[CANCER RESEARCH 64, 5787–5794, August 15, 2004]

Antisense Targeting Protein Kinase C α and β_1 Inhibits Gastric Carcinogenesis

Xiao-Hua Jiang,[1,2] Shui-Ping Tu,[1,2] Jian-Tao Cui,[2] Marie C. M. Lin,[3] Harry H. X. Xia,[2] Wai Man Wong,[2] Annie On-On Chan,[2] Man Fung Yuen,[2] Shi-Hu Jiang,[1] Shiu-Kum Lam,[2] Hsiang-Fu Kung,[3] Jae Won Soh,[4] I. Bernard Weinstein,[4] and Benjamin Chun-Yu Wong[2]

[1]Department of Gastroenterology, Rui-jin Hospital, Shanghai, P.R. China; [2]Department of Medicine and [3]Institute of Molecular Biology, University of Hong Kong, Hong Kong; and [4]Department of Medicine and Herbert Irving Comprehensive Cancer Center, College of Physicians and Surgeons, Columbia University, New York, New York

ABSTRACT

Protein kinase C (PKC) family, which functions through serine/threonine kinase activity, is involved in signal transduction pathways necessary for cell proliferation, differentiation, and apoptosis. Its critical role in neoplastic transformation and tumor invasion renders PKC a potential target for anticancer therapy. In this study, we investigated the effect of targeting individual PKCs on gastric carcinogenesis. We established gastric cancer cell lines stably expressing antisense PKCα, PKCβ_1, and PKCβ_2 cDNA. These stable transfectants were characterized by cell morphology, cell growth, apoptosis, and tumorigenicity *in vitro* and *in vivo*. PKCα-AS and PKCβ_1-AS transfectants showed a different morphology with flattened, long processes and decreased nuclear:cytoplasmic ratio compared with the control cells. Cell growth was markedly inhibited in PKCα-AS and PKCβ_1-AS transfectants. PKCα-AS and PKCβ_1-AS cells were more responsive to mitomycin C- or 5-fluorouracil-induced apoptosis. However, antisense targeting of PKCβ_2 did not have any significant effect on cell morphology, cell growth, or apoptosis. Furthermore, antisense inhibition of PKCα and PKCβ_1 markedly suppressed colony-forming efficiency in soft agar and in nude mice xenografts. Inhibition of PKCα or PKCβ_1 significantly suppressed transcriptional and DNA binding activity of activator protein in gastric cancer cells, suggesting that PKCα or PKCβ_1 exerts their effects on cell growth through regulation of activator protein activity. These data provide evidence that targeting PKCα and PKCβ_1 by antisense method is a promising therapy for gastric cancer.

INTRODUCTION

Protein kinase C (PKC) comprises a family of serine/threonine kinases that plays a key role in the signal transduction pathways. It consists of at least 12 isoforms with different tissue expressions, substrate specificity, and subcellular localization that are related to specialized cell functions, including cell proliferation, differentiation, and apoptosis (1, 2). The members of the classical PKCs (α, β_1, β_2, and γ) bind phorbol esters and are Ca^{2+} dependent. The novel PKCs (δ, ϵ, η, and θ) do not depend on Ca^{2+} but bind phorbol esters. The third subfamily includes the atypical PKCs (ζ, ι, λ, and μ), which do not bind to either Ca^{2+} or phorbol ester (3, 4). Ever since the tumor-promoting phorbol esters were shown to activate PKCs (5), numerous studies have focused on understanding the role of this family in the process of tumorigenesis. However, a potential drawback to use of phorbol esters is their low selectivity for PKC over other protein kinases, as well as their lack of isoform specificity.

Overexpression and down-regulation of PKC isoforms in different cell lines displayed various results, depending on the isoform and the cell type used for the exogenous expression (6–10). For example, transfection of PKCβ_1, PKCϵ, and PKCγ into fibroblasts induced a transformed phenotype, including increased growth rate, high saturation densities, anchorage-independent growth, and enhanced tumorigenicity in nude mice (6–8). However, overexpression of PKCβ_1 in colon carcinoma cells caused tumor growth inhibition (9), and Chinese hamster ovary cells transfected with PKCδ exhibited a significantly reduced proliferation rate on 12-*O*-tetradecanoylphorbol-13-acetate treatment (10). Despite these variable phenotypes obtained in different cell lines, PKCα has been suggested to play an important role in tumorigenesis and tumor progression (11–14). Furthermore, this isoform also has been implicated in several cancer-related processes, such as invasion and metastasis (11, 15). Overexpression of PKCα in MCF-7 breast cancer cells and glioma cells led to a more aggressive phenotype (11, 13). Antisense of PKCα inhibits cell proliferation *in vitro* and tumorigenicity *in vivo* in nude mice xenografts of human glioblastoma and lung cancer cells (12, 14). Microinjection of antibodies against PKCα also inhibits cell growth and differentiation of neuroblastoma cells (16).

Among all of the PKC isoforms, PKCα and PKCβ have been suggested to play an important role in the carcinogenesis and metastasis of gastrointestinal cancers. For example, vaccinia virus-mediated overexpression of PKCα strongly increased CPP32 activity and apoptosis in the detached MKN-45 and MKN-74 cells (17). Moreover, several recent studies showed that PKCβ inhibitors executed antiangiogenic and antitumor effect in gastrointestinal cancers (18–20). Consistent with these results, our previous studies showed that PKCα was the most abundant isoform in gastric epithelial cells (21, 22), and PKCβ_1 acted as a survival mediator in nonsteroidal anti-inflammatory drug-induced apoptosis in gastric cancer cells (22, 23). Despite these results, the exact role of PKC isoforms in gastric tumorigenicity is largely unknown.

In this study, we established gastric cancer cell lines stably expressing antisense PKCα or PKCβ. Characterization of these cells with regard to proliferation rate and tumorigenicity *in vitro* and *in vivo* was performed. Our results suggest that inhibition of PKCα and PKCβ_1 exerts inhibitory effects on the transformed phenotype of gastric cancer cells. Antisense mediated suppression of PKCα and PKCβ_1 inhibits tumor growth in gastric cancer xenografts in nude mice.

MATERIALS AND METHODS

Cell Culture. Gastric cancer cell line MKN-45 (Riken Cell Bank, Tsukuba, Japan) was maintained in RPMI 1640 medium (Life Technologies, Rockville, MD) containing 10% FCS (Life Technologies), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). Two mM 5-fluorouracil (5-FU) and 10 mg/ml mitomycin C (MMC; Pfizer, New York, NY) were solubilized in DMSO and stored at 4°C.

Plasmid Construction. Plasmids pHACE-PKCα-WT, pHACE-PKCβ_1-WT, and pHACE-PKCβ_2-WT were generated (24). To construct antisense PKC plasmid, the full-length cDNA encoding PKCα, PKCβ_1, and PKCβ_2 cDNA were subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) in the antisense orientation after digestion with *Xho*I and *Eco*RI in pHACE-PKC-WT plasmids (24).

Gene Transfection and Establishment of Stable Cell Lines. Cells were seeded in six-well plates to 70–80% confluence. The cells were transfected with 4 μg/well PKC plasmids using Lipofectamine 2000 (Invitrogen). After

Received 4/29/03; revised 4/7/04; accepted 6/16/04.

Grant support: Simon K. Y. Lee Gastroenterology Research Fund, Queen Mary Hospital and Gastroenterological Research Fund, University of Hong Kong.

Notes: X-H. Jiang and S-P. Tu contributed equally to this work.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Benjamin C. Y. Wong, Department of Medicine, University of Hong Kong, Queen Mary Hospital, Hong Kong. Phone: 852-2855-4541; Fax: 852-2872-5828; E-mail: bcywong@hku.hk.

transfection for 4 h, cells were changed to normal medium and allowed to recover overnight. The cells then were trypsinized and split into 1:10 and seeded into new six-well plates. Forty-eight h after transfection, transfected cells were grown in RPMI containing G418 at 0.8 mg/ml until all of the nontransfected cells were dead (~2 weeks). Resistant clones were selected separately using cloning cylinders or pooled together and maintained in RPMI containing 0.2 mg/ml G418 for further study.

Assay of Anchorage-Dependent Cell Growth. Parent and stable transfected cells (1×10^5) were seeded in six-well plates. Cells from triplicate wells were collected every other day. Cell numbers were determined using a Coulter counter (Coulter Electronics, Miami, FL). The number of cells per well is reported as the average ± SD at the indicated days after plating.

Assay of Anchorage-Independent Cell Growth. Parent and stable transfected cells (1×10^4) were plated in 1.5 ml of 0.33% agar medium over 2 ml of 0.5% agar medium as described previously (25). Colonies were scored at 14 days, fixed with 70% ethanol, stained with Coomassie Blue, and counted under a dissection microscope. Only those colonies containing at least 50 cells were considered to be viable survivors.

Acridine Orange Staining. Cells were fixed in 4% formalin/PBS, stained with 10 μg/ml acridine orange (Sigma, St. Louis, MO), and visualized under fluorescence microscope (26). Apoptotic cells were defined as cells showing cytoplasmic and nuclear shrinkage, chromatin condensation, or fragmentation morphologically. At least 300 cells/field were counted to determine the apoptotic index or aberrant nuclei.

Transfection and Luciferase Assay of Activator Protein Activity. Activator protein (AP-1) reporter plasmid was constructed by inserting collagenase promoter region (−73 to +67 containing one AP-1 binding site) into luciferase reporter vector pGL-3-basic (Promega, Madison, WI; Ref. 27). For transient transfection experiments, cells were seeded in 12-well plates to 70–80% confluence. The cells were transfected with 0.8 μg/well AP-1 reporter plasmid using Lipofectamine 2000 (Invitrogen). PRL-CMV vector (0.01 μg/well; Promega) was cotransfected as internal control. After transfection for 4 h, cells were changed to normal medium and allowed to recover overnight. Cells then were incubated in media in the absence or presence of 100 nM phorbol 12-myristate 13-acetate (PMA) for an additional 1 h. Transfected cells were collected and lysed, and the firefly and renilla luciferase activities were measured using the Dual-Luciferase Reporter assay system (Promega) with a model TD-20/20 Luminometer.

Preparation of Cytoplasmic and Nuclear Extract. Nuclear and cytoplasmic extracts were prepared as described by Dignam *et al.* (28). Confluent cells in 10-cm dishes were treated for various times with the indicated effectors. Cells were resuspended in 400 μl of buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A], kept on ice for 15 min, lysed gently with 12.5 μl of 10% NP40, and centrifuged at 2,000 × *g* for 10 min at 4°C. The supernatant was collected and used as the cytoplasmic extracts. The nuclei pellet was resuspended in 40 μl of buffer C [20 mM HEPES (pH 7.9), containing 1.5 mM $MgCl_2$, 450 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A] and agitated for 30 min at 4°C, and the nuclear debris was spun down at 20,000 × *g* for 15 min. The supernatant (nuclear extract) was collected and stored at −80°C until ready for analysis.

Western Blot Analysis. The whole cell lysates were extracted with lysis buffer containing 1% Triton-100, 50 mM sodium chloride, 50 mM sodium fluoride, 20 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% NP40. Western blot analysis was carried out as described previously (21). In brief, equal amounts of cell lysates (20–60 μg) were solubilized in 10% SDS-PAGE and transferred onto nitrocellulose membranes (Sigma). After blocking, the membranes were incubated with the appropriate diluted primary antibody. Proteins were detected by the enhanced chemiluminescence detection system (Amersham, Piscataway, NJ). Antibodies against PKCα (H-7), PKCβ$_1$(c-16), PKCβ$_2$(c-18), PKCξ, PKCι, PKCμ, PKCη, PKCδ, PKCγ, and PKCε, mouse monoclonal antibody β-actin (c-2), and horseradish peroxidase-conjugated antirabbit, antimouse immunoglobulin IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Kinase Activity Assay. Subconfluent monolayers cells (in 100-mm plates) were washed with PBS and resuspended in cold lysis buffer [50 mM Tris/HCL (pH 7.5), 0.3% β-mercaptoethanol, 5 mM EDTA, 10 mM EGTA, and 50 μg/ml phenylmethylsulfonyl fluoride] for 10 min and then were homogenized by repeated aspiration through a 21-gauge needle. Cell debris was removed by centrifugation at 14,000 rpm at 4°C for 15 min. Two μg of anti-PKCα, -PKCβ$_1$, and -PKCβ$_2$ polyclonal antibodies were added to the supernatant and incubated for 1 h at 4°C. Agarose-conjugated protein A was added and incubated for another 1 h. The kinase activity assay was carried out using a PKC enzyme assay kit (Amersham). Twenty-five μl of assay mixture and 0.2 μCi of [γ-^{32}P] ATP were added to each immunoprecipitation sample and incubated for 15 min at 37°C. Peptide binding papers were used to separate phosphorylated peptide. The papers were washed with 5% acetic acid and then transferred to scintillation vials for ^{32}P counting.

Electrophoretic Mobility Shift Assay. Eight μg of nuclear proteins were incubated with 1 μg each of poly(dI-dC) in the presence of 30 fmol of digoxin (DIG)-labeled double-stranded AP-1 probe (5′-CGC TTG ATG ACT CAG CCG GAA-3′; Santa Cruz Biotechnology) for 15 min at room temperature in a total volume of 20 μl using DIG gel shift kit (Roche Diagnostics GmbH, Mannheim, Germany). Oligonucleotide competition experiments were performed in the presence of 50-fold excess of unlabeled oligonucleotides. DNA complexes were resolved from free probe with 4% nondenaturing polyacrylamide gels in 0.5× Tris-borate-EDTA (pH 8.3) and visualized by fluorography.

Confocal Microscopy and Image Analysis. Microscopy was performed using a Zeiss LSM410 laser-scanning confocal microscope (Oberkochen, Germany). All of the images were captured using a 100 × 1.4 numerical aperture objective lens. Image analysis and determination of nuclear:cytoplasmic ratios were carried out using standard functions of the LSM410 software. The ratios were determined by averaging the intensities of 20 random regions within the nucleoplasm and cytoplasm of 50–60 living cells. All of the images were scanned at a fixed gain and black level.

Tumorigenicity in Nude Mice. Single cell suspensions of each of the transfected cell lines and control cell lines were trypsinized and collected. The cell viability was >95% as determined by trypan blue staining. Cells (5×10^6) in a 0.1-ml volume of RPMI were inoculated s.c. into the right flank of 5–6-week-old female BALB/c-*nu/nu* mice (Laboratory Animal Unit, The University of Hong Kong). Institution guidelines were followed in handing the animals. The mice were maintained under sterile conditions. Once palpable tumors were established, tumor volume measurements were taken every 3 days using calipers along two major axes. Tumor volume was calculated as follows: $V = (4/3)\pi R1^2 R2$, where $R1$ is radius 1 and $R2$ is radius 2 and $R1 < R2$. At the end of the experiments, the tumors were excised and kept in 4% formalin or liquid nitrogen for Western blot analysis. The Committee on the Use of Live Animals in Teaching and Research, University of Hong Kong, Hong Kong approved the protocol.

Statistical Analysis. The data shown were mean values of at least three different experiments and expressed as mean ± SD. Student's *t* test was used for comparison. $P < 0.05$ is considered statistically significant.

RESULTS

Generation of Gastric Cancer Cells Stably Expressing Antisense PKCs. MKN-45 human gastric cancer cells were transfected with the control pcDNA, pcDNA-PKCα antisense, pcDNA-PKCβ$_1$ antisense, or pcDNA-PKCβ$_2$ antisense plasmids. Empty vector pcDNA-transfected cell clones were named TC and, together with the parental WD cells, served as controls in this study. After G418 selection, 10 clones each of PKCα-antisense, PKCβ$_1$-antisense, and PKCβ$_2$-antisense cells were picked, spread, and collected. The protein expression of specific PKC isoform in these antisense transfectants was further detected by Western blot analysis using anti-PKC antibodies. One representative clone of each type (named α-AS.3, β$_1$-AS.5, and β$_2$- AS.2), which showed the highest degree of inhibition of corresponding PKC isoform, consequently was selected for further study. Fig. 1 shows the protein expression of corresponding PKC isoform in the representative clone of each type. Transfection with the control vector did not alter PKC expression when compared with the WD cells, whereas introduction of antisense to PKCα, PKCβ$_1$, or

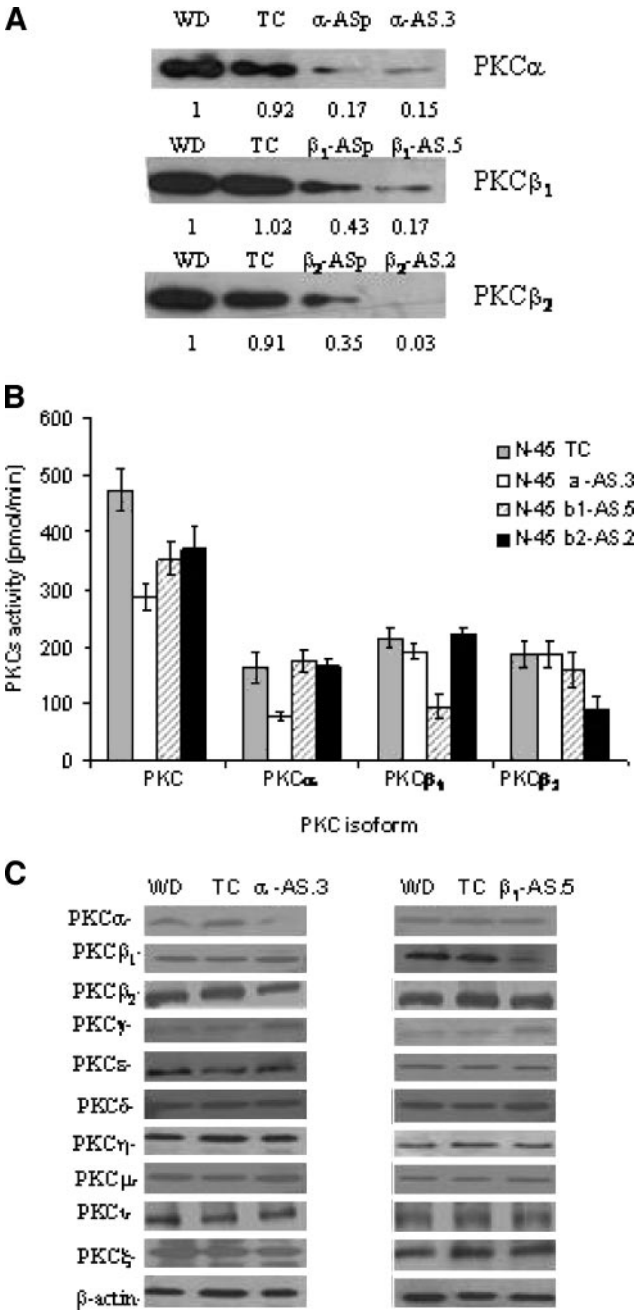

Fig. 1. The protein expression and kinase activity of PKC in PKC- α, β_1, and β_2 antisense-transfected cells. *A*, PKC expression in antisense-transfected cells. Control parental cells (WD), empty vector-transfected cells (TC), antisense PKC-transfected cells from single representative clone (α-AS.3, β_1-AS.5, and β_2-AS.2), and pooled antisense PKC-transfected cells, which were derived from three clones of each type showing the most inhibition of PKCα, PKCβ_1, or PKCβ_2 expression, respectively (α-ASp, β_1-ASp, and β_2-ASp), were incubated with antibodies to PKC isoforms α, β_1, and β_2. The density of PKC isoforms was further detected by video densitometry. *B*, PKC catalytic activity in antisense-transfected cells. The total PKC catalytic activity or specific isoform activity was determined by the protein kinase assay as described in "Materials and Methods" in TC, α-AS.3, β_1-AS.5, or β_2-AS.2 cells. Results were expressed as the means of three independent experiments ± SE. *C*, protein expression of other PKCs in antisense-transfected cells. Proteins extracted from α-AS.3, β_1-AS.5, TC, and parental cells WD were incubated with antibodies to PKCα, PKCβ_1, PKCβ_2, PKCγ, PKCϵ, PKCδ, PKCη, PKCμ, PKCι, and PKCξ. The experiments have been repeated three times, and the representative blot is shown in the figure.

PKCβ_2 resulted in a marked reduction in expression of the corresponding endogenous PKC protein. The protein expression of PKCα, PKCβ_1, and PKCβ_2 was reduced by 85%, 83%, and 97%, respectively, in α-AS.3, β_1-AS.5, and β_2-AS.2 cells when compared with the parental cells. Conversely, 3 of 10 clones of each type, which showed most inhibition of PKCα, PKCβ_1, or PKCβ_2 expression, respectively, were pooled (named α-ASp, β_1-Asp, and β_2-ASp, correspondingly) to minimize cell heterogeneity. These pooled cells revealed a reduction of PKCα, PKCβ_1, and PKCβ_2 expression by 83%, 57%, and 65%, respectively (Fig. 1). To substantiate that the antisense PKC constructs suppress the PKC catalytic activity, the PKCs were immunoprecipitated from lysates of PKCα-AS.3, PKCβ_1-AS.5, or PKCβ_2-AS.2 cells, and the resulting immunocomplexes were subjected to *in vitro* kinase assay. As shown in Fig. 1*B*, the total PKC activity was decreased by 39.9%, 25.7%, and 21.7% in PKCα-AS.3, PKCβ_1-AS.5, and PKCβ_2-AS.2 cells, respectively. Because total PKC activity is not necessarily down-regulated by the inhibition of single PKC isoform, we further determined the specific PKC isoform activity in the corresponding antisense-treated cells. Our results showed that PKCα-, PKCβ_1-, and PKCβ_2-specific catalytic activity were decreased by 52.8%, 56.4%, and 51.7%, respectively, in the corresponding cells. Thus, the expression and catalytic activation of PKC of interest were strikingly inhibited in our established antisense-transfected cells. To further determine the specificity of our antisense constructs, we determined the expression of other PKC isoforms besides the PKC of interest. As shown in Fig. 1*C*, the expression of other PKCs remained unchanged in PKCα-AS.3 and PKCβ_1-AS.5 cells. Similar results were obtained in PKCβ_2-AS.2 cells (data not shown).

Effects of Antisense PKCs on Cell Morphology and Cell Growth. To characterize the gastric cancer cells that stably express antisense PKCs, we first examined the morphologic features of these cells. When compared with the WD control cells, the PKCα-AS.3 and PKCβ_1-AS.5 but not the PKCβ_2-AS.2 cells exhibited a lengthened and flatter morphology. Dendritic-like cytoplasmic processes were visible by phase-contrast microscope (Fig. 2*A*). Importantly, the PKCα-AS.3 and PKCβ_1-AS.2 cells exhibited a decreased nuclear:cytoplasmic ratio. We determined the nuclear:cytoplasmic ratio by measuring the intensities of 20 random regions in the cytoplasm and nucleus of 50–60 living cells. The plot of frequency of cells with nuclear:cytoplasmic ratios is shown in Fig. 2*B*. Fig. 2*Ba* showed that the control TC cells had nuclear:cytoplasmic ratios in the range of 2–6, with the highest frequency of cells having the ratio of 3. The average nuclear:cytoplasmic ratio was calculated to be ~3. In contrast, PKCα-AS.3 and PKCβ_1-AS.5 (Fig. 2, *Bb* and *Bc*) had lower nuclear:cytoplasmic ratios, ranging from 0–5, with an average ratio of ~1.5. However, no significant differences in the nuclear:cytoplasmic ratios were detected between PKCβ_2-AS.2 and control cells (Fig. 2*Bd*). We also found that the cell density was significantly lower in the PKCα-AS.3 and PKCβ_1-AS.5 cells. At confluence, the PKCα-AS.3 and PKCβ_1-AS.5 cells reached cell densities that were only 25% and 57%, respectively, that of the control TC cells. Thus, the saturation density of PKCα-AS.3 and PKCβ_1-AS.5 cells was 1×10^4 and $2.3 \times 10^4/cm^2$, respectively, whereas it was $4 \times 10^4/cm^2$ for the TC cells. We then examined possible effects on the rate of cell proliferation. Growth curves indicated that the WD and TC control cells displayed rapid growth rates, whereas the growth rate of the PKCα-AS.3 and PKCβ_1-AS.5 cells was markedly reduced (Fig. 3*A*). Thus, on day 7 the number of cells in the PKCα-AS.3 and PKCβ_1-AS.5 cultures were 23.7 ± 0.4% and 44.5 ± 0.23%, respectively, when compared with the TC cells. PKCβ_2-AS.2 cells grew more slowly at day 1 and day 3 but showed no significant difference in cell growth after day 5 compared with the control cells (Fig. 3*A*).

Effects of Antisense PKCs on Drug-Induced Apoptosis. Many cancer chemotherapeutic drugs, including 5-FU and MMC, inhibit tumor growth through induction of apoptosis. We compared the apoptotic response to these two drugs in our series of cell lines. The

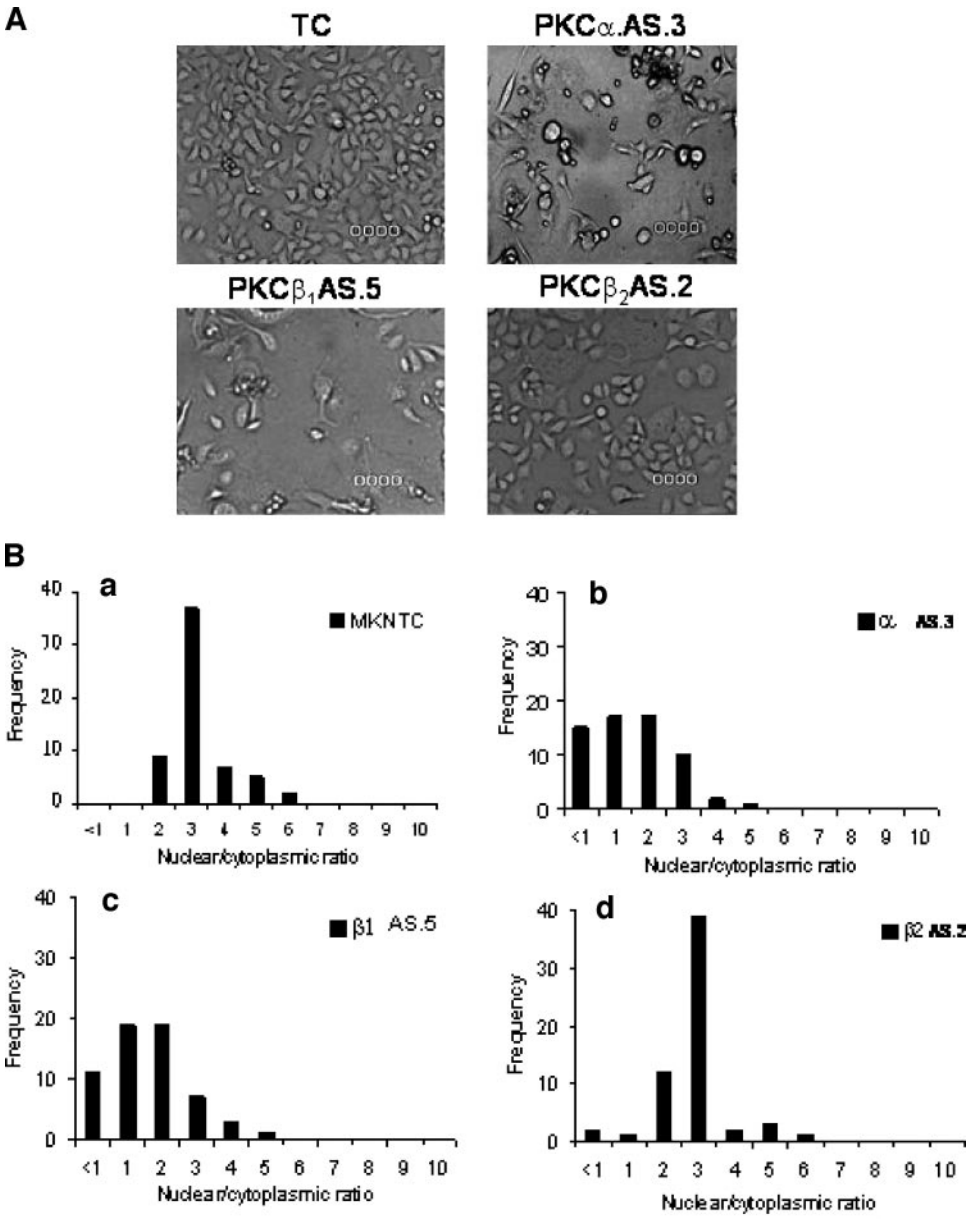

Fig. 2. Morphologic changes in PKC-antisense transfectants. *A*, TC, PKCα-AS.3, PKCβ_1-AS.5, and PKCβ_2-AS.2 were grown to confluence in 10% fetal bovine serum RPMI 1640 and photographed with a phase-contrast microscope. *B*, nuclear:cytoplasmic ratios of antisense-transfected cells. The nuclear:cytoplasmic ratios were measured as described in "Materials and Methods" in TC, PKCα-AS.3, PKCβ_1-AS.5, and PKCβ_2-AS.2 cells.

cells were exposed to 5-FU or MMC for 24 h and then scored for apoptosis by acridine orange staining. As shown in Fig. 4, PKCα-AS.3 and PKCβ_1-AS.5 cells displayed a significant increase in spontaneous apoptosis compared with the TC cells (8.3 $\pm$ 1.5% *versus* 2.1 $\pm$ 0.1% and 8.9 $\pm$ 1.2% *versus* 2.4 $\pm$ 0.2%). When exposed to MMC and 5-FU, PKCα-AS.3 and PKCβ_1-AS.5 but not the PKCβ_2-AS.2 cells showed significant increase of apoptosis compared with the control cells.

Effects of Antisense PKCα and PKCβ_1 on Anchorage-Independent Growth and Tumorigenicity. Next we analyzed the colony-forming ability of the PKC transfectants in semisolid medium because loss of anchorage-independent growth often correlates with tumorigenicity. As shown in Fig. 3*B*, the PKCα-AS.3 cells displayed almost complete loss of colony-forming efficiency, and the PKCβ_1-AS.2 cells had an ~50% decrease when compared with the WD and TC control cells. In view of these results, we examined whether the reduction in PKCα and PKCβ_1 expression in our AS derivatives of gastric cancer cells might affect their tumorigenicity in nude mice. We injected 5×10^6 WD, TC, PKCα-ASp, and PKCβ_1-ASp cells s.c. into athymic nude mice and monitored the mice for tumor appearance and growth. PKCα-ASp and PKCβ_1-ASp cells are pooled representative clones, which suppress PKCα and PKCβ_1 expression by 83% and 57%, respectively. The WD and TC cells caused rapid formation and growth of tumors, although the rates were somewhat slower in the TC cells. The four control mice were killed after 8 weeks or when the tumor burden was >5% of body weight. In contrast, only two of four mice injected with PKCα-ASp cells developed slow-growing tumors and after a long latent period (Fig. 5, *A* and *B*). The other two mice remained tumor free for the 3-month duration of the experiment. At the end of the experiments, the volume of the tumors in mice injected with PKCα-ASp was <5% that of the tumors obtained with the TC cells (Fig. 5*B*). Our results also showed that all of the mice injected with PKCβ_1-ASp cells developed tumors; however, these tumors grew much slower and appeared after a long latent period compared with those of TC-injected mice. At the end of the study, all of the tumors were removed and dissociated, and Western blot analysis was performed on representative specimens to confirm the persistent effect of the antisense PKCs. As shown in Fig. 5*C*, PKCα or PKCβ_1

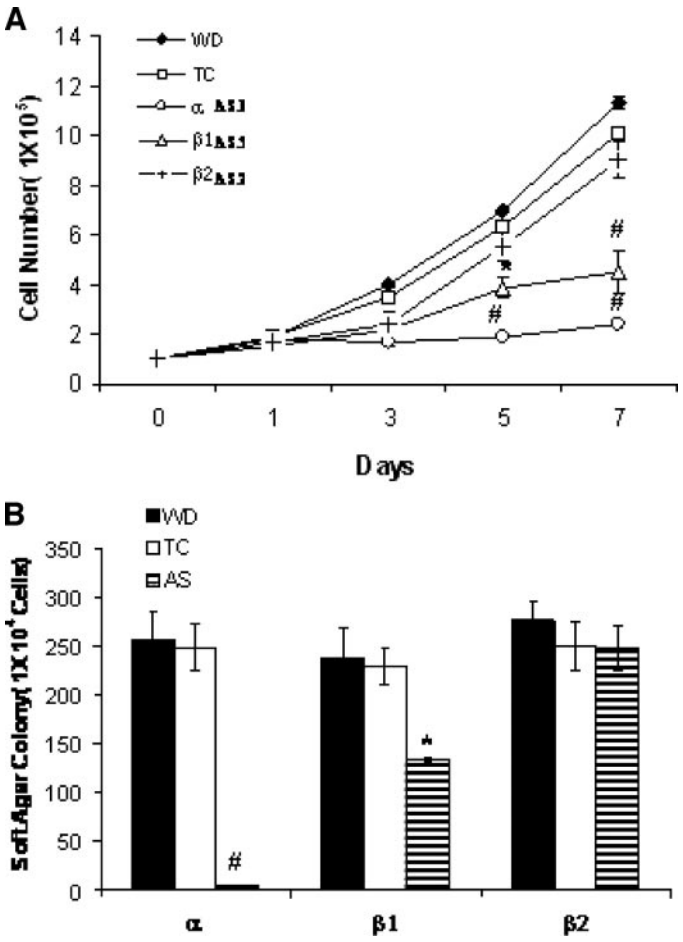

Fig. 3. Cell growth assay and colony-forming ability of PKC antisense transfectants. *A*, cell growth assay in antisense-transfected cells. Cells (1×10^5) were plated onto 60-mm tissue culture dishes in RPMI supplemented with 10% fetal bovine serum. Cells were harvested and counted in a hemocytometer at 48-h intervals. All of the results were expressed as the mean of three independent experiments ± SE. *B*, anchorage-independent cell growth in antisense-transfected cells. Cells were plated in 0.3% agar and analyzed for their ability to form colonies in soft agar as described in "Materials and Methods." *, $P < 0.05$; #, $P < 0.01$ as compared with control cells. The results represent the average ± SD of three experiments; each was performed in duplicate.

expression was significantly suppressed in the tumors obtained from nude mice injected with PKCα-ASp or PKCβ_1-ASp cells, respectively, when compared with tumors obtained from the nude mice injected with control cells.

Antisense PKCα and PKCβ_1 Inhibit the AP-1 Activity. To further investigate by which mechanism antisense PKCs suppress gastric tumorigenesis, we detected the effect of antisense PKCα and PKCβ_1 on AP-1 transcriptional activity. Our results showed that transient expression of antisense PKCα and PKCβ_1 significantly inhibited basal and PMA-induced AP-1 transcriptional activity (Fig. 6*A*). In contrast, antisense inhibition of PKCβ_2 did not affect the transcriptional activity of AP-1 (data not shown). To substantiate the result that inhibition of PKCα or PKCβ_1 suppresses AP-1 activity, we determined the transcriptional activity of AP-1 in stable transfectants of PKCα-AS.3 and PKCβ_1-AS.5. The results showed that basal AP-1-mediated transcriptional activity was significantly lower in PKCα-AS.3 and PKCβ_1-AS.5 cells than in TC and parental cells (Fig. 6*B*). PMA-induced AP-1 transcriptional activity also was dramatically decreased in PKCα-AS.3 and PKCβ_1-AS.5 cells. Consistent with previous results, PKCβ_2-AS.2 cells displayed no difference in AP-1 transcriptional activity compared with control cells (data not shown). Furthermore, AP-1 DNA binding activity as an index of activation was measured by electrophoretic mobility shift assay in the antisense-manipulated cell lines. As shown in Fig. 6*C*, PMA-induced AP-1 DNA binding activity was significantly decreased in PKCα-AS.3 and PKCβ_1-AS.5 cells compared with control cells. Competition with a 50-fold excess of the nonradioactive AP-1 oligonucleotide abolished the appearance of AP-1 complex, indicating that the AP-1 DNA binding activity present in these cells was specific for the AP-1 target sequence.

DISCUSSION

In this study, we characterized the role of PKCα, PKCβ_1, and PKCβ_2 in the proliferation, apoptosis, and tumorigenicity in gastric

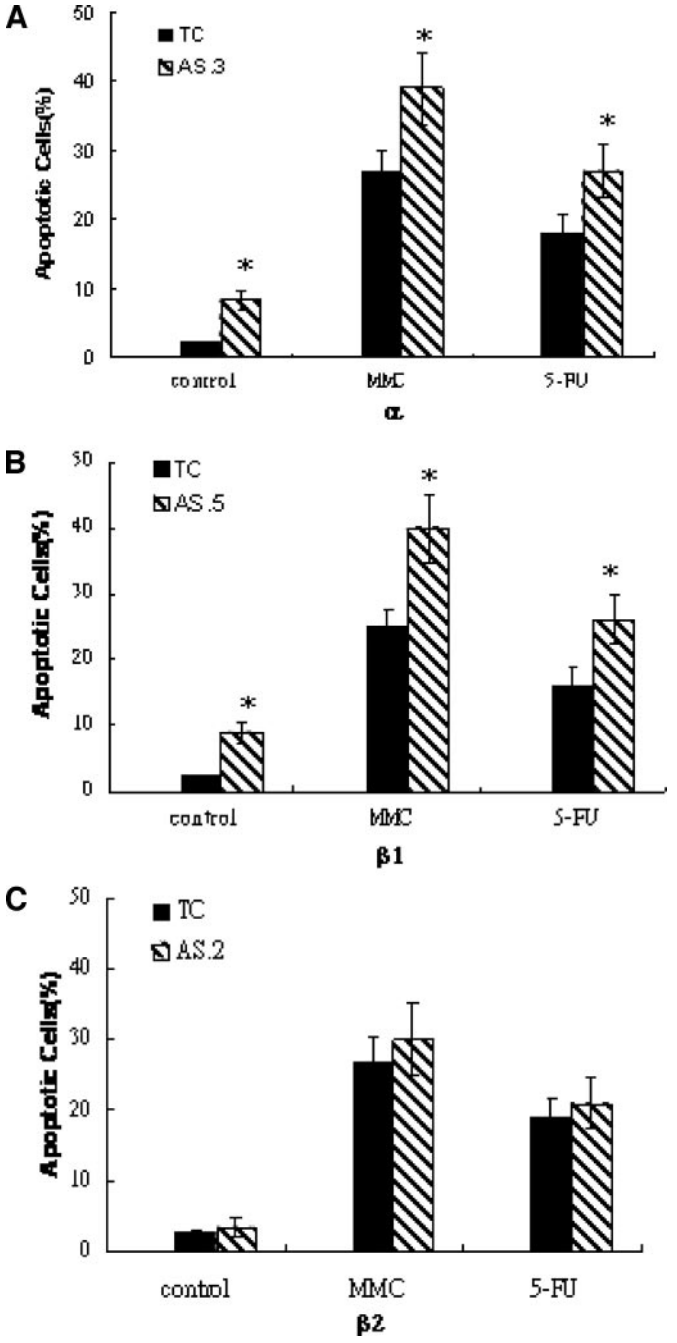

Fig. 4. Apoptotic response of PKC-antisense transfectants to MMC and 5-FU. Control cells and transfected cells were exposed to 10 μg/ml MMC and 2 μM 5-FU for 24 h. Apoptosis was determined by acridine orange staining; *, $P < 0.05$, as compared with control cells. *A*, PKCα; *B*, PKCβ_1; *C*, PKCβ_2. All of the results were expressed as the mean of three independent experiments ± SE.

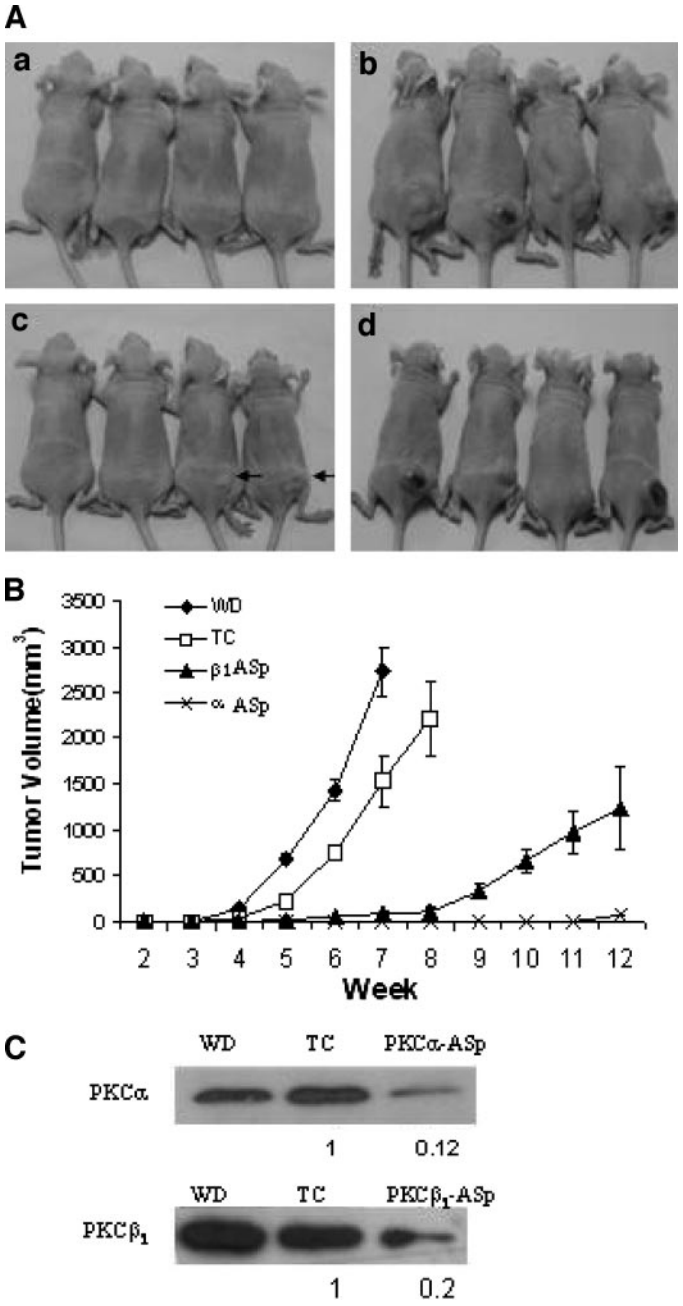

Fig. 5. Tumor growth of human gastric cancer cells stably expressing neovector or antisense PKC in nude mice. *A*, a total of 5×10^6cells were injected into the flank region of nude mice. Tumor development was followed up to 3 months, and mice then were sacrificed. *A*, PBS; *B*, TC; *C*, α-ASp; *D*, β_1-ASp. *B*, Tumor growth curve of stable PKC-antisense transfectants in human gastric cancer xenografts. A total of 5×10^6 cells were injected into the flank region of nude mice. Tumor development was followed up to 3 months, and mice then were sacrificed. Cross-sectional tumor diameters were measured externally, and the approximate tumor volume was calculated as described in "Materials and Methods." The results represent the average ± SD of four developed tumors in the experimental mice. *C*, PKCα and PKCβ_1 protein expression in gastric cancer xenografts. At the termination of nude mice experiments, the tumors developed by each cell line were removed and dissociated, and Western blot analysis was performed.

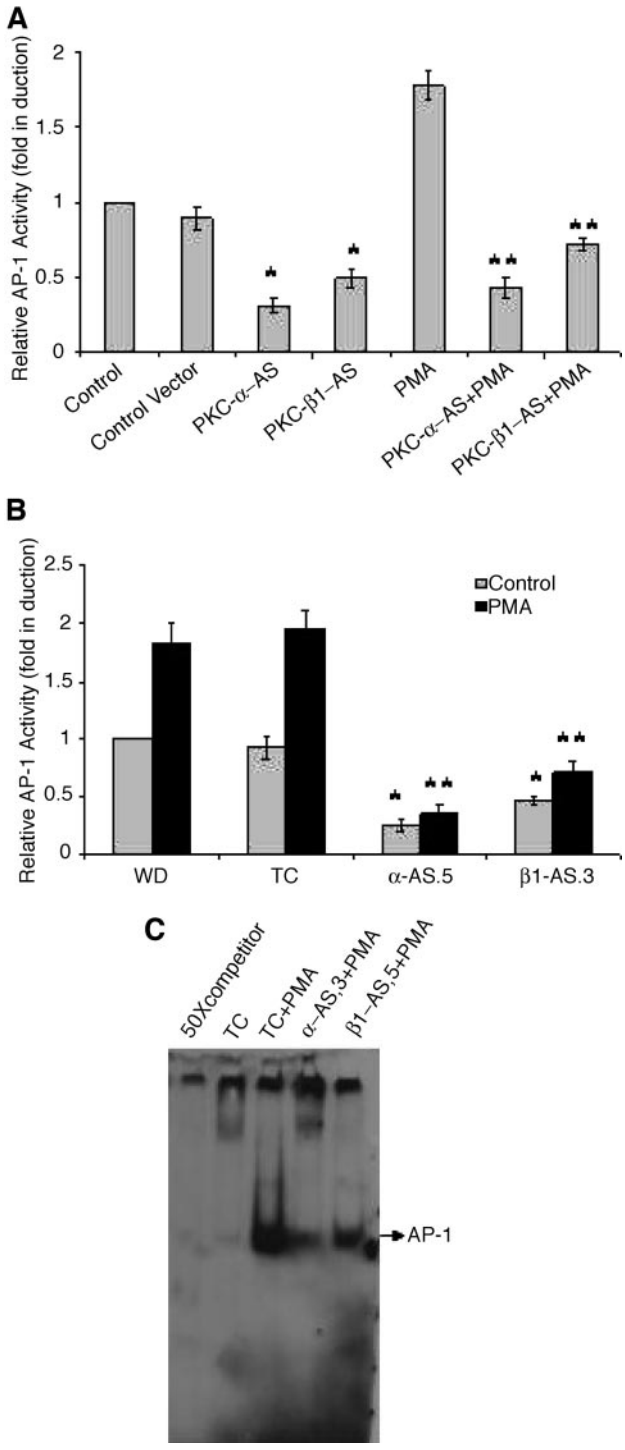

Fig. 6. Antisense PKCα and PKCβ_1 suppressed AP-1 transcriptional and binding activity. *A*, MKN-45 cells were cotransfected with expressing AP-1 luciferase reporter gene and antisense PKCα or PKCβ_1 plasmids. After 24 h, cells were treated without or with 100 nM PMA for another 1 h. Cells were analyzed for luciferase activity. The firefly luciferase reading was normalized to renilla luciferase reading. Results were expressed as the means of three independent experiments ± SE; *, $P < 0.01$, compared with the group treated with control vector; **, $P < 0.01$ compared with control group treated with PMA. *B*, α-AS.3, β_1-AS.5, TC, and parental WD cells were transfected with expressing AP-1 luciferase reporter gene. After 24 h, cells were treated without or with 100 nM PMA for another 1 h. Cells were analyzed for luciferase activity. The firefly luciferase reading was normalized to renilla luciferase reading. Results were expressed as the means of three independent experiments ± SE; *, $P < 0.01$, compared with TC transfectants; **, $P < 0.01$, compared with TC transfectants treated with PMA. *C*, effects of PKCα and PKCβ_1 inhibition on the DNA binding activity of AP-1. Nuclear extracts were prepared and analyzed in an electrophoretic mobility shift assay with DIG-labeled AP-1. Equal amounts (6 μg) of nuclear protein were loaded in each lane. In *Lane 1*, a 50× excess of unlabeled oligonucleotide was added before the addition of DIG-labeled probe.

cancer. We found, for the first time, that PKCα and PKCβ_1, especially the former isoform, played a critical role in maintaining the transformed phenotype of gastric cancer cells. Inhibition of PKCα or PKCβ_1 resulted in cell growth inhibition, enhanced sensitivity to chemotherapeutic drugs, and suppression of *de novo* gastric tumor formation in gastric xenografts in nude mice.

PKC, especially classical PKCs, plays an important role in carci-

nogenesis and progression of gastric cancer (18, 19, 21). To provide further insight into the specific biological roles of classical PKCs, inhibition of PKCα, PKCβ_1, and PKCβ_2 via transfection of antisense was used in the present study. We excluded PKCγ, which generally is considered to be exclusively expressed in the central nervous system (3). We chose the well-characterized human gastric cancer MKN-45 cells, which are highly tumorigenic and express classical PKCs except PKCγ (data not shown). The results indicated that our approach yielded an effective blockage in the synthesis of specific PKC protein and catalytic activity.

We showed that gastric cancer cells transfected with antisense PKCα were impaired markedly in growth and proliferation, and there were significant changes in morphology with lower nuclear:cytoplasmic ratio, which indicates a static status. Antisense PKCα transfectants also exhibited a dramatic decreased colony-forming efficiency in soft agar and reduction of tumor formation in nude mice. The most remarkable result was that only 50% of the PKCα-ASp (pooled antisense PKCα transfectant)-inoculated mice developed tumor at the end. The tumors that were formed also grew slower and were smaller compared with the control group. These results indicate that in contrast to the parental cells, antisense PKCα transfectants exhibited a partial reversal of the transformed phenotype. Therefore, PKCα is not only essential for tumor growth and progression but also seems to play a critical role in maintaining the tumorigenic capacity in gastric cancer.

Although several recent studies showed that PKCβ inhibitors executed antiangiogenic and antitumor effect in gastrointestinal cancer (18–20), there is no study investigating the specific role of two PKCβ isoforms in carcinogenesis and progression of gastric tumor. In this study, we first showed that PKCβ_1-AS-transfected cells showed aberrant cell morphology, suppression of cell growth, and decreased colony-forming ability *in vitro*, whereas the effect was not as profound as in PKCα-AS cells. More importantly, the size and growth rate of gastric tumor xenografts in PKCβ_1-ASp-inoculated nude mice were significantly reduced compared with those in the control group. Surprisingly, we failed to observe any significant change of PKCβ_2-AS cells in cell morphology, cell growth, and cell transformation in the present study. Although PKCβ_1 and PKCβ_2 are derived from a single gene by alternative splicing, they are differentially involved in cell growth, apoptosis, and cell transformation (29, 30). For example, expression of PKCβ_2 in the colon of transgenic mice leads to hyperproliferation and increased susceptibility to colon carcinogenesis (31, 32), whereas PKCβ_1 seemed to act as survival mediators in response to chemotherapeutic agent-induced apoptosis in gastric cancer (22, 23). Nevertheless, our present study clearly showed that PKCβ_1 but not PKCβ_2 plays an important role in gastric carcinogenesis *in vitro* and *in vivo*.

Because PKCα and PKCβ have been implicated in the apoptotic pathway (33, 34), we further investigated and found that the spontaneous apoptotic rates in PKCα-AS and PKCβ_1-AS cells were approximately three times higher than those in the control cells (8.3% *versus* 2.1% and 8.9% *versus* 2.4%), whereas PKCβ_2-AS cells showed no significant difference in spontaneous apoptosis compared with the control. We also challenged PKCα-AS and PKCβ_1-AS cells with MMC and 5-FU. Our results showed that the apoptotic rates in these cell lines were significantly higher than those in the control cells. However, the difference we observed was not as dramatic as the difference shown in other studies using PKC inhibitors (33, 35). For example, Schwartz *et al.* (33) showed that MMC alone induced apoptosis in 40% of the gastric cancer cells, whereas the combination of PKC inhibitor safingol and MMC induced apoptosis in 83% of the cells. Together, these results indicate that other PKC isoforms, besides PKCα or PKCβ_1, may play critical roles in the regulation of apoptosis in gastric cancer.

AP-1 transcriptional factor is known to be the major target of PKC signaling. We further detected AP-1 transcriptional activity and DNA binding activity in PKCα-AS and PKCβ_1-AS cells. Our results showed that inhibition of PKCα or PKCβ_1, but not PKCβ_2, dramatically decreased basal and PMA-stimulated AP-1 activity. In the light of our recent study, which showed that the AP-1/c-Jun NH_2-terminal kinase pathway played a critical role in gastric carcinogenesis (36), our current results indicate that inhibition of AP-1 activity contributes to the PKC-mediated regulation of gastric carcinogenesis.

In conclusion, this study shows a critical role for PKCα and PKCβ_1 in the regulation of cell growth and tumorigenicity of gastric cancer. Thus, targeting of PKCα and/or PKCβ_1 by pharmacologic inhibitors or antisense methods may possibly become part of anticancer therapies for the management of gastric cancer, whereas the effect of combination therapy with PKCα and PKCβ_1 inhibition is worth further investigation.

REFERENCES

1. Nishizuka Y. Protein kinase C and lipid signaling for sustained cellular responses. FASEB J 1995;9:484–96.
2. Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 1992;258:607–11.
3. Nishizuka Y. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 1988;334:661–5.
4. Hug H, Sarre TF. Protein kinase C isoenzymes: divergence in signal transduction? Biochem J 1993;291:329–43.
5. Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkiawa U, Nishizuka Y. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J Biol Chem 1982;257:7847–51.
6. Borner C, Ueffing M, Jaken S, Parker PJ, Weinstein IB. Two closely related isoforms of protein kinase C produce reciprocal effects on the growth of rat fibroblasts. J Biol Chem 1995;270:78–86.
7. Housey GM, Johnson MD, Hsiao WLW, et al. Overproduction of protein kinase C causes disordered growth control in rat fibroblasts. Cell 1988;52:343–54.
8. Persons DA, Wilkison WO, Bell RM. Altered growth regulation and enhanced tumorigenicity of NIH 3T3 fibroblasts transfected with protein kinase C-I cDNA. Cell 1988;52:447–58.
9. Choi PM, Tchou-Wong KM, Weinstein IB. Overexpression of protein kinase C in HT29 colon cancer cells causes growth inhibition and tumor suppression. Mol Cell Biol 1990;10:4650–7.
10. Watanable T, Ono Y, Taniyama Y, et al. Cell division arrest induced by phorbol ester in CHO cells overexpressing protein kinase C-δ subspecies. Proc Natl Acad Sci USA 1992;89:10159–63.
11. Ways DK, Kukoly CA, DeVente J, et al. MCF-7 breast cancer cells transfected with PKC-α exhibit altered expression of other protein kinase C isoforms and display a more aggressive neoplastic phenotype. J Clin Invest 1995;95:1906–15.
12. Ahmad S, Glazer RI. Expression of the antisense cDNA for protein kinase C alpha attenuates resistance in doxorubicin-resistant MCF-7 breast carcinoma cells. Mol Pharmacol 1993;43:858–62.
13. Mandil R, Ashenazi E, Blass M, et al. Protein kinase Cα and protein kinase Cδ play opposite roles in the proliferation and apoptosis of glioma cells. Cancer Res 2001; 61:4612–9.
14. Wang XY, Repasky E, Liu HT. Antisense inhibition of protein kinase Cα reverses the transformed phenotype in human lung carcinoma cells. Exp Cell Res 1999;250:253–63.
15. Liu B, Maher RJ, Hannun YA, Porter AT, Honn KV. 12(S)-HETE enhancement of prostate tumor cell invasion: selective role of PKCα. J Natl Cancer Inst 1994;86: 1145–51.
16. Leli U, Parker PJ, Shea TB. Intracellular delivery of protein kinase C-α or -σ isoform-specific antibodies promotes acquisition of a morphologically differentiated phenotype in neuroblastoma cells. FEBS Lett 1992;297:91–4.
17. Okuda H, Adachi M, Miyazawa M, Hinoda Y, Imai K. Protein kinase Cα promotes apoptotic cell death in gastric cancer cells depending upon loss of anchorage. Oncogene 1999;18:5604–9.
18. Schwartz GK, Jang J, Kelsen D, Albino AP. Protein kinase C: a novel target for inhibiting gastric cancer cell invasion. J Natl Cancer Inst 1993;85:402–7.
19. Teicher BA, Menon K, Alvarez E, Liu P, Shih C, Faul MM. Antiangiogenic and antitumor effects of a protein kinase Cβ inhibitor in human hepatocellular and gastric cancer xenografts. In Vivo 2001;15:185–93.
20. Cesaro P, Raiteri E, Demoz M, et al. Expression of protein kinase C β1 confers resistance to TNFα and paclitaxel-induced apoptosis in HT-29 colon carcinoma cells. Int J Cancer 2001;93:179–84.

21. Zhu GH, Wong BC, Eggo MC, Yuen ST, Lai KC, Lam SK. Pharmacological inhibition of protein kinase C activity could induce apoptosis in gastric cancer cells by differential regulation of apoptosis-related genes. Dig Dis Sci 1999;44:2020–6.
22. Zhu GH, Wong BC, Slosberg ED, et al. Overexpression of protein-β1 isoenzyme suppresses indomethacin-induced apoptosis in gastric epithelial cells. Gastroenterology 2000;118:507–14.
23. Jiang XH, Lam SK, Lin MCM, et al. Novel target for induction of apoptosis by cyclo-oygeanse-2 inhibitor SC236 through a protein kinase C β_1-dependent pathway. Oncogene 2002;21:6113–22.
24. Soh JW, Lee EH, Prywes R, Weinstein IB. Novel roles of specific isoforms of protein kinase C in activation of the c-fos serum response element. Mol Cell Biol 1999;19: 1313–24.
25. Li JJ, Westergaad D, Ghosh P, Colburn NH. Inhibitors of both nuclear factor-κ B and activator protein-1 activation block the neoplastic transformation response. Cancer Res 1997;57:3569–76.
26. Jiang XH, Wong BCY, Lin MCM, et al. Functional p53 status dictates the differential responses of gastric cancer cells to triptolide-induced apoptosis and AP-1 and nuclear factor-κB activations. Oncogene 2001;20:8009–18.
27. Whitmarsh AJ, Davis RJ. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. J Mol Med 1996;74:589–607.
28. Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 1983;11:1475–89.
29. Ono Y, Kikkawa U, Ogita K, et al. Expression and properties of two types of protein kinase C: alternative splicing from a single gene. Science 1987;236:1116–20.
30. Deacon EM, Ponracz J, Griffiths G, Lord JM. Isoenzymes of protein kinase C: differential involvement in apoptosis and pathogenesis. Mol Pathol 1997;50:124–31.
31. Gokmen-Polar Y, Murray NR, Velasco MA, Gatalica Z, Fields AP. Elevated protein kinase Cβ II is an early promotive event in colon carcinogenesis. Cancer Res 2001;61:1375–81.
32. Murray NR, Weems C, Chen L, et al. Protein kinase Cβ II and TGFβ RII in omega-3 fatty acid-mediated inhibition of colon carcinogenesis. J Cell Biol 2002;157:915–20.
33. Schwartz GK, Haimovitz-Friedman A, Dhupar SK, et al. Potentiation of apoptosis by treatment with the protein kinase C-specific inhibitor safingol in mitomycin C-treated gastric cancer cells. J Natl Cancer Inst 1995;87:1394–9.
34. Pongracz J, Tuffley W, Johnson GD, et al. Changes in protein kinase C isoenzyme expression associated with apoptosis in U937 myelomonocytic cells. Exp Cell Res 1995;218:430–8.
35. Teicher BA, Menon K, Alvarez E, Galbreath E, Shih C, Faul MM. Antiangiogenic and antitumor effects of a protein kinase Cβ inhibitor in human HT-29 colon carcinoma and human CaKi1 renal cell carcinoma xenografts. Anticancer Res 2001; 21:3175–84.
36. Wong BCY, Jiang XH, Lin MCM, et al. Cyclooxygenase-2 inhibitor (SC-236) suppresses activator protein-1 through c-Jun NH2-terminal kinase. Gastroenterology 2004;126:136–47.