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Reduction of PKCα Decreases Cell Proliferation, Migration, and Invasion of Human Malignant Hepatocellular Carcinoma

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Abstract Protein kinase C (PKC) superfamily play key regulatory roles on the development of cancer. However, the exact role of these enzymes in human hepatocellular carcinoma (HCC) has not been well established. Using the RT-PCR and Western blotting to analyze the levels of PKC isoforms mRNA and protein in the five different differentiated hepatoma cell lines, we found that PKCα was highly expressed in the poor-differentiated HCC cell lines (SK-Hep-1 and HA22T/VGH) as compared with that in the well-differentiated HCC cell lines (PLC/PRF/5, Hep3B, and HepG2). When treated with PKCα antisense oligonucleotides (ODN), both HA22T/VGH and SK-Hep-1 cells lines showed the reduction of PKCα expression, as well as a deceleration in the growth rate and in the level of cyclin D1, but the increase in the levels of p53 and $p21^{WAF1/CIP1}$. Moreover, the reduction of PKCα expression also inhibited the migratory and invasive potential of both HA22T/VGH and SK-Hep-1 cells lines, and revealed a down-regulation of several migration/invasion-related genes (MMP-1, u-PA, u-PAR, and FAK). These phenomenon were also confirmed by DNA-based small interfering RNA (siRNA) PKCα and PKCα/β specific inhibitor Go6976. Thus, the results indicated that PKCα may be associated with regulation of cell proliferation/migration/invasion in human poorly differentiated HCC cells, suggesting a role for the PKCα in the malignant progression of human HCC. J. Cell. Biochem. 103: 9–20, 2008.

Key words: protein kinase Cα (PKCα); hepatocellular carcinoma; antisense oligonucleotides; small interfering RNA (siRNA)

Protein kinase C (PKC) is an important family of signaling molecules that regulate the proliferation, differentiation, transformation, and apoptosis in cells [Newton, 1995]. The 10 PKC isoforms are divided into conventional (cPKCs:α, βI, βII, and γ), novel (nPKCs: δ, ε, η, and θ), and atypical (aPKCs: ζ and ι/λ) subclasses, depending on their requirement for Ca^{2+}, phosphatidylserine and diacylglycerol [Ohno and Nishizuka, 2002]. PKC plays an important role in carcinogenesis [O'Brian et al., 1989; Kopp et al., 1991; Alvaro et al., 1992; Couldwell et al., 1992; Kusunoki et al., 1992; Tsai et al., 2000]. Changes in PKC activity and PKC isoform expression patterns occur in a variety of tumors. Moreover, PKC may also be involved in tumor promotion/progression [Sharif and Sharif, 1999; Gilhooly et al., 2001; Weichert et al., 2003]. PKC isoform expression alterations have also been demonstrated in the invasion and metastasis of malignant neoplasms. These isoforms contribute to malignant phenotype progression by attachment to the extracellular matrix or basement membrane components, matrix degradation by proteolytic enzymes, and migration through the digested matrix [Gomez et al., 1999].

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Understanding the roles of individual PKC isoforms in haptocarcinogenesis is emerging. In the initiation stage, the levels of PKCα and PKCβ decrease and that of PKCε increases [Lee et al., 1998]. Although the level of PKCα has been demonstrated to be elevated in the promotion stage, findings in PKCβ, δ, and ζ level changes were not consistent in different studies [da Costa et al., 1993; La Porta et al., 1993; La Porta and Comolli, 1994, 1995; Corton et al., 1999]. PKCα and PKCβ level elevation has been associated with the progression of liver cancer, whereas the PKCδ level decreases at this stage [La Porta et al., 1997]. Although changes in PKC isoforms are important for liver cancer promotion/progression, the exact role of these enzymes in human hepatocellular carcinoma (HCC) is remains unclear.

Antisense oligonucleotide (ODN) has been successfully used in many human cell models to inhibit protein synthesis of specific PKC isoforms [Fisher et al., 1993], and has also been employed in studies of specific PKC isoforms in carcinogenesis, tumor progression, and metastasis. For example, antisense of PKCα inhibits cell proliferation in vitro and tumorigenicity in vivo in nude mice xenografts of human glioblastoma and lung cancer cells [Dennis et al., 1998; Jiang et al., 2004; Grossman et al., 2005]. In HCC HepG2 cells, the absence of PKCα activity leads to a reduction in cell growth and induces its apoptosis [Zhu et al., 2005]. These findings suggest that PKCα may be involved in the tumorigenesis of the various cancers, including liver cancer. However, the different in PKCα expression in human hepatocarcinogenesis remains unclear.

In order to evaluate the role of PKCα in human HCC, we used RT-PCR to compare PKC isoform mRNA and protein levels in various differentiations of five human HCC cell lines. We found that PKCα expression significantly increased in the poorly differentiated cell lines as compared to the well-differentiated cell lines. In addition, use antisense ODN PKCα and DNA-based small interfering RNA (siRNA) PKCα to study the functional relevance of the downregulated PKCα expression in HCC cells, our data found that PKCα may play an important role in the regulation of cell proliferation, migration, and invasion in poorly differentiated HCC cells.

MATERIALS AND METHODS

Materials

Anti-PKCα, β, δ, ε, η, θ, ζ, and ι monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). Anti-PKCγ polyclonal antibody was bought from Gibco BRL, Life Technologies, Inc. (Rockville, MD). Anti-p53, anti-$p21^{WAF1/CIP1}$, anti-cyclin D1 and α-tubulin polyclonal antibodies was bought from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-labeled anti-mouse secondary antibody was purchased from Promega (Madison, WI). All primers and antisense ODNs was purchased Mission Biotech (Taipei, Taiwan).

Cell Culture

HA22T/VGH (BCRC No. 60168), PLC/PRF/5 (BCRC No. 60223), Hep3B (BCRC No. 60434), and HepG2 (BCRC No. 60025) were purchased from the Bioresources Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan) and SK-Hep-1 from the American Type Culture Collection (Rockville, MD). The poorly differentiated HA22T/VGH and SK-Hep-1 cell lines and the well-differentiated PLC/PRF/5, Hep3B, and HepG2 cell lines [Aden et al., 1979; Chang et al., 1983]. These cell lines were cultured with DMEM (Gibco BRL) supplemented with 100 μM non-essential amino acid, 2 mM glutamate, 10% fetal bovine serum (FBS), 100 units/ml penicillin G, and 100 μg/ml streptomycin (Sigma Chemicals Co., St. Louis, MO) in a humidified atmosphere containing 5% CO_2 at 37°C.

Oligonucleotide Synthesis

The oligonucleotide primers used in RT-PCR were as follows: the primer sequences of PKCα, PKCδ and PKCζ described previously [Nagata et al., 1996]; PKCβI, PKCβII, PKCγ, PKCε, PKCη, PKCθ and PKCι described previously [Oshevski et al., 1999]; MMP-1 and MMP-3 described previously [Kunapuli et al., 2004]; MMP-2, MMP-9, and MT1-MMP described previously [Chu et al., 2004]; MMP-7 described previously [Ozaki et al., 2000]; u-PA, u-PAR, and t-PA described previously [De Petro et al., 1998]; Rac1 described previously [Lang et al., 2005]; RhoA described previously [Moran et al., 2002]; β_2-MG as a internal

control and described previously [Kondoh et al., 1999]. FAK (GenBank accession no. L13616): 5′-GTGTGAGGGAGAAGTATGAG-3′ (sense) and 5′-ATCTGTAGACTGGAGACAGG-3′ (antisense). The FAK primers were designed using the Biology Workbench 3.2 software (San Diego Super Computer Center, http://workbench.sdsc.edu/).

RNA Isolation and RT-PCR Analysis

Total RNA was isolated from cell specimens by the guanidinium thiocyanate-phenol method. The extract integrity was assessed by 1.5% agarose gel electrophoresis and RNA was visualized by ethidium bromide staining. The total amount of RNA was determined spectrophotometrically. RT-PCR assay was performed according to De Petro et al. [1998] with slight modifications. An aliquot of total RNA (1 μg) was reverse transcribed. The RT product (2 μl) was diluted with the PCR buffer (50 mM KCl, 10 mM Tris–HCl, and 2 mM $MgCl_2$) to a final volume of 50 μl, containing 0.5 μM dNTPs (final concentration, 0.8 mM) and 0.5 unit of Super-Therm Taq DNA polymerase (Southern Cross Biotechnology, Cape Town, South Africa). PCR was performed on a GeneAmp PCR system 2400 (Applied Biosystems, Foster City, CA). For each experiment, the number of PCR cycles was titrated to avoid reaching the amplification plateau. Twenty-five cycles of PCR was used for β_2-MG, 27 for PKCα, PKCδ, PKCζ, MMP-1 and MMP-7, 30 for PKCε and PKCι, 33 for PKCβI, PKCβII, PKCγ, MMP-3, MMP-2, MMP-9, u-PA, u-PAR, t-PA, Rac1 and FAK, and 35 for PKCη and PKCθ. The PCR products were analyzed by 1.5% agarose gel electrophoresis and direct visualization after SYBR Green I (Cambrex Bio Science Rockland, Inc., Rockland, ME) staining. The agarose gels were scanned and analyzed using the Kodak Scientific 1D Imaging System (Eastman Kodak Company, New Haven, CT). To avoid the PCR products to the plateau phase, in our preliminary data we had tested different amount of RNA (0.1, 0.5, 1, and 2 μg) and different cycle number (21, 23, 25, 27, 29, 31, 33, and 35 cycles). Both strands were sequenced for each PCR product from at least two independent PCR reactions. Sequences were compared with the gene data bank by means of BLAST search (National Center of Biotechnology information, NCBI, Bethesda, MD).

Western Blotting

The whole cell lysates were lysed with a lysing buffer (50 mM Tris/HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 1 mM PMSF, 1 mM NaF, 1 mM sodium orthovanadate, 1% (v/v) 2-mercaptoethanol, 1% (v/v) Nonidet P40, 0.3% sodium deoxycholate). Each sample (40 μg) was subjected to 10% SDS-PAGE and blotted onto a polyvinylidene fluoride membrane (Millipore, Belford, MA). After blocking, the membrane was incubated with the specific anti-PKC isoform antibody (1:1,000), anti-p53 antibody (1:500), anti-$p21^{WAF1/CIP1}$ (1:500), anti-cyclin D1 (1:1,000) or α-tubulin antibody (1:2,000). The blots were then incubated with HRP-conjugated anti-mouse antibody (1:3,000) at room temperature for 1 h. Proteins were detected by the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

Cell Proliferation Assay

Cell proliferation was determined by the yellow tetrazolium MTT assay [Sobottka and Berger, 1992]. The optical density at 570 nm was then measured using a spectrophotometer. Cells at the log phase were used to calculate the doubling time according to the equation doubling time $(h) = [\log 2 \times (24 \times \text{No. of days})]/[\log \text{Density}_{\text{final}} - \log \text{Density}_{\text{initial}}]$.

Antisense Knockout Assay

The antisense knockout assay was performed according to Shen et al. [1999] and the following antisense and sense (as a control) sequences were used: PKCα described previously [Shen et al., 1999]; PKCι (antisense 5′-TGTGGGA-CATGGAGCTGCTG-3′, sense 5′-CAGCAG-CACCATGTCCCACA-3′). The ODN sequence of PKCι is in the regions 251–270 of human PKCι mRNA (GenBank accession no. NM_002740). They were formed for targeting the AUG region and had no more than four contiguous intrastrand base pairs or four contiguous G:C pairs.

Cells were plated at 70% density 24 h before antisense treatment. The cells were washed in triplicate with serum free DMEM and incubated with antisense (0, 0.5, 1.0, 2.0, or 5.0 μM) in serum-free DMEM containing 10 μg/ml lipofectin (Life Technologies, Grand Island, NY) at 37°C. The medium was changed to 10%

FBS DMEM medium 6 h later before culturing at 37°C for 24 or 48 h.

Flow Cytometric Analysis

Cell growth was determined by flow cytometry [Detjen et al., 2000]. Cells were treated with sense or antisense ODN (5 μM) for 48 h and aliquots of 10^6 cells were fixed in 70% ethanol at −20°C overnight. The fixed cells were then washed with PBS and incubated in PBS containing 100 mg/ml RNase A, 0.1% Triton X-100, 1 mM EDTA and 1.5 mg/ml propidium iodide (Sigma) at room temperature for 30 min. Cell-cycle analysis was performed on the FACSCalibur flow cytometer and the Cellquest software (Becton Dickinson, San Jose, CA).

siRNA-PKCα Plasmid Construction

We constructed the siPKCα-expressing plasmid vector using the pcDNA-HU6 vector (denoted by Dr. J. Tsai Chang, Institute of Toxicology, College of Medicine, Chung Shan Medical University, Taichung, Taiwan) as the vector backbone according to Chang [2004]. The sequence of the 19-nucleotide siRNA-PKCα duplex from the human PKCα gene (GenBank accession no. NM_002737) was designed using the BLOCK-iT™ RNAi Design available at http://www.invitrogen.com and corresponded to the coding regions 492–510 relative to the first nucleotide of the start codon. The sequences designed to produce hairpin RNAs identical to the oligonucleotide siRNA duplex sequences are follows: sense: 5′-CGC GTC CTG TTG TAT GAA ATT TCA AGA GAA-3′ and antisense: 5′-AAA AAG CGT CCT GTT GTA TGA AAT TCT CTT GAA-3′. To generate siRNA duplex, sense and antisense oligonucleotides (40 μM) were annealed by incubating the mixed oligonucleotides in the PCR thermocycler using the following profile: 37°C for 30 min and 65°C for 15 min. The completed siRNA duplex was then cloned into the pcDNA-HU6 vector in frame of the *Bam*HI and *Hin*dIII sites. The insert was screened by PCR with HU6 primer and confirmed by sequencing with HU6 primer.

Transient Transfection

Transfections were performed using lipofectin. Cells seeded at 60-mm dish were cultured in DMEM supplemented with 10% FCS at 37°C for 24 h. After incubation, the cells were rinsed with serum-free MEM before adding 1 ml MEM containing 15 μg/ml Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA) and 5 μg of the indicated plasmid. The cells were then incubated at 37°C for 6 h before adding 1 ml MEM supplemented with 20% FCS to the medium. After incubation at 37°C for 18 h, the medium was replaced with fresh 10% FCS-DMEM and the cells were incubated at 37°C for 24 h. The cells were then lysed for Western blotting.

Migration and Invasion Assays

The cells were treated with indicated sense or antisense ODN (5 μM) was detached by trypsinization 48 h later and then washed in triplicate in serum-free DMEM. For the migration assay described previously [Saurin et al., 2002], the cells were incubated for 6 h for HA22T/VGH cells, for 12 h for SK-Hep-1 cells and for 24 h for other cell lines. For the invasion assay described previously [Saurin et al., 2002], the cells were incubated for 8 h for HA22T/VGH cells, for 24 h for SK-Hep-1 cells and for 24 h for other cell lines.

Statistical Analysis

Data were expressed as mean ± SEM and analyzed using analysis of variance (ANOVA). Student's *t*-test was used in two-group comparisons. The association between the various factors was determined using the Pearson correlation. $P < 0.05$ was considered to be statistically significant.

RESULTS

PKC Isoform Expression in Human HCC Cell Lines

Using semiquantitative RT-PCR, eight PKC isoforms were shown to have detectable expression in five human HCC cell lines (Fig. 1A). The mRNA level of PKCα was higher in the poorly differentiated SK-Hep-1 and HA22T/VGH cells than in the well-differentiated HepG2, Hep3B, and PLC/PRF/5 cells. A higher PKCι mRNA level was observed in HA22T/VGH, SK-Hep-1, and PLC/PRF/5 cells. However, PKCζ exhibited an opposite trend. For PKCβI and PKCε, the highest mRNA levels were found in SK-Hep-1 cells and PKCβII in PLC/PRF/5 cells. A higher PKCδ mRNA level was observed in HA22T/VGH, SK-Hep-1, Hep3B, and HepG2 cells and PKCη in SK-Hep-1 and PLC/PRF/5 cells (Fig. 1A). The mRNAs of PKCγ and PKCθ were undetectable in all cell lines (data not shown). These mRNA expression patterns were

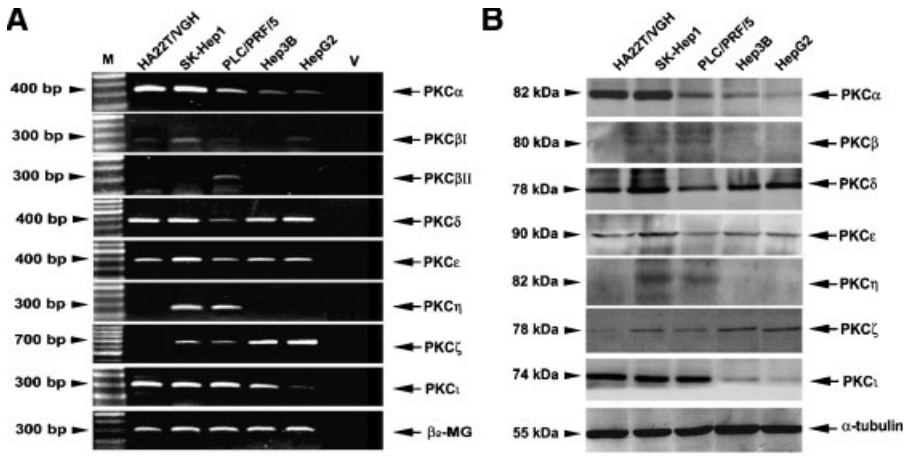

Fig. 1. PKC isoform expression in five human HCC cell lines. **A**: The mRNA levels of PKCα, βI, βII, δ, ε, η, ζ, and ι were detected by RT-PCR as described in Materials and Methods.β_2-MG was used as an internal control. V, negative control for RT-PCR without RT; M, DNA size marker. **B**: The protein levels of PKC isoforms were detected by Western blotting as described in Materials and Methods. Arrows indicate the specific PKC isoform and its molecular weight signed on the left site. The results were based on three independent experiments showing the same expression pattern.

consistent with the protein expression levels determined using Western blotting (Fig. 1B).

A positive correlation was found between the mRNA level and cell doubling time in PKCα ($r = 0.74$, $P < 0.01$) and PKCι ($r = 0.88$, $P < 0.001$). A significant negative correlation was found in PKCζ ($r = -0.77$, $P < 0.01$). For the remaining five detectable isoforms, no significant correlation between the mRNA level and cell doubling times was found.

Antisenses PKCα and siPKCα and Go6976 Effect on Cell Proliferation

Furthermore, we employed antisense ODN PKCα and PKCι to examine the effects of these isoforms on cell proliferation in human HCC cell lines. Antisense ODN PKCα showed a dose-dependent reduction in cell proliferation in HA22T/VGH (Fig. 2A) and the cell doubling time increased from 106.3% of the control (32.9 h) for 1 μM antisense ODN to 161.5% for 2 μM antisense. Moreover, cell proliferation was completely inhibited by 5 μM antisense ODN. At the 5 μM antisense ODN PKCα concentration, the doubling time of SK-Hep-1 cells was increased to 146.6% of the control cells (32.4 h), control cells for PLC/PRF/5 cells increased to 119.5% and for Hep3B cells to 123.9%. No inhibition of cell proliferation was observed in the HepG2 cells (99.1% of the control). Moreover, when treated with 5 μg/ml siPKCα, HA22T/VGH cells revealed the increase in cell doubling time to 135.5% of the control cells. Go6976, a selective PKCα/β inhibitor, at a gradient concentration of 1,000–2,000 nM also significantly inhibited HA22T/VGH cell proliferation from 70% to 80% of the control group. In the antisense ODN PKCι-treated groups, there were no effects on cell proliferation of the HA22T/VGH cells (Fig. 2B). Similarly, these findings were also observed in SK-Hep-1 cells (data not shown).

To verify the effectiveness of antisense ODN in depleting the expression of specific PKC proteins, we examined the PKCα and PKCι levels in total HA22T/VGH cell lysates 48 h after antisense ODN treatment using Western blot analysis. Antisense ODN PKCα (5 μM) and PKCι (5 μM) effectively reduced the levels of the corresponding isoforms compared with the control or sense ODN-treated groups (Fig. 2C,D). The antisense ODN PKCα and PKCι did not inhibit the other non-targeted isoforms.

Because growth inhibition may reflect alterations in the cell cycle, we determined the effects of antisense ODN PKCα or antisense ODN PKCι on cell cycle distributions by flow cytometry. Cell cycle analysis was also performed for the human HCC cells at 48 h after sense or antisense ODN treatment. In HA22T/VGH cells, the G_0/G_1 phase proportion increased from 58% in the control to 72% in the antisense ODN PKCα-treated group (Fig. 3A). The S phase proportion decreased from 24% in the control group to 12% in the antisense ODN

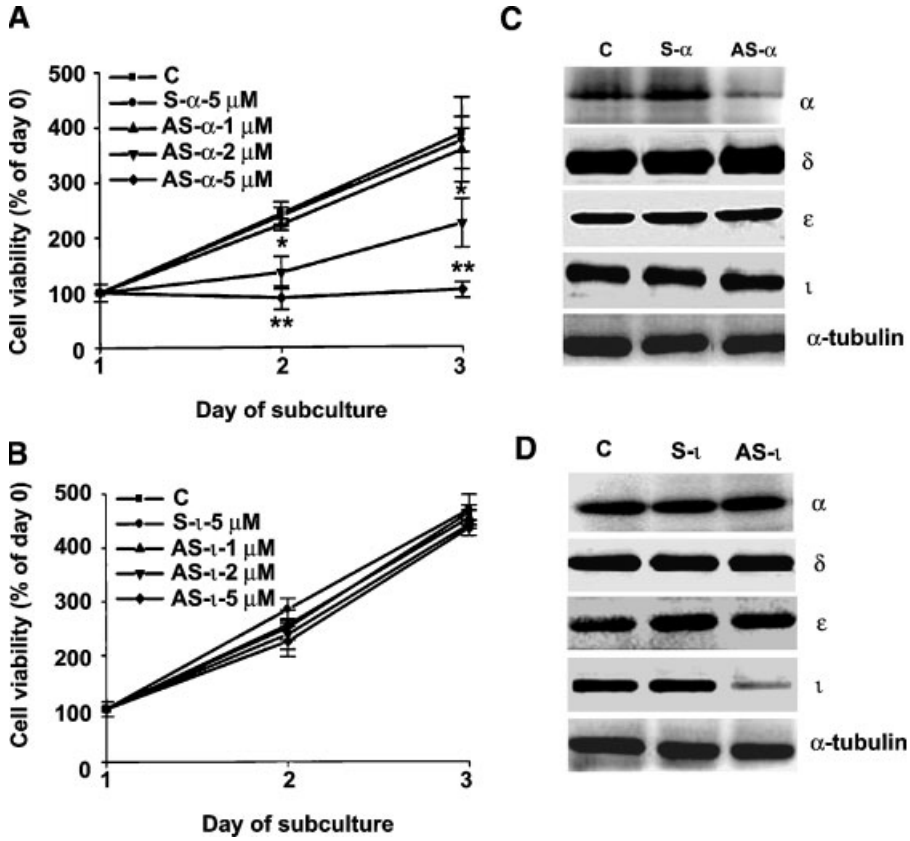

Fig. 2. Effect of antisense ODN PKCs on cell growth. The HA22T/VGH cells were transfected with either 1–5 μM PKCα (**A**) or PKCι (**B**) antisense ODN (AS-α or As-ι) or 5 μM sense ODN (S-α or S-ι). C, the untreated group was designed as control. Cell growth was determined 1–3 days after subculture using the MTT assay as described in Materials and Methods. Absorbance values obtained from untreated cells on day 0 after subculture were taken as 100%. The specific PKC isoform protein reduction by 5 μM PKCα (**C**) or PKCι (**D**) antisense ODN (AS-α or As-ι) or 5 μM sense ODN (S-α or S-ι) was measured on day 3 after subculture using Western blot as described in Materials and Methods. Data are presented as means ± SE of three replicates from two independent experiments. $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control.

PKCα-treated cells. However, no apparent changes were observed in the antisense ODN PKCι-treated group (Fig. 3B). Moreover, these phenomenon were also observed in SK-Hep-1 cells, and both two antisense ODN produced no effects on the HepG2 cell cycle (data not shown). These results indicate that antisense ODN PKCα suppresses cell growth, at least in part, by inhibiting the $G_1 \rightarrow S$ transition in the cell cycle.

Antisense PKCα Effect on the Expression of p53, $p21^{WAF1/CIP1}$, and Cyclins D1

To identify the molecular basis for antisense ODN PKCα induced G_0/G_1 arrest, we examined the effects of antisense ODN PKCα on the expressions of the $p21^{WAF1/CIP1}$, p53, and cyclin D1 in HA22T/VGH cells using a Western blotting method. As shown in Figure 3C, the protein expression levels of PKCα and cyclin D1 were decreased significantly at 48 h after antisense ODN PKCα treatment. In contrast, the expression of $p21^{WAF1/CIP1}$ and p53 was upregulated. These observations were also present in SK-Hep-1 cells (data not shown). The results, together with the cell cycle analysis, indicate that accumulation of $p21^{WAF1/CIP1}$ and p53 might be responsible for antisense ODN PKCα-induced growth arrest at the G_0/G_1 phase.

Antisenses PKCα and siPKCα and Go6976 Effect on the HCC Cell Migration and Invasion

To determine the role of PKCα in cell migration and invasion, migration and invasion assays were performed on the HCC cell lines. For invasion assays, the polycarbonate membranes were coated with the reconstitute

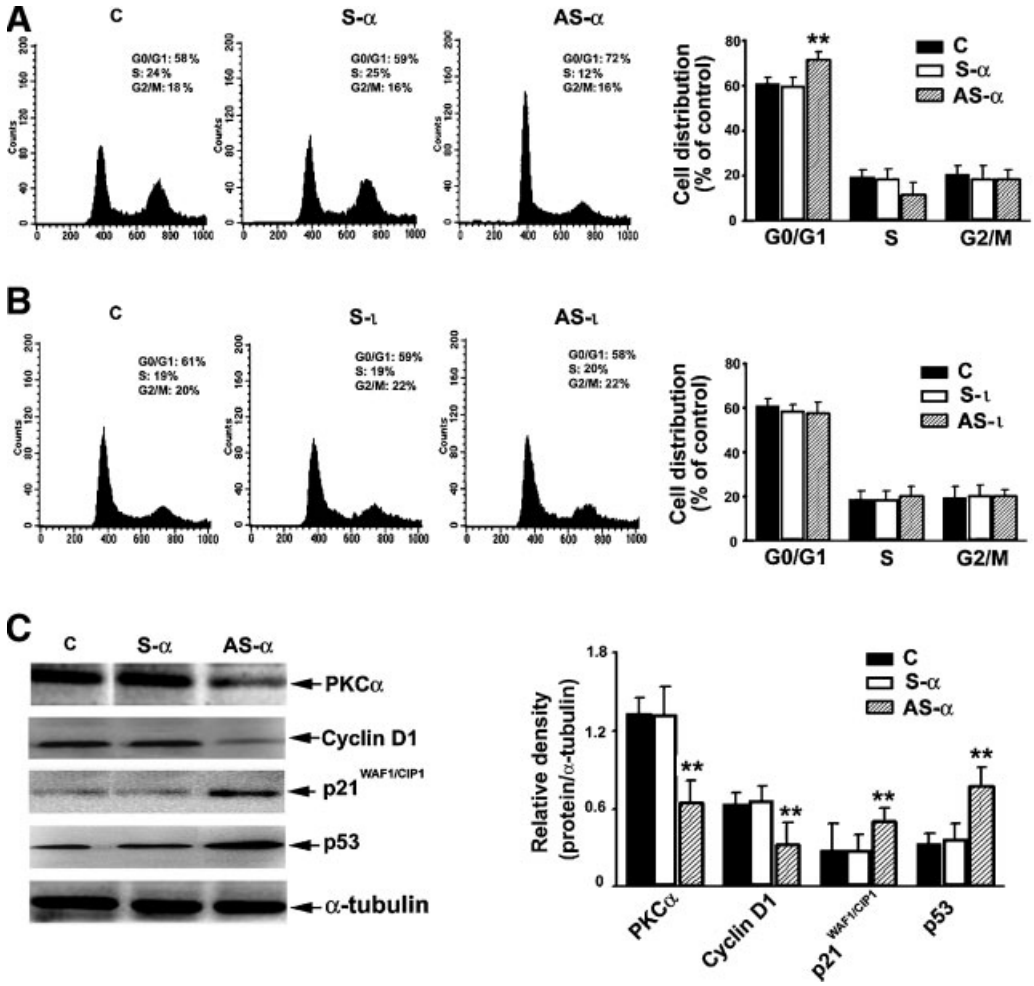

Fig. 3. Antisense ODN PKCs effect on cell cycle arrest of HA22T/VGH cells. **A**: Flow cytometry of cell was treated with 5 μM PKCα antisense ODN (AS-α) or 5 μM PKCα sense ODN (S-α) and assessed for their distribution in the cell cycle as described in Materials and Methods. C, the untreated cultures were designated as controls. The data represent one of three independent experiments with similar results. **B**: Flow cytometry of cell was treated with 5 μM PKCι antisense ODN (AS-ι) or 5 μM PKCι sense ODN (S-ι) and assessed as described above. Values are means ± SE of the percentage of cells in the G1 (open bars), S (closed bars), and G2/M (hatched bars) phases of the cell cycle from three independent experiments. **C**: Expression of PKCα, cyclin D1, p53, and $p21^{WAF1/CIP1}$ after AS-α treatment. HA22T/VGH cells were cultured in the presence of S-α or AS-α for 48 h. Western blotting (**left panel**) was performed as described in Materials and Methods. Relative fold (**right panel**) was determined by normalization with α-tubulin. Values from three similar experiments are pooled and the means and standard errors of the means (SEM) are presented. $^{**}P < 0.01$ versus control.

basement membrane Matrigel. In control cells, the membrane of cells crossing the uncoated filters were about twofold to that of cells passing through the Matrigel-coated filters with longer incubation time indicating that the Matrigel lay constitutes a barrier that had to be actively penetrated by the invasive cells. Antisense ODN PKCα inhibited cell migration and invasion in HA22T/VGH cells by 54% and 55%, respectively, compared with the control or sense ODN groups (Fig. 4A,B, left panel). In the SK-Hep-1 cells, antisense ODN PKCα reduced cell migration and invasion by 55% and 49%, respectively (data not shown). Moreover, siPKCα also decreased cell migration and invasion in HA22T/VGH cells by 50% and 51%, respectively (Fig. 4A,B, middle panel), and Go6976 (1000 nM) decreased by 41% and 39%, respectively (Fig. 4A,B, right panel). This observation suggests that PKCα has a role in cell migrative/invasiveness.

Antisense PKCα and siPKCα Effect on the Migration/Invasion-Related Genes

Using semiquantitative RT-PCR, the migration/invasion-related genes of MMP-1, MMP-3,

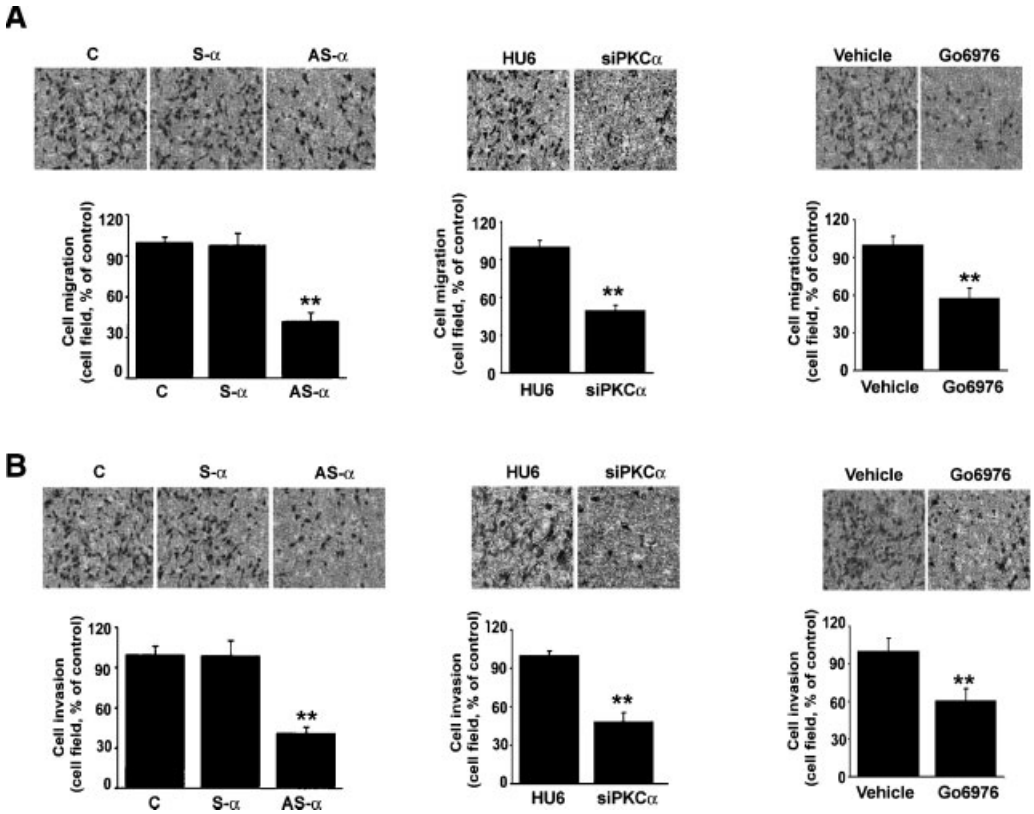

Fig. 4. Antisense ODN PKCα and siPKCα and Go6976 effect on cell migration and invasion in HA22T/VGH cells. The migration (**A**), and invasion (**B**) assays were performed on cell cultures treated with antisense ODNs PKCα (5 μM) (AS-α), sense ODNs PKCα (5 μM) (S-α), pcDNA-HU6 vector (5 μg/ml) (HU6), siRNA-PKCα (5 μg/ml) (siPKCα), vehicle (DMSO), or Go6976 (1,000 nM), as described in Materials and Methods. C, Untreated cultures were designated as controls. $^{**}P < 0.01$; compared with the control (n = 6).

MMP-7, MMP-9, MT1-MMP, u-PA, u-PAR, t-PA, FAK, Rac1, and RhoA were shown to have detectable expression in five human HCC cell lines (Fig. 5A). The five mRNA levels of MMP-1, MMP-3, u-PA, u-PAR, and FAK were higher in the poorly differentiated SK-Hep-1 and HA22T/VGH cells as compared to that in the well-differentiated HepG2, Hep3B, and PLC/PRF/5 cells. A higher Rac-1 and RhoA mRNA level was observed in HA22T/VGH, SK-Hep-1, PLC/PRF/5, and Hep3B cells, and MMP-7 in PLC/PRF/5 and HepG2 cells. As for MT1-MMP, MMP-9, and t-PA, their mRNA levels were found only in SK-Hep-1 cells.

Among these genes, the MMP-1 ($r = 0.94$), MMP-3 ($r = 0.88$), u-PA ($r = 0.92$), u-PAR ($r = 0.74$), and FAK ($r = 0.97$) levels were found to be significantly correlated with that of PKCα ($P < 0.01$). To determine whether the reduction of PKCα affect these five genes, we treated HA22T/VGH cells with 5 μM antisense ODN PKCα for 48 h and monitored the mRNA levels of these genes. The antisense ODN PKCα resulted in a decrease in the mRNA levels of MMP-1, u-PA, u-PAR, and FAK as compared to that in the sense ODN treated group, but not effect on the MMP-3 expression (Fig. 5B). These phenomena were also observed in the siPKCα treated group as compared with that in the HU6 control group (Fig. 5C). In SK-Hep-1 cells, the decreased level of MMP-1, u-PA, u-PAR, and FAK was also observed in the PKCα antisense ODN-treated group (data not shown). These results indicated that PKCα was associated with the expressions of MMP-1, u-PA, u-PAR, and FAK in the poorly differentiated HCC cells.

DISCUSSION

Based on many studies, PKCα has been found to be the most important PKC isoforms for the formation and progression of malignancies in various kinds of cell lines [Hofmann, 2004]. In the present study, we found that PKCα was higher in the poorly differentiated SK-Hep-1 and HA22T/VGH cells, and antisense ODN

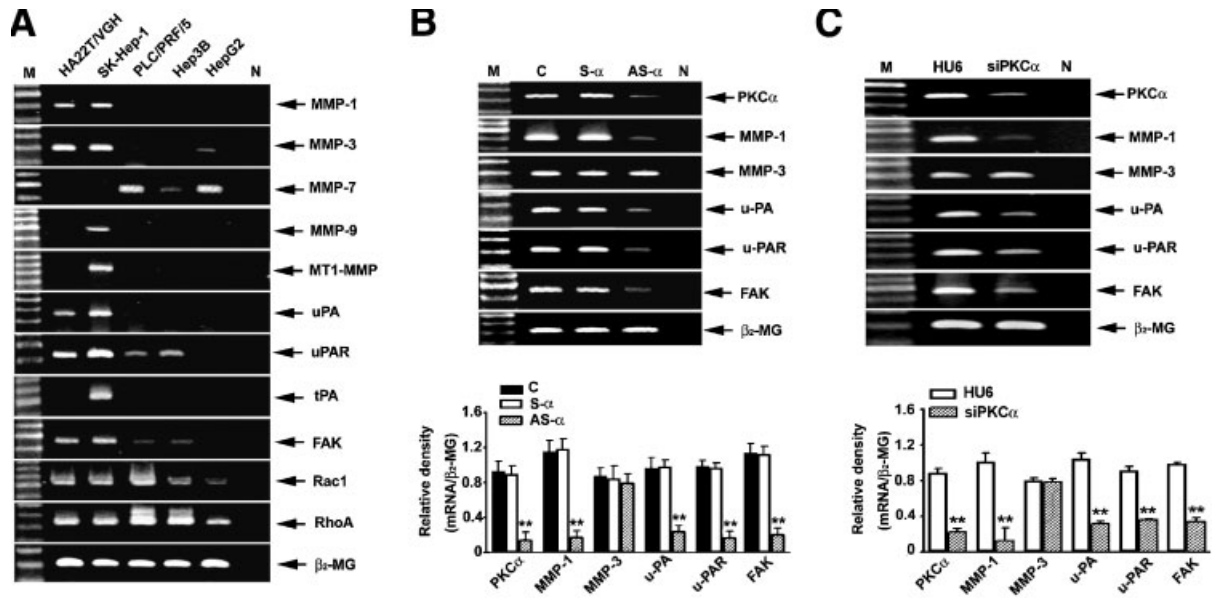

Fig. 5. Antisense ODN PKCα and siPKCα effect on the expressions of the migration/invasion-related genes in human HCC cells. **A**: The mRNA levels of MMP-1, MMP-3, MMP-7, MMP-9, MT1-MMP, u-PA, u-PAR, t-PA, FAK, Rac1, and RhoA were detected in five human HCC cell lines by RT-PCR as described in Materials and Methods. **B**: Antisense ODN PKCα (**left panel**) and siPKCα (**right panel**) inhibit the expressions of the migration/invasion-related genes. RT-PCR analysis of HA22T/VGH cells that were transfected with sense ODN PKCα (5 μM) (S-α), antisense ODN PKCα (5 μM) (AS-α), pcDNA-HU6 vector (5 μg/ml) (HU6), or siRNA-PKCα (5 μg/ml) (siPKCα). **C**: Quantitation of the RT-PCR in (B) by densitometry. PKCα, MMP-1, u-PA, u-PAR, and FAK mRNA levels were normalized to β_2-MG. β_2-MG was used as an internal control. N, negative control for RT-PCR without RT; M, DNA size marker. $^{**}P < 0.01$ versus control.

PKCα and siPKCα and antagonist Go6976 significantly decreased the proliferation, migration and invasion in these cell lines. Some evidences also provide an antiapoptotic/proproliferative function for PKCα in many tumor cells [Lin et al., 2000; Fournier et al., 2001; Mandil et al., 2001; Matassa et al., 2003]. Thus, we suggested that PKCα may be associated with the regulation of cell proliferation/migration/invasion in the poorly differentiated HCC cells.

PKCα appears to play an important role in the cell cycle progression [Besson and Yong, 2000]. Cell cycle progression is regulated by cyclin-dependent kinase (CDK) family members and tumor suppressors, such as p53, cyclin D1, and $p21^{WAF1/CIP1}$. The $p21^{WAF1/CIP1}$ gene, which transcribes one of the cell cycle inhibitors, is shown to contain p53-response elements. In response to DNA damage caused by radiation or chemicals, $p21^{WAF1/CIP1}$ is rapidly induced in a p53-dependent G_1 cell cycle arrest [el-Deiry et al., 1994; Di Leonardo et al., 1994]. Whereas, PKCα and PKCθ suppression-mediated growth arrest induce the expression of cell cycle inhibitor $p21^{WAF1/CIP1}$ by p53-independent mechanisms in Swiss3T3 fibroblasts cells [Deeds et al., 2003]. However, tetrandrine, an antitumor alkaloid, induces G_1 arrest in nocodazole- and serum-starved synchronized HT29 cells by convergent mechanisms, including down-regulation of cyclin D1 and up-regulation of p53/$p21^{WAF1/CIP1}$ [Meng et al., 2004]. Our data agreed with the later findings that PKCα antisense ODN-induced G1 arrest in the poorly differentiated HCC cell lines may be associated with the altered expressions of $p21^{WAF1/CIP1}$, p53, and cyclin D1.

The interesting finding of our study is the discovery of migration and invasion of PKCα in the poorly differentiated HCC cells. Other reports also demonstrate that elevating the level of PKCα promotes migration and invasion of endothelial cells [Harrington et al., 1997] and colon carcinoma cells [Martiny-Baron et al., 1993], and is correlated with the invasive capacity of U-87 glioma cells [Cho et al., 1999]. By the way, our results of the present study suggest for the first time that the PKCα was associated with the expressions of MMP-1, u-PA, u-PAR, and FAK in the poorly differentiated HCC cells (Fig. 5B,C). These genes have been correlated with the greater metastases and poorer prognoses in several types of cancer [Owens et al., 1995; Kornberg, 1998; Reuning

et al., 1998; Inoue et al., 1999; Ito et al., 1999; Judson et al., 1999; Sunami et al., 2000; Itoh et al., 2004] The downregulation of these four genes by the decreased PKCα may be the molecular basis for the inhibitory effects on cell migration and invasion.

In contrast to other study reports that the absence of PKCα activity leads to a reduction in cell growth and induces its apoptosis in HCC HepG2 cells [Zhu et al., 2005], although the poorly differentiated HCC cells transfected with antisense ODN PKCα were impaired markedly in cell growth, antisense ODN PKCα was no effect on the HepG2 cells. The discrepancy may be due to the different in cell condition, because depletion of PKCα may decrease the Bcl-xL content and lead to the vulnerability of cell apoptosis in a serum starvation condition [Takehara et al., 2001; Hsieh et al., 2003], and there may also be due to different thresholds of PKCα loss [Whelan and Parker, 1998].

Moreover, the siPKCα or Go6976 treatment also confirmed that PKCα may be associated with the changes in tumor cell physiological and pathological processes. Therefore, we suggested that PKCα may play an important role in the malignant progression of human HCC cells. In future studies, elucidation of the mechanisms by which this enzyme influences migration/invasion-related gene in poorly differentiated HCC cells will help to identify the links between PKCα signal transduction pathways and cellular migration and invasion in poorly differentiated HCC cells.

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