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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Advance Access publication May 6, 2009

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 induction of MMP-9 is particularly important for the invasiveness of breast cancers. We investigated the inhibitory effect of kalopanaxsaponin A (KPS-A; 3-O-β-D-l-rhamnopyranosyl-[1→2]-α-L-arabinopyranosyl]-hederagenin) is an oleanane triterpene saponin 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-jB and AP-1. These authors contributed equally to this work.

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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; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NF-jB, nuclear factor-jB; PCNA, proliferating cell nuclear antigen; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TIMP, tissue inhibitor of metalloproteinase.
protein kinase C (PKC)6-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 (DMEM/F12 (DMEM/F12: 1:1), fetal bovine serum (FBS), antibiotic–antimycotic (10,000 IU/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, G66976, rottlerin and PMA were purchased from Calbiochem (La Jolla, CA). The following antibodies were purchased from their respective sources: p38 and phosphop38 MAPKs (New England Biolabs, Beverly, MA); total/phospho form of Akt, ERK1/2, c-jun N-terminal kinase (JNK) and IκBα, PKCα, β1, 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% CO2.

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. (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) [3H]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, **C3/P < 0.01 versus PMA alone-treated cells. Data represent the mean ± SE of three independent experiments.
KPS-A inhibits PMA-induced invasion in MCF-7 cells

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

Results

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

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 a primary antibody significantly inhibited PMA-induced invasion cell (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.

![Fig. 2. KPS-A inhibits PMA-induced MMP-9 activation and TIMP-1 expression in MCF-7 cells. (A)](http://carcin.oxfordjournals.org/)

![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)](http://carcin.oxfordjournals.org/)
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 IkBα 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-IkBα 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 membrane 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 NF-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.
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...
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 the 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.

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 17b-estradiol (E2) 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.
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 IkBα 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, which is one of the 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. PKC activates classical (α, β, γ and δ) and novel (ε, η and θ) PKCs by binding to the C1 domain of these isoforms (35). PKCz leads to PMA-stimulated growth of MCF-7 cells through ERK and JNK signaling (36), and impairment of PKCz potentiates heregulin-induced apoptosis in SKBr3 breast cancer cells (37). However, recent studies demonstrated that PKCδ, not PKCz, 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 PKCz 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 PKC/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.

Funding

Korea Research Foundation, Korean Government (KRF-2005-005-J05902); BioGreen 21 Program, Rural Development Administration, Republic of Korea (20050401-034-695-183-02-00).

Acknowledgements

Conflict of Interest Statement: None declared.

References


