PKC Delta (PKCδ) Promotes Tumoral Progression of Human Ductal Pancreatic Cancer

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Objective: Our objective was to study the role of protein kinase C delta (PKCδ) in the progression of human pancreatic carcinoma.

Methods: Protein kinase C delta expression in human ductal carcinoma (n = 22) was studied by immunohistochemistry. We analyzed the effect of PKCδ overexpression on in vivo and in vitro properties of human ductal carcinoma cell line PANC1.

Results: Human ductal carcinomas showed PKCδ overexpression compared with normal counterparts. In addition, in vitro PKCδ-PANC1 cells showed increased anchorage-independent growth and higher resistance to serum starvation and to treatment with cytotoxic drugs. Using pharmacological inhibitors, we determined that phosphatidylinositol-3-kinase and extracellular receptor kinase pathways were involved in the proliferation of PKCδ-PANC1. Interestingly, PKCδ-PANC1 cells showed a less in vitro invasive ability and an impairment in their ability to migrate and to secrete the proteolytic enzyme matrix metalloproteinase-2. In vivo experiments indicated that PKCδ-PANC1 cells were more tumorigenic, as they developed tumors with a significantly lower latency and a higher growth rate with respect to the tumors generated with control cells. Besides, only PKCδ-PANC1 cells developed lung metastasis.

Conclusion: Our results showed that the overexpression of PKCδ in PANC1 cells induced a more malignant phenotype in vivo, probably through the modulation of cell proliferation and survival, involving phosphatidylinositol-3-kinase and extracellular receptor kinase signaling pathways.

Key Words: human pancreatic adenocarcinoma, PKCδ, invasion, PI3K/AKT, ERK

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Ductal adenocarcinoma of the pancreas, which comprises 90% of all human pancreatic cancers, is an extremely lethal disease with an overall 5-year survival rate of only 3% to 5% and a median survival time after diagnosis of less than 6 months.[1] Conventional cancer treatments have little impact on disease course, making pancreatic cancer the fourth leading cause of cancer death in both men and women. Studies in molecular biology have greatly increased understanding of the pathogenesis of this disease. However, novel therapeutic strategies are needed, and these could arise from defining the factors and signaling pathways that stimulate unrestrained proliferation of ductal pancreatic cancer.[2] Recently, members of the protein kinase C (PKC) family have emerged as novel modulators of transformation and cell cycle progression of pancreatic cancers.[3] However, the functional relevance of each PKC isoform is unclear.

Protein kinase C is a multigene family of related serine/threonine kinases that play key roles in proliferation and apoptosis regulation. Moreover, several PKCs have been associated with tumor progression. However, knowledge of the molecular mechanisms through which PKC might contribute to these processes is still vague.

Protein kinase C isoforms are classified according to their cofactor requirements into 3 groups: classical (c), novel (n), and atypical. The classical isoforms (PKCα, PKCβ1, PKCβ2, and PKCγ) are stimulated by calcium, diacylglycerol (DAG), and phospholipids; the novel isoforms (PKCδ, PKCε, PKCη, and PKCΘ) are calcium independent; and the atypical isoforms (PKCζ and PKCλ) are calcium and DAG independent. The classical and novel isoforms respond to phorbol esters. Furthermore, each PKC isozyme displays a unique tissue distribution, subcellular localization, and substrate specificity.[4]

The mechanisms involved in PKC activation have been extensively studied.[5] The increase in plasma membrane DAG levels functions as the trigger for the intracellular relocalization and reversible recruitment of nPKC or cPKC to the plasmatic membrane. There, PKCs undergo a conformational change that exposes binding sites for substrates and anchoring/scaffolding proteins and results in kinase activation. After that, PKCs undergo a series of transphosphorylation and autophosphorylation events that are required for activation and stability. In addition, in response to either phorbol esters or receptor stimulation, PKC isoenzymes can redistribute to the nuclear membrane or to organelles such as the mitochondria or the Golgi apparatus.[4,6] Although this differential redistribution is key to allowing the access to isozymes-specific substrates and ultimately conferring functional selectivity, the mechanisms that direct localization are not fully understood. At molecular level, each PKC isoenzyme is able to modulate several signaling pathways such as nuclear factor κB, extracellular receptor kinase (ERK)/mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT), and p38 MAPK, which could explain the diverse effects of activated PKCs in different models.[7,8]

There is a large body of evidence linking PKC to tumor progression. It is generally proposed that PKC activation positively affects motility, invasion, and metastasis[9,10] through

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the regulation of integrins or extracellular matrix enzymes such as matrix metalloproteinase-9 or members of the urokinase type plasminogen activator (uPA) pathway.[11,12] However, protein kinase C delta (PKCδ) seems to have contradictory roles in tumor progression according to cell type.[7,13] This isoform can act as a tumor suppressor and also as a positive regulator of cell cycle progression. To make things worse, PKCδ has been reported to either stimulate or inhibit invasion and apoptotic programs.[14,15]

In the present work, we detected that PKCδ was overexpressed in human ductal carcinomas compared with their normal counterparts. Moreover, we demonstrated that the human pancreatic carcinoma cell line PANC1 expresses a low basal level of PKCδ. Our aim was, using a transfection stable approach, to overexpress PKCδ isoenzyme in this model and to analyze its effect on in vivo and in vitro properties associated with tumor progression. The overexpression of PKCδ induced a more malignant phenotype when PANC1 cells were inoculated into nude mice. Moreover, in vitro studies revealed that PKCδ overexpression enhanced survival under stress conditions and promoted anchorage-independent growth in PANC1 cells while simultaneously impairing invasion and enzyme production. Furthermore, we supply experimental data indicating that some of the effects induced by PKCδ on these cells could be mediated by ERK and PI3K/AKT pathways.

MATERIALS AND METHODS

Reagents and Antibodies

Medium for cell culture, agarose, geneticin (G418), and Lipofectamine Plus were obtained from Life Technologies, Inc (Rockville, Md). Fetal calf serum (FCS) was from GEN (Buenos Aires, Argentina). Acrylamide, phorbol 12-myristate 13-acetate (PMA), PD98059, and LY294002 were from Sigma (St Louis, Mo). All other reagents for polyacrylamide gel electrophoresis (PAGE) were obtained from Bio-Rad (Richmond, Calif). Hybond-P membranes for blotting and chemiluminescence reagents (ECL) were from Amersham (Aylesbury, UK). Plasminogen was purchased from Chromogenix (Molndal, Sweden), and gelatin was purchased from Sigma Co (St Louis, Mo). Human urokinase was a gift from Serono (Buenos Aires, Argentina). Triton X-100 was obtained from J. T. Baker, Inc (Phillipsburg, NJ).

Monoclonal anti-PKCα, anti-PKCβ, anti-PKCδ, and anti-PKCζ antibodies were purchased from BD Biosciences (San Diego, Calif). Monoclonal antibodies for ERK and phospho-ERK (pERK) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). The antibody used against Ki67 was from Dako (Carpinteria, Calif).

Monoclonal antibodies for AKT and phospho-AKT (pAKT, Ser 473) were purchased from Cell Signaling Technology (Beverly, Mass). Horseradish peroxidase–conjugated antirabbit or antimouse antibodies were obtained from Sigma.

Human Tumors

The expression of PKCδ was studied in 22 paraffin-embedded ductal pancreatic tumors, obtained from the Hospital Italiano de Buenos Aires. Tissue specimens were obtained from surgical material (n = 13) or biopsies (n = 9) from untreated patients at initial diagnosis. The study included samples from 12 men (median age, 62 years; range 46–75 years) and 10 women (median age, 65; range, 50–73 years).

Cell Line

Human pancreatic ductal carcinoma cell line was cultured at 37°C in RPMI 1640 (Gibco; Invitrogen Corp, Carlsbad, Calif), supplemented with 10% FCS and 80 μg/mL gentamicin in a humidified air atmosphere with 5% CO_2.

Expression Vectors, Transfection, and Selection

Human pancreatic ductal carcinoma cells were stably transfected with 5 μg of pMTH-PKCδ, a mammalian expression vector encoding for PKCδ, using Lipofectamine Plus. Human pancreatic ductal carcinoma cells transfected with empty vector (pMTH) were used as control. Forty-eight hours after transfection, cells were selected with 500 μg/mL of G418. After selection, approximately 30 resistant clones were pooled to avoid clonal variations. Transfected cell lines, PKCδ-PANC1 or pMTH-PANC1, were maintained for 10 to 12 passages before use.

Western Blot

Semiconfluent monolayers were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed with 1% Triton X-100 in PBS by scraping with a teflon scrapper. Samples were denatured by boiling in sample buffer with 5% β-mercaptoethanol and run in 10% sodium dodecyl sulfate-PAGE. Fifty micrograms of protein were 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 (Amersham Biosciences, Buckinghamshire, UK). The intensity of the bands was quantified with a digital GS-700 densitometer and Molecular Analyst software (Bio-Rad, Calif).

When both the phosphorylated and the total expression of a molecule were studied, the same membrane was blotted with the antibody against the phosphorylated form and subsequently stripped and probed in a similar fashion with the total antibody as mentioned above.

Subcellular Fractionation

Protein kinase C delta PANC1 and pMTH-PANC1 cells were cultured in 6-cm dishes in RPMI 1640 supplemented with 10% FBS and G418 (250 μg/mL) for 48 hours and treated with PMA (100 nmol/L) for 1, 3, 5, 10, and 15 minutes. After stimulation, cells were washed with cold PBS once, kept in ice and collected in 300 μL of cell lysis buffer (20 mmol/L Tris-HCl, 5 mmol/L ethylene glycol tetraacetic acid, and protease inhibitors, pH 7.4) using a cell scraper. Cell suspensions were sonicated in ice and used for cellular fractionation experiment by ultra centrifugation (55,000 rpm, 30 minutes). Protein kinase C delta in cytosol (supernatant), membrane (pellet), and total (not centrifuged) samples was detected by Western blot using anti-PKCδ antibody at 1:1000 dilutions in 5% bovine serum albumin.

In Vitro Behavior of PKCδ-Transfected Cells

Growth Properties

Anchorage-Dependent Growth

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 RPMI 1640 supplemented with 10% FCS and 80 μg/mL gentamicin. Cell growth was indirectly assessed with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (Promega, Madison, Wis), according to the vendor's indications, every 24 hours for 4 days. Alternatively,

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proliferation was evaluated by cell counting using a hemocytometer and trypan blue exclusion test.

Anchorage-Independent Growth

For soft agar assays, 24-well plates were prepared with 1-mL base feeder layer of 0.6 % agar in complete medium and a semisolid top layer (0.4% agar) containing log phase–growing monodispersed cells (2×10^5 cells/dish). Seven days after seeding, cultures were fixed by adding 10% formaldehyde in PBS, and the number of colonies with more than 10 cells was counted using an inverted microscope.

In a similar experiment and to analyze the involvement of different signaling pathways in this behavior, cells were cultured in the continuous presence of the MAPK kinase-1 (MEK1) inhibitor PD98059 (50 μmol/L) or the PI3K inhibitor LY294002 (20 μmol/L).

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 overnight with 2 to 6 μmol/L doxorubicin (Dox) (Glenmark Pharmaceuticals SA, Mumbai, India), washed twice with PBS and subsequently incubated in a medium with 10% FCS for 48 hours. Cell viability was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay according to the manufacturer's instructions.

The presence of cells in pre-G0/G1 phase of the cell cycle was measured by flow cytometry. Floating cells were collected and adherent cells were detached by trypsinization and joined to floating ones, washed with PBS, and fixed in 70% ethanol. After staining with 100 μg/mL propidium iodide, cell cycle profile was analyzed.

Invasion Assay

Transwell cell culture chambers (Corning, Mass) were used for invasion assay. Eight-micrometer pore membranes were previously coated with 0.1% gelatin on the lower side and with a thin layer of reconstituted basement membrane (Matrigel [BD Biosciences, San Jose Calif], 250 μg/mL) on the upper face of the chamber. The lower chamber contained human cellular fibronectin (16 μg/mL; Sigma) in 0.5 mL RPMI 1640, as chemoattractant. Cells (2×10^4) were seeded in the upper chamber, and 48 hours later, cells on the upper surface of the filter were completely removed by wiping them with a cotton swab. Finally, membranes were fixed in Carnoy's fixative and stained with Hoescht 33258 (Sigma Co, St. Louis, Mo). The nuclei of the cells that invaded Matrigel, passed through the pores, and reattached to the lower surface of the filter were considered as invasive ones and counted in ×400 fields under a fluorescence microscope (Eclipse E400; Nikon, Melville, NY).

Migration Assay

To determine the effects of PKCδ overexpression on PANC1 cell motility, a wound-healing assay was performed. Briefly, wounds of approximately 400 μm wide were made in confluent monolayers of the different transfectant cultures. Cells were then allowed to migrate into the cell-free area for a period of 24 hours. The same spot was photographed at time 0 and at 24 hour. The migratory area was analyzed using the Image-ProPlus 4.5 software (Media Cybernetics Inc, Bethesda, Calif). Cell migration was expressed as the difference between the wounded areas at both times.

Cytoskeleton Analysis

The actin cytoskeleton was studied using labeled phalloidin-fluorescein isothiocyanate (FITC) and fluorescence microscopy. Vimentin was analyzed by immunofluorescence using a specific antibody plus a second antibody labeled with FITC. In all cases, nuclei were stained with 4′,6-diamidino-2-phenylindole.

Production of Proteases

Preparation of Conditioned Media (CM)

Secreted uPA and metalloproteinase (MMP) activities were evaluated in conditioned media (CM). Briefly, semiconfluent cell monolayers growing in 35-mm plastic Petri dishes were extensively washed with PBS. Serum-free medium (1 mL) was then added for 24 hours. Conditioned media were individually harvested, the remaining monolayers were lysed with 1% Triton-X100-PBS, and cell protein content was determined (Bio-Rad protein assay). Conditioned media samples were centrifuged (600*g*, 10 minutes), and the supernatant was aliquoted and stored at −40°C. Samples were used only once after thawing.

Quantification of uPA Activity

To determine uPA activity, a radial caseinolysis assay was used as previously described.[16] Briefly, 4-mm wells were punched in plasminogen-rich casein-agarose gels and 10 μL of CM were seeded. Gels were incubated for 24 hours at 37°C in humidified atmosphere. The diameter of lytic zones was measured, 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.

Detection of Metalloproteinases (MMP) Activity

Metalloproteinase enzymatic activity was determined by quantitative zymography.[17] Conditioned media were run on 9% sodium dodecyl sulfate-PAGE gels containing 1 mg/mL of gelatin under nonreducing conditions. After electrophoresis, gels were washed for 30 minutes using 2.5% Triton X-100 and subsequently incubated for 48 hours at 37°C in a buffer containing 0.25 mol/L Tris-HCl (pH 7.4), 1 mol/L NaCl, and 25 mmol/L $CaCl_2$. Metalloproteinase activity was confirmed using gels incubated in EDTA-containing buffer (40 mmol/L). After incubation, gels were fixed and stained with 0.5% Coomassie brilliant blue. Gelatinolytic bands were measured using a digital densitometer GS-700. Data were expressed as arbitrary units (AU) and normalized to the original cell culture protein content.

In Vivo Behavior of PKCδ-Transfected Cells

Animals

All the experiments were carried out using 2-month-old nude mice obtained from the Animal Care Area from the Comisión Nacional de Energía Atómica. They were housed 5 per cage, kept under an automatic 12-hour light/12-hour darkness schedule, and given sterile pellets and tap water ad libitum. All animal studies were conducted in accordance with the highest standards of animal care as outlined in the National Institutes of Health guide for the care and use of laboratory animals.

Tumorigenicity

Protein kinase C delta-PANC and pMTH-PANC 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 = 20) were inoculated subcutaneously (sc) into the left flank with 6×10^6 cells in 0.3 mL of

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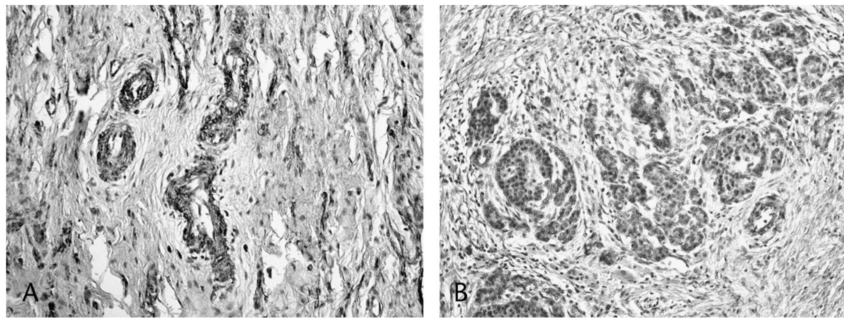

FIGURE 1. Immunohistochemical PKCδ staining of human ductal pancreatic carcinoma. A and B, Microscopic images of 2 different tumors showing specific PKCδ staining at cytoplasmic level (A: ×200; B: ×400).

RPMI 1640. Latency was defined as the time between sc injection of tumoral cells and the palpation of external tumors. The 3 largest perpendicular diameters were recorded twice a week to evaluate tumor growth. Mice were killed, and tumors were fixed in 10% formalin and embedded in paraffin. Sections of 5 μm were stained with hematoxylin and eosin for histopathological studies. The fraction of cycling cells in sc tumors was analyzed using immunohistochemistry against Ki67 antigen and recording the number of stained cells per field (×400).

To investigate the presence of spontaneous metastases, organs were removed and fixed in Bouin solution and then examined under dissection using a stereoscopic microscopy.

Immunohistochemistry and Immunofluorescence

For immunohistochemistry, human pancreatic cancer and PANC1 tumor specimens grown in mice were fixed in 10% formalin immediately after removal and processed to paraffin

TABLE 1. PKCδ Expressions According to the Main Features With Clinical Relevance in Pancreatic Cancer

Parameter*	PKCδ-Positive Cases/Total (%)
Sex	
M	7/12 (58.3)
F	6/10 (60.0)
Age	
≤60	6/9 (66.7)
61–69	2/5
>70	5/7 (71.5)
Stage	
I	2/4
II	5/7 (71.5)
III/IV	4/7 (57.1)
T	
I/II	2/5
III/IV	9/13 (69.2)
N	
0	7/11 (63.6)
1	4/7 (57.1)
Differentiation Grade	
1	3/4
2	6/10 (60.0)
3	3/5

*Where columns do not sum up, data were missing or unknown.

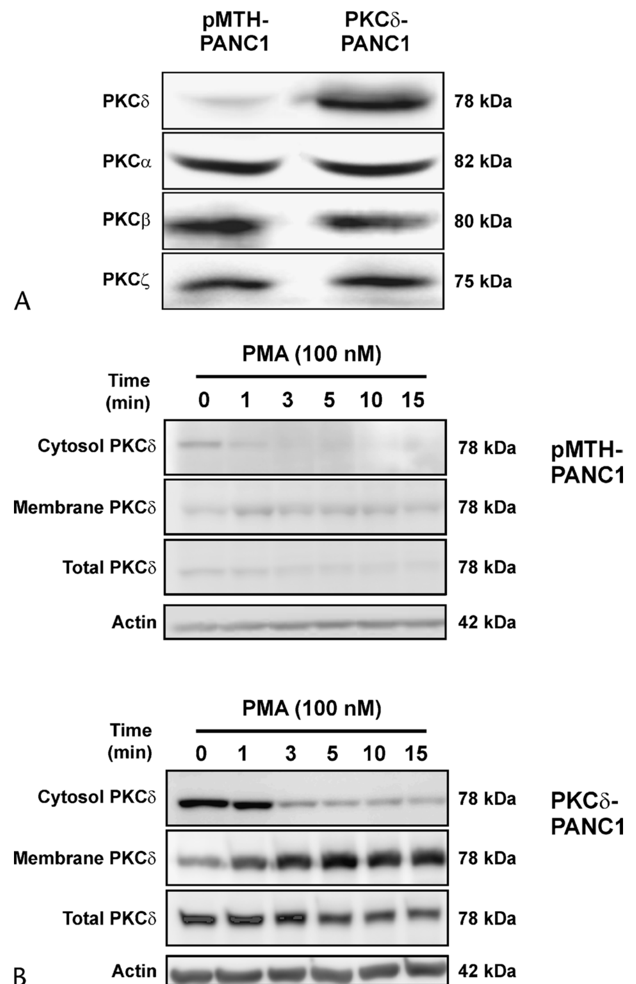

FIGURE 2. Expression of PKC isoforms in PANC1 cells. A, Western blot developed by PKC monoclonal antibodies in human PANC1 cells. A 78-kDa band is revealed corresponding to PKCδ present in PKCδ-PANC1 cells, which was slightly present in cells transfected with the vector alone (pMTH). PANC1 cells expressed other PKC isoforms, an expression which did not vary after the stable PKCδ transfection. B, Protein kinase C delta translocation from the cytosol to the membrane in PANC1-PKCδ and control cells after phorbol ester treatment.

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blocks. Representative sections (5 μm thick) were placed on positively charged slides and microwaved in citrate buffer (pH=6) to recover antigenicity. Tissues were treated with 30 volumes of H_2O_2 for 15 minutes to eliminate endogenous peroxidase and blocked for 1 hour with 2.5% skim milk in PBS. Primary antibodies against PKCδ or Ki-67 diluted in PBS were incubated overnight at 4°C. After washing with PBS, samples were incubated with biotinylated antimouse or antirabbit antibody respectively and then with streptavidin-peroxidase conjugate (Vector Laboratories, Inc, Burlingame, Calif). The immunoreactive product was visualized with a substrate solution of 3-3′ diaminobenzidine.

For immunofluorescence, cells were seeded on glass cover slides. After 48 hours in culture, cells were washed with PBS and fixed with 3.7% buffered formaldehyde. Cells were incubated overnight with the following antibodies: p21 (sc 6246, Santa Cruz Biotechnology, Santa Cruz, Calif), p16 (sc 468, Santa Cruz Biotechnology), p27 (sc 528, Santa Cruz Biotechnology), and Cyclin D1 (sc 8396, Santa Cruz Biotechnology). Next, cells were incubated with the corresponding secondary antibody coupled with FITC (1:200; Zymed Lab, South San Francisco, Calif). Nuclei were counterstained with DAPI for 1 minute before mounting with Vectashield (Vector Laboratories, Inc). Cells were analyzed with a fluorescence microscope.

Statistical Analysis

All assays were performed in triplicate and independent experiments were repeated at least twice. The significance of differences between groups was calculated by applying Student, analysis of variance (ANOVA), or χ^2 tests, as indicated. $P < 0.05$ was considered to be significant.

RESULTS

PKCδ Is Up-Regulated in Human Ductal Pancreatic Carcinomas

We studied PKCδ expression in 22 samples of human ductal pancreatic carcinomas. Protein kinase C delta immunolabeling

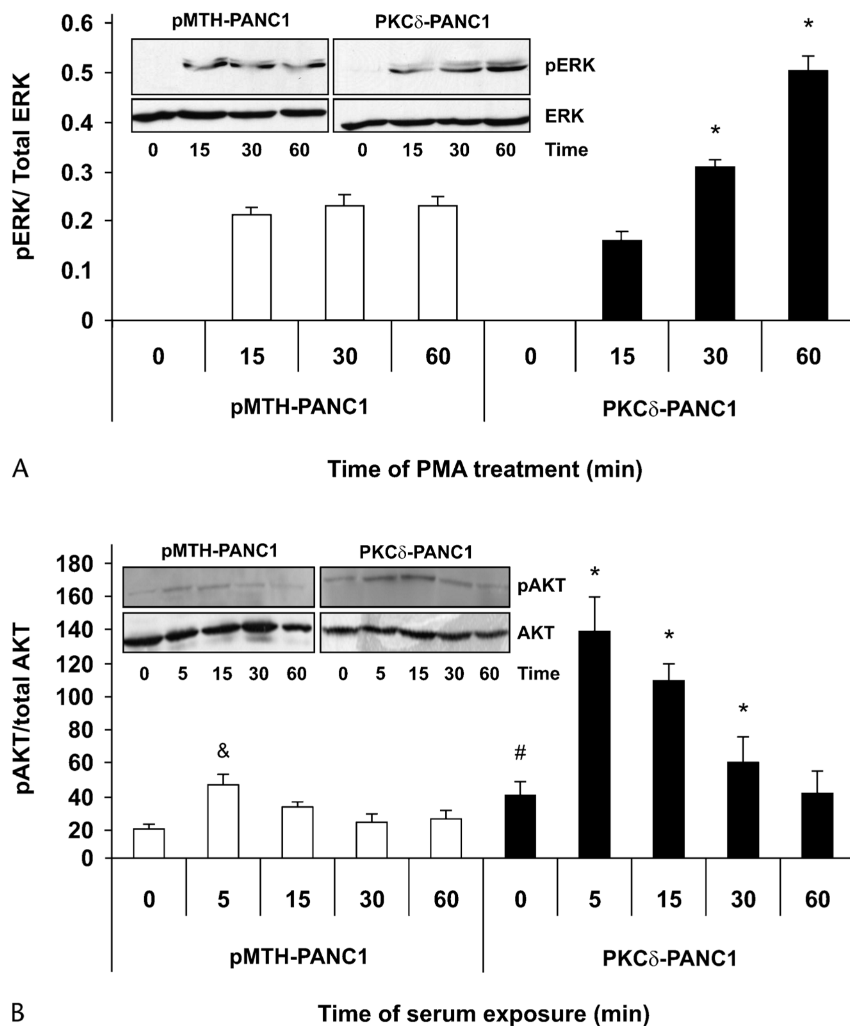

FIGURE 3. Activation of AKT and ERK MAPK pathways by PKCδ overexpression. A, Western blot revealed with pERK and total ERK antibodies. At basal level, both PKCδ-PANC1 and pMTH-PANC1 cells showed similar total ERK levels and almost undetectable pERK. However, after PMA treatment, pERK was significantly more activated in PKCδ-PANC1 (*t* test, **P* < 0.05 vs pMTH-PANC1 at 30 and 60 minutes). B, Western blot processed with pAKT and total AKT antibody. Protein kinase C delta PANC1 showed a slightly higher basal level of pAKT, which reached higher levels than pMTH-PANC1 cells, upon serum stimulation. Total AKT was similar in both cells (*t* test: **P* < 0.05 vs PKCδ-PANC1 without serum exposure; # and &, *P* < 0.05 vs pMTH-PANC1 at 0 minute of serum exposure).

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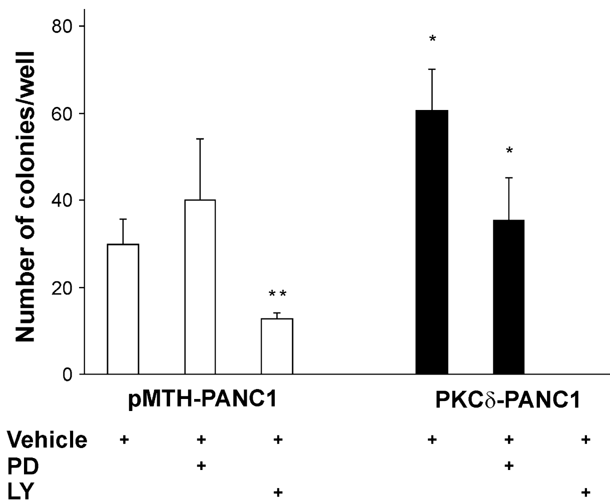

FIGURE 4. Anchorage-independent growth of PANC1 cells. PANC1 transfected cells with PKCδ formed a significantly higher number of colonies in soft agar. When colonies grew in the presence of pharmacological inhibitors of signaling pathways, it was observed that PD98059 (inhibitor of ERK1/2 pathway) prevented the increase of the number of PKCδ-PANC1 colonies. As shown, PI3K also participates in the anchorage-independent growth of PANC1 cells. Data are expressed as the mean ± SD of triplicate determinations (ANOVA, Scheffe test: $^{**}P < 0.01$ vs pMTH-PANC1 cells and $^{*}P < 0.05$ vs PKCδ-PANC1 cells).

showed a granular or a diffuse staining at cytoplasm level. We found that 2 tumors were negative, 7 expressed a low amount of PKCδ, and 13 samples expressed moderate or high PKCδ levels (Fig. 1).

In 19 cases, it was possible to study the expression of the enzyme in normal peritumoral ductal cells with complete preservation of its histoarchitecture. In 68.4% (13/19) of these cases, tumor cells presented a higher expression than its adjacent normal counterpart. In addition, 31.6% (6/19) tumors showed a similar PKCδ expression between tumoral and their respective normal duct cells.

Then, we analyzed whether PKCδ immunostaining could be associated with the main clinicopathological features in pancreatic cancer (Table 1). We did not find any association with patients' age, sex, tumor size, presence of metastatic lymph nodes, or histological differentiation grade.

Stable Expression of PKCδ in the Human Ductal Pancreatic Carcinoma Cell Line PANC1 (PKCδ-PANC1)

To study the role of PKCδ in the pancreatic cancer, we used a well-established cell model for this disease, PANC1. First, we studied the expression of PKC isoforms in this cell line. As shown in Figure 2A, these cells are able to express PKCα, PKCβ, PKCζ, and very low levels of PKCδ. To study the role of PKCδ in the modulation of tumor progression, we transfected PANC1 cells with an expression vector encoding PKCδ or with the vector alone as control. After selection with G418, antibiotic-resistant clones were screened for PKCδ expression by Western blot. The level of expression of PKCδ in the transfected cells was more than 10 times higher than in the nontransfected ones (Fig. 2A). On the other hand, PKCδ transfection did not alter the expression of other PKC isozymes present in PANC1 cells. The transfection approach did not modify the epithelial polyhedric morphology of PANC1 cells when grown as monolayers (data not shown).

The high expression of PKCδ was also confirmed by immunofluorescence showing that PKCδ-PANC1 presented a remarkable cytoplasm granular staining in more than 85% of cells, whereas pMTH-PANC1 cells were almost negative (data not shown). The treatment of PKCδ-PANC1 cells with the phorbol ester PMA induced the translocation of PKCδ from the cytosol to the membrane between 1 and 3 minutes after activation (Fig. 2B). Also, using immunofluorescence, we observed that the PMA treatment induced the translocation of PKCδ to the nuclear compartment (data not shown). These results suggested that PKCδ ectopically expressed in PANC1 is phorbol ester responsive and functionally active.

Activation of PI3K/AKT and ERK1/ERK2 by PKCδ

Next, we analyzed whether PKCδ overexpression modulated p42/p44 ERK MAPK, a crucial molecule in the mitogenic signaling pathway. Control and PKCδ-PANC1 cells showed similar low basal levels of phosphorylated (active) ERK. However, 30 to 60 minutes after PMA treatment, PKCδ-PANC1 cells showed a significant higher level of pERK than control cells. The same treatment in control cells induced a less degree of ERK activation, reaching the maximum value at 15 minutes. No changes were observed in the total ERK levels (Fig. 3A).

We also studied whether PKCδ was able to activate the PI3K/AKT pathway, a crucial mediator of biological properties associated with cancer progression. We found that basal pAKT levels were slightly higher in PKCδ-PANC1 cells relative to

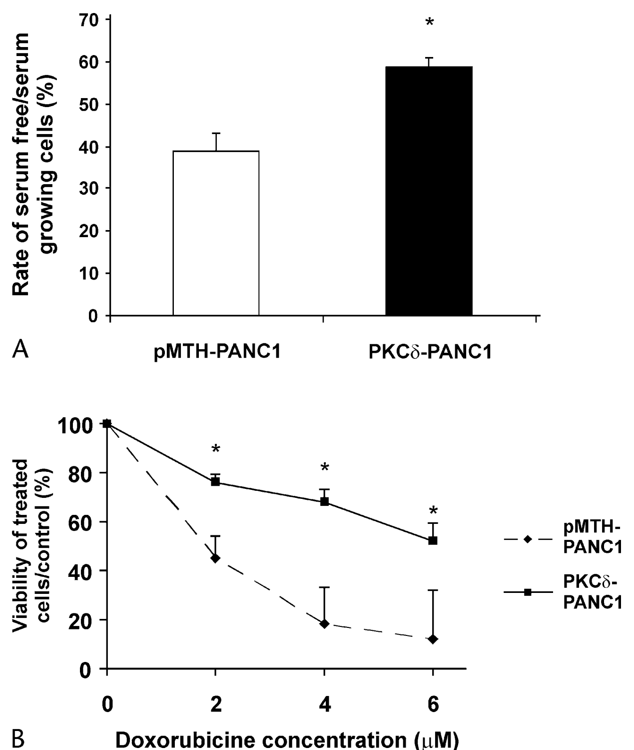

FIGURE 5. Cell death induced by stress conditions. A, Cells growing in the absence of serum. Protein kinase C delta PANC1 were more resistant to starving conditions (*t* test, $^{*}P < 0.05$). B, As shown, PKCδ-PANC1 are more resistant to treatment with the cytotoxic drug Dox (*t* test, $^{*}P < 0.05$ PKCδ-PANC1 cells vs pMTH-PANC1 cells at each Dox concentration).

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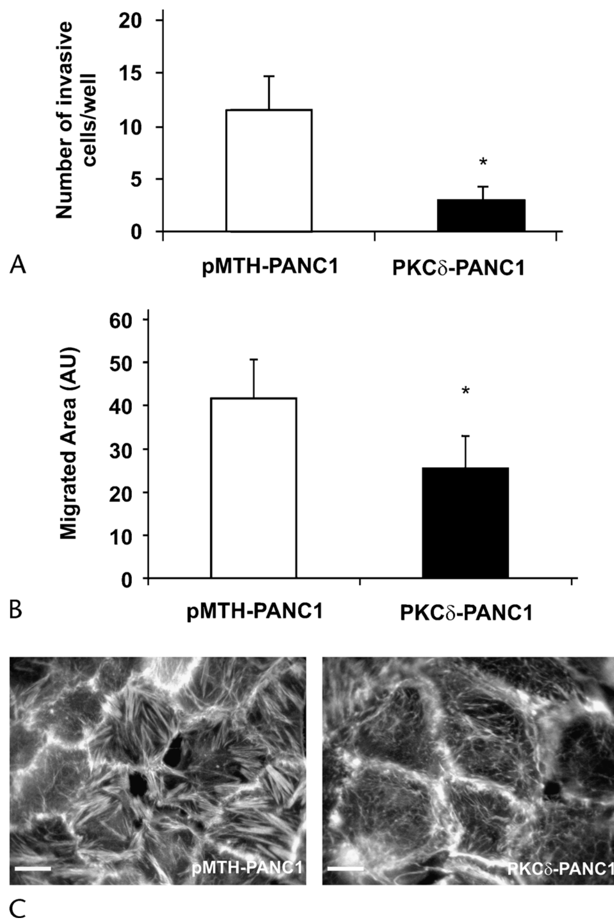

FIGURE 6. Effect of PKCδ overexpression on cellular properties associated with tumor progression. A, Protein kinase C delta PANC1 cells showed a reduced invasion ability with respect to control pMTH-PANC1 cells. Data are expressed as the mean ± SD of triplicate determinations (*t* test, $*P < 0.05$). B, Protein kinase C delta PANC1 showed a reduced ability to migrate when a wound assay was used (*t* test, $*P < 0.05$). C, Fluorescence microscopy using phalloidin-FICT showed that PKCδ overexpression decreased the number of actin stress fibers while increasing the cortical microfilaments distribution (×1000).

control cells. The addition of serum to serum-starved PANC1 cells induced a higher activation of AKT in PKCδ-PANC1 cells (Fig. 3B).

Effects of PKCδ Expression on the In Vitro Behavior of PANC1 Cells. 1-PKCδ Does Not Modulate the Growth of Unsynchronized Monolayers But Enhances Growth in Anchorage-Independent Conditions

To determine whether PKCδ modulates growth in PANC1 cells, proliferation curves were obtained for a period of 4 days. No difference in the population doubling time between PKCδ-PANC1 and pMTH-PANC1 cells was observed when cells were grown as unsynchronized monolayer cultures attached to the plastic surface (population doubling time, 35.5 ± 0.9 hours vs 31.5 ± 4.0 hours for PKCδ-PANC1 and pMTH-PANC1, respectively).

On the other hand, as the ability to grow in an anchorage-independent way represents an important indicator of cell transformation, cells were seeded in soft agar. We observed that the transfection of PKCδ induced a significant increase (about 50%) in the ability of PANC1 cells to form colonies in soft agar (Fig. 4).

To investigate the signaling pathways involved in the modulation of anchorage-independent growth, the same assay was performed in the presence of ERK1/2 and PI3K pharmacological inhibitors (Fig. 4). We determined that the increase in the number of PKCδ-PANC1colonies was inhibited when the ERK1/2 pharmacological inhibitor PD98059 was added to the culture medium without affecting control cells. Moreover, LY294002, a PI3K-specific inhibitor, induced a reduction of about 58% in the number of pMTH-PANC1 colonies while it completely impaired the development of PKCδ-PANC1 colonies. These results suggest the involvement of both ERK1/2 and PI3K/AKT pathways in the enhancement of anchorage-independent growth induced by PKCδ in PANC1 cells.

2-PKCδ-PANC1 Cells Are More Resistant to Cell Death Induced by Stress Conditions

The ability of tumor cell populations to expand in number is determined by the balance between proliferation and death. The acquisition of death resistance constitutes an essential feature in malignant transformation and represents a hallmark for most types of cancer. This prompted us to explore whether

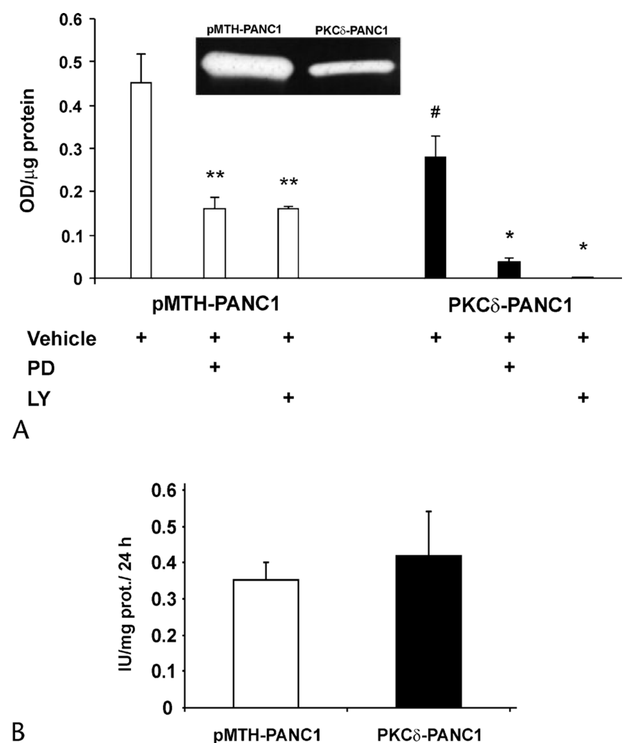

FIGURE 7. Proteolytic enzymes associated with the invasive phenotype. A, Quantitative zymography indicated that PKCδ-PANC1 cells secreted lower amount of MMP2. Also shown is the effect of pharmacological inhibitors of MEK, and PI3K pathways ([#]PKCδ-PANC1 vs pMTH-PANC1; (Anova-Scheffe, [#]PKCC-PANC1 vs pMTH-PANC1, * and $**P < 0.05$ vs the corresponding control). Inset: Zymography showing 2 gelatinolytic bands (MW ~65 kDa) of representative samples of PKCδ-PANC1–secreted media (right) and pMTH-PANC1–secreted media (left). B, Radial caseinolysis showed that the overexpression of PKCδ did not modulate the uPA activity in PANC1 cells.

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TABLE 2. In Vivo Tumor Growth Parameters of PKCδ-PANC1 and pMTH-PANC1 Cells

	pMTH-PANC1	PKCδ-PANC1
Incidence	5/10 (50%)	10/12 (83.3%)*
Latency median (range), days	66.0 (66–73)	36.5 (31–53)†
Growth rate, mm^3/d	1.34 ± 0.75	4.96 ± 1.83‡
Metastatic ability	No	Yes

Protein kinase C delta PANC1 tumors presented a significantly lower latency and a higher growth rate than control tumors.

*χ^2 test; borderline significance, $P = 0.09$.

†Mann-Whitney *U* test, $P < 0.01$.

‡*t* test, $P < 0.001$.

PKCδ could modulate the survival capacity of PANC1 cells in response to different stress conditions such as serum starvation or the presence of the cytotoxic agent Doxorubicine (Dox).

As shown in Figure 5A, PKCδ cells were more resistant to cell death induced by serum deprivation, indicating that PKCδ could be associated with a lower dependence on FCS factors. When floating and attached cells, after 48 hours of serum withdrawal, were collected and analyzed together by flow cytometry, an approximately 6-fold decrease in the number of sub-G0/G1 figures in PKCδ-PANC1 population compared with pMTH-PANC1 was seen (3.73% of apoptotic figures vs 22.7% in control cells).

Moreover, Figure 5B shows that PKCδ-PANC1 cells were more resistant to the cytotoxic effect of Dox treatment than vector transfectant cells. For example, at a dose of 6 μmol/L Dox, PKCδ-PANC1 cells were approximately 4 times more resistant to the cytotoxic effect than pMTH-PANC1 cells.

3-PKCδ Impairs the In Vitro Invasive and Migratory Ability and Induces the Redistribution of Actin Cytoskeleton

Matrigel assays using transwell chambers were used to determine the effect of PKCδ on the invasive potential of PANC1 cells. We observed that the stable overexpression of PKCδ significantly reduced the invasive ability of PANC1 to cross the ex vivo matrix analog (Fig. 6A).

Wound migration assays showed that PKCδ-PANC1 had a reduced ability to migrate compared with control cells (Fig. 6B). To understand the impaired migratory capacity of PKCδ-PANC1, we analyzed actin cytoskeleton distribution, a factor influencing migration and invasion of tumoral cells. We observed that PKCδ-PANC1 cells presented a decrease of stress fibers compared with PANC1 control cells (Fig. 6C). On the other hand, no alteration in the expression and/or distribution of the intermediate filament vimentin was observed (data not shown).

4-PKCδ Does Not Modulate uPA But Impairs the Ability of PANC1 Cells to Secrete MMP2

It is known that proteases are disregulated during pathological events such as tumor invasion. Among the main proteases associated with the invasive capacity of tumor cells are the MMPs and the serine protease uPA. Zymographic assay followed by a densitometric analysis indicated that PANC1 cells secrete mainly MMP2. As shown in Figure 7, PKCδ-PANC1 cells secreted significantly lower amounts of catalytically active MMP2 (approximately 2-fold) than the control cells. It was observed that the impairment of MEK1 and PI3K pathways reduced MMP2 secretion in both cell lines when different pharmacological inhibitors of the main signaling pathways were used. However, a further analysis indicated that the impairment was more marked in PKCδ-PANC1 cells. Thus, while LY 294002, a PI3K specific inhibitor, reduced more than 60% of MMP2 secretion in pMTH-PANC1, it almost totally impaired the secretion in PKCδ-PANC1.

On the other hand, the overexpression of PKCδ did not modulate uPA secretion by PANC1 cells as evaluated by radial caseinolysis of CM (Fig. 7B).

PKCδ Promotes In Vivo Tumor Growth of PANC1 Cells

Protein kinase C delta PANC1 and pMTH-PANC1 cells were inoculated sc into nude mice to determine their tumorigenicity, latency, and growth rate. Although PKCδ-PANC1 cells developed sc tumors in a higher percentage of mice than pMTH-PANC1 cells, the statistical analysis indicated that this difference shows only a borderline significance (Table 2). On the other hand, PKCδ-PANC1 tumors presented a significantly lower latency and a higher growth rate than control tumors (Table 2; Fig. 8A). Ninety-six days after cell inoculation, the mean volume of PKCδ-PANC1 tumors was about 4 times larger than PANC1 control tumors. Interestingly, only PKCδ-PANC1 developed lung metastasis in approximately 30% of the animals.

Associated with this behavior, the histopathological analysis showed a higher number of mitotic figures and a significantly higher number of cycling cells in PKCδ-PANC1 cells versus control cells as revealed by staining with Ki-67 (Fig. 8B).

DISCUSSION

As PKC enzymes have key roles in determining the fate of cells in relation to growth, survival, and invasion abilities, their deregulation may be associated with tumorigenesis and cancer

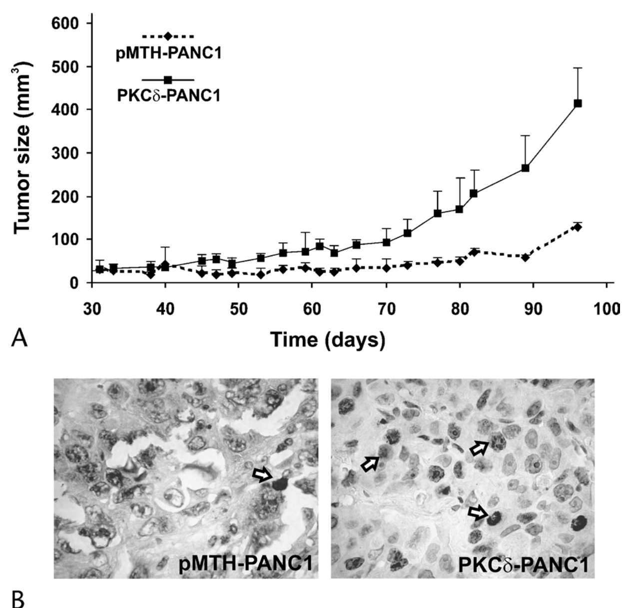

FIGURE 8. Effect of PKCδ on in vivo growth of PANC1 cells. A, Protein kinase C delta PANC1 and pMTH-PANC1 cells grown as subcutaneous tumors in nude mice. The results of 2 independent experiments are shown. The growth rate of PKCδ-PANC1 cells is higher than that shown by control cells. B, Immunostaining of sc tumors against Ki67 antigen. Arrows indicate Ki-67 positive cells.

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progression. However, a great variability was found in PKC-mediated responses in cancer cells, related to both biological outcome and underlying mechanisms, attributed to differences in the cell type–specific pattern of PKC isoenzyme expression. Without any doubt, the role of the novel PKCδ is the most controversial. Therefore, it is of great interest to understand the function of this molecule in cancer and to identify the signaling regulatory pathways utilized by this isoenzyme.

Our aim was to study the role of PKCδ in human pancreatic cancer. First, we demonstrated that this isoenzyme is up-regulated in human ductal carcinomas, similar to the findings of El-Rayes et al,[3] suggesting its role as a tumor-promoting molecule in this tissue. In this regard, PKCδ has also been found overexpressed in colon tumors but down-regulated in malignant gliomas, bladder carcinomas, and endometrial tumors.[4,18,19]

Taking into account our observation about the possible role for PKCδ as a promoter of cancer progression and with the aim to perform functional studies, we undertook a molecular approach to overexpress PKCδ in the human ductal carcinoma cell line PANC1, which presents a low expression of this isoenzyme. We have demonstrated that the stable expression of PKCδ in PANC1 cells was biologically functional, because after PMA stimulation, it translocated to the plasmatic membrane. Also, specific PKC downstream signaling pathways became activated such as those involving ERK/MAPK and AKT. In fact, PKCδ-PANC1 cells had a higher constitutive phosphorylation of AKT than vector transfected cells, which were even more phosphorylated upon specific activation.

Then, we analyzed whether PKCδ was able to modulate in vitro growth, a property that is known to be the result of an intricate balance between proliferation and cell death. Although a great number of studies suggest that PKCδ suppresses proliferation, some reports have demonstrated a positive role for PKCδ in cell proliferation, both in normal and transformed or cancer cell lines.[10,20] We observed that PKCδ enhanced PANC1 cell proliferation but only when cells grew in an anchorage-independent manner, whereas no difference was observed when cells were grown as unsynchronized monolayers. Specifically, the ability of cells to grow in an anchorage-independent way appears to be a fundamental characteristic of cancer cells. Similar to our results, other authors have found that PKCδ could modulate the growth of cells in semisolid agar. For instance, Liao et al[21] have reported that a dominant negative PKCδ could block anchorage-independent growth of transformed rat embryo fibroblasts, and Kiley et al[22] determined that PKCδ overexpression in mammary metastatic cell lines significantly increased anchorage-independent growth. Preliminary results from our laboratory indicated that the overexpression of PKCδ induced the enhancement of the expression of several growth factors such as insulin like growth factor (IGF), epidermal growth factor, and fibroblast growth factor, that could be, at least in part, responsible for their higher growth ability.

To further analyze the signaling pathways involved in PKCδ modulation of anchorage-independent proliferation, we used a pharmacological approach. We observed that both ERK1/2 and PI3K/AKT pathways were involved in the enhancement of PKCδ-PANC1 growth in soft agar. The pharmacological blockage of this last pathway completely impaired the growth of PANC1 colonies in soft agar but only in the context of PKCδ overexpression. Therefore, it is possible that anchorage-independent proliferation becomes absolutely dependent on the PI3K/AKT pathway when PKCδ is up-regulated.

Acquisition of cell death resistance is an early mechanism associated with malignant transformation.[23] Protein kinase C delta isoform is a critical component of the cellular stress response and both proapoptotic and antiapoptotic effects have been reported.[9,10,24–26] We found that PKCδ-PANC1 cells were more resistant than control cells to cell death induced by serum starvation or by doxorubicin. In this regard, there are some controversies in the involvement of PKCδ in DNA damage induced by cytotoxic drugs. In fact, similar to our results, PKCδ was reported to act as a prosurvival factor in the MCF-7 human breast cancer cell line in vitro,[27] but in human non–small cell cancer cells, PKCδ overexpression increases chemotherapy-induced apoptosis,[28] and in prostate cancer cells, PKCδ causes apoptosis via the release of death receptor ligands and the activation of an autocrine proapoptotic loop.[25] Thus, the function of PKCδ varies considerably with cell types and with the apoptotic stimuli. It is probable that the enhanced activation of PI3K/AKT signaling pathway found in PKCδ-PANC1 cells could be responsible for the prosurvival activity. In addition, it is possible that some secreted cytokines induced by PKCδ overexpression could activate, in an indirect way, different survival pathways. For instance, we have recently found that PKCδ-PANC1 cells secreted a higher amount of IGF (Mauro L, data not shown), a growth factor known to promote survival, through the activation of insulin growth factor receptor 1 in the plasmatic membrane, which in turn can phosphorylate insulin receptor substrate 1 and lead to the activation of several downstream molecules such as AKT.[29]

The progression of a tumor from in situ to an invasive phenotype is a major prerequisite for cancer metastasis.[23] There is little information linking PKCδ to invasion and, moreover, few rigorous studies have been carried out to dissect the mechanisms involved. This fact has prompted us to analyze the effect of PKCδ overexpression on the invasive behavior of human ductal pancreatic PANC1 cells. Surprisingly, we found that the overexpression of PKCδ induced in vitro less invasive and migratory phenotypes. Our results are similar to those found by other authors in breast cancer cells or mouse embryonic fibroblasts, isolated from PKCδ null mice models, where PKCδ also negatively modulated migration.[30] Inhibition of migration was also consistent with our finding that PKCδ-PANC1 cells presented less stress fibers and a redistribution of actin to the cortex.

As mentioned previously, the secretion of extracellular proteases, in particular MMPs, a group of zinc-dependent extracellular matrix–degrading enzymes, plays an important role in cancer invasion.[31] It has been shown that increased MMP expression is correlated with the progression of various types of tumors, including pancreatic cancer.[32] Coincidently, with Zervos et al,[33] we found that MMP2 is the principal MMP expressed by PANC1 cells. We determined by using a standard zymography procedure that the in vitro less invasive PKCδ-PANC1 cells secreted a significantly lower amount of active MMP2. On the other hand, no modulation in the level of secreted uPA in PKCδ-PANC1 was observed. In other cellular models, the overexpression of PKCδ was also associated with a reduced ability to secrete proteolytic enzyme.[13]

To analyze the signaling pathways involved in the inhibitory effect of PKCδ on MMP2 production, we used a pharmacological approach. Neither the blockage of MEK nor that of PI3K reversed the PKCδ effect. Unexpectedly, the blockage of any of these pathways reduced even more MMP2 activity, being the effect induced by the blockage of PI3K, which is much more striking in the PKCδ-PANC1 cells than the pMTH-PANC1 cells. Our results give new evidence about the elusive role of PI3K/AKT in invasion. As the invasion results

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from the balance between proinvasive and anti-invasive pathways, we hypothesize that while activated, PI3K/AKT promotes invasion of pancreatic cancer cells, similar to Tanno's results using the same model,[34] other molecular pathway(s) activated by PKCδ could inhibit invasion. Thus, when the proinvasive activity induced by PI3K/AKT is blocked, the balance slopes toward a higher inhibition of invasion. In addition, recent reports indicate that the ser-thr AKT family is composed of 2 members with different activities[35,36]: AKT1 that can generate contradictory messages, increasing soft agar growth, but inhibiting invasion; and AKT2 that stimulates both growth and invasion. Preliminary results using phospho-MAPK kinase antibody array indicated that although both AKT-activated isoforms are increased, the enhancement of pAKT1 seems to be the most striking one.

As the stable overexpression of PKCδ in PANC1 cells increased anchorage-independent growth and cell survival but impaired in vitro cell invasion, it was a challenge to establish the real contribution of the PKCδ isoenzyme to cancer progression. For this reason, PKCδ-PANC1 and pMTH-PANC1 cells were inoculated as xenografts in the subcutaneous flank of nude mice. We observed that PKCδ promoted the in vivo tumoral growth of PANC1 cells and their ability to develop spontaneous lung metastasis. The discrepancy between in vitro and in vivo results could be attributed to the complex mechanisms that regulate proteolytic secretion and the invasion ability of tumoral cells. The higher invasive and metastatic behavior of pancreatic cells overexpressing PKCδ could be due to the interplay of other enzymes and their natural inhibitors that could induce a more malignant phenotype in vivo. In fact, uPA activity, which was not reduced by PKCδ overexpression, could initiate in an in vivo context a proteolytic cascade responsible for the metastatic phenotype.

CONCLUSIONS

Pancreatic ductal adenocarcinoma is the fourth most common cause of cancer-related mortality. This tumor 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.[37] We demonstrated a higher expression of PKCδ in human ductal pancreatic samples, implying that PKCδ could be an additional genetic or epigenetic alteration involved in pancreatic cancer progression. Our experimental approach in a human ductal carcinoma cell line supports this conclusion, as the overexpression of PKCδ makes PANC1 cells grow faster in vivo and acquire a metastatic phenotype. Mechanistic studies suggest that this behavior is related to a higher in vitro anchorage-independent growth and to an enhanced resistance to different apoptotic stimulus, involving at least AKT and ERK signaling pathways.

A growing number of studies suggest that PKC would be a plausible drug target for treatment of certain human cancers, and our results support this concept for pancreatic cancer. However, more studies are necessary before using. The blockage of PKCδ would induce the reduction of cell growth but at the same time might promote migration, enzyme secretion, and invasion. Therefore, it is possible that the final result would be dependent on the particular genetic background of the treated cancer cell. Therefore, without hesitation, the relative role of individual PKC enzymes in cancers is just beginning to be known.

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