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Kalopanaxsaponin A inhibits PMA-induced invasion by reducing matrix metalloproteinase-9 via PI3K/Akt- and PKCδ-mediated signaling in MCF-7 human breast cancer cells

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Induction of matrix metalloproteinase (MMP)-9 is particularly important for the invasiveness of breast cancers. We investigated the inhibitory effect of kalopanaxsaponin A (KPS-A) on cell invasion and MMP-9 activation in phorbol 12-myristate 13-acetate (PMA)-treated MCF-7 human breast cancer cells. KPS-A inhibited PMA-induced cell proliferation and invasion. PMA-induced cell invasion was blocked in the presence of a primary antibody of MMP-9, and KPS-A suppressed the increased expression and/or secretion of MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1. Using specific inhibitors, we confirmed that PMA-induced cell invasion and MMP-9 expression is primarily regulated by nuclear factor-kappa B (NF-κB) activation via phosphatidylinositol 3-kinase (PI3K)/Akt and activator protein-1 (AP-1) activation via extracellular signal-regulated kinase (ERK)1/2. KPS-A decreased PMA-induced transcriptional activation of NF-κB and AP-1 and inhibited PMA-induced phosphorylation of ERK1/2 and Akt. Treatment with the protein kinase C (PKC)δ inhibitor rottlerin caused a marked decrease in PMA-induced MMP-9 secretion and cell invasion, as well as ERK/AP-1 activation, and KPS-A reduced PMA-induced membrane localization of PKCδ. Furthermore, oral administration of KPS-A led to a substantial decrease in tumor volume and expression of proliferating cell nuclear antigen, MMP-9, TIMP-1 and PKCδ in mice with MCF-7 breast cancer xenografts in the presence of 17β-estradiol. These results suggest that KPS-A inhibits PMA-induced invasion by reducing MMP-9 activation, mainly via the PI3K/Akt/NF-κB and PKCδ/ERK/AP-1 pathways in MCF-7 cells and blocks tumor growth and MMP-9-mediated invasiveness in mice with breast carcinoma. Therefore, KPS-A may be a promising anti-invasive agent with the advantage of oral dosing.

Introduction

Breast cancer is the malignancy that is most frequently diagnosed in women, and its incidence is rapidly increasing in all industrialized countries. Because of local invasion and metastasis, neither radiation therapy nor chemotherapy substantially increases the length or quality of life of patients with advanced breast cancer. Therefore, the control of invasion and metastasis is an important therapeutic strategy, and the development of effective anti-invasive agents for breast cancer would be very probably to improve treatment.

Metastasis is one of the major causes of mortality in cancer patients and occurs as a complex multistep process involving cancer cell adhesion, invasion and migration. Although a number of proteinases are involved in tissue lysis, the secretion of matrix metalloproteinases (MMPs) is crucial in cancer cell metastasis, because MMPs are responsible for the degradation of environmental barriers, such as the extracellular matrix and basement membrane (1,2). Among human MMPs, MMP-2 (also known as gelatinase-A and 72-kDa type IV collagenase) and MMP-9 (also known as gelatinase-B and 92-kDa type IV collagenase) have been correlated with the malignancy of various tumors and with poor survival in patients with breast cancer (1,2). MMP-2 is commonly constitutively present in tissues, and it is maximally expressed in malignant neoplasms as part of the host response to the presence of neoplastic cells, rather than as part of an initial response to invasion (3). In contrast, synthesis and secretion of MMP-9 can be stimulated by a variety of growth factors and inflammatory cytokines during pathological processes and by agents such as phorbol 12-myristate 13-acetate (PMA) (4–7). It has been reported that induction of MMP-9 is particularly important for the invasiveness of human cancers, including breast cancer (8–12), thereby blockade of MMP-9-mediated invasion suppresses the metastasis of breast cancer cells into other organs (13,14).

The activity of MMP-9 in various tumor cells is tightly controlled, with regulation occurring primarily at the transcription level (15). The promoter of human *MMP-9* contains *cis*-acting regulatory elements that bind with transcription factors such as nuclear factor-kappa B (NF-κB; −600 bp) or activator protein-1 (AP-1; −533 bp and −79 bp) (16,17). NF-κB and AP-1 are ubiquitous eukaryotic transcription factors and can be induced by multiple stimuli. NF-κB, a heterodimer of p50 and p65, is sequestered in the cytoplasm due to its association with the inhibitory protein IκBα under normal conditions. Stimulation by inflammatory cytokines or tumor promoters leads to the dissociation of IκBα from NF-κB through the ubiquitin/proteasome-dependent pathway following its phosphorylation. The released NF-κB translocates into the nucleus and binds to the promoter region of *MMP-9*, leading to gene expression (18). On the other hand, AP-1 is a nuclear transcription factor comprising homodimers and heterodimers of members of the Fos and Jun families (19). NF-κB- and/or AP-1-dependent MMP-9 expression is regulated by mitogen-activated protein kinases (MAPKs) and by the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, depending on the cell type and on the type of stimuli (10,16,20–22). Therefore, these upstream molecules that regulate MMP-9 expression or enzymatic activity can be used as a target for treating breast cancer metastasis. Recent studies to detect new anti-metastatic agents have demonstrated that plant-derived compounds with chemopreventive potential inhibit the invasiveness of several types of cancer by modifying MMP-9 expression (16,23–26).

Kalopanaxsaponin A (KPS-A; 3-O-[L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl]-hederagenin) is an oleanane triterpene saponin found in *Kalopanax pictus* Nakai (Araliaceae), a plant that has been traditionally used for the treatment of rheumatoidal arthritis and diabetes mellitus in East Asian countries (27,28). KPS-A displays cytotoxicity in J82, T24, Colon 26 and 3LL cancer cells (29), significantly reduces LL/2 tumor growth in mice and apparently prolongs the life span of mice with Colon 26 and 3LL Lewis lung carcinomas (27).

In this study, we investigated the inhibitory effect of KPS-A on cell invasion and MMP-9 activation in PMA-treated MCF-7 human breast cancer cells. KPS-A reduced MMP-9 expression by blocking the activation of NF-κB and AP-1 transcription factors via PI3K/Akt- and

Abbreviations: AP-1, activator protein-1; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; JNK, c-jun N-terminal kinase; KPS-A, kalopanaxsaponin A; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NF-κB, nuclear factor-kappa B; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TIMP, tissue inhibitor of metalloproteinase.

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protein kinase C (PKC)δ-mediated extracellular signal-regulated kinase (ERK)1/2 signaling. In a mouse xenograft model of MCF-7 cells, KPS-A suppressed tumor growth and the expression of invasive biomarkers.

Materials and methods

Materials

KPS-A was generously provided by Professor Hee-Juhn Park (Sangji University, Wonju, Korea) (Figure 1A) and was dissolved in dimethyl sulfoxide, followed by dilution with culture medium. Dulbecco's modified Eagle's medium/F12 (DMEM/F12, 1:1), fetal bovine serum (FBS), antibiotic–antimycotic (10 000 U/ml penicillin G sodium, 10 000 μg/ml streptomycin sulfate and 25 μg/ml amphotericin B), phosphate-buffered saline (PBS) and 0.25% trypsin–ethylenediaminetetraacetic acid were purchased from Gibco BRL (Rockville, MD). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and dimethyl sulfoxide were purchased from Sigma–Aldrich (St Louis, MO). PD98059, SB203580, SP600125, LY294002, GF109203X, Gö6976, rottlerin and PMA were purchased from Calbiochem (La Jolla, CA). The following antibodies were purchased from their respective sources: p38 and phospho-p38 MAPKs (New England Biolabs, Beverly, MA); total/phospho form of Akt, ERK1/2, c-jun N-terminal kinase (JNK) and IκBα (Cell Signaling Technology, Denver, MA); MMP-9, c-Fos, c-Jun, p50, p65, PKCα, PKCβ1, PKCδ, tissue inhibitor of metalloproteinase (TIMP)-1 and Lamin A/C (Santa Cruz Biotechnology, CA); β-actin (Sigma–Aldrich); proliferating cell nuclear antigen (PCNA) (DAKO Diagnostics, Ontario, Canada); horseradish peroxidase-conjugated secondary antibodies (Amersham Life Science, Little Chalfont, UK) and biotinylated anti-mouse/anti-rabbit immunoglobulin G (H + L) and horseradish peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, CA).

Cell culture

The estrogen receptor (ER)-positive human breast cancer cell line MCF-7 was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM:F12 (1:1) supplemented with 10% FBS and 1% antibiotic–antimycotic at 37°C in a humidified atmosphere of 5% CO_2.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

MCF-7 cells (5×10^3 cells/well) were plated into a 96-well culture plate with 10% FBS–DMEM and left overnight to adhere. The cells were cultured in serum-free medium containing 0–10 μg/ml KPS-A in the absence or presence of 0.5 μM PMA for 24 and 48 h, respectively. The cells were then incubated with a 5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution for an additional 4 h at 37°C. The formazan product was dissolved with 200 μl dimethyl sulfoxide. Absorbance was measured at 570 nm in a microplate reader (Bio-Rad, Hercules, CA).

5-Bromo-2′-deoxyuridine incorporation assay

MCF-7 cells (5×10^3 cells/well) were seeded onto a 96-well culture plate and incubated overnight at 37°C. The cells were treated with serum-free medium containing 0–10 μg/ml KPS-A and 0.5 μM PMA for 24 h, followed by labeling with 10 μM 5-Bromo-2′-deoxyuridine for an additional 4 h. The cells were fixed with 200 μl FixDenat solution (Roche Diagnostics, Mannheim, Germany) for 30 min at −20°C. DNA synthesis in proliferating cells was measured using the 5-Bromo-2′-deoxyuridine labeling and detection Kit III (Roche Diagnostics) according to the manufacturer's protocol. Absorbance was measured at 450 nm using a microplate reader.

Invasion assay

MCF-7 cells were cultured in 10% FBS–DMEM:F12 (1:1) with 10 μCi/ml [^{3}H]-thymidine for 24 h. The 8 μm pore size polycarbonate nucleopore filter inserts in a 24-well transwell chamber (Corning Costar, Cambridge, MA) were coated with 30 μg/well Matrigel (Becton Dickinson, Lincoln Park, NJ). The [^{3}H]thymidine-labeled cells (5×10^4 cells) were seeded into the upper part of the Matrigel-coated filter, and serum-free DMEM/F12 with 0.5 μM PMA was added to the lower part with 0–10 μg/ml KPS-A, 1 μg/ml anti-MMP-9 antibody, 50 μM PD98059, 20 μM SB203580, 50 μM LY294002, 40 μM SP600125, 2 μM GF109203X, 2 μM Gö6976 or 0.5 μM rottlerin for 48 h.

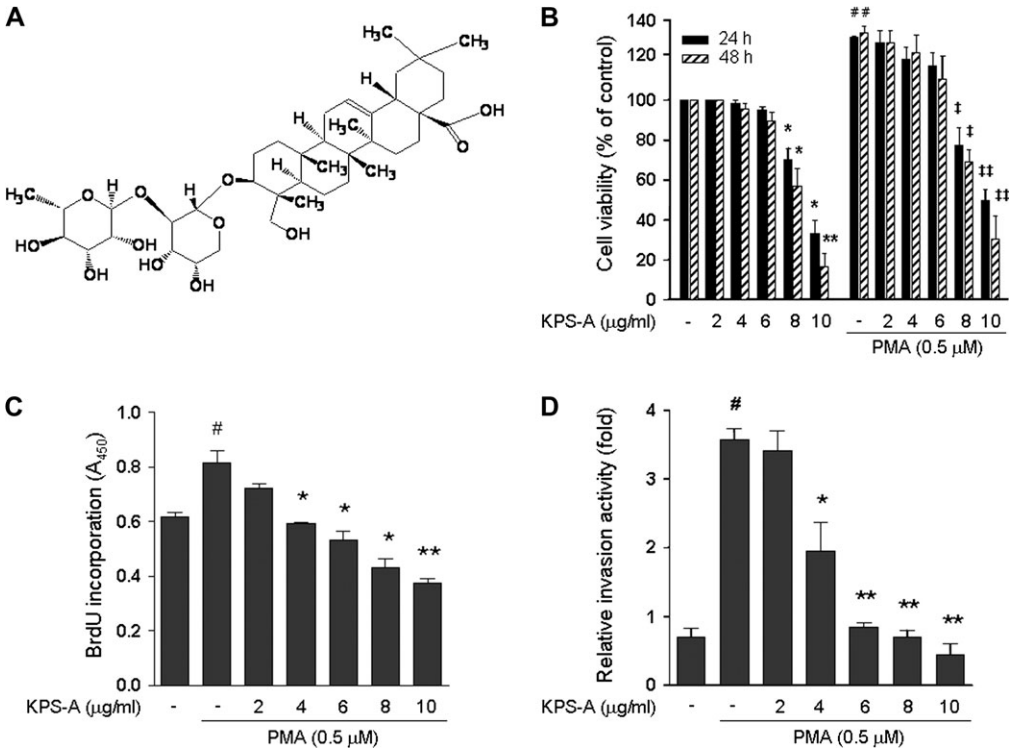

Fig. 1. KPS-A suppresses PMA-induced proliferation and invasion of MCF-7 cells. (**A**) Chemical structure of KPS-A. (**B**) MCF-7 cells were treated with 0–10 μg/ml KPS-A in the absence or presence of 0.5 μM PMA for 24 and 48 h, and cell viability was measured using an 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay; $^{\#}P < 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle alone-treated cells, $^{‡}P < 0.05$, $^{‡‡}P < 0.01$ versus PMA alone-treated cells. (**C**) Cells were treated with KPS-A and/or PMA for 24 h, and the amount of the newly synthesized DNA in proliferating cells was measured by a 5-Bromo-2′-deoxyuridine (BrdU) incorporation assay; $^{\#}P < 0.01$ versus vehicle alone-treated cells, $^{*}P < 0.01$, $^{**}P < 0.001$ versus PMA alone-treated cells. (**D**) [^{3}H]thymidine-labeled MCF-7 cells were treated with or without PMA and KPS-A for 48 h. Invasion activities were determined by a Matrigel-coated *in vitro* invasion assay and expressed as changes in invasion relative to control; $^{\#}P < 0.001$ versus vehicle alone-treated cells, $^{*}P < 0.01$, $^{**}P < 0.001$ versus PMA alone-treated cells. Data represent the mean ± SE of three independent experiments.

The radioactivity of the cells that invaded through the Matrigel into the lower chamber was counted using a LS6500 liquid scintillation counter with liquid scintillation cocktail (Beckman Coulter, Fullerton, CA). Results are expressed as the changes in invasion relative to control.

Western blot analysis

To examine the expression and/or activation of MMP-9, TIMP-1, MAPKs and Akt, cells were pretreated with KPS-A for 2 h or PKC inhibitors for 1 h and then stimulated with 0.5 μM PMA for 24 h (MMP-9 and TIMP-1) or 20 min (MAPKs and Akt). Total lysates were prepared as described previously (6). Cytoplasmic and nuclear fractions to examine the activation of transcription factors were obtained from cells treated with PMA for 1 h (NF-κB) or 2 h (AP-1) after 2 h pretreatment with KPS-A (30). In addition, cytosolic and membrane fractions to identify the localization of PKC isotypes were also prepared with cells treated with PMA for the indicated time, or cells pretreated with KPS-A for 2 h and then stimulated with PMA for 15 min (20). Equal amounts of protein (50 μg) were separated on 10% sodium dodecyl sulfate–polyacrylamide gels. The proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membrane was blocked with 5% skim milk in PBS-containing 0.1% Tween-20 and then incubated with each primary antibody (1:1000) against specific proteins in 5% skim milk overnight at 4°C. The blots were incubated with a 1:3000 dilution of the respective horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature and were again washed with PBS-containing 0.1% Tween-20. The targeted proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Life Science) according to the protocol of the manufacturer.

Gelatin zymography

MCF-7 cells (1×10^5 cells) were incubated in serum-free medium including 0.5 μM PMA for 24 h, following pretreatment with 0–10 μg/ml KPS-A for 2 h, or specific inhibitors of MAPKs (PD98059, SB203580 and SP600125), PI3K (LY294002) and PKC isotypes (GF109203X, Gö6976 and rottlerin) for 1 h, respectively. The conditioned medium was collected and protein concentration was determined by the Bradford method (Bio-Rad). Equal amounts of protein (10 μg) were separated on 8% sodium dodecyl sulfate–polyacrylamide gels-containing 0.1% (wt/vol) gelatin. The gel was washed with 2.5% Triton X-100 for 30 min at room temperature and then incubated in a buffer containing 10 mM $CaCl_2$, 0.01% NaN_3 and 50 mM Tris–HCl (pH 7.5) for 16 h at 37°C. The gel was stained with 0.2% Coomassie Brilliant Blue and photographed on a light box. MMP-9 gelatinolytic activity was detected as clear bands in a dark blue background.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assay was performed using a DNA–protein binding detection kit (Promega, Madison, WI) according to the manufacturer's protocol. MCF-7 cells were pretreated with 5 μg/ml KPS-A for 2 h, or specific inhibitors of MAPKs (PD98059, SB203580 and SP600125), PI3K (LY294002) and PKC (GF109203X, Gö6976 and rottlerin) for 1 h, followed by treatment with 0.5 μM PMA for 1 h or 2 h to determine DNA-binding activity of NF-κB and AP-1, respectively. Nuclear extract was prepared as described previously (30). Double-stranded oligonucleotides containing the NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) or AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′) consensus sequences were end labeled with Klenow [γ-^{32}P]adenosine triphosphate (3000 Ci/mmol) using T4 polynucleotide kinase and purified with a NICK column (Amersham Pharmacia Biotechnology, Piscataway, NJ). The eluted solution was used as probes for electrophoretic mobility shift assay. Nuclear extracts (10 μg) were incubated with the binding buffer [10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid, 4% (vol/vol) glycerol and 1 μg/μl poly dI-dC] for 10 min at room temperature, then treated with 0.5 pmol-labeled probe for 20 min. The DNA–protein complex was separated on a 6% polyacrylamide gel in 0.5× Tris–Borate–ethylenediaminetetraacetic acid buffer at 10 V for 3 h. Gels were dried and exposed to Kodak-XAR film.

Transient transfection and luciferase reporter assays

Transcriptional activities of NF-κB and AP-1 were measured by luciferase reporter assay using the pNF-κB-Luc and pAP-1-Luc reporter plasmid (Clontech, Palo Alto, CA). MCF-7 cells (1×10^5 cells/well) were seeded into 6-well plates. The cells at 70–80% confluence were cotransfected with 1 μg of NF-κB or AP-1 reporter constructs and 0.5 μg pSV-β-galactosidase for 8 h in serum- and antibiotics-free Opti-MEM (Gibco BRL) with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). The transfected cells were pretreated with KPS-A at the indicated concentrations for 2 h and then incubated with 0.5 μM PMA for 8 h. Luciferase and β-galactosidase activities were assayed according to the manufacturer's protocol (Promega), using a microplate spectrofluorometer (Molecular Devices, Palo Alto, CA). Luciferase activity was normalized by β-galactosidase activity in cell lysate and expressed as an average of three independent experiments.

Xenograft assays in nude mice

Female Balb/C athymic nude mice (5-week-old mice; Central Lab Animal, Seoul, Korea) were maintained at 20–22°C on a 12 h light–dark cycle. Animal studies were performed in accordance with experimental protocols that were approved by the animal ethics committee of Yonsei University College of Dentistry (Seoul, Korea). MCF-7 cells (1×10^7 cells/0.2 ml PBS) cultured in 10% FBS–DMEM/F12 were subcutaneously injected into the right flanks of the mice. To stimulate the growth of MCF-7 cells, a 17β-estradiol pellet (Innovative Research of America, Sarasota, FL) was implanted into the intracapular region before the injection of MCF-7 cells. Two days later, the mice were administered three times/week with KPS-A in 0.1 ml PBS (5 and 10 mg/kg body wt) by oral gavage for 23 days, and the control group received PBS alone. Tumor volume was measured with a digital electric caliper and calculated by the following formula: $(\text{width in mm})^2 \times (\text{length in mm})/2$. The mice were killed under anesthesia. The tumors were collected for immunohistochemical studies.

Immunohistochemical analysis

The collected MCF-7 xenograft tumors were fixed with 4% paraformaldehyde solution for 24 h and then embedded in paraffin. Serial tissue sections (4 μm thick) were prepared and mounted onto slides. The sections were deparaffinized in xylene, rehydrated in alcohol and treated with 3% (vol/vol) H_2O_2 for 10 min at room temperature to suppress endogenous peroxidase activity. The antigen retrieval was performed by microwave treatment of the sections for 10 min in 10 mM sodium citrate buffer (pH 6.0). After rinsing three times with PBS for 5 min, the sections were incubated with 10% normal goat serum for 20 min at room temperature to block non-specific binding sites and reacted overnight at 4°C with a primary antibody against PCNA, MMP-9, TIMP-1 and PKCδ at a dilution of 1:100 in PBS. The sections were rinsed with PBS and incubated with biotinylated anti-mouse/anti-rabbit immunoglobulin G (H + L) (1:100 dilution in 1% bovine serum albumin) at room temperature for 30 min, followed by exposure to horseradish peroxidase-conjugated streptavidin (1:200 dilution in 1% bovine serum albumin) at room temperature for 30 min. The sections were reacted with 0.02% 3,3′-diaminobenzidine as chromogen and then counterstained with hematoxylin.

Statistical analysis

Statistical analysis was performed using InStat statistical software (GraphPad Software, San Diego, CA). The results are presented as mean ± SE. The statistical significance of differences between groups was analyzed via repeated measures of one-way analysis of variance followed by Student's *t*-test. A P value <0.05 was considered to be significant.

Results

KPS-A inhibits PMA-induced proliferation and invasion of MCF-7 cells

We first determined the effect of KPS-A on PMA-induced proliferation of MCF-7 cells. PMA treatment for 24 and 48 h significantly increased the viability of MCF-7 cells, but KPS-A at >6 μg/ml reduced cell viability in the absence or presence of PMA (Figure 1B). When the amount of the newly synthesized DNA was quantified by measuring the incorporated 5-bromo-2′-deoxyuridine in MCF-7 cells treated with PMA and KPS-A for 24 h, KPS-A dose dependently inhibited PMA-induced DNA synthesis (Figure 1C). Next, we examined whether KPS-A could inhibit PMA-induced invasion in MCF-7 cells. PMA caused a 3.6-fold increase in the invasion of MCF-7 cells, but PMA-induced cell invasion was inhibited by KPS-A in a dose-dependent manner (Figure 1D). Moreover, KPS-A significantly inhibited PMA-induced cell invasion at non-cytotoxic doses of 6 μg/ml or less.

KPS-A inhibits PMA-induced invasion of MCF-7 cells by reducing MMP-9 activation and TIMP-1 expression

To confirm whether MMP-9 activity is involved in increased PMA-induced invasion of MCF-7 cells, the cells were treated with PMA and/or a primary antibody of MMP-9. Addition of an MMP-9 primary antibody significantly blocked PMA-induced cell invasion (Figure 2A). We next studied the effect of KPS-A on MMP-9 expression in cells and its activity in the conditioned media. Western blot analysis and gelatin zymography revealed that the basal level of MMP-9 in MCF-7 cells was low but that its protein expression and secretion were markedly induced by PMA treatment. KPS-A inhibited

increased MMP-9 expression (Figure 2B) and secretion (Figure 2C) in PMA-treated MCF-7 cells. In addition, PMA-induced TIMP-1 expression was also blocked by KPS-A treatment (Figure 2B). These results indicate that KPS-A alleviates cell invasion by reducing MMP-9 expression and secretion as well as TIMP-1 expression in PMA-treated MCF-7 cells.

PMA increases cell invasion and MMP-9 activity through PI3K/Akt- and ERK-mediated activation of transcription factors in MCF-7 cells

To explore signaling pathways regulating the invasiveness of PMA-treated MCF-7 cells, cells were treated with PD98059, SB203580, SP600125 and LY294002, which are specific inhibitors of ERK1/2, p38 MAPK, JNK and PI3K, respectively. PMA-stimulated cell invasion was suppressed by all the inhibitors but was significantly inhibited by the ERK1/2 and PI3K inhibitors (Figure 3A). PMA-induced MMP-9 secretion (Figure 3B) and DNA binding of NF-κB (Figure 3C) were also inhibited by MAPK inhibitors and the PI3K inhibitor, but DNA binding of AP-1 was abolished only by the ERK1/2 inhibitor. These results indicate that PMA stimulates cell invasion and MMP-9 secretion by modulating PI3K/Akt-mediated NF-κB activation and ERK-mediated AP-1 activation.

KPS-A inhibits PMA-induced transcriptional activity of MMP-9 and phosphorylation of ERK1/2 and Akt in MCF-7 cells

To determine whether MMP-9 expression was regulated at a transcriptional level by KPS-A, we performed a promoter assay using transiently transfected MCF-7 cells with a luciferase reporter gene linked to the MMP-9 promoter sequence, including NF-κB and

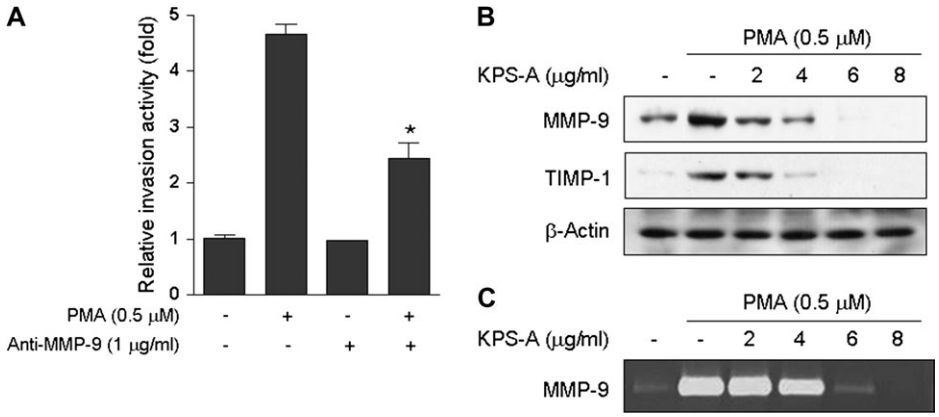

Fig. 2. KPS-A inhibits PMA-induced MMP-9 activation and TIMP-1 expression in MCF-7 cells. (**A**) [^{3}H]thymidine-labeled MCF-7 cells were incubated in Matrigel-coated transwells with or without PMA and anti-MMP-9 polyclonal antibody for 48 h. Invasion activities were determined by radioactivity of the invaded cells and expressed as changes in invasion relative to control conditions. Data represent the mean ± SE of three independent experiments; $^*P < 0.01$ versus PMA alone-treated cells. (**B**) Cells were pretreated with KPS-A for 2 h and then stimulated with PMA for 24 h. Western blot analysis was performed with MMP-9 and TIMP-1-specific antibodies. (**C**) Cells were pretreated with KPS-A for 2h followed by PMA stimulation for 24 h. MMP-9 enzyme activity in the conditioned medium was analyzed by gelatin zymography.

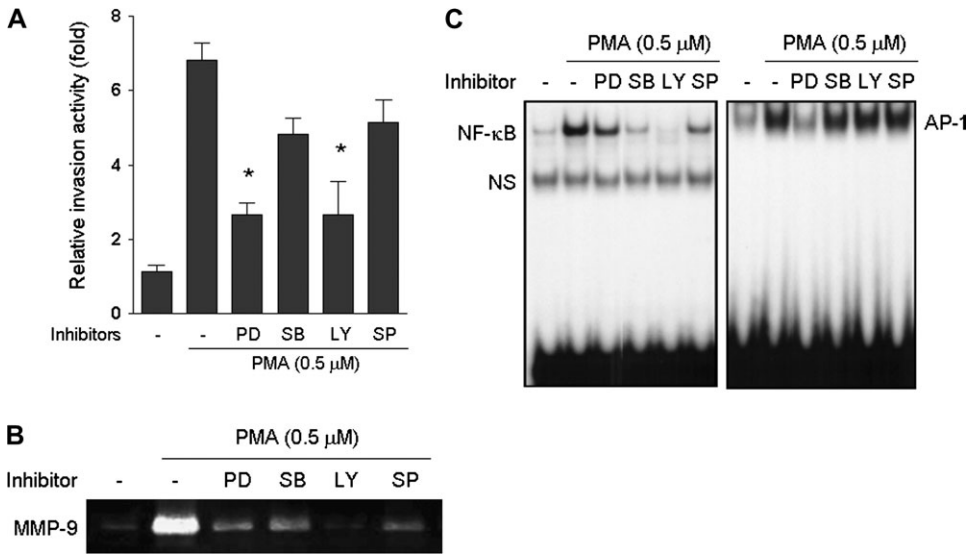

Fig. 3. PMA enhances cell invasion and MMP-9 secretion via PI3K/Akt/NF-κB and ERK1/2/AP-1 pathways in MCF-7 cells. (**A**) [^{3}H]thymidine-labeled MCF-7 cells were seeded into the upper part of a Matrigel-coated filter, and MAPK [PD98059 (PD, 50 μM), SB203580 (SB, 20 μM) and SP600125 (SP, 40 μM)] or PI3K [LY294002 (LY, 50 μM)] inhibitors were added to the lower chamber of the Transwell apparatus in the presence of PMA for 48 h. Invasion activities were determined by radioactivity of the invaded cells and expressed as changes in invasion relative to control conditions. Data represent the mean ± SE of three independent experiments. $^*P < 0.01$ versus PMA-treated cells. (**B**) Cells were treated with specific inhibitors of MAPKs or PI3K for 1 h and then stimulated with PMA for 24 h. Gelatine zymography was performed with the conditioned medium to detect MMP-9 activity. (**C**) Cells were pretreated with specific inhibitors of MAPKs or PI3K for 1 h and then stimulated with PMA for 1 and 2 h. Electrophoretic mobility shift assay was performed as described in Materials and Methods; NS, non-specific.

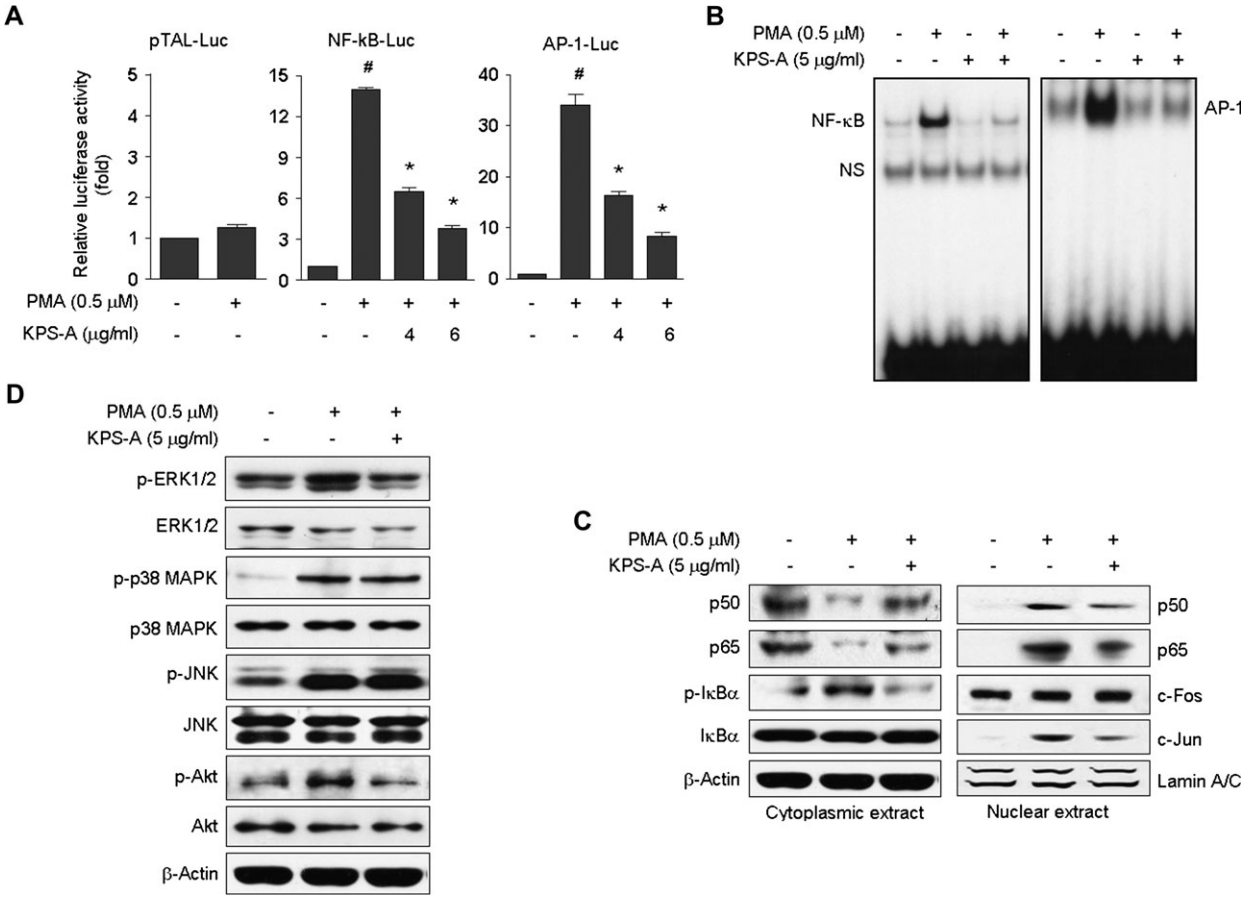

Fig. 4. KPS-A inhibits PMA-induced transcriptional activation of MMP-9 and phosphorylation of ERK1/2 and Akt in MCF-7 cells. (**A**) Cells were transfected with pTAL-Luc, pNF-κB-Luc or pAP-1-Luc. The transfected cells were treated with KPS-A for 2 h and then PMA for 8 h. Data for the NF-κB- and AP-1-dependent luciferase activity were normalized to β-galactosidase activity from cotransfection of pRSV β-galactosidase. The data represent the mean ± SE of three independent experiments; $^{\#}P < 0.001$ versus unstimulated cells, $^{*}P < 0.01$ versus PMA alone-treated cells. (**B**) Cells were pretreated with KPS-A for 2 h and then stimulated with PMA for 1 h for NF-κB or 2 h for AP-1. Prepared nuclear extract was reacted with radioactive oligonucleotides containing the NF-κB or the AP-1 motif of the MMP-9 promoter. Reacted bound complexes were separated by 6% non-denaturating polyacrylamide electrophoresis; NS, non-specific. (**C**) Cells were pretreated with KPS-A for 2 h followed by PMA stimulation for 1 or 2 h. Western blotting was performed to determine the nuclear levels of NF-κB (p50 and p65) and AP-1 (c-Fos and c-Jun) subunits as well as the cytoplasmic levels of NF-κB subunits, IκBα and pIκBα. (**D**) Cells were incubated with KPS-A for 2 h followed by PMA stimulation for 20 min, and the levels of total/phospho MAPKs and Akt were determined by western blotting.

AP-1-binding site motifs. NF-κB-dependent luciferase activity was increased 13.5-fold and AP-1-dependent luciferase activity was increased 34-fold in PMA-stimulated MCF-7 cells, compared with unstimulated cells. KPS-A treatment decreased the NF-κB- and AP-1-dependent luciferase activities induced by PMA in a dose-related manner (Figure 4A). To further evidence the inhibitory effect of KPS-A on the transcriptional activity of MMP-9, we examined the effect of KPS-A on the DNA-binding activity of NF-κB and AP-1 by electrophoretic mobility shift assay. The DNA-binding activity of NF-κB was maximally induced at 1 h and returned to control levels 4 h after PMA stimulation, whereas AP-1 DNA-binding activity was significantly enhanced at 2 h and remained so for 8 h (data not shown). Building upon these results, when MCF-7 cells were treated with PMA for 1 h or 2 h after pretreatment with KPS-A for 2 h, PMA-induced increases in the DNA-binding activity of NF-κB and AP-1 were substantially inhibited by KPS-A (Figure 4B). In western blot analysis, PMA stimulated the phosphorylation of IκBα in cytoplasm and, thereby, the nuclear translocation of NF-κB subunits p50 and p65. In the case of AP-1, c-Jun expression was considerably augmented, but c-Fos expression was only negligibly induced in PMA-treated cells. However, KPS-A reduced the cytoplasmic level of phospho-IκBα and the nuclear levels of NF-κB subunits and c-Jun (Figure 4C). Moreover, KPS-A inhibited the phosphorylation of ERK1/2 and Akt, but not p38 MAPK or JNK, in PMA-treated MCF-7 cells (Figure 4D). Our data suggest that KPS-A inhibits the activation of NF-κB and AP-1 by blocking Akt and ERK1/2 activation in PMA-treated MCF-7 cells.

KPS-A inhibits memebrane localization of PMA-induced PKCδ

To determine whether PMA causes the activation of any PKC isotypes in MCF-7 cells, we analyzed the levels of PKCα, PKCβ1 and PKCδ in cytosol and membrane fractions. PMA stimulated translocation from the cytosol to the membrane of PKCδ only, after 10 min of stimulation, although PKCα and PKCβ1 isotypes were also expressed in MCF-7 cells (Figure 5A). Furthermore, to confirm whether PKCδ, but not PKCα and PKCβ1, is involved in PMA-induced MMP-9 activation and cell invasion, MCF-7 cells were exposed to PMA together with PKC inhibitors. PMA-induced MMP-9 secretion and cell invasion were blocked by a specific PKCδ inhibitor rottlerin and by a broad inhibitor of PKC GF109203X, but not by a specific PKCα and PKCβ1 inhibitor Gö6976 (Figure 5B). Interestingly, rottlerin and GF109203X inhibited PMA-induced phosphorylation of ERK1/2 (Figure 5C) and DNA-binding activity of AP-1, not NK-κB (Figure 5D). PMA-induced membrane localization of PKCδ was blocked by pretreatment with KPS-A for 2 h (Figure 5E). These results indicate that PMA mainly stimulates MMP-9-mediated cell invasion through PKCδ-triggered ERK/AP-1 activation in MCF-7 cells, and KPS-A suppresses PMA-induced PKCδ activation.

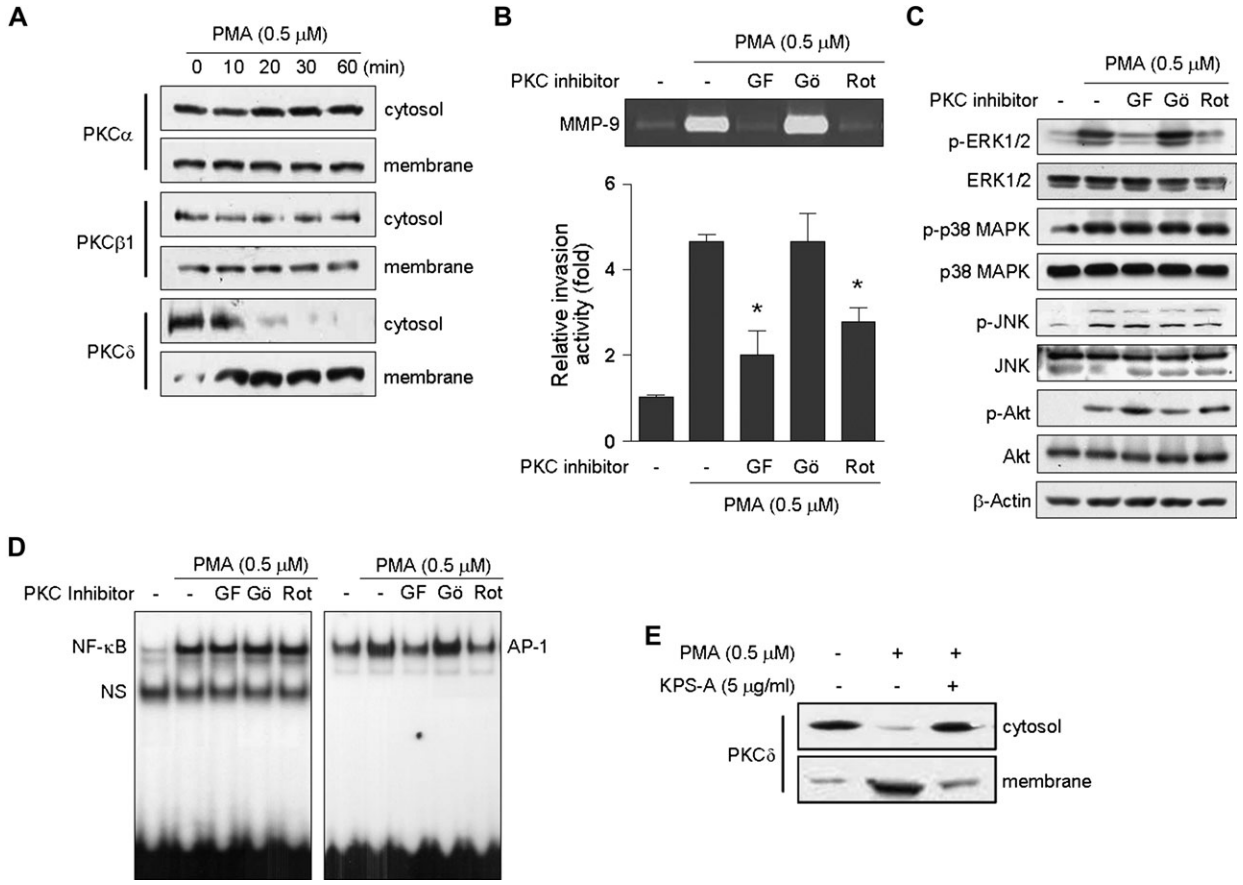

Fig. 5. KPS-A inhibits PMA-induced PKCδ activation. (**A**) MCF-7 cells were treated with PMA, and the levels of PKC isotypes were analyzed in cytosol and membrane fractions by western blotting. (**B**, top) Cells were pretreated for 1 h with GF109203X (GF, 2 μM), Gö6976 (Gö, 2 μM) or rottlerin (Rot, 0.5 μM) followed by PMA stimulation for 24 h. The MMP-9 activity in the conditioned media was analyzed by gelatin zymography; bottom, [^{3}H]thymidine-labeled MCF-7 cells were incubated in serum-free media with PKC inhibitors and PMA for 48 h. Invasion activities were measured using the Matrigel-coated transwell assay. Data represent the mean ± SE of three independent experiments; $^*P < 0.01$ versus PMA alone-treated cells. (**C**) Cells were stimulated with PMA for 20 min after pretreatment with PKC inhibitors for 1 h, and the levels of total/phospho MAPKs and Akt were determined by western blotting. (**D**) Cells were pretreated with specific PKC inhibitors for 1 h and then stimulated with PMA for 1 and 2 h. Electrophoretic mobility shift assay was performed as described in Materials and Methods; NS, non-specific. (**E**) Cells were pretreated with KPS-A for 2 h and then stimulated with PMA for 15 min. Western blot analysis was performed to detect the PKCδ level in cytosol and membrane fractions.

KPS-A inhibits breast cancer growth and MMP-9 expression in athymic nude mice

To verify *in vitro* anti-invasive activity of KPS-A, we investigated its inhibitory effect on tumor growth and MMP-9 expression in mice with MCF-7 breast cancer xenografts. Tumor volume increased markedly for 23 days in mice inoculated with MCF-7 cells in the presence of 17β-estradiol pellets, but orally administered KPS-A resulted in a significant dose-related inhibition of tumor growth (Figure 6A). Furthermore, immunohistochemical analysis indicated that while the expression of PCNA, MMP-9, TIMP-1 and PKCδ in the tumors was noticeably higher in MCF-7 cell-injected mice, the expression of these proteins was inhibited by orally administered KPS-A (Figure 6B). Therefore, KPS-A can suppress MMP-9-mediated invasion of breast cancer in mice.

Discussion

The current study was designed to estimate the anti-invasive potential of KPS-A and to explore the molecular mechanisms underlying its activity. We first evaluated the inhibitory effect of KPS-A on PMA-induced invasion in MCF-7 cells. PMA is a well-known inflammatory stimulus and tumor promoter that activates almost all PKC isozymes by direct binding. It causes dramatic PKC-mediated induction of invasiveness in ER-positive MCF-7 human breast cancer cells, which are usually weakly invasive (31). Our data show that KPS-A inhibited PMA-induced cell proliferation and invasion. As described in previous studies (16,26,32), treatment with PMA ranging from 10 nM to 1 μM stimulated MMP-9 secretion in a dose-related manner (data not shown). We confirmed that PMA-induced cell invasion was blocked in the presence of a primary antibody of MMP-9, evidence that MMP-9 plays a central role in the PMA-induced invasion of MCF-7 cells. In PMA-treated MCF-7 cells, KPS-A suppressed the increased expression and secretion of MMP-9. In addition, PMA-induced TIMP-1 expression was inhibited by KPS-A. Although TIMPs were thought to inhibit tumor growth and MMP-induced proteolysis of the surrounding matrix, a growing body of evidence suggests that TIMP-1 is a multifunctional protein that inhibits apoptosis in breast epithelial cells and breast carcinoma cells and therefore may promote tumor growth and development (33,34).

PMA increases the invasiveness of various types of cancer cells by activating MMP-9 via transcription factors and the PKC, MAPKs and PI3K/Akt pathways (20,25,31). However, the distinct mechanisms regulating PMA-induced MMP-9 expression in different cell types are not defined clearly. To gain a comprehensive understanding of the PMA-induced signaling cascade underlying MMP-9 expression in MCF-7 human breast cancer cells, we assessed the effects of specific inhibitors of three MAPKs and PI3K on PMA-induced invasion, MMP-9 activity in the conditioned medium and DNA binding of

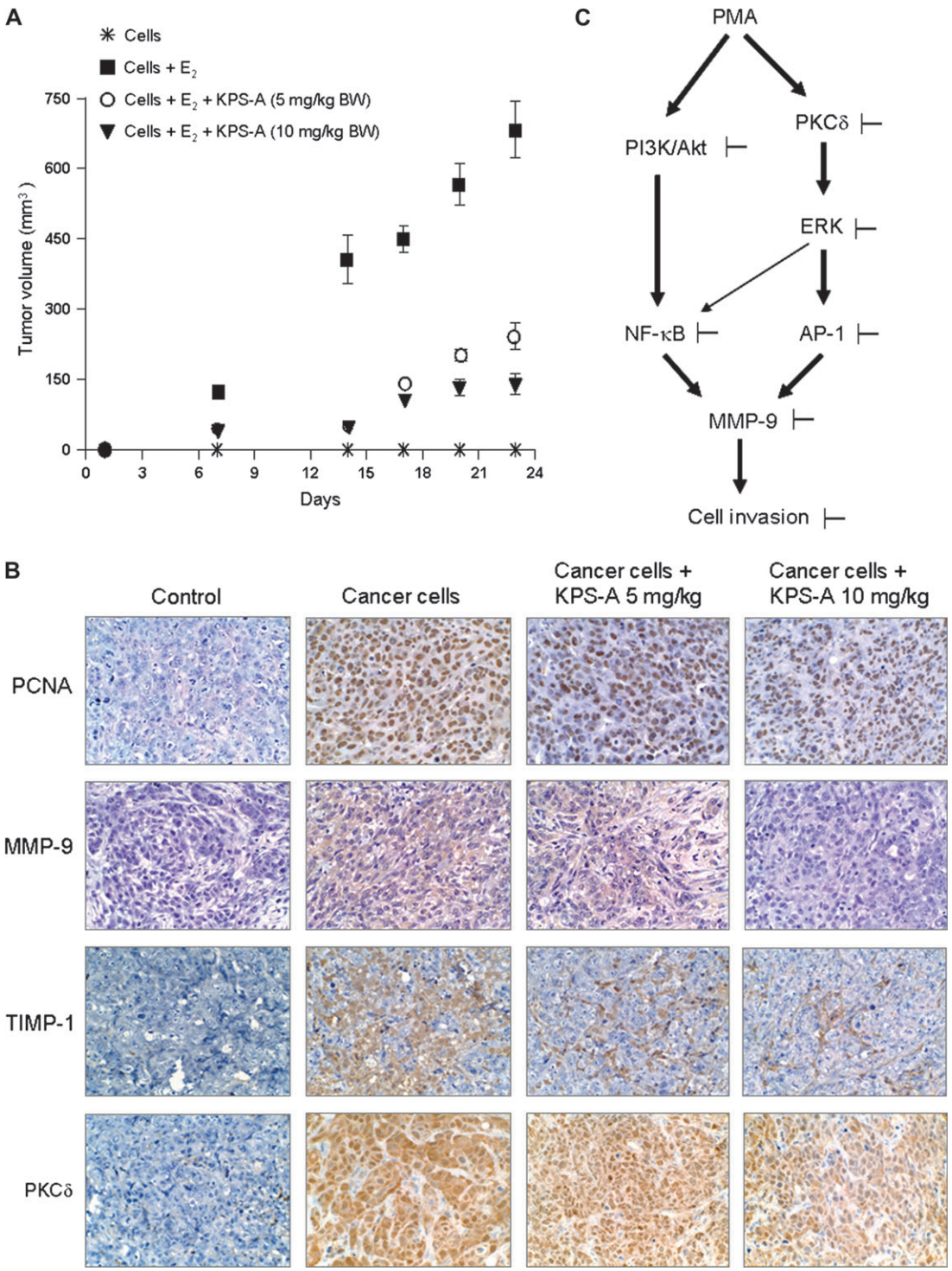

Fig. 6. KPS-A inhibits the growth and invasiveness of MCF-7 breast cancer in nude mice. (**A**) MCF-7 cells were single injected into the right flank of each female Balb/C nude mouse with a 17β-estradiol (E_2) pellet ($n = 5$). Oral administration of KPS-A was carried out three times a week for 23 days and tumor volume was calculated. (**B**) Immunohistochemical analysis for PCNA, MMP-9, TIMP-1 and PKCδ was performed on tumor tissue sections from cancer cell alone-injected mice and cancer cell-injected mice with oral dosing of KPS-A [5 and 10 mg/kg body wt (BW)], using specific antibodies. Original magnification was ×200. (**C**) Molecular mechanisms underlying anti-invasive activity of KPS-A in PMA-treated MCF-7 cells.

transcription factors. PMA-induced invasion was more significantly inhibited by treatment with an ERK1/2 inhibitor (PD98059) or a PI3K/Akt inhibitor (LY294002), than it was with a p38 MAPK inhibitor (SB203580) or a JNK inhibitor (SP600125). PMA-induced MMP-9 secretion and DNA binding of NF-κB was inhibited to some extent by all of the MAPK inhibitors and was completely inhibited by the PI3K/Akt inhibitors, whereas DNA binding of AP-1 was abolished only by an ERK1/2 inhibitor. These results indicate that cell invasion and MMP-9 expression is mainly regulated by NF-κB activation via PI3K/Akt and by AP-1 activation via ERK1/2, although p38 MAPK and JNK may also partially contribute to PMA-induced cell invasion and MMP-9 expression by activating NF-κB.

We found that KPS-A decreased PMA-induced transcriptional and DNA-binding activity of NF-κB and AP-1. KPS-A inhibited the level of phosphorylated IκBα in the cytoplasm and the translocation of NF-κB subunits p50 and p65 to the nucleus in PMA-treated MCF-7 cells. KPS-A also suppressed PMA-induced expression of c-Jun, rather than c-Fos, both of which are members of the AP-1. In our further study to test the effect of KPS-A on MAPKs and Akt activation in PMA-treated MCF-7 cells, KPS-A suppressed PMA-induced phosphorylation of ERK and Akt, key pathways in PMA-induced cell invasion via MMP-9 expression. These results demonstrate that KPS-A reduces MMP-9 expression by blocking NF-κB activation via PI3K/Akt as well as AP-1 activation via ERK1/2 and consequently inhibits MMP-9-mediated cell invasion in MCF-7 human breast cancer cells.

Activation of PKC by PMA involves the translocation of PKC isoforms to the plasma membrane, causing proliferation, differentiation, malignant transformation and cell death in cancer cells. PMA activates classical (α, βI, βII and γ) and novel (δ, ϵ, η and θ) PKCs by binding to the C1 domain of these isoforms (35). PKCα leads to PMA-stimulated growth of MCF-7 cells through ERK and JNK signaling (36), and impairment of PKCα potentiates heregulin-induced apoptosis in SKBr3 breast cancer cells (37). However, recent studies demonstrated that PKCδ, not PKCα, plays a critical role in MMP-9 induction in MCF-7 cells. PKCδ mediates PMA-induced MMP-9 secretion through the Ras/Raf/MEK/ERK pathway (6) and platelet-induced invasion and MMP-9 secretion (38). PKCδ protects against tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in breast cancer cells (39). Therefore, PKC isoforms are promising targets for the prevention and treatment of breast cancer. In this study, PMA stimulation resulted in the translocation of PKCδ from the cytosol to the cell membrane, whereas translocation of PKCα and PKCβ1 was not observed. Treatment with a non-cytotoxic dose of a PKCδ inhibitor (rottlerin) and a broad PKC inhibitor (GF109203X), but not a PKCα and PKCβ1 inhibitor (Gö6976), caused marked inhibition in PMA-induced activation of ERK1/2 and AP-1, as well as PMA-induced MMP-9 secretion and cell invasion. These data indicate that PMA-activated PKCδ mediates MMP-9 expression and cell invasion via ERK1/2 and AP-1. As expected, KPS-A reduced PMA-induced membrane localization of PKCδ.

To confirm *in vitro* anti-invasive activity of KPS-A, we estimated its inhibitory effect on tumor growth and MMP-9 expression in MCF-7 breast cancer xenografts of mice. 17β-Estradiol pellets were implanted into mice because tumor growth was not observed in the absence of 17β-estradiol pellets (data not shown). Like PMA, 17β-estradiol activates PKCδ by direct binding in ER-positive breast cancer cells (40–43). Oral administration of KPS-A led to a substantial inhibition of tumor growth in a dose-related manner. PCNA, MMP-9, TIMP-1 and PKCδ expression was inhibited in the tumor tissues of mice orally administered with KPS-A. These results indicate that KPS-A suppresses the breast cancer growth and MMP-9-mediated invasiveness in mice.

In conclusion, KPS-A inhibited PMA-induced invasion by reducing MMP-9 activation mainly through the PI3K/Akt/NF-κB and PKCδ/ERK/AP-1 pathways in MCF-7 human breast cancer cells (Figure 6C). Oral administration of KPS-A suppressed tumor growth and MMP-9-mediated invasiveness in mice implanted with MCF-7 cells in the presence of 17β-estradiol. It is possible that targeting the signaling molecules that regulate MMPs expression might be a more effective means of therapeutically inhibiting MMPs. Therefore, KPS-A is a promising anti-invasive agent; in addition to reducing MMP-9, it primarily targets the signaling molecules PKCδ and PI3K/Akt that regulate MMP-9 and has the added advantage of oral dosing.

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