Abstract. Cell invasion is required for neoplastic metastasis. Matrix metalloproteinase-9 (MMP-9), which degrades the extracellular matrix, is a major component in the process of cancer cell invasion. Sulfuretin is one of the major flavonoids isolated from *Rhus verniciflua*. Sulfuretin has been used to reduce oxidative stress, platelet aggregation, the inflammatory response and mutagenesis. However, the effect of sulfuretin on breast cancer metastasis is unknown. In this study, we investigated the inhibitory effect of sulfuretin on 12-0-tetradecanoylphorbol-13-acetate (TPA)-induced MMP-9 expression and cell invasion in MCF-7 cells. Sulfuretin inhibited TPA-induced transcriptional activation of nuclear factor-κB (NF-κB). We demonstrated that sulfuretin mediated the inhibition of TPA-induced MMP-9 expression and that cell invasion in MCF-7 cells involved suppression of the NF-κB pathway. Therefore, inhibiting MMP-9 expression by sulfuretin may have therapeutic potential for controlling breast cancer invasiveness.

Introduction

Breast cancer is one of the leading causes of cancer-related death in women (1). Despite successful treatment of the primary malignancy, relapse and subsequent metastatic spread can still occur at distant sites in the body through the bloodstream or lymphatic channels, including bone, lung, liver, kidney, thyroid and brain (2). Invasion and metastasis are fundamental processes and are the major causes of morbidity and mortality in patients with breast cancer. These processes require degradation of the extracellular matrix (ECM), which provides biochemical and mechanical barriers to cancer cell movement (3). The ECM consists of type IV collagen, laminin, heparan sulfate proteoglycan, nidogen and fibronectin (4). Degradation of the ECM requires extracellular proteinases, of which the matrix metalloproteinases (MMPs) play a critical role in breast cancer. Among the MMP family, gelatinases A (72 kDa gelatinase, type IV collagenase, MMP-2) and B (92 kDa gelatinase, type IV collagenase, MMP-9) play critical roles in ECM degradation and cell migration, leading to tumor cell invasion in breast cancer (4,5). MMP-9 is a key enzyme for degrading type IV collagen, which is a major component of the basement membrane. Elevated MMP-9 levels are functionally linked to elevated metastatic potential in many types of tumors, including brain (6), prostate (7), bladder (8), and breast (9,10). A variety of stimuli, including growth factors (e.g., fibroblast growth factor-2, epidermal growth factor and hepatocyte growth factor), cytokines (e.g., tumor necrosis factor-α), oncogenes (e.g., Ras) and 12-O-tetradecanoylphorbol-13-acetate (TPA) are involved in metastasis (11-14). Among these stimulators, TPA is a well-known selective activator of protein kinase C (11) and stimulates MMP-9 synthesis and secretion during MCF-7 cell invasion (9,10). Consequently, inhibiting MMP-9 expression and/or its upstream regulatory pathways is critical for treating malignant tumors, including breast carcinoma. NF-κB and...
AP-1 are transcription factors important in the regulation of MMP-9, as the MMP-9 gene promoter contains binding sites for both factors (15). Studies have shown that the mitogen-activated protein kinase (MAPK) signaling pathway is important for AP-1 activation, and that NF-κB activation requires IκB kinase, ERK, JNK and p38 MAPK, depending on the cell type (14,16-18).

Sulfuretin is a major flavonoid isolated from the heartwood of Rhus verniciflua Stokes (RVS), which has been used to reduce oxidative stress (19), platelet aggregation (20), the inflammatory response (21) and mutagenesis (22). A recent study found that sulfuretin inhibits cytokine-induced β-cell damage and prevents streptozotocin-induced diabetes by suppressing the NF-κB pathway (23). Therefore, it was hypothesized that sulfuretin may have anticancer properties that inhibit cell invasion. In the present study, sulfuretin was evaluated for its potential activity against TPA-induced cell invasion and MMP-9 expression in MCF-7 cells, and the related molecular mechanisms were investigated. Our results demonstrated that sulfuretin suppresses TPA-induced MMP-9 expression by blocking NF-κB activation but not AP-1 activation.

Materials and methods

Cells and materials. MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics at 37˚C in a 5% CO₂ incubator. Sulfuretin was purchased from Symrise GmbH & Co. (Holzminden, Germany). TPA, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and anti-β-actin antibodies were obtained from Sigma (St. Louis, MO, USA). Antibodies for p38, p-p38, JNK, p-JNK, ERK and p-ERK were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies for MMP-9, p50, p65, proliferating cell nuclear antigen (PCNA), and horseradish peroxidase (HRP)-conjugated IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for p38, p-p38, JNK, p-p38, JNK, NF-κB p65, and β-actin antibodies were obtained from BioRad (Richmond, CA, USA).

Determination of cell viability. The effect of sulfuretin on MCF-7 cell viability was determined using an established MTT assay. Briefly, 3x10⁴ cells were seeded in wells and incubated at 37°C for 24 h to allow attachment. The attached cells were untreated or treated with 1, 5, 10, 30, and 50 μM sulfuretin for 24 h at 37°C. The cells were washed with PBS prior to adding MTT (0.5 mg/ml PBS) and incubated at 37°C for 30 min. Formazan crystals were dissolved with dimethyl sulfoxide (100 μl/well) and detected at 570 nm using a Model 3550 microplate reader (Bio-Rad, Richmond, CA, USA).

Western blot analysis. MCF-7 cells (5x10⁴) were pretreated with 10 and 30 μM sulfuretin for 1 h and then incubated with TPA for 24 h at 37°C. Cells were lysed with ice-cold M-PER® Mammalian Protein Extraction reagent (Pierce Biotechnology, Rockford, IL, USA), and the protein concentration in the lysate was determined using the Bradford method (24). Samples (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 10% acrylamide and transferred to Hybond® nylon membranes (GE Healthcare Life Sciences, Buckinghamshire, UK) using a western blot apparatus. Each membrane was blocked for 2 h with 2% bovine serum albumin or 5% skim milk and then incubated overnight at 4°C with 1 μg/ml of a 1:2000 dilution of primary antibody. HRP-conjugated IgG (1:2000 dilution) was used as the secondary antibody. Protein expression levels were determined by signal analysis using an image analyzer (Fuji-Film, Tokyo, Japan).

Gelatin zymography assay. Conditioned media were collected after a 24 h stimulation, mixed with non-reducing sample buffer, and electrophoresed on a polyacrylamide gel containing 0.1% (w/v) gelatin. The gel was washed at room temperature for 30 min with 2.5% Triton X-100 solution, and subsequently incubated at 37°C for 16 h in 5 mM CaCl₂, 0.02% Brij and 50 mM Tris-HCl (pH 7.5). The gel was stained for 30 min with 0.25% (w/v) Coomassie Brilliant Blue in 40% (v/v) methanol/7% (v/v) acetic acid and photographed using an image analyzer (Fuji-Film). Proteolysis was imaged as the white zone in a dark blue field. Densitometric analysis was performed using Multi Gauge Image Analysis software (Fuji-Film).

Quantitative real-time polymerase chain reaction (PCR). Total RNA was extracted from cells using a FastPure™ RNA kit (Takara, Shiga, Japan). The RNA concentration and purity were determined by absorbance at 260/280 nm. cDNA was synthesized from 1 μg total RNA using a PrimeScript™ RT Reagent kit (Takara). MMP-9 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression was determined by real-time PCR using the ABI PRISM 7900 Sequence Detection system and SYBR® Green (Applied Biosystems, Foster City, CA, USA). The primers were: MMP-9 (NM 004994) sense, CCTGGAGACCTGAGAACCATACT; antisense, CCACCCGAGTGTACCACTAGC and GAPDH (NM 002046) sense, ATGGAATCCCTCACCACCTT; antisense, CGCCCCCAC TTGATTTTGG. All results were normalized to the GAPDH housekeeping gene to control for variation in mRNA concentrations. Relative quantification was performed using the comparative ∆∆Ct method according to the manufacturer's instructions.

Preparation of the nuclear extract. MCF-7 cells (2x10⁵) were treated with sulfuretin in the presence or absence of TPA for 4 h. Cells were immediately washed twice, scraped into 1.5 ml of ice-cold PBS (pH 7.5), and pelleted at 1,500 x g for 3 min. Cytoplasmic and nuclear extracts were prepared from the cells using the NE-PER® Nuclear and Cytoplasmic Extraction reagent (Pierce Biotechnology).

Electrophoretic mobility shift assay (EMSA). Activation of NF-κB and AP-1 was assessed with a gel mobility shift assay using nuclear extracts. An oligonucleotide containing the κ-chain (κB, 5'-AGTTGAGGGGACTTTCCCAGGC-3') or AP-1 (5'-CGCTTGATGAGTCAGCCGGAA-3') binding sites was synthesized and used as a probe for the gel retardation assay. The two complementary strands were annealed and labeled with [α-32P]dCTP. Labeled oligonucleotides (10,000 cpm),...
10 µg of nuclear extract and binding buffer (10 mM Tris-HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng poly(dI·dC) and 1 mM dithiothreitol) were then incubated for 30 min at room temperature in a final volume of 20 µl. The reaction mixtures were analyzed by electrophoresis on 4% polyacrylamide gels in 0.5X Tris-borate buffer. The gels were dried and examined by autoradiography. Specific binding was controlled by competition with a 50-fold excess of cold κB and AP-1 oligonucleotide.

**Invasion assay.** The invasion assay was carried out in 24-well chambers (8-µm pore size) coated with 20 µl Matrigel-diluted DMEM. The Matrigel coating was rehydrated in 0.5 ml DMEM for 30 min immediately before the experiments. Cells (2x10^5) were added to the upper chamber with chemoattractant in the bottom well. Conditioned medium (0.5 ml) was added to the lower compartment of the invasion chamber, and the chambers were incubated for 24 h. After the incubation, cells on the upper side of the chamber were removed using cotton swabs, and cells that had migrated were fixed and stained with toluidine blue solution. Invading cells were counted in five random areas of the membrane using a light microscope. Data are the means ± standard errors from three individual experiments performed in triplicate.

**Statistical analysis.** The statistical analysis was performed using analysis of variance and Duncan’s test. Differences with a p<0.05 were considered statistically significant.

**Results**

**Sulfuretin suppresses TPA-induced MMP-9 activation in MCF-7 cells.** The chemical structure of sulfuretin is shown in Fig. 1A. To verify the effect of sulfuretin on cell viability, cells were seeded in 96-well culture plates at a density of 1x10^5 cells/well. The cytotoxic effect of sulfuretin on MCF-7 cells was analyzed using the MTT assay. Treatment of MCF-7 cells with the indicated concentrations of sulfuretin for 24 h did not result in any significant change in cell viability (Fig. 1B). Therefore, we performed the subsequent experiments using the optimal non-toxic concentrations (10 and 30 µM) of sulfuretin. Western blot analysis, real-time PCR and zymography were performed in MCF-7 cell-containing samples to investigate the effect of sulfuretin on TPA-induced MMP-9 expression. The western blot analysis revealed that sulfuretin treatment of MCF-7 cells blocked upregulation of TPA-induced MMP-9 protein expression (Fig. 2A). Real-time PCR revealed a TPA-induced increase in the MMP-9 level in MCF-7 cells, and
that sulfuretin blocked TPA-induced MMP-9 upregulation in a dose-dependent manner (Fig. 2B). A zymography analysis was carried out to determine the effect of sulfuretin on TPA-induced MMP-9 secretion. The analysis demonstrated that treatment of MCF-7 cells with TPA increased MMP-9 secretion, while sulfuretin significantly diminished the TPA-induced MMP-9 secretion (Fig. 2C). These results were consistent with the potent sulfuretin-mediated inhibition of TPA-induced MMP-9 expression in MCF-7 cells.

**Sulfuretin inhibits TPA-induced NF-κB DNA binding activity but not the AP-1 and MAPK pathway.** To clarify the mechanism involved in the sulfuretin-mediated inhibition of MMP-9 expression, the effects of sulfuretin on TPA-induced activation of NF-κB and AP-1 were evaluated using EMSA. As shown in Fig. 3A and B, TPA substantially increased NF-κB and AP-1 binding activity. Pretreatment with sulfuretin inhibited TPA-stimulated NF-κB binding activity but not that of AP-1. These results were consistent with the view that sulfuretin specifically blocks NF-κB activation in MCF-7 cells. In the western blot analysis, TPA stimulated the phosphorylation of IKKαβ and IκBα in the 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, while c-Fos expression was only negligibly induced in the TPA-treated cells. Based on our results, the increased p-IKKαβ, p-IκBα and translocation of p65 and p50 as a result of TPA stimulation was significantly suppressed by sulfuretin treatment (Fig. 3D and E). We confirmed that TPA induced the phosphorylation of c-Jun, a major subunit of AP-1, and sulfuretin had no effect on c-Jun phosphorylation (Fig. 3E). The effect of sulfuretin on TPA-induced phosphorylation and activation of MAPKs was investigated to determine the inhibitory effect of sulfuretin on MAPK. Sulfuretin had no effect on MAPK phosphorylation 30 min after TPA treatment (Fig. 3C). These results suggest that the regulation of TPA-induced MMP-9 expression by sulfuretin does not involve the MAPK pathway.

**Effect of sulfuretin on TPA-induced MCF-7 cell invasion in vitro.** Upregulation of MMP-9 expression contributes to
the increased invasiveness of cancer cells (25,26). An *in vitro* invasion assay was used to investigate the inhibitory effects of sulfuretin on the invasive ability of MCF-7 cells. Treatment with TPA increased MCF-7 cell invasion when compared with that in untreated control cells, as determined by a Matrigel invasion assay. Incubating MCF-7 cells with TPA resulted in a 10-fold increase in cell invasiveness. However, treatment with sulfuretin diminished TPA-induced cell invasion by 65% (Fig. 4).

**Discussion**

Studies involved in the development of effective anti-invasive strategies have focused mainly on the utilization of natural bioactive agents in MCF-7 cells. In the present study, we isolated sulfuretin from the heartwood of *Rhus verniciflua* Stokes (RVS) and examined its effects on TPA-induced MMP-9 expression and cellular invasion in MCF-7 cells. RVS exerts a remarkable spectrum of biological activities affecting basic cellular functions, and the identification of sulfuretin as an active principle agent has been demonstrated (19,22,23,27-34). In previous reports, sulfuretin exerted anticancer and antiproliferative effects in various types of cells (28,30). Additionally and consistent with these results, our previous observations indicated that sulfuretin inhibits MMP expression and inflammatory responses by interfering with the transcriptional activation of NF-κB (23,27,35). However, no study has reported on the anti-invasive effects of sulfuretin in MCF-7 cells. Thus, we designed this study to estimate the anti-invasive potential of sulfuretin and to explore the molecular mechanisms underlying its activity.

Metastasis is the primary cause of breast cancer mortality. Tumor metastasis is a multistep process by which a subset of individual cancer cells disseminates from a primary tumor to distant secondary organs or tissues. This process involves cell proliferation, ECM degradation, cell migration and tumor growth at metastatic sites (10,36). Tumor cell invasion is an early step in the metastatic cascade, representing the beginning of the transition from the benign stage to malignancy. Tumor invasion is morphologically associated with a distorted edge of the primary tumor where individual or cohorts of tumor cells actively invade the ECM surrounding the primary tumor (37). MMP-9 has been regarded as a major critical molecule during tumor invasion and metastasis. MMP-9 activation is particularly associated with tumor progression and invasion, including that in mammary tumors (38). Inflammatory cytokines, growth factors and phorbol esters stimulate MMP-9 by activating different intracellular-signaling pathways in breast cancer cells (39-41). The inhibitory effect on MMP-9 expression is important for developing a therapeutic experimental model of tumor metastasis.

NF-κB and AP-1 are transcription factors important in regulating MMP-9, as the MMP-9 gene promoter contains NF-κB and AP-1 binding sites (15). AP-1, which belongs to the bZIP group of DNA-binding proteins, associates to form a variety of homodimers and heterodimers through a combination of signaling events, leading to increased activity of proteins that directly potentiate Jun and Fos family members or activate transcription factors that regulate c-jun and c-fos expression (42-45). NF-κB comprises a family of inducible transcription factors that regulate host inflammatory and immune responses (46). Diverse signal transduction cascades
mediate stimulation of the NF-κB pathway (46). NF-κB and AP-1 elements are centrally involved in TPA-mediated MMP-9 gene induction (36,47). Additionally, the MAPK signaling pathway is important for NF-κB activation, which requires IκB kinase depending on cell type (14,16-18,48). However, our results showed that sulfuretin inhibited activation of NF-κB but not MAPK or AP-1 in MCF-7 cells. This result suggests that sulfuretin inhibited TPA-induced MMP-9 expression through the NF-κB pathway.

These experiments confirmed that TPA-stimulated cell invasion was suppressed by sulfuretin. Data obtained from the Matrigel invasion assay showed that sulfuretin inhibited the TPA-induced invasive potential of MCF-7 cells (Fig. 4). These data suggest that the inhibition cell invasion caused by sulfuretin was correlated with inhibition of MMP-9 expression and the NF-κB signaling pathway.

In conclusion, our results demonstrated that sulfuretin is a potent inhibitor of TPA-induced MMP-9 expression, and that it strongly blocked the NF-κB signaling pathway in breast carcinoma cells. This is the first study to show that sulfuretin suppresses TPA-stimulated cancer cell invasion by inhibiting MMP-9 expression. We also detailed the molecular mechanisms of the NF-κB pathway in breast cancer cells responsible for this inhibitory effect. Thus, sulfuretin may be a potential candidate for preventing breast tumor invasion and metastasis in vivo.

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References