ARTICLE COVERSHEET

LWW_CONDENSED-FLA(7.75X10.75)

SERVER-BASED

Template version : 10.2
Revised: 02/18/2013

Article : MPA21775
Creator : jbutac
Date : Thursday March 14th 2013
Time : 23:30:02
Number of Pages (including this page) : 12

Modulation of Pancreatic Tumor Potential by Overexpression of Protein Kinase C β1

Stéfano M. Cirigliano, MSc, Laura V. Mauro, PhD,* Valeria C. Grossoni, PhD,* Lucas L. Colombo, MD, PhD,* Miriam J. Diament, MSc,* Marcelo G. Kazanietz, PhD,† Elisa D. Bal de Kier Joffé, MD, PhD,* Lydia I. Puricelli, PhD,* and Alejandro J. Urtreger, PhD**

Objective: This study aimed to investigate whether the overexpression of protein kinase C β1 (PKCβ1) is able to modulate the malignant phenotype displayed by the human ductal pancreatic carcinoma cell line PANC1.

Methods: PKCβ1 overexpression was achieved using a stable transfection approach. PANC1-PKCβ1 and control cells were analyzed both in vitro and in vivo.

Results: PANC1-PKCβ1 cells displayed a lower growth capacity associated with the down-regulation of the MEK/ERK pathway and cyclin expression. Furthermore, PKCβ1 overexpression was associated with an enhancement of cell adhesion to fibronectin and with reduced migratory and invasive phenotypes. In agreement with these results, PANC1-PKCβ1 cells showed an impaired ability to secrete proteolytic enzymes. We also found that PKCβ1 overexpressing cells were more resistant to cell death induced by serum deprivation, an event associated with G0/G1 arrest and the modulation of PI3K/Akt and NF-κB pathways. Most notably, the overexpression of PKCβ1 completely abolished the ability of PANC1 cells to induce tumors in nude mice.

Conclusions: Our results established an important role for PKCβ1 in PANC1 cells suggesting it would act as a suppressor of tumorigenic behavior in pancreatic cancer.

Key Words: human pancreatic adenocarcinoma, PKCβ1, tumor growth inhibition

Abbreviations: CM - conditioned media, DAG - diacylglycerol, Dox - doxorubicin, ERK - extracellular signal–regulated kinase, FCS - fetal calf serum, GSK3β - glycogen synthase kinase 3 β, MAPK - mitogen-activated protein kinase, MMP - matrix metalloproteinase, NF-κB - nuclear factor-κB, PAGE - polyacrylamide gel electrophoresis, PBS - phosphate-buffered saline, PKC - protein kinase C, PMA - phorbol 12-myristate 13-acetate, PI3K - phosphatidylinositol-3-kinase, RPMI - Roswell Park Memorial Institute medium, SDS - sodium dodecyl sulfate, uPA - urokinase-type plasminogen activator

(*Pancreas* 2013;00: 00–00)

From the *Research Area, Institute of Oncology "Angel H. Roffo," University of Buenos Aires, Buenos Aires, Argentina; and †Department of Pharmacology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA.

Received for publication July 2, 2012; accepted February 28, 2013.

Reprints: Alejandro J. Urtreger, PhD, Research Area, Institute of Oncology "Angel H. Roffo," Av San Martín 5481, (C1417DTB) Buenos Aires, Argentina (e-mail: urtreger@yahoo.com.ar).

This work was supported by the PICT-11217 BID 1728 OC-AR grant from the ANPCyT.

The authors declare no conflict of interest.

Elisa D. Bal de Kier Joffé, Lucas L. Colombo, Lydia I. Puricelli, and Alejandro J. Urtreger are members of the Scientific Research Career of the National Council for Scientific and Technical Research (CONICET).

Lydia I. Puricelli and Alejandro J. Urtreger equally contributed to the direction of this study.

Pancreatic cancer has the poorest prognosis of any common gastrointestinal malignancy, with a 5-year overall survival of less than 5% and a median survival time after diagnosis of less than 6 months.[1] Because of the intrinsic aggressive nature of pancreatic cancers, the difficulties in diagnosis, and the limited therapeutic options, pancreatic cancer is the fourth leading cause of cancer death overall in the United States.[2] Despite the significant advances in the molecular basis and pathogenesis of the pancreatic cancer, there is a great need to identify novel "druggable" targets.[3] Members of the protein kinase C (PKC) family have emerged in the last years as important modulators of transformation and malignant progression of pancreatic cancers.[4,5]

Protein kinase C comprises a family of at least 11 lipid-dependent serine/threonine kinases that influence a wide range of cellular functions, including proliferation, differentiation, and apoptosis.[6–8] On the basis of their structural similarities and cofactor dependence PKC isoforms have been grouped into 3 families[9,10]: classical PKC isozymes (α, β, and γ), which can be stimulated by Ca^{2+} and the lipid second messenger diacylglycerol (DAG) or phorbol esters; novel PKC isozymes (δ, ε, η, and Θ), which can be activated by DAG or phorbol esters but are Ca^{2+} independent; and atypical PKC isozymes (ζ and λ/ι), which are unresponsive to Ca^{2+}, DAG, and phorbol esters. Alterations in the expression of PKC isozymes have been reported in numerous human cancers.[11–13] Moreover, there is a large body of evidence linking PKCs with signaling pathways involved in cancer progression and metastatic dissemination of cancer cells.[14,15] Indeed, members of the various PKC families have been associated with altered expression of matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA).[14–16] These enzymes play crucial roles in normal tissue remodeling; however, they display altered expression or activity in cancer cells, therefore contributing to the enhanced cell motility and invasiveness that characterized the metastatic phenotype.

The role of PKCβ1 in malignant transformation is controversial. PKCβ1 overexpression in rat fibroblasts results in transformed cells that exhibit anchorage-independent growth properties and form tumors when inoculated into nude mice.[17] PKCβ1 is also involved in the attenuation of apoptotic responses in gastric and hepatocellular carcinomas.[18,19] In contrast, PKCβ1 overexpression reduced the tumorigenic potential of colon cancer and breast cancer cells,[16,20] and also induced growth arrest and apoptosis of myeloid cells.[21] There are only few studies addressing the expression levels of PKCβ1 in human ductal pancreatic cancer, and its role in the progression of the disease is almost unknown.[22]

The main goal of the present work was to analyze whether aberrant expression of PKCβ1 alters the malignant phenotye of a human pancreatic tumor–derived cell line (PANC1). As an experimental approach, we stably overexpressed this classical PKC isozyme in pancreatic cells and analyzed the phenotypic consequences of such overexpression, both in vivo and in vitro. Our experiments revealed that PKCβ1 abrogates the malignant

phenotype of PANC1 when cells were inoculated into nude mice. Signaling studies demonstrated that PKCβ1 downregulates the activation of the MEK/ERK pathway, leading to profound effects on the ability of PANC1 cells to proliferate, adhere, migrate, and secrete proteases associated with the invasive behavior. In addition, PKCβ1 overexpression had prominent effects on survival of pancreatic cancer cells through the modulation of phosphatidylinositol-3-kinase (PI3K)/Akt and nuclear factor (NF)-κB pathways.

MATERIALS AND METHODS

Reagents and Antibodies

Culture medium, agarose, Geneticin, and Lipofectamine Plus, were obtained from Life Technologies Inc (Rockville, Md). Fetal calf serum (FCS) was from GEN (Buenos Aires, Argentina). Plasminogen was purchased to Chromogenix (Molndal, Sweden) and gelatin from Sigma Co (St Louis, Mo). Human urokinase was a gift from Serono Argentina S.A. (Buenos Aires, Argentina). Triton X-100 was obtained from J. T. Baker (Phillipsburg, NJ). Monoclonal anti-PKCα, β, ε, and ζ antibodies were purchased from BD Biosciences (San Diego, Calif). The anti-PKCε (anti-ε-tag) antibody was obtained from Life Technologies Inc. Monoclonal antibodies for ERK, phospho-ERK (pERK), NF-κB (p65), and IκBα and polyclonal antibodies for actin, cyclin D1, cyclin D2, and p21 were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). Monoclonal antibodies for Akt, phospho-Akt (pAkt, Ser 473), glycogen synthase kinase 3 β (GSK3β), and phospho-GSK3β (pGSK3β, Ser9) were purchased from Cell Signaling Technology (Danvers, Mass). Horseradish peroxidase conjugated antimouse antibodies was obtained from Sigma Co. Hybond-P membranes for blotting and chemiluminescence reagents (ECL) were from GE Healthcare Bio-Sciences (Little Chalfont, UK). Acrylamide and all other reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad (Richmond, Calif).

Cell Line and Culture Conditions

PANC1, a human pancreatic ductal carcinoma cell line (ATCC, Manassas, Va) was cultured at 37°C in RPMI1640 medium supplemented with 10% FCS and 80 μg/mL gentamicin in a humidified air atmosphere with 5% CO_2.

Expression Vectors, Transfection, and Selection

PANC1 cells were stably transfected with 5 μg of pMTH-PKCβ1, a mammalian expression vector encoding for PKCβ1, using Lipofectamine Plus (PANC1-PKCβ1). Cells transfected with the empty vector (pMTH) were used as control (PANC1-vector). All the expression constructs possess an ε-tag insert sequence that encodes for a KGFSYFGEDLMP peptide, derived from the last 12 amino acids of PKCε.[23] The ε-tagged proteins can be readily detected with a commercially available antibody specific for the ε-peptide. Forty-eight hours after transfection, cells were selected with 500 μg/mL of G418. After a selection period of 15 days, approximately 30 resistant clones were pooled to avoid clonal variations.[16]

Western Blot

Semiconfluent monolayers were washed twice with ice-cold PBS and then lysed with 1% Triton X-100 in PBS by scraping with a Teflon scraper. Samples were denatured by boiling in sample buffer with 5% β-mercaptoethanol and run in 10% SDS-PAGE. Fifty microgram of protein was loaded in each lane. Gels were blotted to Hybond-P membranes. After incubation for 1 hour in PBS containing 5% skim milk with 0.1% Tween-20, membranes were incubated with the first antibody overnight at 4°C, and then for 1 hour with a secondary antibody coupled to horseradish peroxidase. Detection was performed by chemiluminescence. Bands were digitalized with a Foto/Analyst Express System (Fotodyne Inc, Hartland, Wis) and signal intensity was quantified with Gel-Pro Analyzer software. When both the phosphorylated and total forms of a protein were studied, the same membrane was blotted initially with the antibody against the phosphorylated form, subsequently stripped, and finally probed with the antibody against the total protein.

Determination of NF-κB and IκB Expression Levels

NF-κB levels in cytoplasm and nucleus and IκB levels in cytoplasm were analyzed by Western blot. Nuclear and cytoplasmic fractions were separated using NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce Biotechnology, Rockford Ill). Briefly, subconfluent monolayers growing in 100-mm Petri dishes were trypsinized and centrifuged (500*g*, 5 minutes). The supernatant was discarded and the pellet was resuspended in the cytoplasmic extraction reagent I buffer (CERI). Then CERII buffer were added and the mixture was centrifuged (13,000*g*, 5 minutes). Supernatant corresponds to the cytoplasmic protein fraction, and the remaining pellet was resuspended in nuclear extraction reagent buffer (NER) to obtain the nuclear protein fraction. Protein content in each fraction was determined, and samples were aliquoted and stored at −80°C, and used only once after thawing.

NF-κB-Dependent Reporter Gene Expression Assay

PANC1-PKCβ1 and PANC1-vector cells were transiently cotransfected with NF-κB-Luc reporter plasmid and *Renilla* Luciferase Control Reporter Vector (pRL-CMV; Promega, Madison, Wis) in a 10:1 ratio, using Fugene and following standard protocols. Briefly, 4×10^5 cells were transfected in suspension and then 5×10^4 cells were seeded onto individual wells of 24-well plates, subjected or not to serum starvation, and lysed 48 hours later. Luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega) and normalized to the constitutive *Renilla* luciferase activity.

Cell Proliferation

Population doubling time was determined during the exponential growth phase of unsynchronized monolayer cultures. Briefly, 3×10^3 cells/well were seeded onto 96-well plates in RPMI1640 supplemented with 10% FCS and 80 μg/mL gentamicin. Cell viability was assessed at different times after seeding using the MTS assay (Celltiter 96 Non Radioactive Proliferation Assay; Promega) according to vendor's indications.[5]

Susceptibility to Cell Death

Subconfluent monolayers growing in 96-well plates were extensively washed with PBS and subjected to serum starvation (48 hours), or treated for 2 hours with 2 to 6 μM doxorubicin (Dox), washed twice with PBS, and subsequently incubated in medium with 10% FCS for 48 hours. A combined starvation-Dox treatment was also performed. In this case, cells were starved for 24 hours and then treated with Dox as previously mentioned. Cell viability was evaluated with the MTS assay.

Analysis of Cell Cycle Distribution by Flow Cytometry

Cells were serum starved overnight and then washed with PBS, detached, fixed with 75% ice-cold ethanol and stained with 50 μg/mL propidium iodide. DNA content was analyzed

by flow cytometry using an Epics Elite ESP Coulter cytometer (Beckman Coulter, Fullerton, Calif).

Adhesion Assay

Petri dishes (35 mm) were coated with human fibronectin (16 μg/mL) (Sigma Co) for 1 hour at room temperature. To avoid the adhesion to uncoated surfaces, wells were further blocked with BSA (1 μg/mL) for 1 hour at room temperature. Suspensions containing 4×10^5 cells were seeded in FCS-free culture media and incubated at 37°C. After 60 minutes, medium was removed and adherent cells were washed twice with PBS, trypsinized, and counted using a hemocytometer. The adhesion efficiency was expressed as the percentage of the total number of seeded cells.

Invasion Assay

Transwell cell culture chambers (Corning, Lowell, Mass) were used for invasion assay. Filters (8-μm membrane pores) were coated with 0.1% gelatin on the lower side and with a thin layer (250 μg/mL) of Matrigel (Becton Dickinson Labware, Bedford, Mass) on the upper side. The lower chamber contained 0.5 mL of culture media supplemented with human fibronectin (16 μg/mL) (Sigma Co) as chemoattractant. Cells (2×10^4) were seeded in the upper chamber and 48 hours later the upper surface of the filter was removed and wiped using a cotton swab. Finally, membranes were fixed in Carnoy and stained with Höechst 33258. Cells that invaded Matrigel, passed through the pores, and reattached on the lower surface of the filter were considered as invasive and their fluorescent nuclei were counted using an epifluorescence microscope ×400 (Eclipse E400, Nikon).

Migratory Capacity

Wounds of approximately 400-μm width were made in subconfluent monolayers, and cells were then allowed to migrate into the cell-free area for a period of 24 hours in low FCS culture media (2%). The same spot was photographed at time 0 and 24 hours, and the migratory area was determined using the Image-ProPlus 4.5 software. Cell migration was expressed as the percentage of the area occupied by the migratory cells in the original cell-free wounded area.

Quantification of uPA and MMPs Activities of Conditioned Media

Secreted uPA, MMP-2, and MMP-9 activities were evaluated in conditioned media (CM) as previously described.[15] Briefly, semiconfluent cell monolayers growing in 35-mm plastic Petri dishes were extensively washed with PBS. Serum-free medium (1 mL) was added and incubation was continued for 24 hours. Conditioned media were individually harvested, the remaining monolayers were lysed with 1% Triton-X100 in PBS, and cell protein content was determined using the Bio-Rad Protein Assay. Samples of CM were stored at −40°C and used only once after thawing.

To determine uPA activity, a radial caseinolysis assay was used, as previously described.[24] Briefly, 4-mm wells were punched in the plasminogen-rich casein-agarose gels and 10 μL of CM were loaded. Gels were incubated for 24 hours at 37°C in a humidified atmosphere. The diameter of lytic zones was determined, and the areas of degradation were referenced to a standard curve of purified urokinase (0.1–50 IU/mL) and normalized to the original cell culture protein content.

Matrix metalloproteinase activity was determined on substrate-impregnated gels, as previously described by Aguirre Ghiso et al.[25] Briefly, CM samples were run on 9% SDS polyacrylamide slab gels containing 1 mg/mL of gelatin, under nonreducing conditions. After electrophoresis, gels were washed for 30 minutes in 2.5% Triton X-100 and subsequently incubated for 48 hours at 37°C in a buffer containing 0.25 M Tris-HCl pH 7.4, 1 M NaCl, and 25 mM $CaCl_2$. Nonspecific activity was detected using gels incubated in the same buffer solution but supplemented with 40 mM EDTA. After incubation, gels were fixed and stained with Coomassie Brilliant Blue G-250. Gelatinolytic bands were digitalized with a Foto/Analyst Express System (Fotodyne Inc) and signal intensity was quantified with Gel-Pro Analyzer software. Data were expressed as arbitrary units and normalized to the original cell culture protein content.

Animal Studies

In vivo experiments were carried out using 2-month old BALB/c nude mice obtained from the Animal Care Area of "Comisión Nacional de Energía Atómica." Mice were housed 5 per cage, kept under an automatic 12-hour light/12-hour darkness schedule, and provided with sterile pellets and tap water ad libitum. All animals studies were conducted in accordance with the standards of animal care as outlined in the NIH Guide for the Care and Use of Laboratory Animals.

Analysis of tumor cell growth and metastasis was performed essentially as described.[16] Briefly, PANC-PKCβ1 and PANC-pMTH cells were harvested from subconfluent cultures during the exponential growth phase by treatment with trypsin-EDTA, washed thoroughly with RPMI, and resuspended in the same medium. Nonanesthetized nude mice (n = 10) were inoculated subcutaneously (sc) into the left flank with 6×10^6 cells in 0.3 mL RPMI. The size of the tumors was measured with a sliding caliper twice a week by measuring length (L) and width (W), and volume was calculated using the formula $V = ¾ (\pi \times L \times 2W)$, where L is the longest and W is the shortest diameter. Mice were killed 80 days after tumor inoculation and tumors fixed in 10% formalin and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin for histopathological studies. The lungs, liver, kidney, and spleen were also fixed and examined for the presence of metastatic nodules.

Statistical Analysis

All assays were performed in triplicate, and independent experiments repeated at least twice. Statistical differences between groups were calculated by applying Student t or Kruskal-Wallis tests, as indicated. A value of $P < 0.05$ was considered to be significant.

RESULTS

Overexpression of PKCβ1 in PANC1 Cells

To investigate whether the overexpression of the classical PKCβ1 isozyme can modulate the malignant phenotype potential of PANC1 cells, a human ductal pancreatic tumor-derived cell line, we used a stable transfection approach. Overexpression of PKCβ1 was achieved by transfecting PANC1 cells with the expression vector pMTH-PKCβ1 (tagged with an ε-epitope tag, see Olah et al[23]) followed by selection with G418. To avoid clonal variations, G418-resistant clones of PKCβ1 overexpressing cells (PANC1-PKCβ1) were pooled 2 weeks after transfection. The same cell line transfected with the empty vector (pMTH) was used as control (PANC1-vector). Western blot analysis using the anti-ε-tag antibody revealed the presence of an 80-kd band corresponding to the ε-tagged PKCβ1 in PANC1-PKCβ1 cells but not in cells transfected with the vector alone (Fig. 1A, upper panel). F1 Using this stable transfection approach, we achieved 2.4-fold increase in PKCβ1 expression levels, relative to vector transfected cells (Fig. 1A, second panel, B). Overexpression of PKCβ1 did not alter the expression of α, ε, and ζ PKC isozymes expressed by PANC1 cells (Fig. 1A, B). PANC1 cells express very low levels

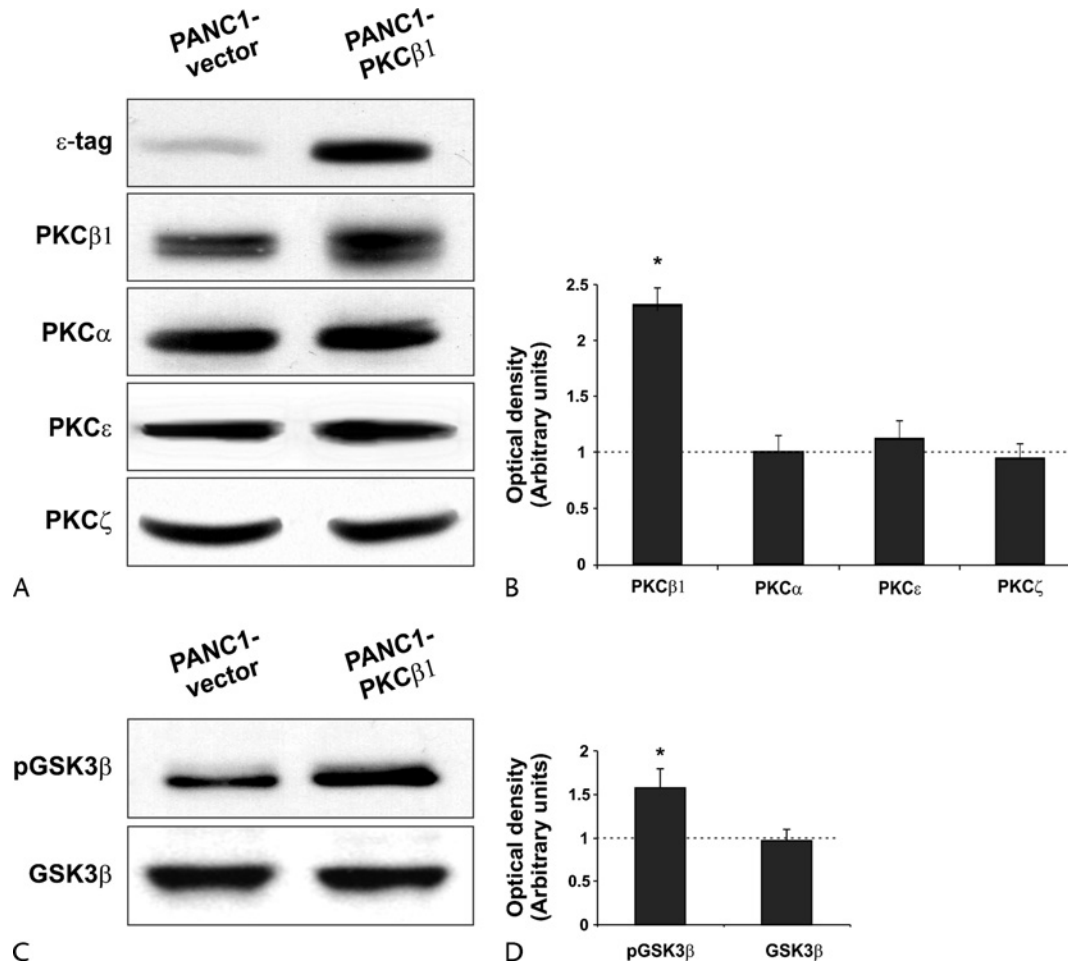


FIGURE 1. Panel A, Expression of PKCβ1 in PANC1 cells. in PANC1 cells. Whole cell lysates prepared from PANC1 cells transfected with either pMTH-PKCβ1 or pMTH vector alone were resolved on 10% SDS-PAGE and blotted with anti-ε-tag antibodies or with antibodies against individual PKC isozymes (50 μg protein/lane). Panel B, Densitometric analysis of panel A. Western blot bands corresponding to 3 independent experiments were measured with a digital densitometer. Results were expressed in arbitrary units as relative to vector-transfected cells (*dotted line*). Data were expressed as the mean (SD). *$P < 0.05$ versus PANC1-vector cells (Student *t* test). Panel C, PKCβ1 overexpression induces the phosphorylation of GSK3β at serine 9. PANC1 cells transfected with either pMTH-PKCβ1 or pMTH vector were resolved on 10% SDS-PAGE and blotted with specific phospho-GSK3β or total GSK3β antibodies (50 μg protein/lane). Panel D, Densitometric analysis of panel C. Western blot bands corresponding to 3 independent experiments were measured with a digital densitometer. Results were expressed in arbitrary units as relative to vector-transfected cells (*dotted line*). Data were expressed as the mean (SD). *$P < 0.05$ versus PANC1-vector cells (Student *t* test).

of PKCδ [5] and the transfection procedure did not alter these endogenous levels (data not shown). Pooled PKCβ1-transfected PANC1 cells from other independent transfection experiments showed similar PKC expression levels and biological properties as described later.

As it is known that active PKCβ phosphorylates GSK3β at serine 9,[26] next, we evaluated the status of GSK3β phosphorylation in our cell lines. Figure 1C and D shows a significant increase in pGSK3β levels in PKCβ1 overexpressors as compared to control transfected cells. Total GSK3β levels remained unchanged. From these data, we conclude that the transfection of PKCβ1 into PANC1 cells results in an efficient expression of a functional PKCβ1.

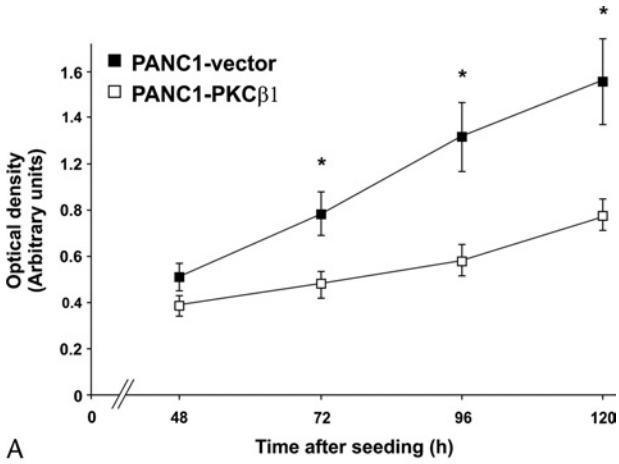
Effect of PKCβ1 on PANC1 Cell Proliferation

Analysis of proliferation revealed that PKCβ1 overexpression induced a marked increase in the population doubling time of PANC1 unsynchronized monolayers as compared to control cells (96.1 [5.2] vs 60 [3.4] hours for PANC1-PKCβ1 and PANC1-vector, respectively, $P < 0.05$) (Fig. 2A). F2

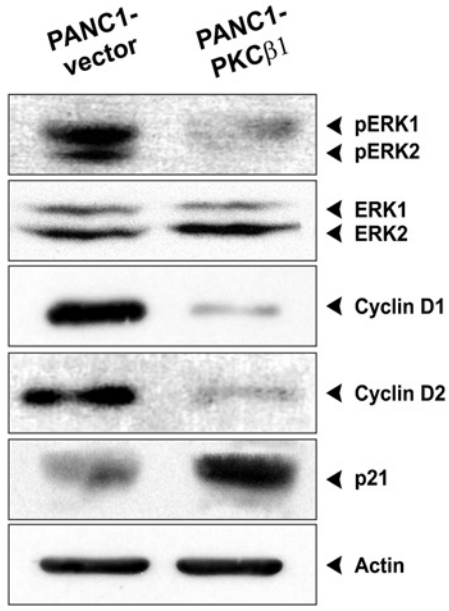
Next, we analyzed whether PKCβ1 overexpression modulated p42/p44 ERK MAPK, a crucial component of the mitogenic signaling pathways. PANC1-PKCβ1 cells, exhibited an important reduction in phosphorylated (active) p44/p42 ERK MAPK levels as compared to control cells (Fig. 2B). Moreover, Western blots demonstrated a significant reduction in the expression of cyclins D1 and D2, which are key regulators of cell cycle progression, together with elevated expression of the cyclin dependent kinase inhibitor $p21^{cip/waf1}$ (Fig. 2B).

Effect of PKCβ1 on Cell Survival

The ability of tumor cell populations to expand in number is determined by the balance between proliferation and death.



A



B

FIGURE 2. Panel A, Effect of PKCβ1 on cell proliferation. on cell proliferation. Cell growth was indirectly assessed by MTS assay at different times during the exponential growth phase of unsynchronized PANC1 cultures. Each data point represents the mean (SD) of quadruplicate determinations. *$P < 0.05$ versus PANC1-PKCβ1 cells (Student t test). At least 3 independent experiments were performed with similar results. Panel B, Regulation of ERK1/2 MAPK and cell cycle–associated proteins by PKCβ1 overexpression. Cell lysates prepared from PANC1-PKCβ1 or vector transfected cells were subject to Western blot analysis using specific against pERK, ERK, cyclin D1, cyclin D2, and p21. Results are representative of 3 independent experiments.

The acquisition of resistance toward cell death constitutes an essential feature in malignant transformation and represents a hallmark for most types of cancer.[27] This prompted us to explore whether PKCβ1 could modulate the survival capacity of PANC1 cells in response to either serum deprivation or the cytotoxic agent Dox. As shown in Figure 3A, PKCβ1-overexpressing cells were more resistant to cell death induced by serum starvation. Flow cytometry analysis revealed that FCS depletion induces the arrest of PKCβ1 cells in the G0/G1 phase of the cell cycle, thus favoring cell survival under this stress condition (Table 1). No modulation of PI3K/Akt or NF-κB survival pathways could be detected in PKCβ1 overexpressing cells under constitutive conditions; however, a significant increase in pAkt levels and in NF-κB activity, evaluated using a gene reporter assay, could be detected 48 hours after FCS depletion (Fig. 3B, C). In addition, nuclear NF-κB levels were also increased in PANC1-PKCβ1 cells concomitantly with a significant reduction in cytoplasmic IκBα levels under serum starved conditions (Fig. 3D). On the other hand, the overexpression of PKCβ1 did not confer any survival advantage when cells were exposed to the cytotoxic drug Dox. Moreover, PANC1-PKCβ1 cells were more sensitive to Dox treatment than control transfectants (Fig. 3E). However, when cell were serum-starved previously to the Dox treatment, PKCβ1 overexpressors were more resistant to the cytotoxic drug than control cells. This phenomenon could be mediated by the induction of cell cycle arrest together with the activation of the Akt and NF-κB pathways, already observed in the absence of FCS (Fig. 3F).

Role of PKCβ1 in the Modulation of Adhesion, Migration, and Invasion

Next, we analyzed the ability of the overexpressed PKCβ1 to alter the adhesive potential of PANC1 cells. We found that PKCβ1 transfectants display a significant increase in the fibronectin-adhesive capacity relative to control (vector-transfected) cells (Fig. 4A).

To analyze whether PKCβ1 overexpression modulates the migratory properties of PANC1 cells, we performed a "wound" assay. We found that PKCβ1-transfected cells showed a significantly lower migratory potential than control cells (Fig. 4B, C). The ability of PANC1 cells to invade through Matrigel, using a transwell chamber, was also examined. Our results demonstrated that the stable transfection of PKCβ1 significantly reduced the invasion ability of PANC1 cells (Fig. 4D). Our findings argued that PKCβ1 is able to negatively regulate both migration and invasiveness of pancreatic PANC1 cancer cells.

Role of PKCβ1 in Protease Secretion

It is well established that proteases are involved in migratory and invasive processes. As we have found that PKCβ1 negatively modulates these properties, we decided to examine whether secreted activity of proteolytic enzymes was altered as a consequence of PKCβ1 overexpression. Interestingly, overexpression of PKCβ1 in PANC1 cells significantly decreased uPA activity in the CM (Fig. 5A). Furthermore, PKCβ1 overexpression completely abrogated the secretion of MMP-9, whereas it significantly reduced secreted MMP-2 activity, as determined by zymography (Fig. 5B and inset).

PKCβ1 Inhibits In Vivo Tumor Growth of PANC1 Cells

To determine whether overexpression of PKCβ1 altered tumorigenic properties of PANC1 cells, we carried out experiments in nude mice. Notably, although PANC-vector cells efficiently formed tumors in nude mice, PANC-PKCβ1 cells were unable to grow in vivo. Control cells formed tumors that grew slowly but steadily over time reaching a median volume of 60 mm^3 at 80 days after inoculation (Fig. 6). Histopathological analysis revealed that in all cases control mice developed very poorly differentiated adenocarcinomas constituted by solid cords of heterogeneous epithelioid cells almost devoid of stroma around them. Moderate local invasiveness, with the tumoral cells invading dermis and

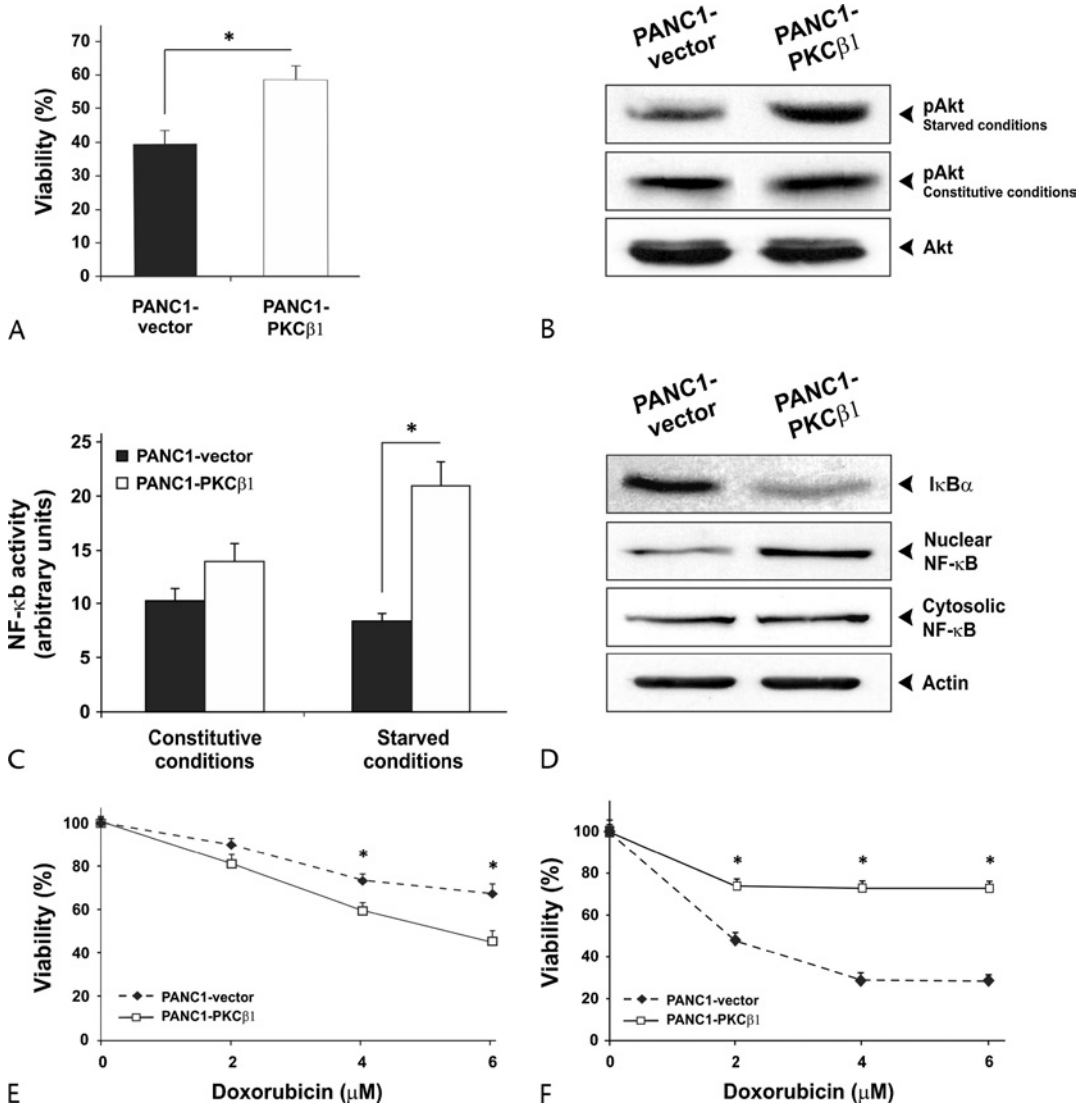


FIGURE 3. Panel A, Cell death susceptibility induced by serum starvation. Cells were deprived of serum and viability was evaluated 48 hours later using the MTS assay. Data were expressed as the mean (SD) of 3 independent experiments. *$P < 0.05$ versus PANC1-vector cells (Student *t* test). Panel B, Evaluation of phospho-Akt levels in PKCβ 0.05 versus PANC1-vector cells (Student *t* test). Panel B, Evaluation of phospho-Akt levels in PKCβ1 overexpressors. Cell lysates prepared from control and PKCβ1 overexpressing cells were subject to Western blot analysis using anti-pAkt and anti-Akt antibodies. Total Akt level was used as protein loading control. Results are representative of 3 independent experiments. Panel C, NF-κB gene reporter assay. PANC1-PKCβ1 and PANC1-vector cells were cotransfected with NF-κB-Luc reporter plasmid and a *Renilla* luciferase vector as control. Luciferase activities were determined 48 hours later as described in Materials and Methods section and data were normalized to the constitutive *Renilla* luciferase activity. All samples showed similar *Renilla* luciferase activity levels. Data expressed as the mean (SD) are representative of 3 independent experiments. *$P < 0.05$ versus PANC1-vector cells (Student *t* test). Panel D, Evaluation of NF-κB and IκBα expression in PKCβ1 overexpressors. Nuclear and cytoplasmic protein fractions corresponding to PANC1-vector and PANC1-PKCβ1 cells were resolved on 10% SDS-PAGE and blotted with anti-NF-κB (p65) and anti-IκBα antibodies (only cytoplasmic fraction). Lanes were loaded with 50-μg protein for cytoplasmic determinations and 20-μg protein for nuclear determinations. A representative experiment is shown. Panel E, Cell death susceptibility to cytotoxic drug treatment. Cells were treated with different doses of Dox (2–6 μM) for 2 hours. Cell viability was evaluated 48 hours later using the MTS assay. Values were compared to those obtained in both cell lines without treatment, which were set as 100% survival. Data expressed as the mean (SD) are representative of at least 3 independent experiments. *$P < 0.05$ versus PANC1-PKCβ1 cells treated with the same dose of Dox (Student *t* test). Panel F, Cell death susceptibility to combined serum starvation and cytotoxic drug treatment. Cells were deprived of serum for 24 hours and then treated with different doses of Dox (2–6 μM) for 2 hours. Cell viability was evaluated 24 hours later using the MTS assay. Values were compared to those obtained in both cell lines without Dox treatment, which were set as 100% survival. Data expressed as the mean (SD) are representative of at least 3 independent experiments. *$P < 0.05$ versus PANC1-vector cells treated with the same dose of Dox (Student *t* test).

TABLE 1. Analysis of Cell Cycle Distribution of PANC1-PKCβ1 and PANC1-Vector Cells After 48 Hours of FBS Deprivation

	PANC1-Vector, %	PANC1-PKCβ1, %
G0/G1	45.5 (0.76)	86.7 (1.0)
S	4.0 (0.32)	2.8 (0.4)
G2/M	49.8 (1.7)	9.9 (1.9)

muscular tissues, was observed in all cases (data not shown). Neither PANC1-PKCβ1 nor control cells developed metastatic nodules in the lungs, liver, kidney, or spleen.

DISCUSSION

Protein kinase C enzymes have key roles in cancer cell growth, survival, and motility, and their deregulation is often associated with tumor development and cancer progression.[28] However, a great variability in PKC-mediated responses and their underlying mechanisms has been found in cancer cells. Some of these differences have been attributed to the cell type–specific pattern of PKC-isozymes expression. Whereas the classical PKCβ isozyme has been implicated in several types of cancer including prostate, colon, and hematological cancers, its precise role during the tumorigenic process of pancreatic cancer is not clear.[5] There is scarce information regarding PKCβ1 expression levels in pancreatic cancer.[22,29] Evans and coworkers have described that there are no differences in PKCβ1 expression

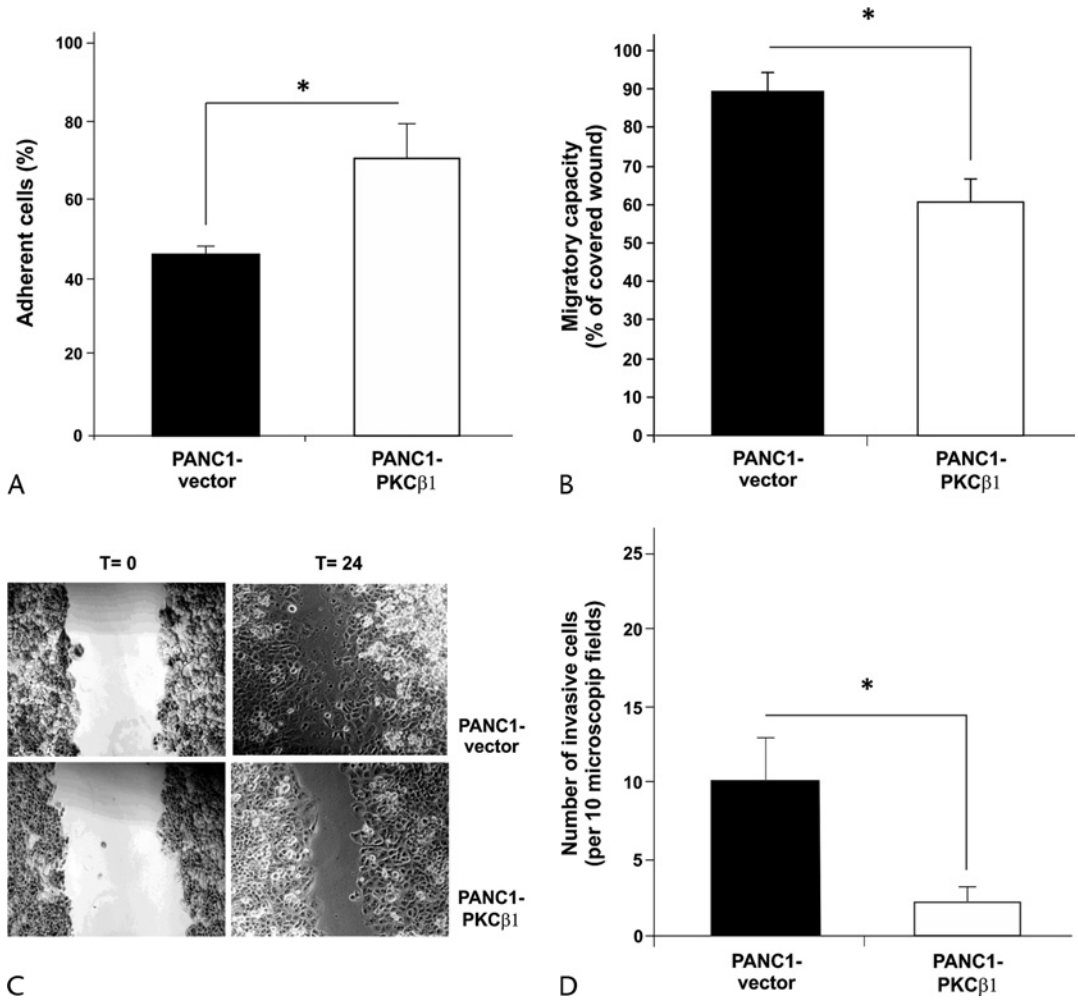


FIGURE 4. Panel A, Effect of PKCβ11 on adhesive capacity. Control and PKCβ1 transfectants were assayed for their adhesive capacity to fibronectin as described in ''Materials and Methods.'' The rate of adhesion was expressed as the percentage of the total number of cells seeded. Data expressed as the mean (SD) are representative of 3 independent experiments. *$P < 0.05$ versus PANC1-vector cells (Student *t* test). Panel B, Effect of PKCβ1 on migratory potential. Subconfluent monolayers were ''wounded'' at time 0 and cells were allowed to migrate into the cell-free area for 12 hours. The same spot was photographed at different times and cell migration was quantified by calculating the percentage of the area occupied by cells that migrated into the original cell-free wounded area. Data expressed as the mean (SD) are representative of 3 independent experiments *$P < 0.05$ versus PANC1-vector cells (Student *t* test). Panel C, Representative photographs of wounded monolayers are shown. Panel D, Effect of PKCβ1 on cell invasion. Invasiveness was assessed using Transwell culture chambers coated with Matrigel. The nuclei of those invasive cells that reattached the lower surface of the filter were counted under an epifluorescence microscope. Data expressed as the number (SD) of invasive cells per ten ×400 microscopic fields are representative of 3 independent experiments. *$P < 0.05$ versus PANC1-vector cells (Student *t* test).

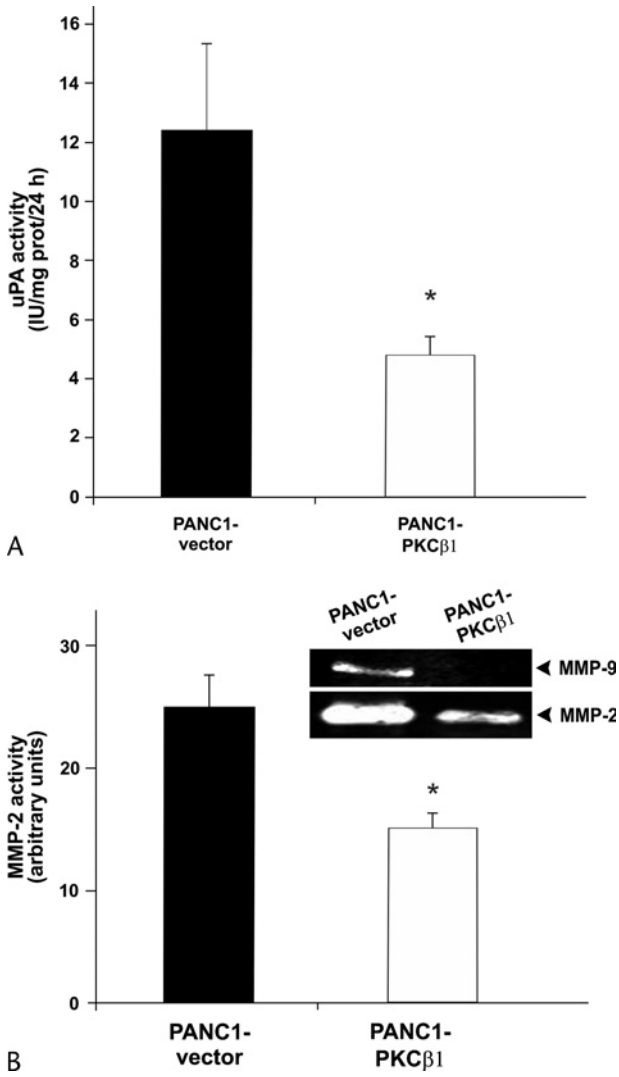


FIGURE 5. Panel A, Quantification of uPA activity by radial caseinolysis. uPA-secreted activity was quantified in CM from PKC transfectants and control cell cultures. Data are expressed as the mean (SD) of triplicate determinations. Three independent experiments gave similar results. *$P < 0.01$ versus PANC1-vector cells (Student *t* test). Panel B, Quantification of MMPs activity by zymography. MMPs secreted to CM from PKC- and vector-transfected cultures were analyzed. MMP-2 lytic bands were digitalized with a Foto/Analyst Express System and signal intensity was quantified with Gel-Pro Analyzer software. Data are expressed as the mean (SD) in arbitrary units. *P 0.01 versus PANC1-vector cells (Student *t* test). Panel B, Quantification of MMPs activity by zymography. MMPs secreted to CM from PKC- and vector-transfected cultures were analyzed. MMP-2 lytic bands were digitalized with a Foto/Analyst Express System and signal intensity was quantified with Gel-Pro Analyzer software. Data are expressed as the mean (SD) in arbitrary units. *$P < 0.05$ versus control cells (Student *t* test). Results are representative of 3 independent experiments. *Inset*: representative MMP-2 and MMP-9 gelatin zymograms are shown.

between pancreatic duct cancer and non malignant major duct epithelia. On the contrary, normal minor ducts presented higher PKCβ1 expression as compared with pancreatic cancer.[22] To our knowledge, there are no other studies in the literature which evaluate the role of PKCβ1 in human pancreatic tumor progression.

Here, we describe that the overexpression of PKCβ1 in pancreatic carcinoma cells leads to a complete suppression of the in vivo tumor growth. This loss of the tumorigenic potential could be related to the ability of PKCβ1 to impair the proliferative capacity of the cells. It is well known that mitogenesis is often associated with p42/44 ERK/MAPK activation, and accordingly, inhibition of the ERK pathway results in most cases in reduced cell proliferation.[30] In addition, the link between the reduction ERK1/2 signaling and decreased cyclin D1 expression was previously described by several authors.[30,31] Our studies in PANC1 cells show that PKCβ1 overexpression is associated with pERK1/2 activity down-modulation and the reduction in cyclins D1 and D2 expression, together with an increased expression of the cell cycle inhibitor $p21^{CIP/WAF1}$. All these molecules are critical components of the cell cycle machinery responsible for G1/S transition, therefore suggesting that PKCβ1 overexpression exerts an important effect on the regulation of this phase of the cell cycle.

Signaling through PI3K and its downstream effector Akt has been widely implicated in cell survival.[32] In our study, we observed that PKCβ1 overexpression conferred resistance to cell death induced by serum deprivation and caused a substantial activation of Akt. Similar findings were observed for NF-κB. It has been reported that this transcription factor drives survival responses through the regulation of several genes expression such as Bcl-2 and caspase inhibitors. These observations, together with an increased accumulation of cells in the G0/G1 phase observed in PANC1-PKCβ1, may explain the elevated cell death resistance to this stress condition.

The progression of a tumor in situ to an invasive tumor is a major prerequisite to cancer metastasis.[27] There is little information linking PKCβ1 to invasion and moreover, no rigorous studies have been carried out to dissect the mechanisms involved. This fact has prompted us to analyze the effect of PKCβ1 overexpression on the invasive behavior of human ductal pancreatic PANC1 cells. We found that overexpression of PKCβ1 in PANC1 cells induced less migratory and invasive migratory phenotypes in vitro. Furthermore, it is important to note that the secretion of extracellular proteases also plays an important role in invasive/motile events of cancer cells.[33] Proteases have other functions rather than simply degrading ECM facilitating invasion. These functions include the modulation

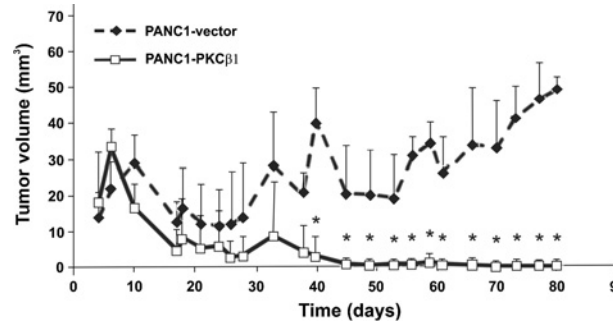


FIGURE 6. In vivo tumor growth of PANC1-transfected cells. PANC1 control and PKCβ1 transfectants were harvested from subconfluent cultures and inoculated transfectants were harvested from subconfluent cultures and inoculated sc in the left flank of nude mice. The size of the 2 perpendicular diameters was recorded and used to calculate tumor volume. Tumor growth was followed up for 80 days. The figure shows the results of 1 experiment representative of 3 independent assays. *$P < 0.01$ versus PANC1-vector cells (Kruskal-Wallis test).

of cell migration,[34] the release of growth factors from the ECM (a well-described reservoir of such factors[35]), and the promotion of angiogenesis.[35,36] It has been shown that increased MMP expression correlates with the progression of various types of tumors, including pancreatic cancer.[37] Coincidently with Zervos et al,[38] we found that MMP-2 is the principal MMP expressed by PANC1 cells. We speculate that the decreased invasive potential of PANC1-PKCβ1 cells could be a consequence of the reduced secretion of MMP-2 and uPA and the complete loss of MMP-9 activity. As ERK/MAPK is known to regulate protease production in cancer cells,[39] it is conceivable that the reduction observed in proteases secretion in PANC1-PKCβ1 cells might be a consequence of the decrease in pERK levels. Akt has also been described as a negative regulator of motility in cancer cells,[40,41] so we cannot rule out a contribution of the PI3K/Akt pathway as well to the reduced motility observed in PANC1-PKCβ1 cells.

PKCβ has been proposed as a target for cancer therapy, and there are several clinical trials for the PKCβ inhibitor enzastaurin.[42–44] A phase II study in pancreatic cancer patients demonstrated that the addition of enzastaurin to gemcitabine seemed not to improve survival.[45] However, in pancreatic cancer xenograft models, the combination of enzastaurin with radiotherapy reduced microvessel density and resulted in increase growth delay, which encourages further research in clinical trials.[46]

Pancreatic ductal adenocarcinoma is thought to arise from proliferative premalignant lesions of the ductal epithelium through a series of genetic alterations. These include activating mutations in the K-Ras gene and the loss of several tumor suppressor genes. Regarding PKC isoenzymes, in a previous work,[5] we have demonstrated using human samples and experimental models, that PKCδ could be an additional genetic or epigenetic alteration involved in pancreatic cancer progression, acting as an oncogene. The findings presented in this study indicate that PKCβ1 could have a crucial role in human pancreatic cancer progression, as its overexpression completely abolished the in vivo growth of ductal pancreatic cancer cells. So, our results could have implication for understanding the role of PKCβ1 in pancreatic cancer progression. Without any doubt, the balance among the activities of the different PKC isoforms, including the particular genetic background of each pancreatic tumor would determine the fate of the tumoral cell.

AQ1

REFERENCES

1. Yang GY, Wagner TD, Fuss M, et al. Multimodality approaches for pancreatic cancer. *CA Cancer J Clin*. 2005;55:352–367.
2. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2007. *CA Cancer J Clin*. 2007;57:43–66.
3. Ko AH. Future strategies for targeted therapies and tailored patient management in pancreatic cancer. *Semin Oncol*. 2007;34:354–364.
4. Ali AS, Ali S, El-Rayes BF, et al. Exploitation of protein kinase C: a useful target for cancer therapy. *Cancer Treat Rev*. 2009;35:1–8.
5. Mauro LV, Grossoni VC, Urtreger AJ, et al. PKC Delta (PKCdelta) promotes tumoral progression of human ductal pancreatic cancer. *Pancreas*. 2010;39:e31–e41.
6. Dekker LV, Parker PJ. Protein kinase C—a question of specificity. *Trends Biochem Sci*. 1994;19:73–77.
7. Kiley SC, Clark KJ, Duddy SK, et al. Increased protein kinase C delta in mammary tumor cells: relationship to transformation and metastatic progression. *Oncogene*. 1999;18:6748–6757.
8. Nishizuka Y. Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J*. 1995;9:484–496.
9. Mellor H, Parker PJ. The extended protein kinase C superfamily. *Biochem J*. 1998;332(Pt 2):281–292.
10. Leskow FC, Krasnapolski MA, Urtreger AJ. The pros and cons of targeting protein kinase C (PKC) in the management of cancer patients. *Curr Pharm Biotechnol*. 2011;12:1961–1973.
11. Gokmen-Polar Y, Murray NR, Velasco MA, et al. Elevated protein kinase C betaII is an early promotive event in colon carcinogenesis. *Cancer Res*. 2001;61:1375–1381.
12. Regala RP, Weems C, Jamieson L, et al. Atypical protein kinase C iota is an oncogene in human non-small cell lung cancer. *Cancer Res*. 2005;65:8905–8911.
13. Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. *Nat Rev Cancer*. 2007;7:281–294.
14. Grossoni VC, Falbo KB, Mauro LV, et al. Protein kinase C delta inhibits the production of proteolytic enzymes in murine mammary cells. *Clin Exp Metastasis*. 2007;24:513–520.
15. Urtreger AJ, Grossoni VC, Falbo KB, et al. Atypical protein kinase C-zeta modulates clonogenicity, motility, and secretion of proteolytic enzymes in murine mammary cells. *Mol Carcinog*. 2005;42:29–39.
16. Grossoni VC, Todaro LB, Kazanietz MG, et al. Opposite effects of protein kinase C beta1 (PKCbeta1) and PKCepsilon in the metastatic potential of a breast cancer murine model. *Breast Cancer Res Treat*. 2009;118:469–480.
17. Pan Q, Bao LW, Kleer CG, et al. Protein kinase C epsilon is a predictive biomarker of aggressive breast cancer and a validated target for RNA interference anticancer therapy. *Cancer Res*. 2005;65:8366–8371.
18. Jiang XH, Lam SK, Lin MC, et al. Novel target for induction of apoptosis by cyclo-oxygenase-2 inhibitor SC-236 through a protein kinase C-beta(1)-dependent pathway. *Oncogene*. 2002;21:6113–6122.
19. Teicher BA, Menon K, Alvarez E, et al. Antiangiogenic and antitumor effects of a protein kinase C beta inhibitor in human hepatocellular and gastric cancer xenografts. *In Vivo*. 2001;15:185–193.
20. Goldstein DR, Cacace AM, Weinstein IB. Overexpression of protein kinase C beta 1 in the SW480 colon cancer cell line causes growth suppression. *Carcinogenesis*. 1995;16:1121–1126.
21. Macfarlane DE, Manzel L. Activation of beta-isozyme of protein kinase C (PKC beta) is necessary and sufficient for phorbol ester-induced differentiation of HL-60 promyelocytes. Studies with PKC beta-defective PET mutant. *J Biol Chem*. 1994;269:4327–4331.
22. Evans JD, Cornford PA, Dodson A, et al. Expression patterns of protein kinase C isoenzymes are characteristically modulated in chronic pancreatitis and pancreatic cancer. *Am J Clin Pathol*. 2003;119:392–402.
23. Olah Z, Lehel C, Jakab G, et al. A cloning and epsilon-epitope-tagging insert for the expression of polymerase chain reaction-generated cDNA fragments in *Escherichia coli* and mammalian cells. *Anal Biochem*. 1994;221:94–102.
24. Urtreger AJ, Aguirre Ghiso JA, Werbajh SE, et al. Involvement of fibronectin in the regulation of urokinase production and binding in murine mammary tumor cells. *Int J Cancer*. 1999;82:748–753.
25. Aguirre Ghiso JA, Kovalski K, Ossowski L. Tumor dormancy induced by downregulation of urokinase receptor in human carcinoma involves integrin and MAPK signaling. *J Cell Biol*. 1999;147:89–104.
26. Spalding AC, Zeitlin BD, Wilder-Romans K, et al. Enzastaurin, an inhibitor of PKCbeta, enhances antiangiogenic effects and cytotoxicity of radiation against endothelial cells. *Transl Oncol*. 2008;1:195–201.
27. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646–674.
28. Grossoni VC, Falbo KB, Kazanietz MG, et al. Protein kinase C delta enhances proliferation and survival of murine mammary cells. *Mol Carcinog*. 2007;46:381–390.
29. Raffaniello RD, Nam J, Cho I, et al. Protein kinase C isoform expression and function in transformed and non-transformed pancreatic acinar cell lines. *Biochem Biophys Res Commun*. 1998;246:166–171.
30. Terada Y, Nakashima O, Inoshita S, et al. Mitogen-activated protein kinase cascade and transcription factors: the opposite role of MKK3/6-p38K and MKK1-MAPK. *Nephrol Dial Transplant*. 1999;14(suppl 1):45–47.
31. Mazzoni E, Adam A, Bal de Kier Joff ED, et al. Immortalized mammary epithelial cells overexpressing protein kinase C gamma acquire a

malignant phenotype and become tumorigenic in vivo. *Mol Cancer Res*. 2003;1:776–787.
32. Zhong M, Lu Z, Foster DA. Downregulating PKC delta provides a PI3K/Akt-independent survival signal that overcomes apoptotic signals generated by c-Src overexpression. *Oncogene*. 2002;21:1071–1078.
33. Noel A, Jost M, Maquoi E. Matrix metalloproteinases at cancer tumor-host interface. *Semin Cell Dev Biol*. 2008;19:52–60.
34. Whitley BR, Palmieri D, Twerdi CD, et al. Expression of active plasminogen activator inhibitor-1 reduces cell migration and invasion in breast and gynecological cancer cells. *Exp Cell Res*. 2004;296: 151–162.
35. Rao JS, Gondi C, Chetty C, et al. Inhibition of invasion, angiogenesis, tumor growth, and metastasis by adenovirus-mediated transfer of antisense uPAR and MMP-9 in non-small cell lung cancer cells. *Mol Cancer Ther*. 2005;4:1399–1408.
36. Stetler-Stevenson WG. Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. *J Clin Invest*. 1999;103: 1237–1241.
37. Juuti A, Lundin J, Nordling S, et al. Epithelial MMP-2 expression correlates with worse prognosis in pancreatic cancer. *Oncology*. 2006;71:61–68.
38. Zervos EE, Shafii AE, Haq M, et al. Matrix metalloproteinase inhibition suppresses MMP-2 activity and activation of PANC-1 cells in vitro. *J Surg Res*. 1999;84:162–167.
39. Aguirre Ghiso JA, Alonso DF, Farias EF, et al. Deregulation of the signaling pathways controlling urokinase production. Its relationship with the invasive phenotype. *Eur J Biochem*. 1999;263:295–304.
40. Toker A, Yoeli-Lerner M. Akt signaling and cancer: surviving but not moving on. *Cancer Res*. 2006;66:3963–3966.
41. Yoeli-Lerner M, Yiu GK, Rabinovitz I, et al. Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. *Mol Cell*. 2005;20:539–550.
42. Querfeld C, Rizvi MA, Kuzel TM, et al. The selective protein kinase C beta inhibitor enzastaurin induces apoptosis in cutaneous T-cell lymphoma cell lines through the AKT pathway. *J Invest Dermatol*. 2006;126:1641–1647.
43. Querfeld C, Kuzel TM, Kim YH, et al. Multicenter phase II trial of enzastaurin in patients with relapsed or refractory advanced cutaneous T-cell lymphoma. *Leuk Lymphoma*. 2009;52:1474–1480.
44. Vansteenkiste J, Ramlau R, von Pawel J, et al. A phase II randomized study of cisplatin-pemetrexed plus either enzastaurin or placebo in chemonaive patients with advanced non-small cell lung cancer. *Oncology*. 2012;82:25–29.
45. Richards DA, Kuefler PR, Becerra C, et al. Gemcitabine plus enzastaurin or single-agent gemcitabine in locally advanced or metastatic pancreatic cancer: results of a phase II, randomized, noncomparative study. *Invest New Drugs*. 2009;29:144–153.
46. Spalding AC, Watson R, Davis ME, et al. Inhibition of protein kinase Cbeta by enzastaurin enhances radiation cytotoxicity in pancreatic cancer. *Clin Cancer Res*. 2007;13:6827–6833.

AUTHOR QUERY

AUTHOR PLEASE ANSWER QUERY

AQ1 = Citations of references have been modified. Please check if correct.

END OF AUTHOR QUERY

Author Reprints

For *Rapid Ordering* go to: www.lww.com/periodicals/author-reprints

Pancreas

Order

Author(s) Name

Title of Article

*Article # *Publication Mo/Yr

**Fields may be left blank if order is placed before article number and publication month are assigned.*

Quantity of Reprints ____ $_____

Covers (Optional) _____ $_____

Shipping Cost $_____

Reprint Color Cost $_____

Tax $_____

Total $_____

REPRINTS ORDERED & PURCHASED UNDER THE AUTHOR REPRINTS PROGRAM MAY NOT BE USED FOR COMMERCIAL PURPOSES

Reprint Pricing

50 copies = $336.00
100 copies = $420.00
200 copies = $494.00
300 copies = $571.00
400 copies = $655.00
500 copies = $732.00

Plain Covers

$108.00 for first 100 copies
$18.00 each add'l 100 copies

Reprint Color

($70.00/100 reprints)

Shipping

Within the U.S. - $15.00 up to the first 100 copies and $15.00 for each additional 100 copies

Outside the U.S. - $30.00 up to the first 100 copies and $30.00 for each additional 100 copies

Tax

U.S. and Canadian residents add the appropriate tax or submit a tax exempt form.

Payment

● MC ● VISA ● Discover ● American Express

Account # / / Exp. Date

Name

Address Dept/Rm

City State Zip Country

Telephone

Signature

Ship to

Name

Address Dept/Rm

City State Zip Country

Telephone

Lippincott Williams & Wilkins
a Wolters Kluwer business

Use this form to order reprints. Publication fees, including color separation charges and page charges will be billed separately, if applicable.

Payment must be received before reprints can be shipped. Payment is accepted in the form of a check or credit card; purchase orders are accepted for orders billed to a U.S. address.

Prices are subject to change without notice.

For quantities over 500 copies contact our Healthcare Dept. For orders shipping in the US and Canada: call 410-528-4396, fax your order to 410-528-4264 or email it to Meredith.Doviak@wolterskluwer.com. Outside the US: dial 44 1829 772756, fax your order to 44 1829 770330 or email it to Christopher.Bassett@wolterskluwer.com.

MAIL your order to:
Lippincott Williams & Wilkins
Author Reprints Dept.
351 W. Camden St.
Baltimore, MD 21201

FAX:
410.528.4434

For questions regarding reprints or publication fees,
E-MAIL:
reprints@lww.com

OR **PHONE:**
1.866.903.6951

For *Rapid Ordering* go to: www.lww.com/periodicals/author-reprints