

Antisense Targeting Protein Kinase C α and β_1 Inhibits Gastric Carcinogenesis

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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 PKC α , PKC β_1 , and PKC β_2 cDNA. These stable transfectants were characterized by cell morphology, cell growth, apoptosis, and tumorigenicity *in vitro* and *in vivo*. PKC α -AS and PKC β_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 PKC α -AS and PKC β_1 -AS transfectants. PKC α -AS and PKC β_1 -AS cells were more responsive to mitomycin C- or 5-fluorouracil-induced apoptosis. However, antisense targeting of PKC β_2 did not have any significant effect on cell morphology, cell growth, or apoptosis. Furthermore, antisense inhibition of PKC α and PKC β_1 markedly suppressed colony-forming efficiency in soft agar and in nude mice xenografts. Inhibition of PKC α or PKC β_1 significantly suppressed transcriptional and DNA binding activity of activator protein in gastric cancer cells, suggesting that PKC α or PKC β_1 exerts their effects on cell growth through regulation of activator protein activity. These data provide evidence that targeting PKC α and PKC β_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 Ca²⁺ dependent. The novel PKCs (δ , ϵ , η , and θ) do not depend on Ca²⁺ but bind phorbol esters. The third subfamily includes the atypical PKCs (ζ , ι , λ , and μ), which do not bind to either Ca²⁺ 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 PKC β_1 , PKC ϵ , and PKC γ 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 PKC β_1 in colon carcinoma cells caused tumor growth inhibition (9), and Chinese hamster ovary cells transfected with PKC δ exhibited a significantly reduced proliferation rate on 12-O-tetradecanoylphorbol-13-acetate treatment (10). Despite these variable phenotypes obtained in different cell lines, PKC α 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 PKC α in MCF-7 breast cancer cells and glioma cells led to a more aggressive phenotype (11, 13). Antisense of PKC α 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 PKC α also inhibits cell growth and differentiation of neuroblastoma cells (16).

Among all of the PKC isoforms, PKC α and PKC β have been suggested to play an important role in the carcinogenesis and metastasis of gastrointestinal cancers. For example, vaccinia virus-mediated overexpression of PKC α strongly increased CPP32 activity and apoptosis in the detached MKN-45 and MKN-74 cells (17). Moreover, several recent studies showed that PKC β inhibitors executed antiangiogenic and antitumor effect in gastrointestinal cancers (18–20). Consistent with these results, our previous studies showed that PKC α was the most abundant isoform in gastric epithelial cells (21, 22), and PKC β_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 PKC α or PKC β . Characterization of these cells with regard to proliferation rate and tumorigenicity *in vitro* and *in vivo* was performed. Our results suggest that inhibition of PKC α and PKC β_1 exerts inhibitory effects on the transformed phenotype of gastric cancer cells. Antisense mediated suppression of PKC α and PKC β_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 μ g/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-PKC α -WT, pHACE-PKC β_1 -WT, and pHACE-PKC β_2 -WT were generated (24). To construct antisense PKC plasmid, the full-length cDNA encoding PKC α , PKC β_1 , and PKC β_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 μ g/well PKC plasmids using Lipofectamine 2000 (Invitrogen). After

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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 $\pm$ 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 μ g/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 μ g/well AP-1 reporter plasmid using Lipofectamine 2000 (Invitrogen). PRL-CMV vector (0.01 μ g/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 μ l 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 μ g/ml leupeptin, 1 μ g/ml aprotinin, and 1 μ g/ml pepstatin A], kept on ice for 15 min, lysed gently with 12.5 μ l of 10% NP40, and centrifuged at $2,000 \times g$ for 10 min at 4°C. The supernatant was collected and used as the cytoplasmic extracts. The nuclei pellet was resuspended in 40 μ l 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 μ g/ml leupeptin, 1 μ g/ml aprotinin, and 1 μ g/ml pepstatin A] and agitated for 30 min at 4°C, and the nuclear debris was spun down at $20,000 \times g$ 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 μ g) 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 PKC α (H-7), PKC β_1 (c-16), PKC β_2 (c-18), PKC ξ , PKC ι , PKC μ , PKC η , PKC δ , PKC γ , and PKC ϵ , 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 μ g/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 μ g of anti-PKC α , -PKC β_1 , and -PKC β_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 μ l of assay mixture and 0.2 μ Ci 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 μ g of nuclear proteins were incubated with 1 μ g 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 μ l 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 $\times$ 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 handling 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: $V = (4/3)\pi R_1^2 R_2$, where R_1 is radius 1 and R_2 is radius 2 and $R_1 < R_2$. 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 $\pm$ 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-PKC α antisense, pcDNA-PKC β_1 antisense, or pcDNA-PKC β_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 PKC α -antisense, PKC β_1 -antisense, and PKC β_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 PKC α , PKC β_1 , or

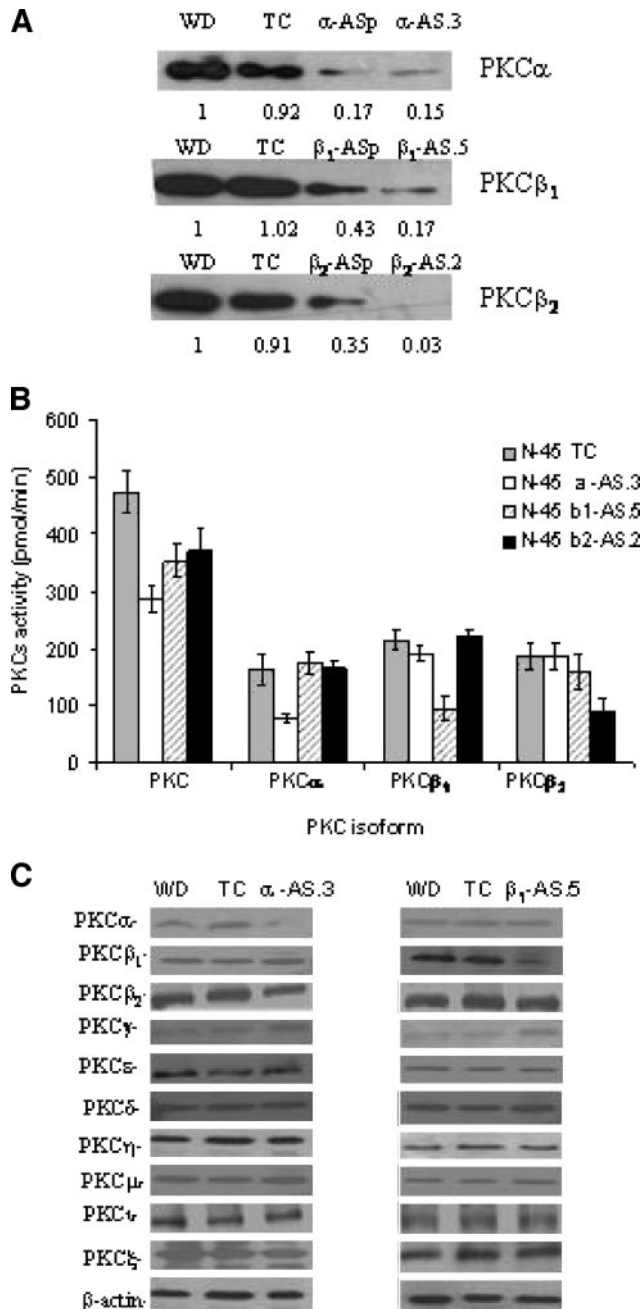


Fig. 1. The protein expression and kinase activity of PKC in PKC- α , β_1 , and β_2 antisense-transfected cells. A, PKC expression in antisense-transfected cells. Control parental cells (WD), empty vector-transfected cells (TC), antisense PKC-transfected cells from single representative clone (α -AS.3, 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 PKC α , PKC β_1 , or PKC β_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 $\pm$ 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 PKC α , PKC β_1 , PKC β_2 , PKC γ , PKC ϵ , PKC δ , PKC η , PKC μ , PKC ι , and PKC ξ . The experiments have been repeated three times, and the representative blot is shown in the figure.

the parental cells. Conversely, 3 of 10 clones of each type, which showed most inhibition of PKC α , PKC β_1 , or PKC β_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 PKC α , PKC β_1 , and PKC β_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 PKC α -AS.3, PKC β_1 -AS.5, or PKC β_2 -AS.2 cells, and the resulting immunocomplexes were subjected to *in vitro* kinase assay. As shown in Fig. 1B, the total PKC activity was decreased by 39.9%, 25.7%, and 21.7% in PKC α -AS.3, PKC β_1 -AS.5, and PKC β_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 PKC α -, PKC β_1 -, and PKC β_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. 1C, the expression of other PKCs remained unchanged in PKC α -AS.3 and PKC β_1 -AS.5 cells. Similar results were obtained in PKC β_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 PKC α -AS.3 and PKC β_1 -AS.5 but not the PKC β_2 -AS.2 cells exhibited a lengthened and flatter morphology. Dendritic-like cytoplasmic processes were visible by phase-contrast microscope (Fig. 2A). Importantly, the PKC α -AS.3 and PKC β_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. 2B. Fig. 2Ba 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, PKC α -AS.3 and PKC β_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 PKC β_2 -AS.2 and control cells (Fig. 2Bd). We also found that the cell density was significantly lower in the PKC α -AS.3 and PKC β_1 -AS.5 cells. At confluence, the PKC α -AS.3 and PKC β_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 PKC α -AS.3 and PKC β_1 -AS.5 cells was 1×10^4 and $2.3 \times 10^4/\text{cm}^2$, respectively, whereas it was $4 \times 10^4/\text{cm}^2$ 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 PKC α -AS.3 and PKC β_1 -AS.5 cells was markedly reduced (Fig. 3A). Thus, on day 7 the number of cells in the PKC α -AS.3 and PKC β_1 -AS.5 cultures were $23.7 \pm 0.4\%$ and $44.5 \pm 0.23\%$, respectively, when compared with the TC cells. PKC β_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. 3A).

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

PKC β_2 resulted in a marked reduction in expression of the corresponding endogenous PKC protein. The protein expression of PKC α , PKC β_1 , and PKC β_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

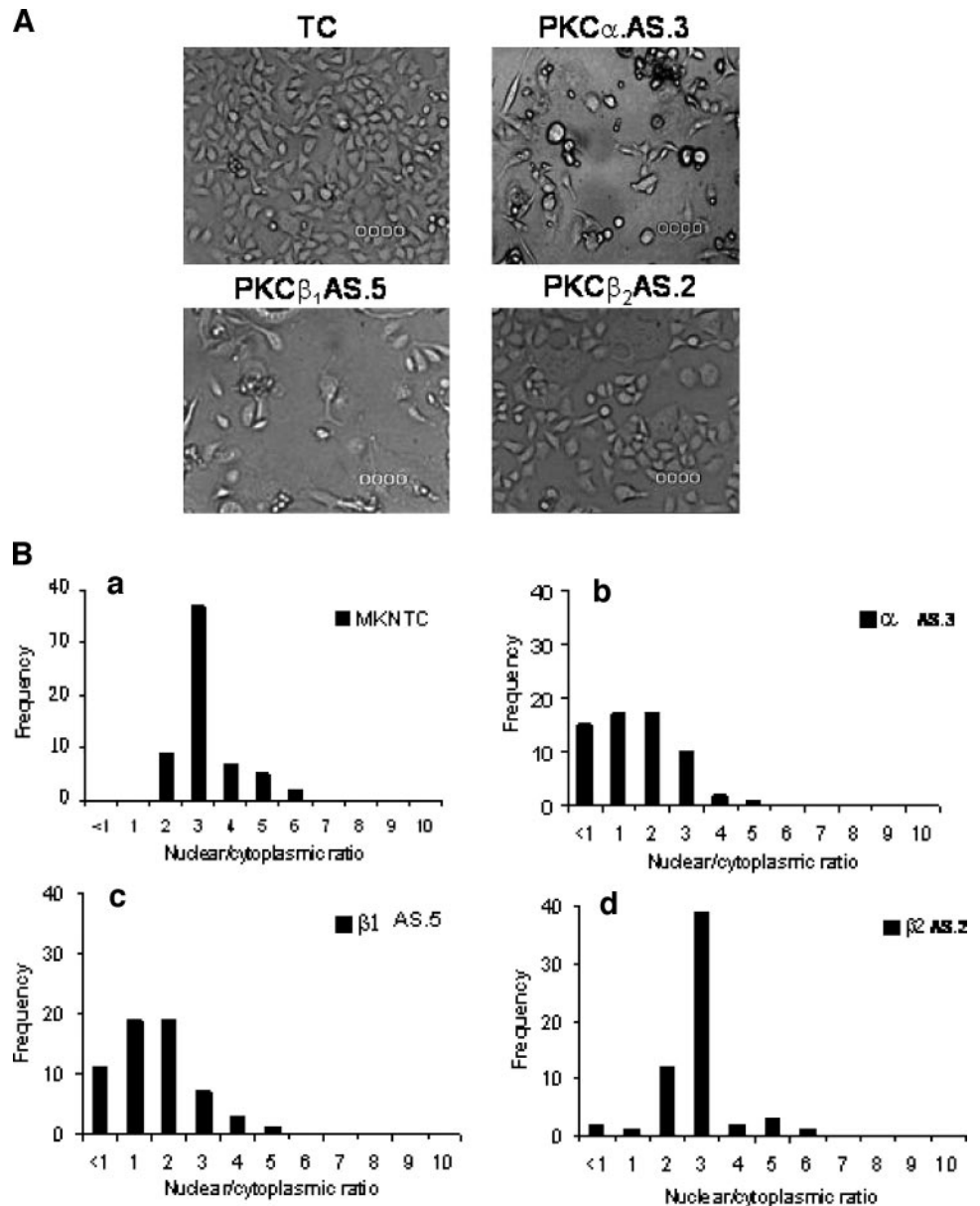


Fig. 2. Morphologic changes in PKC-antisense transfectants. A, TC, PKC α -AS.3, PKC β_1 -AS.5, and PKC β_2 -AS.2 were grown to confluence in 10% fetal bovine serum RPMI 1640 and photographed with a phase-contrast microscope. B, nuclear:cytoplasmic ratios of antisense-transfected cells. The nuclear:cytoplasmic ratios were measured as described in "Materials and Methods" in TC, PKC α -AS.3, PKC β_1 -AS.5, and PKC β_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, PKC α -AS.3 and PKC β_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, PKC α -AS.3 and PKC β_1 -AS.5 but not the PKC β_2 -AS.2 cells showed significant increase of apoptosis compared with the control cells.

Effects of Antisense PKC α and PKC β_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. 3B, the PKC α -AS.3 cells displayed almost complete loss of colony-forming efficiency, and the PKC β_1 -AS.2 cells had an $\sim 50\%$ decrease when compared with the WD and TC control cells. In view of these results, we examined whether the reduction in PKC α and PKC β_1 expression in our AS derivatives of gastric cancer cells might affect their tumorigenicity in nude mice. We injected 5×10^6 WD, TC, PKC α -ASp, and PKC β_1 -ASp cells s.c. into

athymic nude mice and monitored the mice for tumor appearance and growth. PKC α -ASp and PKC β_1 -ASp cells are pooled representative clones, which suppress PKC α and PKC β_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 PKC α -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 PKC α -ASp was $<5\%$ that of the tumors obtained with the TC cells (Fig. 5B). Our results also showed that all of the mice injected with PKC β_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. 5C, PKC α or PKC β_1

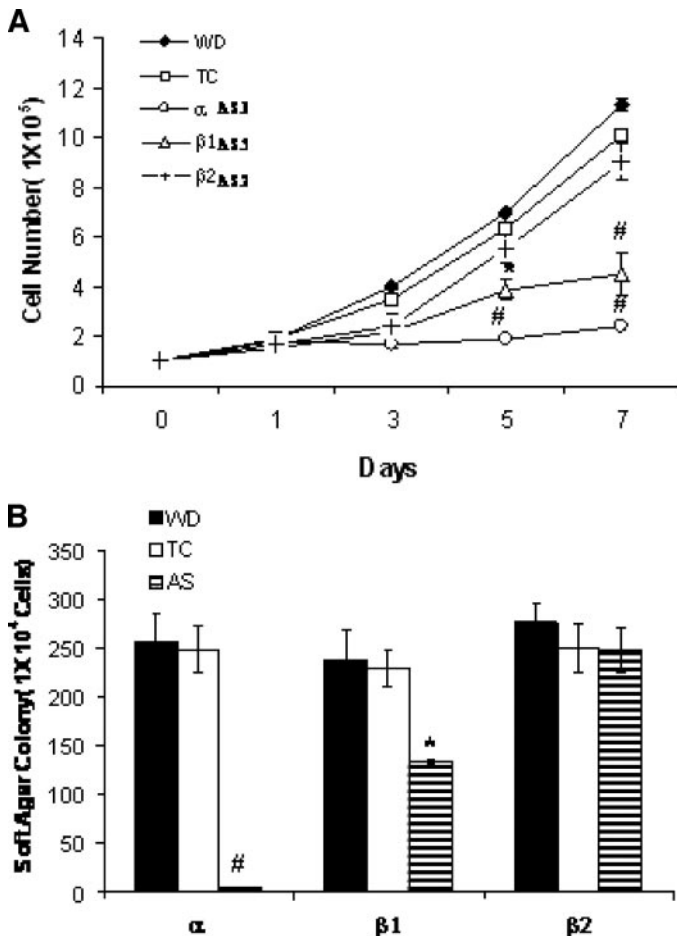


Fig. 3. Cell growth assay and colony-forming ability of PKC antisense transfectants. **A**, cell growth assay in antisense-transfected cells. Cells (1×10^5) were plated onto 60-mm tissue culture dishes in RPMI supplemented with 10% fetal bovine serum. Cells were harvested and counted in a hemocytometer at 48-h intervals. All of the results were expressed as the mean of three independent experiments $\pm$ 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 $\pm$ SD of three experiments; each was performed in duplicate.

expression was significantly suppressed in the tumors obtained from nude mice injected with PKC α -ASp or PKC β_1 -ASp cells, respectively, when compared with tumors obtained from the nude mice injected with control cells.

Antisense PKC α and PKC β_1 Inhibit the AP-1 Activity. To further investigate by which mechanism antisense PKCs suppress gastric tumorigenesis, we detected the effect of antisense PKC α and PKC β_1 on AP-1 transcriptional activity. Our results showed that transient expression of antisense PKC α and PKC β_1 significantly inhibited basal and PMA-induced AP-1 transcriptional activity (Fig. 6A). In contrast, antisense inhibition of PKC β_2 did not affect the transcriptional activity of AP-1 (data not shown). To substantiate the result that inhibition of PKC α or PKC β_1 suppresses AP-1 activity, we determined the transcriptional activity of AP-1 in stable transfectants of PKC α -AS.3 and PKC β_1 -AS.5. The results showed that basal AP-1-mediated transcriptional activity was significantly lower in PKC α -AS.3 and PKC β_1 -AS.5 cells than in TC and parental cells (Fig. 6B). PMA-induced AP-1 transcriptional activity also was dramatically decreased in PKC α -AS.3 and PKC β_1 -AS.5 cells. Consistent with previous results, PKC β_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. 6C, PMA-induced AP-1 DNA binding activity was significantly decreased in PKC α -AS.3 and PKC β_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 PKC α , PKC β_1 , and PKC β_2 in the proliferation, apoptosis, and tumorigenicity in gastric

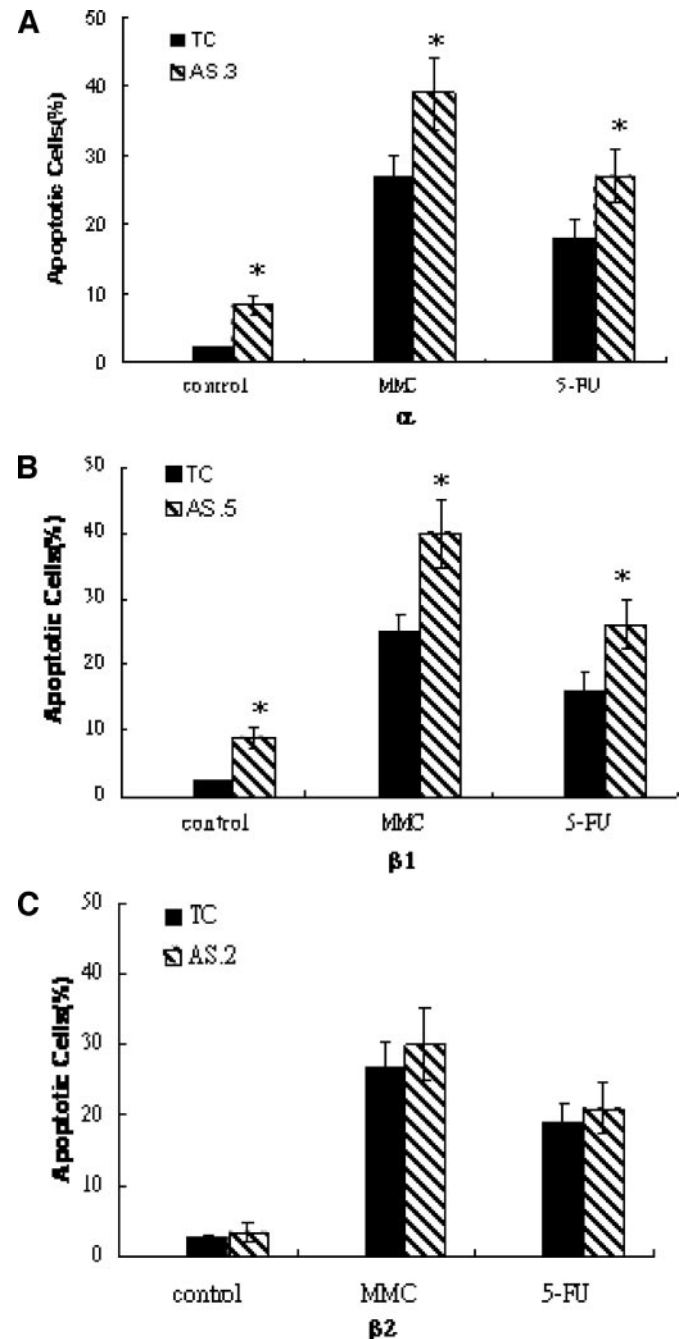


Fig. 4. Apoptotic response of PKC-antisense transfectants to MMC and 5-FU. Control cells and transfectant cells were exposed to 10 μ g/ml MMC and 2 μ M 5-FU for 24 h. Apoptosis was determined by acridine orange staining; *, $P < 0.05$, as compared with control cells. **A**, PKC α ; **B**, PKC β_1 ; **C**, PKC β_2 . All of the results were expressed as the mean of three independent experiments $\pm$ SE.

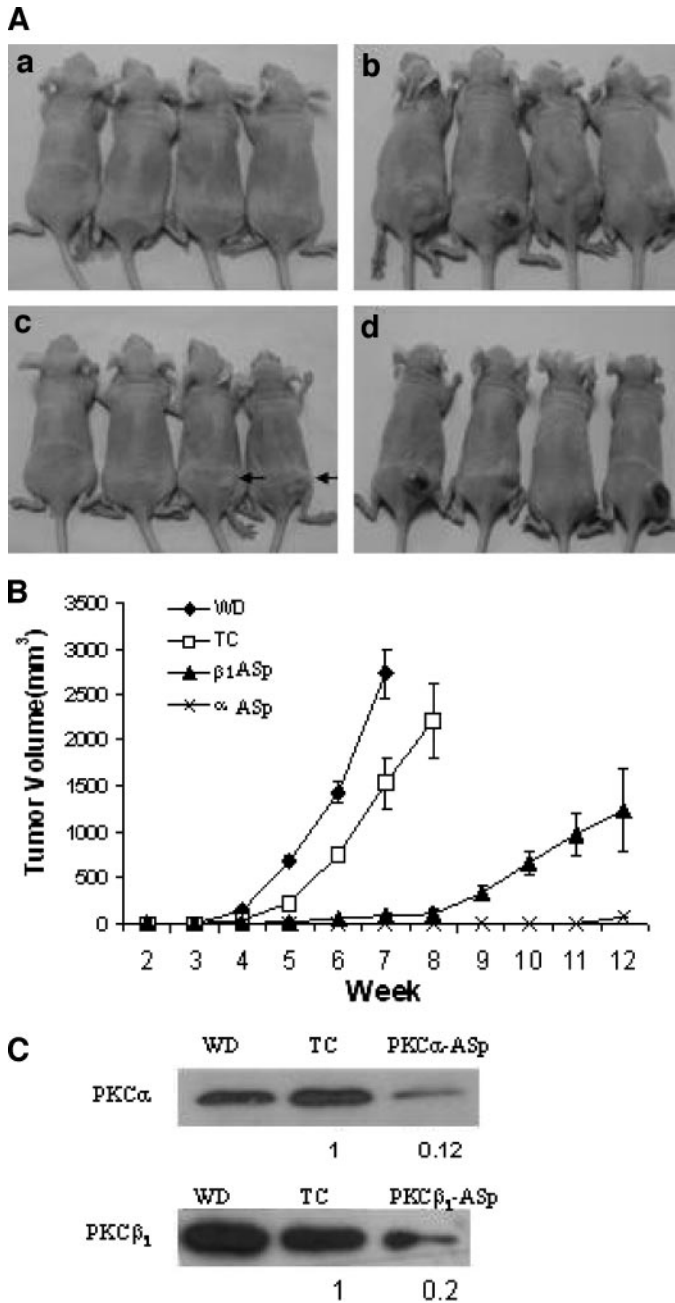


Fig. 5. Tumor growth of human gastric cancer cells stably expressing neovector or antisense PKC in nude mice. A, a total of 5×10^6 cells were injected into the flank region of nude mice. Tumor development was followed up to 3 months, and mice then were sacrificed. A, PBS; B, TC; C, α -ASp; D, β_1 -ASp. 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 $\pm$ SD of four developed tumors in the experimental mice. C, PKC α and PKC β_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.

cancer. We found, for the first time, that PKC α and PKC β_1 , especially the former isoform, played a critical role in maintaining the transformed phenotype of gastric cancer cells. Inhibition of PKC α or PKC β_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-

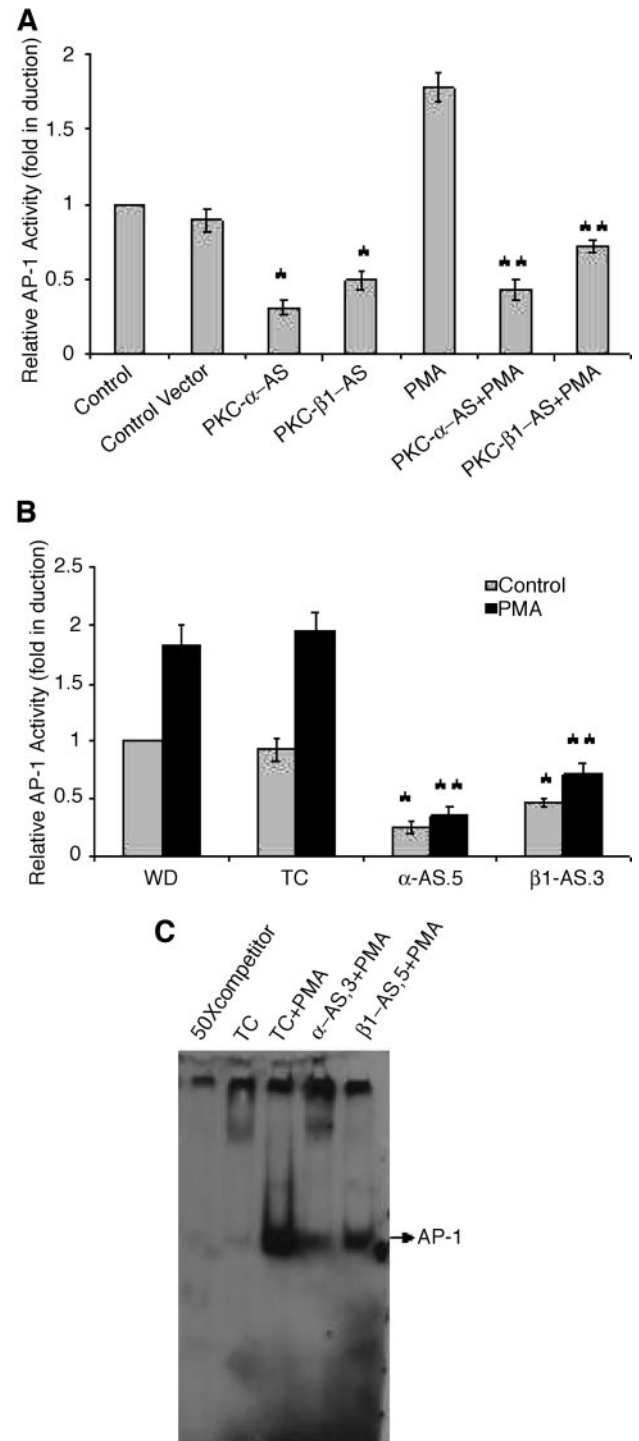


Fig. 6. Antisense PKC α and PKC β_1 suppressed AP-1 transcriptional and binding activity. A, MKN-45 cells were cotransfected with expressing AP-1 luciferase reporter gene and antisense PKC α or PKC β_1 plasmids. After 24 h, cells were treated without or with 100 nM PMA for another 1 h. Cells were analyzed for luciferase activity. The firefly luciferase reading was normalized to renilla luciferase reading. Results were expressed as the means of three independent experiments $\pm$ 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 $\pm$ SE; *, $P < 0.01$, compared with TC transfectants; **, $P < 0.01$, compared with TC transfectants treated with PMA. C, effects of PKC α and PKC β_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 μ g) of nuclear protein were loaded in each lane. In Lane 1, a 50 $\times$ excess of unlabeled oligonucleotide was added before the addition of DIG-labeled probe.

nogenesis and progression of gastric cancer (18, 19, 21). To provide further insight into the specific biological roles of classical PKCs, inhibition of PKC α , PKC β_1 , and PKC β_2 via transfection of antisense was used in the present study. We excluded PKC γ , 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 PKC γ (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 PKC α 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 PKC α 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 PKC α -ASp (pooled antisense PKC α 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 PKC α transfectants exhibited a partial reversal of the transformed phenotype. Therefore, PKC α 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 PKC β inhibitors executed antiangiogenic and antitumor effect in gastrointestinal cancer (18–20), there is no study investigating the specific role of two PKC β isoforms in carcinogenesis and progression of gastric tumor. In this study, we first showed that PKC β_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 PKC α -AS cells. More importantly, the size and growth rate of gastric tumor xenografts in PKC β_1 -ASp-inoculated nude mice were significantly reduced compared with those in the control group. Surprisingly, we failed to observe any significant change of PKC β_2 -AS cells in cell morphology, cell growth, and cell transformation in the present study. Although PKC β_1 and PKC β_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 PKC β_2 in the colon of transgenic mice leads to hyperproliferation and increased susceptibility to colon carcinogenesis (31, 32), whereas PKC β_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 PKC β_1 but not PKC β_2 plays an important role in gastric carcinogenesis *in vitro* and *in vivo*.

Because PKC α and PKC β have been implicated in the apoptotic pathway (33, 34), we further investigated and found that the spontaneous apoptotic rates in PKC α -AS and PKC β_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 PKC β_2 -AS cells showed no significant difference in spontaneous apoptosis compared with the control. We also challenged PKC α -AS and PKC β_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 safinol and MMC induced apoptosis in 83% of the cells. Together, these results indicate that other PKC isoforms, besides

PKC α or PKC β_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 PKC α -AS and PKC β_1 -AS cells. Our results showed that inhibition of PKC α or PKC β_1 , but not PKC β_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 NH₂-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 PKC α and PKC β_1 in the regulation of cell growth and tumorigenicity of gastric cancer. Thus, targeting of PKC α and/or PKC β_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 PKC α and PKC β_1 inhibition is worth further investigation.

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