Inhibition of PKC α reduces the ability of migration of kidney cancer cells but has no impact on cell apoptosis

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Received July 29, 2015; Accepted January 20, 2017

DOI: 10.3892/etm.2017.4258

Abstract. Kidney cancer is among the most important causes of cancer-associated mortality worldwide. The present study aimed to evaluate protein kinase C α (PKC α) expression in kidney cancer tissues and cell lines, and its significance in apoptosis and migration. Expression of PKC α was analyzed using quantitative polymerase chain reaction and western blotting. In addition, the inhibitor of PKC α (calphostin C and GO6976) was used to treat kidney cancer cells. The ACHN cell line was generated with PKC α -small-interfering RNA (siRNA) and a stable expression of PKC α , in order to facilitate the analysis of apoptosis and migration of PKC α during knockdown and inactivation. Flow cytometry was used to determine the rates of apoptosis. Immunohistochemical staining was used to identify the localization of PKC α in renal clear cell carcinoma and normal sections. PKC α expression in normal tissues was found to be greater than in cancerous tissues. Furthermore, apoptosis was not promoted with PKC α inhibitors or PKC α -siRNA treatment, and a decrease of the migration ability was observed following transfection with PKC α -dominant negative. The results indicated that inhibition of PKC α might not contribute to apoptosis progression in kidney carcinoma.

Introduction

Kidney cancer is the one of the leading causes of cancer-related mortality worldwide. Renal clear cell carcinoma (RCC) accounts for ~85% of kidney cancer diagnoses, particularly in the United States (1,2).

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Abbreviations: PKC, protein kinase C; RCC, renal clear cell carcinoma; ADM, adriamycin; PARP, poly-ADP-ribose polymerase; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PI, propidium iodide

Key words: protein kinase C α , kidney carcinoma, apoptosis, migration, inhibitor

Protein kinase C (PKC) represents a family of serine/threonine kinases that are classified into three major groups: Classical (α , β I, β II and γ), novel (δ , ϵ , η and θ) and non-classical (μ , ζ and ι). These kinases may be involved in growth, differentiation, apoptosis, tumor promotion and migration (3-5). As a result, all of these PKC isozymes have distinct functions and sometimes they even have opposing roles in cancer (6,7). For instance, in glioma cells, PKC α enhances cell proliferation, indicating that it may have a role in tumor promotion. However, overexpression of PKC δ inhibits cell proliferation and may be associated with cell apoptosis (8). Previous results have suggested that PKC α may be closely associated to cancer progression (5). Notably, a high level of PKC α expression was strongly associated with a high migratory activity of colon cancer cells, and a translocation of the activated PKC α at the plasma membrane was observed (9). By contrast, cell apoptosis was induced in LNCaP cells of prostate carcinoma upon activation of PKC α , indicating tumor suppressive properties (10). Previous research from our laboratory revealed that high expression and activation of PKC α is associated with tumor progression in superficial bladder carcinoma, and abnormal activation of PKC α may result in endogenous resistance to chemotherapy drugs, such as adriamycin (11). Furthermore, it has been suggested that the unusual translocation of PKC α between the plasma membrane and cytosol could be involved in the progression of kidney carcinoma (12). However, the mechanism of PKC α in kidney cancer and its efficiency thus far remained unclear. Whether PKC α functioned as a tumor promoter or suppressor in kidney cancer was also unclear. Therefore, the present study investigated the expression of PKC α in both clinical specimens and kidney cancer cell lines, and used gene silencing technology in order to identify the function of PKC α in kidney cancer.

Materials and methods

Cell culture. All cell lines were purchased from Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). In total, four different types of kidney cell lines selected: ACHN (Obtained from carcinomatous pleural effusion of kidney cancer), 786-O (renal adenocarcinoma), caki-1 (skin metastasis of kidney cancer) and HKC (immortalized renal tubular epithelial cells). ACHN and 786-O were cultured in RPMI-1640 (Lonza, Verviers Sprl, Verviers, Belgium) basal medium. Caki-1 was grown

in McCoy's 5A medium (Gibco; Thermo Fisher Scientific, Inc., Grand Island, NY, USA), and F12 basal medium (Gibco; Thermo Fisher Scientific, Inc.) was selected for the HKC cells. Cells were also supplemented with 10% fetal bovine serum (FBS; EuroClone SpA, West York, UK) with antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin; both Santa Cruz Biotechnology, Inc., Dallas, TX, USA) in a humidified 5% CO₂ incubator at 37°C.

PKC α inhibitors and antibodies. The PKC α inhibitor GO6976 and calphostin C were obtained from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany) and dissolved in dimethylsulfoxide (Sigma-Aldrich; Merck Millipore). Antibodies against PKC α (sc-8393) and poly-ADP-ribose polymerase 1 (PARP-1; sc-25780) were purchased from Santa Cruz Biotechnology, Inc.). Anti-caspase 3 was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; WH0002597M1) was obtained from Sigma-Aldrich (Merck Millipore) and anti-caspase-9 (WL01551) was supplied by Wanleibio Co., Ltd. (Shenyang, China).

Small-interfering RNA (siRNA), plasmid and cell transfection. The sequences of PKC α siRNA were as follows: Forward, 5'-GUGCCAUGAAUUUGUACUTT-3' and reverse, 5'-AGUAAACAAUUAUGGCACTT-3'. Inactivation of PKC α in ACHN cells was achieved when ACHN cells stably expressed the PKC α -dominant negative (DN; ACHN-DN) PKC α . PKC α -siRNA and ACHN-DN plasmid (2.5 μ g of plasmid DNA) transfections were both performed using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA). Lipofectamine[®] 2000 was used for 12 μ l in a 2-ml transfection system. After 24 h of transfection, the cells were replaced by RPMI-1640 basal medium for 24 h and were then processed in two parts. In the first part the cells were harvested following siRNA transfection and RPMI-1640 basal medium treatment for 24 h; In the second part the cells were continuously treated with G418 (600 μ g/ml) for selecting stable clones for a period of 14 days. The medium was changed every 48 h and colonies of G418-resistant cells were selected.

Samples. In total, 20 patients (aged 31-79 years; 10 men and 10 women) who were diagnosed with primary kidney carcinoma were selected from the Department of Urology in the First Hospital of China Medical University (Shenyang, China) between December 2011 and July 2013. Seven cases had left RCC and thirteen cases had right RCC. The diagnosis was confirmed by pathological examination, and the histological subtype was identified as RCC. In addition, none of the patients accepted chemotherapy or radiation therapy treatments. Samples of the normal control kidney tissue were collected from each patient with a distance of >3 cm from the tumor. A part of tumor tissues and the corresponding normal tissues were quickly frozen in -80°C for protein extraction and the remainder was perfused with phosphate-buffered saline (PBS) and fixed with 4% formalin overnight for paraffin embedment and immunohistochemical staining.

Quantitative polymerase chain reaction (qPCR) analysis. RNAs were extracted with TRIzol reagent (Invitrogen;

Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, and were then quantified using a NanoDrop ND-100 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Reagents were purchased from Takara Bio, Inc. (Otsu, Japan) and were used to detect the expression of PKC α . The reaction was 20 μ l in total and a LightCycler 480 qPCR system (Roche Diagnostics, Basel, Switzerland) was used for the reaction. The conditions were the following: 50°C for 2 min, 95°C for 5 min, 45 cycles of 95°C for 40 sec and 55°C for 30 sec. Data were analyzed using the 2^{- $\Delta\Delta$ ct} method and mRNA expression was normalized against β -actin RNA. The primer sequences for β -actin were as follows: Sense, 5'-CTCCATCCTGGCCTCGCTGT-3' and anti-sense, 5'-GCTGTCACTTCACCGTTCC-3'. In addition, the PKC α mRNA expression was detected using the following primers: Sense, 5'-GGAACCAAGCAGTATT-3' and anti-sense, 5'-GTCCTTCTGAATCCAACAT-3'. The PKC β sequences were: Sense, 5'-AAATGCTCCCTCAACCCT-3' and anti-sense, 5'-TCAAATCCCAATCCCAA-3' and the PKC γ sequences were: sense, 5'-GCGGCTGGAACGATTGGA-3' and anti-sense, 5'-TGGCGGCGGGTGAGATTAC-3'.

Western blotting. The purchased cells were lysed in the culture flask using RIPA buffer containing the protease inhibitor phenylmethanesulfonyl fluoride, then centrifuged at 13,200 $\times$ g for 30 min. Total cellular proteins were extracted from kidney tissues using a protein extraction buffer RIPA containing protease inhibitors. Equal quantities of protein (50 μ g cells and 100 μ g tissues) extracts were subjected to 10 % SDS-PAGE electrophoresis at 220 V and the resolved proteins were transferred onto polyvinylidene fluoride membranes. The membranes were then blocked for 60 min in a 37°C temperature-controlled shaking table, and subsequently incubated with the following primary antibodies: Polyclonal mouse anti-PKC α (1:1,000), polyclonal rabbit anti-PARP-1 (1:1,000), polyclonal rabbit anti-caspase-3 (1:1,000), polyclonal rabbit anti-caspase-9 (1:1,000) and anti-GAPDH (1:2,000) overnight at 4°C. The following day, Tris-buffered saline and Tween-20 was used to remove unbound antibodies and then incorporated with the secondary antibody diluted at 1:2,000 for 60 min in a 37°C temperature-controlled shaking table. The bands were then visualized by chemiluminescence using the EC3 Imaging System (UVP LLC, Cambridge, UK).

Immunohistochemical staining. Fresh kidney tissue samples obtained from the patients (~1.5x1.5x0.2 cm) were perfused with PBS and fixed with 4% formalin overnight. The next day, tissues were embedded in paraffin and then 4- μ m sections were placed on glass slides. Afterwards, antigen retrieval was performed for 2.5 min. Next, an immunohistochemical kit (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) was used, then the slides were incubated with primary antibody (monoclonal mouse anti-PKC α ; 1:100; sc-8393; Santa Cruz Biotechnology, Inc.) overnight at 4°C. Subsequently, 3,3'-diaminobenzidine staining was applied for 3 min then rinsed out immediately. In addition, hematoxylin staining solution was used for nuclear counterstaining, and the dyes were rinsed well under running water for >3 h. Finally, the slides were mounted and analyzed using an upright metallurgical microscope (IX71S8F-3; Olympus Corporation, Tokyo, Japan).

Figure 1. (A) Fifteen cases displayed downregulated expression of protein kinase α in renal clear cell carcinoma tissues, and number 11, 17 and 18 were considered to be outliers ($P=0.001$). (B) PKC α had a lower expression in renal cell carcinoma tissues after abandoning pairs of N/T but no statistical significance was observed ($P=0.07$). PKC α , protein kinase C α ; N/T, normal/tumor tissues.

Figure 2. (A) Western blotting was used to measure the PKC α protein in 20 pairs of kidney tumor tissues and their matched normal tissues. Normal tissues had a higher expression than the kidney tumor tissues ($P=0.006$). (B) Expression of PKC α in kidney N/T was assessed by western blotting (unmatched pairs). PKC α exhibited a higher expression in normal tissues compared with the kidney tumor tissues ($P=0.016$). PKC α , protein kinase C α ; N/T, normal/tumor tissues.

Figure 3. PKC α mRNA expression in four kidney cancer cell lines (ACHN, 786-O, Caki-1 and HKC) was assayed using quantitative polymerase chain reaction. β -actin was used as an internal control. PKC α expression was significantly greater in ACHN and Caki-1 cells than in the non-malignant cells, HKC. HKC expression was used as the basis for comparison against the other cell lines. ACHN vs. HKC, ** $P=0.004$; Caki-1 vs. HKC, * $P=0.033$; 786-O vs. HKC, $P=0.116$. PKC α , protein kinase C α .

Figure 4. Expression of PKC α in ACHN cells was much higher than the other two PKC classical types, PKC β and PKC γ ($P<0.01$). PKC, protein kinase C.

Flow cytometry analysis. After 24 h with the PKC α inhibitors, the cells were washed with PBS and detached with 0.25% trypsin. Subsequently, the cells were centrifuged at 1,200 $\times$ g for 5 min and the supernatant solutions discarded. Next, cells were washed twice with PBS, and the mixture was centrifuged at 1,200 $\times$ g for 5 min. Afterwards, the cell mass was resuspended in 400 μ l PBS and detected with a Annexin V-fluorescein isothiocyanate/propidium iodide kit (Beyotime Institute of Biotechnology, Haimen, China) using a flow cytometer (FACSCalibur Flow Cytometer; BD Pharmingen, San Diego, CA, USA) and following the manufacturer's instructions. Q2 represented late stage apoptotic and Q4 represented early apoptotic cells. In addition, Q2+Q4 was used to detect apoptosis.

Scratch wound healing assay. The migration capacities of ACHN cells and the PKC α -DN-expressing cell line ACHN-DN were investigated. The two types of cells were plated at a density of 1×10^5 cells/well in 24-well plates. Cells were then incubated in RPMI-1640 medium containing 10% FBS for 24 h to 80% confluence, then a scratch was performed using a 200 μ l pipette tip, and the cells were grown for 24 h in serum-free medium. The scratch spaces were then analyzed using an inverted microscope.

Statistical analysis. Statistical analysis was performed using SPSS for windows 13.0 statistical analysis software (SPSS, Inc., Chicago, IL, USA). In addition, the independent and the

Figure 5. Inhibition of PKC α did not promote the apoptosis of kidney cancer cells. (A-D) The effect of the two inhibitors on the apoptosis of human kidney cancer cells was investigated by flow cytometry. Apoptosis rates were determined in ACHN cells lines following treatment with calphostin C and GO6976. In this chart, 'cal' represented calphostin C, vs. 'con', which represented the control. ACHN-con, vs. ACHN-Cal: $P=0.24$. ACHN-con, vs. ACHN-GO6976: $P=0.98$. The apoptosis rate was not different after the two drugs were added. PKC α , protein kinase C α .

Figure 6. Apoptosis-regulated proteins had no apparent changes after the addition of the two inhibitors of PKC α -calphostin C and GO6976. In the chart, 'GO' represented GO6976; 'cal' represented calphostin C. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PARP-1, poly-ADP-ribose polymerase 1; PKC α , protein kinase C α .

Figure 7. 786O-si and Caki1-si both represented the PKC α knockdown cells. Cleaved proteins of caspase-3, -9 and PARP-1 were all absent from the transfected cells, which suggested that PKC α has no impact on suppressing apoptosis in kidney carcinoma. PKC α , protein kinase C α ; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PARP-1, poly-ADP-ribose polymerase 1.

paired t-test were used. $P < 0.05$ was used to indicate a statistically significant difference.

Results

PKC α expression was frequently decreased in human kidney cancer tissues. In total, 18 pairs of cancer tissue and their corresponding normal samples were selected. The results of Fig. 1A indicated that PKC α was significantly downregulated in the majority of kidney carcinoma (15 out of 16) using qPCR, where numbers 17 and 18 were considered to be outliers based on statistical algorithms. Afterwards, the pairs were disrupted to compare normal tissues and tumor tissues, and a high expression rate of PKC α in kidney normal tissues was identified (Fig. 1B). In addition, the results of western blotting revealed that the levels of PKC α were evidently decreased in ~80% kidney tumors (16 in 20) (Fig. 2A). However, kidney cancer tissues still show a lower expression than before, after the pairs were separated (Fig. 2B).

qPCR results demonstrated that the expression of PKC α was upregulated in metastatic cancer cell lines. The expression of PKC α was examined by qPCR in renal tubular epithelial HKC cells, in two types of metastatic cancer cell lines and in the human RCC cell line, 786-O. The results indicated that PKC α was significantly upregulated in ACHN ($P = 0.004$) and Caki-1 ($P = 0.033$) cells compared with the HKC cells (Fig. 3). However, proteins associated with metastasis require further studies in the two cell lines.

Drugs, siRNA and plasmids were used to decrease PKC α , however, there was no increase in apoptosis. Initially, the expression pattern of the PKC classical types family (PKC α , β and γ) was analyzed in ACHN cells. qPCR analysis revealed that PKC α expression was prominent in the ACHN cell line, whereas the expression levels of PKC β and PKC γ was too faint to be detected (Fig. 4), indicating that PKC α may mediate specific and vital functions in kidney cancer cells. Calphostin C and GO6976 were inhibitors of the PKC classical types. Since PKC α occupied the main status, the effect of the other two PKC types was considered to be minimal. In addition, the two drugs simply inhibited PKC α . The effects of calphostin C (100 nM) and GO6976 (500 nM) were investigated by flow cytometry and western blotting, and the results demonstrated that PKC α expression was reduced. Furthermore, three types of apoptosis-regulated proteins were also detected (caspase-3, -9 and PARP-1). PKC inhibitors led to a no significant increase in the apoptosis rates of ACHN cells (Fig. 5), and there were no increasing cleaved bands of the apoptosis-regulated proteins (Fig. 6). Based on the lack of increase of apoptosis-regulated protein expression following treatment with the PKC inhibitors in kidney cancer cells, it was speculated that PKC α may be not be an inhibitor of apoptosis. In order to verify this assumption, Lipofectamine 2000 was used to transfect siRNA in order to knockdown PKC α in ACHN and Caki-1 cells. In addition, it was used to get the kinase inactivation plasmid (ACHN-DN) to inactivate PKC α . A successful knockdown of PKC α was confirmed by western blotting. In addition, no significant effects were identified on basal apoptosis-regulated protein expression

Figure 8. Following inactivation of PKC α kinase in the ACHN cell lines, there was no apoptosis-regulated protein increase. This result proved that when PKC α lost its activity, the viability of kidney cancer cells did not change. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PARP-1, poly-ADP-ribose polymerase 1; PKC α , protein kinase C α .

(Fig. 7). ACHN-DN inactivated PKC α but increased its expression. Meanwhile, the condition of apoptosis-regulated proteins remained the same (Fig. 8). These results suggested that PKC α have not yet been found to have a major impact on suppressing apoptosis in kidney carcinoma.

PKC α protein stained strongly in cytoplasm of normal kidney tubular epithelial cells but not in glomeruli. On account of its higher and more prevalent expression in the kidney, PKC α expression was also investigated by immunohistochemical analysis of RCC and normal sections. It was demonstrated that PKC α protein was expressed in the normal kidney proximal tubular epithelial and distal convoluted tubule cells, and it stained the cytoplasm strongly, whilst being absent or negligible in the glomeruli. In addition, PKC α staining in RCC tissue sections was shown to have a very weak brown staining throughout the cytoplasm and sometimes even negatively stained. Finally, almost all 24 pairs of tissues exhibited similar staining characteristics, where strong staining of PKC α protein was indicated in the cytoplasm of normal kidney tissue and weak staining was observed in RCC tissues (Fig. 9).

Decrease of migration ability following transfection with PKC α -DN. In order to analyze a possible effect of PKC α on kidney cancer cell migration, wound-healing assay was applied. It was revealed that transfection with PKC α -DN significantly inhibited the migration of ACHN cells (Fig. 10).

Figure 9. (A) Representative images from immunohistochemical staining showed that protein kinase α was localized in the cytoplasm in normal kidney proximal tubular and distal convoluted tubule cells, with strong brown staining. (B) In addition, renal clear cell carcinoma tissues were almost negatively stained. N, normal tissue; T, tumor tissue.

Figure 10. (A) Scratches width was compared in order to verify the capability of migration of ACHN and ACHN-DN cells. (B) The results showed that after inhibiting the kinase activity of protein kinase C α , the cells' healing rate decreased (* $P=0.003$).

In addition, an altered morphology of ACHN cells was noted following transfection. ACHN cells acquired a more angular shape compared to the untransfected control cells, and the healing rate was subject to statistical analysis.

Discussion

The present study revealed that the PKC family had different biological effects in different types of tumors. The upregulation or downregulation of PKC and their isoenzyme or their biological effect had previously been studied in human tumor samples (13-17). Downregulation of PKC isozymes was associated with the occurrence and progression of several human cancer types. Previous studies had verified that three types of PKC isozymes, α , β and δ , were predominant in the normal bladder epithelium. Furthermore, PKC β and δ were downregulated in transitional cell carcinomas; however, PKC α increased (18,19).

In the present study, two inhibitors (calphostin C and GO6976) were used to decrease PKC α . As earlier work

confirmed, Ca²⁺ spiking can be prevented by the PKC inhibitor calphostin C (20) due to conventional PKCs (α , β I, β II and γ) being activated by Ca²⁺. Therefore, calphostin C mainly inhibited PKC α , β I, β II and γ . In addition, GO6976 had previously been demonstrated to inhibit PKC isoenzymes α and β I (21-23). In Fig. 4, it was indicated that PKC β and γ had low expression in the kidney cancer cell line, therefore GO6976 and calphostin C mainly inhibited PKC α . Thus, apoptosis-related proteins were selected to evaluate the impact on apoptosis following the addition of the two inhibitors. It was revealed that the expression of apoptosis-related proteins, caspase-3, -9 and PARP-1, had not changed significantly, and no hydrolysis bands had evidently increased. Although the focus was on siRNA and the PKC α inactive plasmid, there was no increase observed in cell apoptosis. Therefore, PKC α was not considered to have a major impact on suppressing apoptosis in kidney carcinoma.

Based on the results on the detection of tumor tissues, PKC α was found to have a high expression in normal kidney

but decreased in tumor tissues. In other words, PKC α may decrease when normal kidney tissue becomes cancerous. Notably, when we compared one patient's tumor tissues with normal ones, PKC α presented a lower expression in tumors. However, when we disrupted the pairs the result demonstrated no statistical significance. These results indicated that the expression of PKC α varied from person to person; therefore, the most accurate method was to compare tumor tissue pairs with the normal control ones.

Fig. 3 indicated that in two kidney cancer transferred cell lines, ACHN and Caki-1, PKC α was markedly expressed. However, more research is required in order to understand the mechanism and metastasis-related proteins in the two cell lines, and whether they are correlated with PKC α . Previous research affirmed that in retinal pigment epithelium cells, the wound healed more slowly in the siRNA-PKC α compared to the non-siRNA group at the three time points (24). This result suggests that PKC α may be important in cell migration. In the results of the present study, following inactivation of PKC α , cell migration was inhibited which suggested that PKC α may also be important in the ability to move in kidney cancer cell lines.

Immunohistochemical staining revealed that the expression of PKC α protein was detected in the normal kidney proximal tubular epithelial and distal convoluted tubule cells, which were predominantly located in the cytoplasm, and exhibited very strong staining. Meanwhile, in tumor sections, PKC α stained very weakly or not at all. This observation was consistent with the results of western blotting. In conclusion, it was shown that the inhibition of PKC α may not be due to a significant gain in expression of apoptotic proteins, and that PKC α does not function by suppressing apoptosis in kidney cancer cells. In addition, PKC α was decreased in tumor tissues of the kidney. Therefore, down-regulation of PKC α may be an early event in the development of kidney carcinoma, but the mechanisms by which PKC α functioned remained elusive. In conclusion, the results of the present study demonstrated that inhibition of PKC α may not contribute to apoptosis progression in kidney carcinoma.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant no. 81172438) and the Doctoral Fund of the Ministry of Education of China (grant no. 20112104110006).

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