin-embedded sections showing expression of 1α(OH)ase in pancreatic ducts and islets. Bottom right panel: no primary antibody control staining. (**C**) Staining for 1α(OH)ase in pancreatic, ductal carcinoma of the breast and pediatric renal carcinoma. Staining was performed on paraffin-embedded tissues as described in Figure 1.

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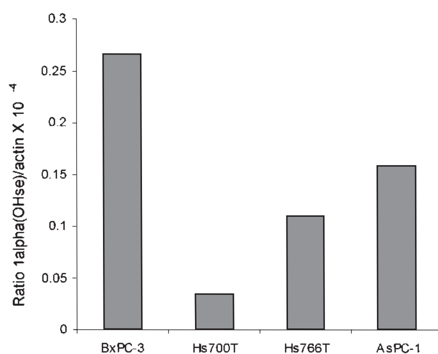
Table I. Comparison of results for staining for expression of 1α(OH)ase in this study and the study by Zehnder *et al*. (41)

	Zehnder *et al*.	Our study
Prostate epith.	ND	+
Pancreas	Islets	Islets, ducts
Kidney	Tubules	Tubules, podocytes
Colon	Epithelia, ganglia	Ganglia only
Placenta	+	ND
Adrenal medulla	+	ND
Skin	+	ND
Liver	ND	Bile ducts
Cancers	ND	+ in pancreatic, breast, renal cell carcinoma

A

	Cell line	Ct Mean	Ct Std. Dev.	SQ Mean	SQ Std. Dev.
1α-(OH)se	BxPC-3	38.1	7.76E-01	1.19E-03	6.20E-04
	Hs700T	41.9	8.41E-01	9.61E-05	5.71E-05
	Hs766T	40.2	8.57E-01	2.89E-04	1.74E-04
	AsPC-1	38.8	9.92E-01	7.73E-04	5.50E-04
β-actin	BxPC-3	15.5	3.19E-01	4.47E+01	1.12E+01
	Hs700T	16.1	1.27E-01	2.75E+01	2.68E+00
	Hs766T	16.3	8.43E-01	2.63E+01	1.30E+01
	AsPC-1	15.4	1.63E-01	4.89E+01	5.98E+00

B



C

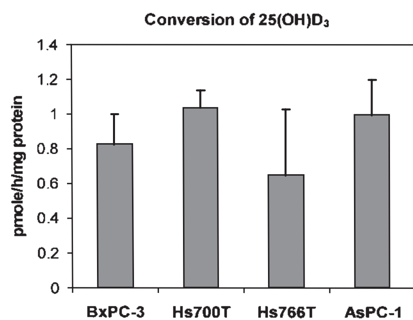
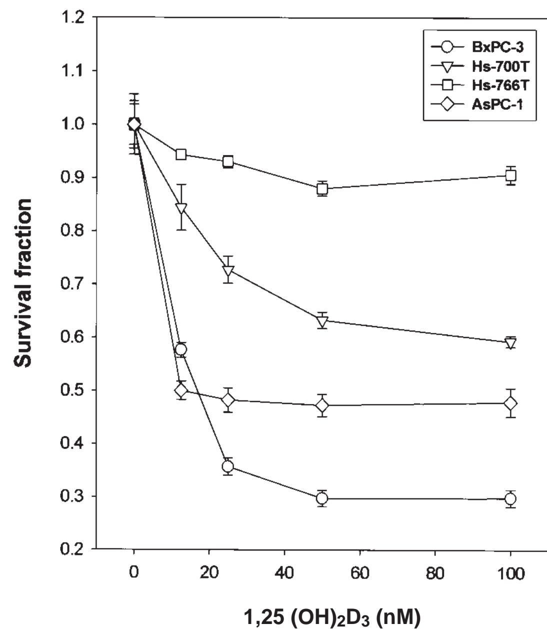


Fig. 3. Pancreatic cell lines express enzymatically active 1α(OH)ase. (**A**) RT–PCR results for 1α(OH)ase and β-actin levels in BxPC-3, Hs700T, Hs766T and AsPC-1 cells. Total RNA (5 μg) was used in cDNA synthesis by reverse transcription. Two micrograms of cDNA was then used for PCR reactions using primers against 1α(OH)ase or human β-actin. Experiments were performed in triplicate and average values for Ct (threshold cycle), and relative starting quantity are reported along with standard deviation values. (**B**) 1α(OH)ase/β-actin ratios of relative standard quantity values for BxPC-3, Hs700T, Hs766T and AsPC-1 cells. (**C**) 1α(OH)ase enzymatic activity in four pancreatic cell lines. Assays were performed on whole cell extracts from the indicated cell lines as described (33). Experiments were performed in triplicate and error bars represent SD of the means.

possibility, we performed reporter assays using plasmid constructs with the VDRE fused upstream of the chloramphenicol acetyltransferase (CAT) gene promoter. As shown in Figure 5, VDR reporter activity was significantly up regulated in all

A



B

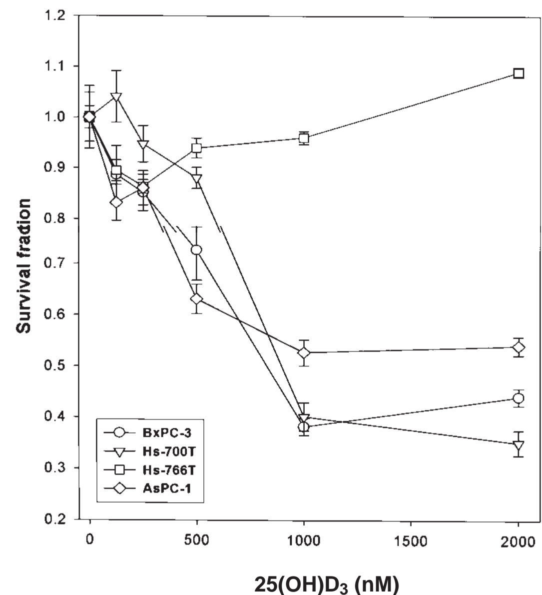


Fig. 4. $1,25(OH)_2D_3$ (**A**) and $25(OH)D_3$ (**B**) inhibit the proliferation of BxPC-3, Hs700T and AsPC-1 cells but not Hs766T pancreatic carcinoma cells. Cells were treated with increasing doses of $1,25(OH)_2D_3$ (10, 25, 50 and 100 nM) or $25(OH)D_3$ (100, 250, 500 and 1000 nM) and MTT assays were performed 6–7 days later. Results represent the average of four experiments and error bars represent ± SEM values.

three cell lines (BxPC-3, Hs700T and Hs766T). Interestingly, the unresponsive cell line Hs766T, exhibited the most robust up-regulation of VDR activity in response to $1,25(OH)_2D_3$. These results suggest strongly that the lack of growth inhibition of the Hs766T cell line is unlikely to result from a defect in the activation of the VDR, and point towards a mechanism that lies downstream of VDR activation.

Regulation of the CDK inhibitors p21 and p27 by $25(OH)D_3$ and $1,25(OH)_2D_3$

To further investigate the mechanism of differential sensitivity to $25(OH)D_3$ and $1,25(OH)_2D_3$ between the cell lines, we

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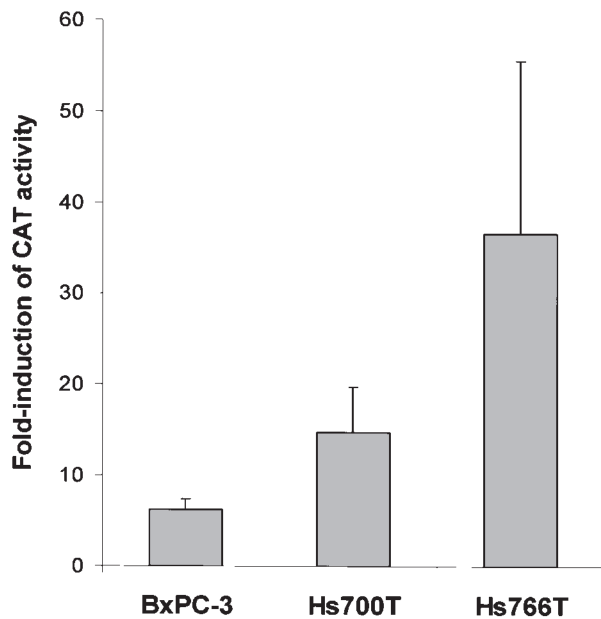

Fig. 5. VDRE reporter activity assay in pancreatic tumor cells. Cells were transfected with a plasmid carrying the VDRE fused upstream of the CAT gene and a plasmid expressing β-gal for normalization. CAT activity was assessed using cellular extracts containing equivalent amounts of β-gal activity. Fold-induction is the percent conversion of chloramphenicol in the presence of $1,25(OH)_2D_3$ divided by the percent conversion observed in cells treated with vehicle only (EtOH).

examined the effects of treatments with these agents on the up-regulation of two cell-cycle inhibitors, p21 and p27. Both p21 and p27 are inhibitors of cyclin-dependent kinase 2 (cdk2), are involved in inducing G_1/S cell-cycle arrest, and have been implicated in the antiproliferative effects of $1,25(OH)_2D_3$ and its analogs (26,34–36). The BxPC-3, Hs700T and Hs766T cell lines were treated with $1,25(OH)_2D_3$ (0.1 μM) or $25(OH)D_3$ (0.1 and 1 μM) for 48 h and the levels of p21 and p27 were analyzed by western transfer and immunoblotting. Blotting for actin was used to normalize the levels of p21 and p27 and correct for loading errors. As shown in Figure 6A, treatment with 0.1 μM $1,25(OH)_2D_3$ induced an up-regulation of p21 levels in BxPC-3 and Hs700T cells. Treatment with the same dose of $25(OH)D_3$ failed to induce a similar up-regulation of p21 in these two cell lines, but a dose of 2 μM $25(OH)D_3$ induced p21 to levels similar to those induced by 0.1 μM $1,25(OH)_2D_3$. Thus, the dose-dependent response of p21 induction by $25(OH)D_3$ followed a trend similar to that of growth inhibition in BxPC-3 and Hs700T. Although both $1,25(OH)_2D_3$, and $25(OH)D_3$ induced a small increase in p21 in Hs766T cells, neither agent was able to induce p21 to levels similar to those achieved in BxPC-3 or Hs700T cells. The Hs766T cell line exhibited substantially reduced basal levels of p21 compared with the other cell lines. The mechanism of this differential regulation of basal p21 levels is currently unknown, but cannot be attributed to p53 status, since both BxPC-3 and Hs766T cells have mutant p53.

In contrast to the induction of p21, p27 levels were up-regulated by $1,25(OH)_2D_3$, and $25(OH)D_3$ only in BxPC-3 cells (Figure 6B). As in the case of p21 induction, a 10-fold higher dose of $25(OH)D_3$ was required to induce a similar up-regulation to that induced by $1,25(OH)_2D_3$. The AsPC-1 cell line exhibited similar results as the BxPC-3 cells with robust up-regulation of p21 levels and more moderate up-regulation of p27 levels (data not shown).

$25(OH)D_3$ induces a G_1/S cell-cycle arrest in pancreatic tumor cell lines

To investigate whether the antiproliferative effects of $25(OH)D_3$ are due to cell-cycle inhibition, the three cell lines were treated with 1 μM $25(OH)D_3$ for 24 or 48 h, and cell-cycle analysis was performed. As shown in Figure 7 and Table II, $25(OH)D_3$ induced a strong G_1/S phase arrest in the BxPC-3 and Hs700T cells but only a slight arrest in the Hs766T cells that was evident only 48 h after treatment. When the percent change in G_1/S ratio at both 24 and 48 h after treatment with $25(OH)D_3$ were combined, it can be seen that the BxPC-3 cells were the most strongly inhibited (175% over EtOH treated controls), the Hs700T were second, with a 115% increase, and the Hs766T were third, with only a 43% increase. Table II summarizes the results from the cell-cycle analysis, MTT assays and p21/p27 immunoblots that demonstrate the correlation between p21/p27 induction, cell-cycle inhibition and antiproliferative effects of $25(OH)D_3$.

The three pancreatic cell lines (BxPC-3, Hs700T or Hs766T) tested thus far have wt-Ki-*ras* gene. Since mutant *Ki-ras* mutations are found in >90% of pancreatic carcinomas (37), it is possible that these cells may not be representative of human pancreatic cancer. The effects of $1,25(OH)_2D_3$ and its analogs on AsPC-1, which expresses mutant Ki-Ras protein, suggest that at least in these pancreatic tumor cells, the presence of *Ki-Ras* mutations does not preclude sensitivity to $25(OH)D_3$. To more directly investigate the possible effects of *Ki-Ras* mutation on the effects of $25(OH)D_3$, we transfected Hs700T cells with a mutant *Ki-ras* allele and obtained a stable cell line, Hs700T.*ras*. Next, we tested whether the Hs700T.*ras* cells have active Ras protein, by analyzing the phosphorylation status of the Akt protein, which is a downstream target of phosphorylation by the activated Ras pathway. The Hs700T.*ras* cells exhibited higher levels of phosphorylation of Akt compared with the parental Hs700T cells (Figure 8A), whereas the levels of total Akt remained unchanged. We then tested the antiproliferative effects of $25(OH)D_3$ on the growth of these cells. As seen in Figure 8B, these cells were growth-inhibited by $25(OH)D_3$ at levels comparable with those in the parental Hs700T cells. The data further support our findings that *Ki-ras* mutations do not appear to inhibit the antiproliferative effects of $25(OH)D_3$, at least in the cell lines tested.

Discussion

The kidneys are the principal site responsible for the synthesis of circulating $1,25(OH)_2D_3$ from its precursor, $25(OH)D_3$. However, expression of 1α(OH)ase, the enzyme that catalyzes this conversion, has been reported in non-renal tissues, including keratinocytes, prostatic cells and recently, the pancreas (38–41). Zehnder *et al.* examined the distribution of 1α(OH)ase in extra-renal tissues (41). They reported specific staining for 1α(OH)ase in skin (basal keratinocytes, hair follicles), lymph nodes (granulomata), colon (epithelial cells and parasympathetic ganglia), pancreas (islets), adrenal medulla, brain (cerebellum and cerebral cortex) and placenta (decidual and trophoblastic cells). They also reported over-expression of the enzyme in disease states including psoriatic skin and sarcoidosis. The present study demonstrates that 1α(OH)ase is expressed in normal and malignant human

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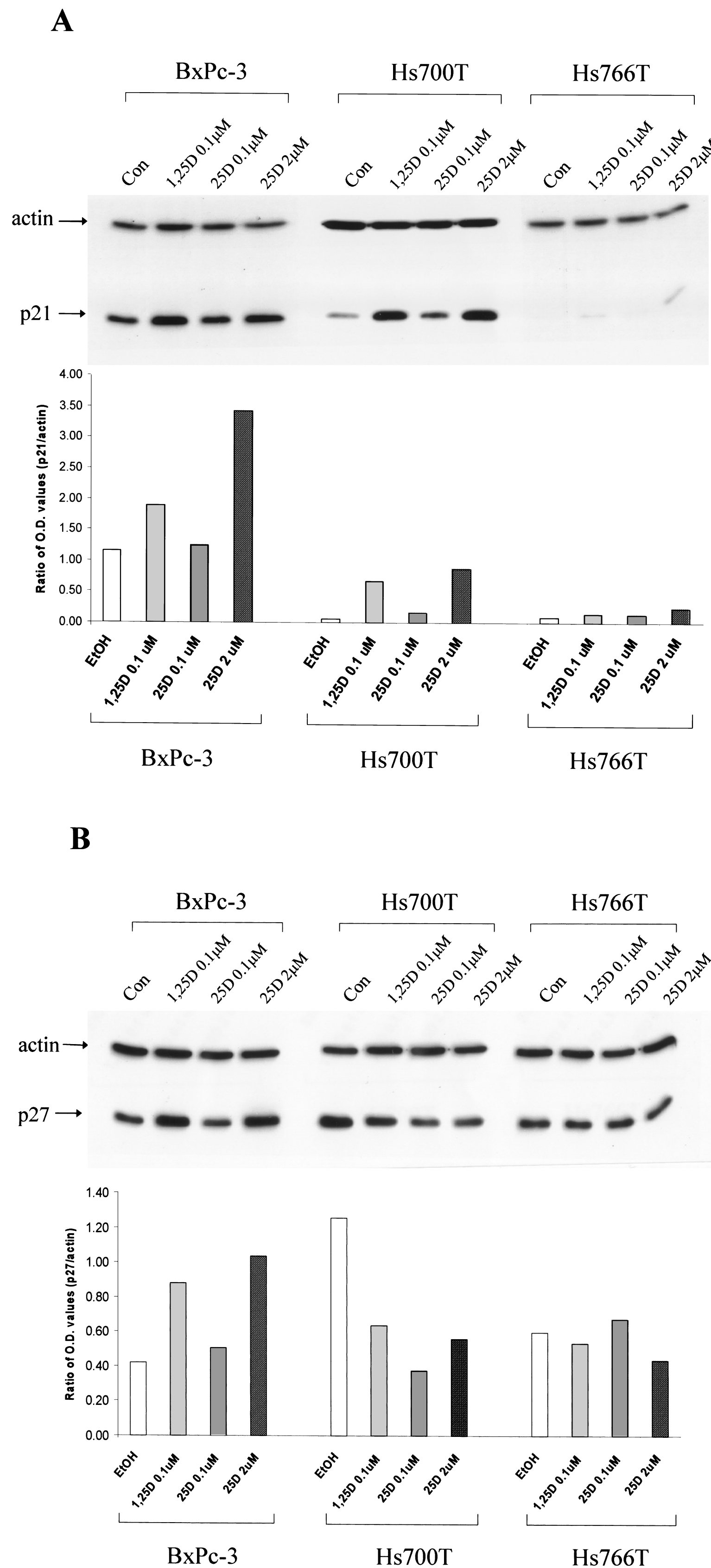

Fig. 6. 25(OH)D_3 induces p21 and p27 accumulation at the same doses that it induces inhibition of cell proliferation. Cells were treated with EtOH (vehicle control), 100 nM 1,25$(OH)_2D_3$ and 100 nM or 2 μM 25(OH)D_3. Cell lysates were obtained at 48 h after treatment from each treatment group. Immunoblotting was performed with a mouse monoclonal antibody against p21 (**A**), or mouse monoclonal antibody against p27 (**B**). Immunoblotting for actin was performed with a mouse monoclonal antibody as a loading control. Bar graphs at the bottom indicate optical density values. Values for the p21 and p27 protein levels were normalized to those for actin. The experiment was performed twice with similar results.

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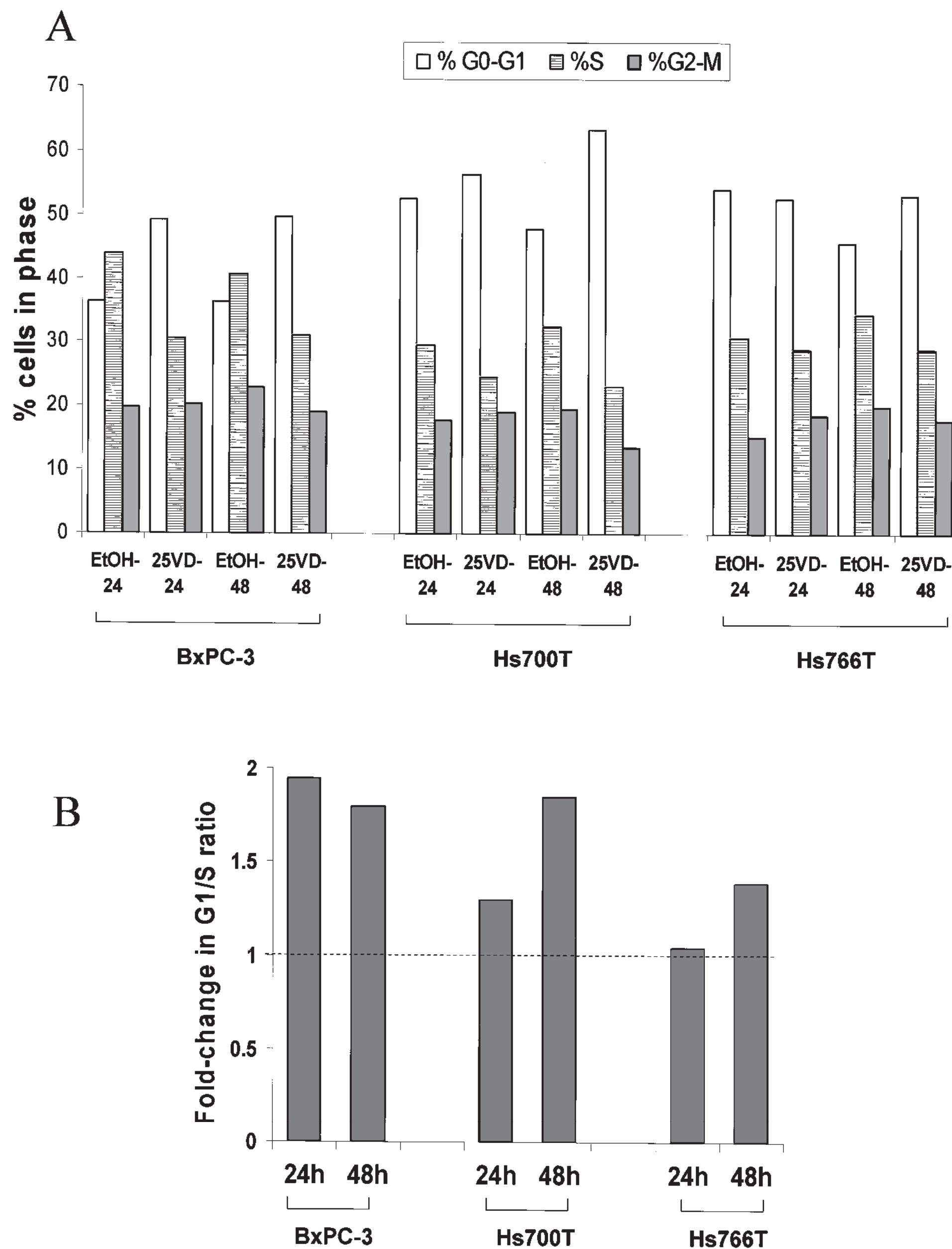

Fig. 7. Effects of $25(OH)D_3$ on the cell cycle distribution of BxPC-3, Hs700T and Hs766T cells. (**A**) Cells were treated continuously with 1 μM $25(OH)D_3$. Twenty-four or forty-eight hours after treatment began, cells were fixed and DNA content was analyzed by flow cytometry. G_1, S and G_2 phases were determined by the ModFit program. The percent cells in each phase is plotted. Results represent the average of two independent experiments. (**B**) The fold-increase in the ratio of cells in G_1/S phases before and after treatment with $25(OH)D_3$.

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pancreatic tissue and in various pancreatic cell lines. Thus, the pancreas joins a growing list of extrarenal tissues in which 1α(OH)ase expression has been documented (29,38–41). To our knowledge, this is the first demonstration of 1α(OH)ase expression in pancreatic tumor cell lines and in pancreatic adenocarcinoma. These findings support the possible therapeutic use of $25(OH)D_3$ for pancreatic cancer. Although Zehnder *et al*. reported 1α(OH)ase expression only in the pancreatic islets (41), we found strong expression in both the islets and the ducts. Although there is some controversy about the cell of origin of pancreatic neoplasms (42), ~90% of malignant pancreatic neoplasms are classified as ductal adenocarcinomas (37,42), a fact which underscores the potential preventive and therapeutic implications of these findings.

Our immunohistochemical detection of 1α(OH)ase expression in pancreatic normal and malignant tissue is not quantitative. However, our data suggest that the levels of expression in tumor cells are sufficient to confer sensitivity to the antiproliferative effects of the prohormone $25(OH)D_3$. Three of four tumor cell lines were growth inhibited by $25(OH)D_3$, at doses that are ~30–50-fold higher than those required for $1,25(OH)_2D_3$, to exert a similar effect. Similar results were reported recently in *ras*-transformed keratinocytes, whose growth was inhibited by 50–60% by 1 μM doses of $25(OH)D_3$ (52). Secondly, all four cell lines demonstrated conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ with similar efficiencies. Notably, the activity found in pancreatic cell lines [average of 0.65–1.2 pmol of $1,25(OH)_2D_3$/mg protein/h], is higher than that found in prostate

Table II. Summary of the effects of $25(OH)D_3$ on the cell cycle analysis, MTT assays and p21/p27 immunoblots

	BxPc-3	Hs700T	Hs766T
Cumulative % change is G_1/S ratio after 24 and 48 h of $25(OH)D_3$	175	115	43
% Growth inhibition by 1 μM $25(OH)D_3$	70	45	10
Induction of p21 and/or p27	p21 and p27	p21	–

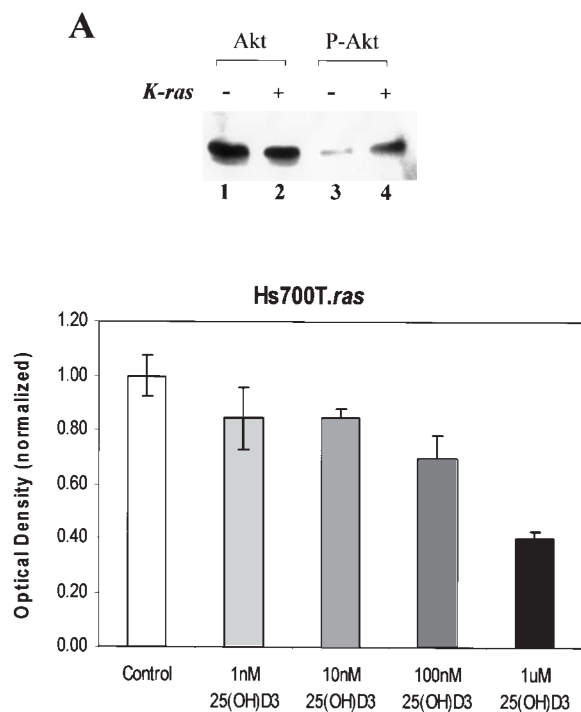

Fig. 8. Pancreatic cells expressing mutant Ki-*ras* are inhibited by $25(OH)D_3$. (**A**) Phosphorylation status of Akt in Hs700T (lanes 1 and 3) and Hs700T.*ras* (lanes 2 and 4). Membrane was incubated with rabbit polyclonal antibodies against total Akt (lanes 1 and 2) or against phospho-Akt (ser473) (lanes 3 and 4). (**B**) Effect of $25(OH)D_3$ on Hs700T.*ras* cells. MTT assays were performed as in Figure 4.

cancer cell lines DU145 and PC-3 (average of 0.31 and 0.07 pmol/mg protein/h, respectively) but is lower than normal and benign prostatic hyperplasia primary cultures and keratinocyte cultures (average of 3.08, 1.05 and 2.1 pmol/mg protein/h, respectively) (29). In general, cell lines have lower activity than primary cultured cells, including keratinocytes, normal prostate cells and renal proximal tubular cells (43). Taken together, the immunohistochemical, biochemical and antiproliferative data indicate that human pancreatic cancer cell lines express functional 1α(OH)ase (29,44).

It is possible that some of the effects of $25(OH)D_3$ are due to its binding to the VDR. However, since the affinity of $25(OH)D_3$ for the VDR is between 1/500 and 1/1000th of the affinity of $1,25(OH)_2D_3$ (45), it is more likely that the effects of $25(OH)D_3$ are due to its conversion to $1,25(OH)D_3$. Furthermore, Huang *et al*. demonstrated recently that targeted deletion of both alleles of 1α(OH)ase from Ras-transfromed keratinocytes abolished most of growth inhibition induced by $25(OH)D_3$ (46). These results strongly suggest that most of the antiproliferative activity of $25(OH)D_3$ requires intracellular conversion of $1,25(OH)_2D_3$ to $25(OH)D_3$.

The differential sensitivity of the four pancreatic cell lines to $25(OH)D_3$ was not due to differences in VDR activity, since the non-responding cell line Hs766T displayed the highest levels of VDR activity as determined by a reporter activity assay. On the other hand, the sensitivity of the four cell lines to the antiproliferative effects of $25(OH)D_3$ tended to correlate with the levels of induction of the cell cycle inhibitors p21 and p27. The exact mechanism by which $1,25(OH)_2D_3$ and its prohormone elicit their antiproliferative responses has not been firmly established. However, strong evidence suggests a central role for p21 and p27 CDKIs in the antiproliferative properties of these agents. First, induction of p21 and p27 has been observed after treatment of other tumor cell lines, such as prostate, breast, colorectal and leukemic tumor cells with $1,25(OH)_2D_3$ or $25(OH)D_3$ and these increases of CDKI levels are consistent with a concomitant inhibition of the cell cycle at the G_1/S interface (26,35,36,47–50). Secondly, reduction of p21 or p27 levels by antisense cDNA strongly inhibited the antiproliferative effects of $1,25(OH)_2D_3$ in prostate and leukemic tumor cells (47). Thirdly, p27 was found to be required for the antiproliferative effects of $1,25(OH)_2D_3$ on mouse embryonic fibroblasts, since primary MEFs from $p27^{-/-}$ mice failed to exhibit inhibition of proliferation with 100 nM $1,25(OH)_2D_3$ (51). Although our data on p21/p27 induction and cell-cycle arrest collectively point towards a critical role for the CDKIs in the antiproliferative effects of $1,25(OH)_2D_3$ and $25(OH)D_3$, other possible mechanisms including the induction of apoptosis and differentiation as possible mediators of these effects cannot be excluded.

Of the four cell lines tested, the Hs766T cells were almost completely unresponsive to the effects of $1,25(OH)_2D_3$ and $25(OH)D_3$. This cell line also failed to increase p27 or p21 levels in response to these treatments, and displayed the lowest increase in G_1/S ratio following 24 and 48 h treatments with these agents. Moreover, the basal levels of p21 in this cell line were substantially lower than those in the other three cell lines. The reason for this decrease in basal p21 levels is not yet clear, but one potential mechanism might involve methylation of the p21 promoter in this cell line. Interestingly, pre-treatment of Hs766T cells with the histone deacetylase inhibitor Trichostatin A restored induction of p21 levels and responsiveness to $1,25(OH)_2D_3$ (Eads *et al*., unpublished results), suggesting that the p21 promoter may indeed be methylated in this cell line. Our results regarding the induction of p21 and p27 are in agreement with those of Kawa *et al*. (26), who reported similar effects of $1,25(OH)_2D_3$ and 22-oxa-1,25-dihydroxyvitamin D_3 on the BxPC-3, Hs700T and Hs766T cell lines.

The role of Ras activating mutations on the effects of $1,25(OH)_2D_3$ and its analogs has been controversial. The activated Ras protein has been suggested to inhibit the function of $1,25(OH)_2D_3$ and its analogs in certain cell lines. For example, transformation of immortalized keratinocytes with the *Ha-ras* oncogene resulted in a partial resistance to the growth inhibitory effects of $1,25(OH)_2D_3$ and this resistance was attributed to a Ha-Ras-induced phosphorylation of the RXR and disruption of the VDR/RXR complex in the malignant keratinocytes (52,53). However, it was shown recently that $25(OH)D_3$ could inhibit the growth of mouse keratinocytes transformed with *Ha-ras in vitro* and *in vivo* in a manner that was completely dependent on the presence of 1α(OH)ase (46). The *in vitro* inhibition occurred

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at doses of 1 μM, which produces significant inhibition in the pancreatic cancer cell lines. Our data with the pancreatic epithelial cell line AsPC-1, which has mutant *Ki-Ras* and the *Ki-ras* transformed Hs700T cells, also suggest that activating Ras mutations in pancreatic tumor cells do not impair the growth-inhibiting properties of 25(OH)D_3. The proliferation of both mutant Ki-Ras-containing cell lines was inhibited by this agent at levels that were comparable with those achieved for the most sensitive cell line with wt-*Ki-ras*, BxPC-3. These results are in agreement with other published studies which describe the antiproliferative effects of 1,25(OH)$_2D_3$ and EB1089 on pancreatic tumor cell lines (25,27,28). The lack of an inhibitory effect of *Ki-ras* mutations on the antiproliferative effects of 25(OH)D_3 may reflect a difference in the activity of this gene in keratinocytes versus pancreatic cells, or to the differential biochemical properties of the two isoforms. For example, Ha-Ras and Ki-Ras were shown to localize in different cellular compartments and to display distinct profiles of activation of downstream effector gene products (54,55).

In summary, we suggest that the presence of 1α(OH)ase in normal and tumor pancreatic cells may enable the development of new forms of vitamin D-based interventions for cancers of this organ. Such interventions are based on the observations that: (i) pancreatic cells express VDR and 1α(OH)ase; (ii) dietary supplementation of rats with vitamin D (cholecalciferol) and calcium reduces the proliferation of pancreatic normal epithelial cells (56); (iii) 1,25(OH)$_2D_3$ and 1,25(OH)$_2D_3$ analogs inhibit the proliferation of pancreatic cancer cells *in vitro* and *in vivo*. The large therapeutic window of 25(OH)D_3 compared with 1,25(OH)$_2D_3$, and the availability and safety of oral formulations of 25(OH)D_3 should enable the rapid entry of this pro-hormone into pre-clinical studies on the treatment and prevention of pancreatic cancer. Finally, because 25(OH)D_3 is produced in the body from vitamin D (i.e. cholecalciferol and ergocalciferol), vitamin D itself—which is very inexpensive and (at non-pharmacologic doses) very safe—could play a role in the prevention of pancreatic cancer. The conversion of vitamin D to 25(OH)D may help explain the recent demonstration that the addition of vitamin D and calcium to the diet in rats dramatically reduced the rate of proliferation of pancreatic epithelial cells that was induced by a 'Western' (i.e. high fat) diet (56).

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