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Antitumor effects of the flavone chalcone: inhibition of invasion and migration through the FAK/JNK signaling pathway in human gastric adenocarcinoma AGS cells

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Abstract Chalcones (benzylideneacetophenone) are cancer-preventive food components found in a human diet rich in fruits and vegetables. In this study, we first report the chemopreventive effect of chalcone in human gastric adenocarcinoma cell lines: AGS. The results showed that chalcone could inhibit the abilities of the adhesion, invasion, and migration by cell–matrix adhesion assay, Boyden chamber invasion/migration assay, and wound-healing assay. Molecular data showed that the effect of chalcone in AGS cells might be mediated via sustained inactivation of the phosphorylation of focal adhesion kinase (FAK) and c-Jun N-terminal kinase 1 and 2 (JNK1/2) signal involved in the downregulation of the expressions of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9). Next, chalcone-treated AGS cells showed tremendous decrease in the phosphorylation and degradation of inhibitor of kappaBα (IκBα), the nuclear level of NF-κB, and the binding ability of NF-κB to NF-κB response element. Furthermore, treating FAK small interfering RNA (FAK siRNA) and specific inhibitor for JNK (SP600125) to AGS cells could reduce the phosphorylation of JNK1/2 and the activity of MMP-2 and MMP-9. Our results revealed that chalcone significantly inhibited the metastatic ability of AGS cells by reducing MMP-2 and MMP-9 expressions concomitantly with a marked reduction on cell invasion and migration through suppressing and JNK signaling pathways. We suggest that chalcone may offer the application in clinical medicine.

Keywords Chalcone · Invasion · Migration · FAK · JNK1/2 · MMP-2 · MMP-9

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Introduction

Gastric cancer is a kind of gastrointestinal tract cancer which is the leading cause of cancer-related mortality in the world. Moreover, approximately 90 % of gastric cancers are adenocarcinomas [1]. Studies have shown that a high intake of smoked, salted, nitrated foods, and carbohydrates, with a low intake of vegetables, fruits, and milk, are linked to cancer incidence. These diets have been shown to significantly increase the risk for gastric cancer [2]. Despite recent advances in gastric cancer treatment, there is still no effective cure for patients with advanced stages of this disease [1]. Increased intake of vegetables and fruits has been associated in part with reduced risks of certain cancers [3]. In recent years, attention has been focused on the anticancer properties of edible plants, which play an important role in the prevention of disease. Chalcones (benzylideneacetophenone) are the intermediate precursors for all flavonoids' synthesis in the phenylpropanoid pathway in plants and are unique in the flavonoid family [4]. Naturally occurring chalcones are found mostly in their hydroxylated forms, and many reports have documented their biologically active properties [5, 6]. For example, chalcone has shown anticancer activity via inhibition of cell proliferation, carcinogenesis, and metastasis [7, 8].

FAK is a non-receptor tyrosine kinase that is a key element in growth factor and integrin signaling. FAK plays

a central role in cell spreading, survival, differentiation, proliferation, metastasis, and tissue remodeling [9–11]. Tumor metastasis occurs by a series of steps and various cytophysiological changes, including damaged intercellular interaction, increased cancer cells and extracellular matrix (ECM) interaction, damaged ECM components, and vessel formation. Excess ECM degradation is one of the hallmarks of tumor invasion and migration and is regulated by extremely complicated mechanisms [12]. PI3K/Akt signal transduction pathway was found to be necessary to mediate the proliferation and metastasis of human non-small cell lung cancer (NSCLC) and is closely associated with the development and progress of various tumors [13]. Akt kinase, an important component of post-survival signaling pathways, is activated via PI3K signaling pathway. [14]. The mitogen-activated protein kinase superfamily members (MAPK) are associated with increased scattering/motility, invasion, proliferation, survival, and morphogenesis [15, 16]. Three major mammalian MAPKs include extracellular signal-regulated kinase 1 and 2 (ERK1/2), JNK1/2, and p38 MAPK. The diverse MAPK members are activated in response to different extracellular stimuli and have distinct downstream targets, thus serving different roles in cellular responses. ERK1/2, JNK1/2, and p38 MAPK play a central role in regulating the expressions of matrix metalloproteinases (MMPs) [17–19]. MMPs, a family of zinc-dependent neutral endopeptidases, have been associated with tumor cell invasion and migration because of their ability to hydrolyze various ECMs. Previous papers have demonstrated that the mechanism underlying the anti-invasive and anti-migrated effects was related to the inhibition of activity and expression of MMPs-2/9, which were involved in the MAPK signaling pathway. Induction of MMP-2 and MMP-9 synthesis involves multiple signaling cascades, especially for the MAPK pathway [20]. The promoter of MMPs is highly conserved and shown to contain multiple functional elements, including NF-κB, AP-1, and EST elements [21, 22].

Many NF-κB activation pathways have been revealed, and all of them rely upon sequentially activated kinase cascades [23]. NF-κB is a ubiquitously expressed transcription factor that regulates many cytokines and various genes. It is involved in immune response, inflammation, and malignant transformation. The active NF-κB complex consists of the p50, p52, p65 (Rel A), Rel B, and c-Rel. The p50 subunit binds DNA, whereas the p65 subunit is responsible for the interaction of NF-κB with its inhibitor, IκB. In most cell types, the p50/p65 heterodimer is located within the cytoplasm complexed to IκB. The complex prevents nuclear translocation and activity of NF-κB [24, 25]. The AP-1 transcription factor is involved in cell proliferation, differentiation, apoptosis, and neoplastic transformation. AP-1 consists of two different gene families: Jun and Fos. The AP-1 is either a homodimer of Jun proteins or a heterodimer of Jun and Fos proteins [26]. In this study, we employed human gastric adenocarcinoma AGS cells to assess the molecular mechanism responsible for the antimetastatic effect of chalcone. We found that chalcone suppresses invasion and migration of AGS cells through inactivating FAK/JNK signaling pathway.

Materials and methods

Reagents and antibodies

Chalcone (Purity ≥95 %) was purchased from Extrasynthese (Genay, France.); DMSO, Tris–HCl, EDTA, SDS, phenylmethylsulfonyl fluoride, bovine serum albumin (BSA), gelatin, crystal violet, leupeptin, Nonidet P-40, deoxycholic acid, and sodium orthovanadate were purchased from Sigma-Aldrich (St. Louis, MO, USA); the protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Dulbecco's phosphate buffer solution (PBS), trypsin–EDTA, and RPMI-1640 medium were purchased from Life Technologies, Inc. (Gibco/BRL, Gaithersburg, MD). Antibody against IκBα, FAK, and PKB/Akt, proteins, and phosphorylated proteins were purchased from Cell Signaling Tech. (Beverly, MA, USA). PI3K, MMP-2, MMP-9, NF-κB (p65), β-actin, and C23 antibodies were purchased from BD Transduction Laboratories (San Jose, CA, USA). The enhanced chemiluminescence (ECL) kit was purchased from Amersham GE Healthcare UK Ltd. (Buckinghamshire, England).

Cell culture

AGS, a human gastric adenocarcinoma cell line, was obtained from BCRC (Food Industry Research and Development Institute in Hsin-Chu, Taiwan). Cells were cultured in a RPMI-1640 medium supplemented with 10 % fetal calf serum, 2 mM L-glutamine, 100 U/ml of penicillin, 100 mg/ml streptomycin-mixed antibiotics, and 1 mM sodium pyruvate. All cell cultures were maintained at 37 °C in a humidified atmosphere of 5 % CO_2-95 % air. The culture medium was renewed every 2–3 days. Adherent cells were detached by incubation with trypsin. For chalcone treatment, the stock solution of chalcone was dissolved in dimethyl sulfoxide (DMSO) and sterilized by filtration through 0.2 μm disk filters. Appropriate amounts of stock solution (1 mg/ml in DMSO) of chalcone were added to the cultured medium to achieve the indicated concentrations.

Cell viability assay

To measure the effect of chalcone on cell viability, the AGS cells (4×10^4 cells/ml) were seeded into a 24-well plate. Cells were treated with or without chalcone under various concentrations for various periods of time (24 and 48 h). At the end of the assay period, cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay, as described previously [27]. Each concentration was repeated three times. After the exposure period, the medium was removed and followed by washing the cells with PBS. Then, the medium was changed and incubated with MTT solution (5 mg/ml)/well for 4 h. The medium was removed, and formazan was solubilized in isopropanol and measured spectrophotometrically at 563 nm. The percentage of viable cells was estimated by comparing with the untreated control cells.

Cell–matrix adhesion assay

After pretreatment with chalcone (0, 0.5, 1, and 2 μg/ml) for 24 h, cells were seeded at a density of 1×10^5 cells/ml in a 24-well plate and coated with 500 μl type IV collagen (10 μg/ml); then, they were cultured for 30 min. Then, non-adherent cells were removed by PBS washes, and adherent cells were fixed in ethanol. After staining with 0.1 % crystal violet, fixed cells were lysed in 0.2 % Triton X-100 and measured spectrophotometrically at 550 nm.

Boyden chamber invasion and migration assay

The ability of AGS cells to pass through filters coated with matrigel (Collaborative Biomedical Products, Bedford, MA) was measured by Boyden chamber invasion assay [28]. Matrigel was diluted to 200 μg/ml with cold-filtered distilled water and applied to the top side of the 8-μm pore polycarbonate filter. Briefly, AGS cells were treated with various concentrations of chalcone. After 24 h, cells were detached by trypsin and resuspended in serum-free medium. Medium containing 10 % fetal bovine serum medium was applied to the lower chamber as chemoattractant, and then, cells were seeded on the upper chamber at a density of 2.5×10^4 cells/ml in 50 μl of serum-free medium. The chamber was incubated for 8 h at 37 °C. At the end of incubation, the cells in the upper surface of the membrane were carefully removed with a cotton swab. Cells invading across the matrigel to the lower surface of the membrane were fixed with methanol and stained with 5 % Giemsa solution. The invading cells on the lower surface of the membrane filter were counted with a light microscope. The data are presented as the average number of cells attached to the bottom surface from randomly chosen fields. Each experiment was carried out in triplicate. To measure the ability of AGS cells on migration, cells were seeded into a Boyden chamber with 8-μm pore polycarbonate filters which were not coated with matrigel. Migrating cells were treated with various concentrations of chalcone. The migration assay was measured as described in the invasion assay.

Wound-healing assay

To determine cell motility determination, AGS cells (1×10^5cells/ml) were seeded in 6-well tissue culture plate and grown to 80–90 % confluence. After aspirating the medium, the center of the cell monolayers was scraped with a sterile micropipette tip to create a denuded zone (gap) of constant width. Subsequently, cellular debris was washed with PBS, and the AGS cells were exposed to various concentrations of chalcone (0, 0.5, 1, and 2 μg/ml). The wound closure was monitored and photographed at 0, 6, 12, 24, and 48 h with an Olympus CKX-41 inverted microscope and an Olympus E-410 camera. To quantify the migrated cells, pictures of the initial wounded monolayers were compared with the corresponding pictures of cells at the end incubation. Artificial lines fitting the cutting edges were drawn on pictures of the original wounds and overlaid on the pictures of cultures after incubation. Migrated cells across the white lines were counted in six random fields from each triplicate treatment, and data are presented as mean ± SD.

Gelatin- and casein-plasminogen zymography

The activities of MMP-2 and MMP-9 in the conditioned medium were measured by gelatin zymography assay as described previously [29]. Samples were mixed with loading buffer and electrophoresed on 8 % SDS–polyacrylamide gel containing 0.1 % gelatin. Electrophoresis was performed at 140 and 110 V for 3 h. Gels were then washed twice in zymography washing buffer (2.5 % Triton X-100 in double-distilled H_2O) at room temperature to remove SDS, followed by incubation at 37 °C for 12–16 h in zymography reaction buffer (40 mM Tris–HCl, 10 mM $CaCl_2$, and 0.02 % NaN_3), stained with Coomassie blue R-250 (0.125 % Coomassie blue R-250, 0.1 % amino black, 50 % methanol, and 10 % acetic acid) for 1 h, and destained with destaining solution (20 % methanol, 10 % acetic acid, and 70 % double-distilled H_2O).

Visualization of u-PA activity was performed by casein-plasminogen Zymography. Briefly, 2 % casein and 20 μg/ml plasminogen were added to 8 % SDS-PAGE gel. Samples with a total protein of about 20 μg were then loaded onto the gels. The u-PA activity of cells treated or untreated with chalcone was measured as described in the gelatin Zymography section.

Western blotting analysis

The preparation of cytosolic and nuclear fractions of the cells was performed as described previously [30]. The Western blotting was performed as follows. The denatured samples (50 μg purified protein) were resolved on 10–12 % SDS-PAGE gels. The proteins were then transferred onto nitrocellulose membranes. Non-specific binding of the membranes was blocked with Tris-buffered saline (TBS) containing 1 % (w/v) nonfat dry milk and 0.1 % (v/v) Tween-20 (TBST) for more than 2 h. Membranes were washed with TBST three times for 10 min and incubated with an appropriate dilution of specific primary antibodies in TBST overnight at 4 °C. Subsequently, membranes were washed with TBST and incubated with appropriate secondary antibody (horseradish peroxidase-conjugated goat antimouse or antirabbit IgG) for 1 h. After washing the membrane three times for 10 min in TBST, the bands' detection was revealed by enhanced chemiluminescence using ECL Western blotting detection reagents and exposed ECL hyperfilm in FUJFILM Las-3000 mini (Tokyo, Japan). Then, proteins were quantitatively determined by densitometry using FUJFILM-Multi Gauge V3.0 software.

Small interfering RNA for FAK

We assessed the effect of inducing RNA interference on FAK using silencer siRNA. Control siRNA is a siRNA sequence that will not cause the specific degradation of any cellular message. AGS cells were transfected with 3.3 nmol of FAK siRNA. FAK siRNA is target-specific 19–25 nt siRNAs designed to knock down gene expression. After siRNA transfection, we determined phospho-JNK in the whole cell lysates by Western blotting assay and determined MMP-2, MMP-9 activity in the conditioned medium by gelatin zymography assay.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted using the total RNA Extraction Midiprep System (Viogene BioTek, Taiwan). Total RNA (2 μg) was transcribed to 20 μl cDNA with 1 μl deoxynucleotide triphosphate (dNTP; dNTP set consists of 2.5 mM aqueous solutions at pH 7.0 of each of dATP, dCTP, dGTP, and dTTP), 1 μl Oligo dT (10 pmol/ml), 1 μl RTase (200 U), 1 μl RNase inhibitor, and 5× reaction buffer. The appropriate primers (sense of MMP-2, 5′-GGC CCTGTCACTCCTGAGAT-3′, nt 1337–1356; antisense of MMP-2, 5′-GGCATCC AGGTTATCGGGGA-3′, nt 2026–2007; sense of MMP-9, 5′-CACTGTCCACCCCTCAGA GC-3′, nt 1305–1324; antisense of MMP-9, 5′-GCCACT TGTCGGCGATAAGG-3′, nt 1804–1785; sense of u-PA, 5′-TTGCGGCCATCTACAGGAG-3′, nt 654–672; antisense of u-PA 5′-ACTGGGGATCGTTATACATC-3′, nt 1205–1124; sense of β-actin, 5′-TCGTGCG TGACATT AAGGAG-3′ nt 250–269; antisense of β-actin, 5′-AGTA CTTGCGCTCAGGAG GA-3′, nt 639–620) were used for polymerase chain reaction (PCR) amplifications. PCR was performed with Platinum Taq polymerase (Invitrogen, San Diego, CA, USA) under the following conditions: 30 cycles of 94 °C for 1 min, 59 °C (MMP-2 and u-PA) or 60 °C (MMP-9 and β-actin) for 1 min, 72 °C for 1 min, followed by 10 min at 72 °C. PCR products were analyzed by agarose gel electrophoresis and visualized by treatment with ethidium bromide.

Electrophoretic mobility shift assay (EMSA)

The DNA-binding activities of NF-κB and AP-1 in nuclear extracts were assessed by EMSA [31] using the Lightshift kit from Pierce (Rockford, Illinois, USA) with biotin-labeled double-stranded AP-1 and NF-κB oligonucleotides. A five-microgram aliquot of nuclear proteins were mixed with either biotin-labeled NF-κB or AP-1 oligonucleotide probe for 15 min at room temperature or with oligonucleotides containing sense of NF-κB, 5′-AGTTGAGGGG ACTTTCCCAGGC-3′, antisense of NF-κB, 3′-TCAAC TCCCCTGAAA GGGTCCG-5′; sense of AP-1, 5′-CGCTT GATGACTCAGCCGGAA-3′, antisense of AP-1, 3′-GC GAACTACTGAGTCGGCCTT-5′. DNA probes were added to 10 μl binding reactions containing double-distilled H_2O, 5 μg nuclear proteins, 1 μl poly (dI-dC), 1 μl biotin-labeled double-stranded NF-κB oligonucleotides, and 2 μl of tenfold binding buffer into a microcentrifuge tube and were incubated for 15 min at room temperature. Specific competition binding assays were performed by adding 200-fold excess of unlabeled probe as a specific competitor. Following protein–DNA complexes' formation, samples were loaded on a 6 % nondenaturing polyacrylamide gel in 0.5× TBE buffer and then transferred to positively charged nitrocellulose membranes (Millipore, Bedford, MA, USA) and cross-linked in a Stratagene crosslinker. Gel shifts were visualized with streptavidin-horseradish peroxidase followed by chemiluminescent detection.

Statistical analysis

Data were expressed as mean ± standard deviation of three independent experiments. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences were established at $P \leq 0.05$.

Results

Cytotoxicity of chalcone to AGS cells

As described, chalcone (see its chemical structure in Fig. 1a) is a natural flavone with pleiotropic activities against cancer growth. In this study, we first examined the effect of chalcone on cell cytotoxicity in AGS cells. As shown in Fig. 1b, chalcone showed a dose- and time-dependent inhibitory effect on the cell viability of AGS cells. Compared to 0 μg/ml, after 24- and 48-h treatment with chalcone at a concentration between 0 and 2 μg/ml was not significantly altered, indicating that chalcone was not toxic to AGS cells at these dosages. When cells were treated with 3–7 μg/ml chalcone for 24 and 48 h, the cell viability was significantly decreased. These results demonstrated that treating with chalcone with doses higher than 2 μg/ml for 24 and 48 h resulted in dose- and time-dependent loss of cell viability in AGS cells, but doses lower than 2 μg/ml for 24 and 48 h did not cause cytotoxicity.

Chalcone suppresses the cell adhesion, invasion, and migration in AGS cells

Previous studies have demonstrated that the cancer metastasis and invasion are highly related to the degradation of ECM, intercellular adhesion, and cellular motility. Firstly, in the cell–matrix adhesion assay, the result showed that the cell adhesion ability of AGS cells was significantly reduced by a 24-h treatment of chalcone. Such reduction was concentration dependent with a 65.5 % decrease ($P < 0.001$) when chalcone was at 2 μg/ml (Fig. 2a). We next assessed the effect of chalcone on cell invasion and migration was investigated using a Boyden chamber and the results showed chalcone induced a dose-dependent decrease in invasion and migration with an increasing concentration of chalcone. According to a quantitative assessment, treatment with 2 μg/ml of chalcone inhibited 60.5 and 55 % of cell invasion and migration after 24 h incubation, respectively, compared with those of the non-chalcone-treated group (Fig. 2b, c). The results demonstrated that chalcone significantly inhibited the invasion and migration of highly metastatic AGS cells. The effect of chalcone on AGS cell migration was further determined by wound-healing assay. As shown in Fig. 2d, an apparent and gradual increase of cells in the denude zone was observed in the cells treated with the control for 0, 6, 12, 24, and 48 h under inverted microscope. According to a quantitative assessment, treatment with 1 and 2 μg/ml of chalcone inhibited 26 and 67.1 % of cell migration after 24 h, respectively; such doses of chalcone inhibited 30.1 and 50.6 % of cell migration at 48 h, respectively. Also,

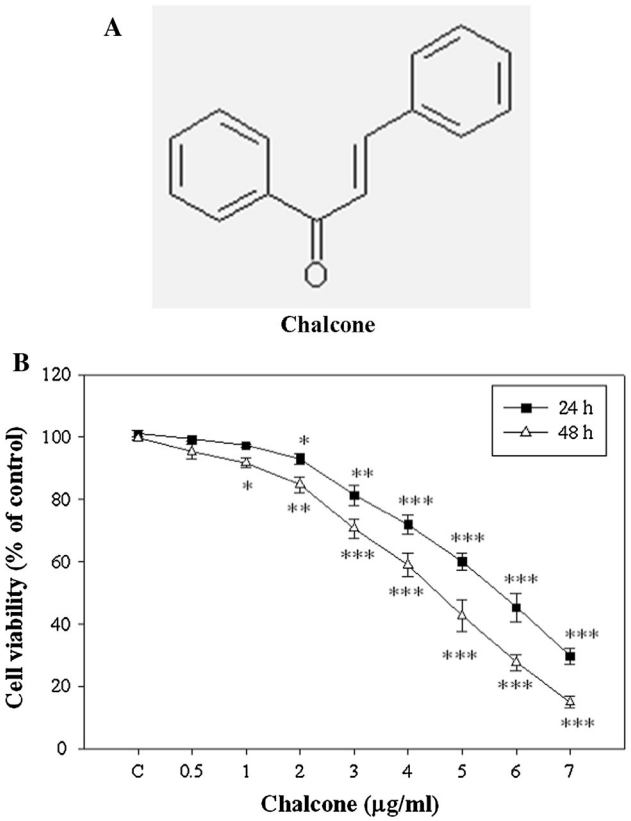

Fig. 1 The effects of chalcone on the viability in AGS cells. **a** Chemical structure of chalcone. **b** Cultured cells were treated with or without chalcone under various concentrations for 24 and 48 h. Thereafter, cell viabilities were determined by MTT assay. The survival cell number was directly proportional to formazan, which was measured spectrophotometrically at 563 nm. Values represent mean ± SD of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the untreated control (dose 0)

compared with the untreated cells, the level of AGS cells' numbers decreased almost twofold when treating with 2 μg/ml chalcone for 48 h. The results demonstrated that chalcone significantly inhibited the invasion and migration of highly metastatic AGS cells.

Chalcone suppresses the MMP-2 and MMP-9 activity and expression in AGS cells

ECM degradation is by overexpression of proteolytic enzyme activity, such as the matrix metalloproteinases. Meanwhile, locomotion of tumor cells into the extracellular matrix and invasion of lymph and blood vessels occur, and the migrated tumor cells concomitantly escape the immunologic system in the circulation, exit to the new tissue, and eventually colonize the distant site [12]. Thus, the effect of chalcone on MMP-2/-9 and u-PA activities was investigated by gelatin- and casein-plasminogen zymography under a condition of serum starvation to explain

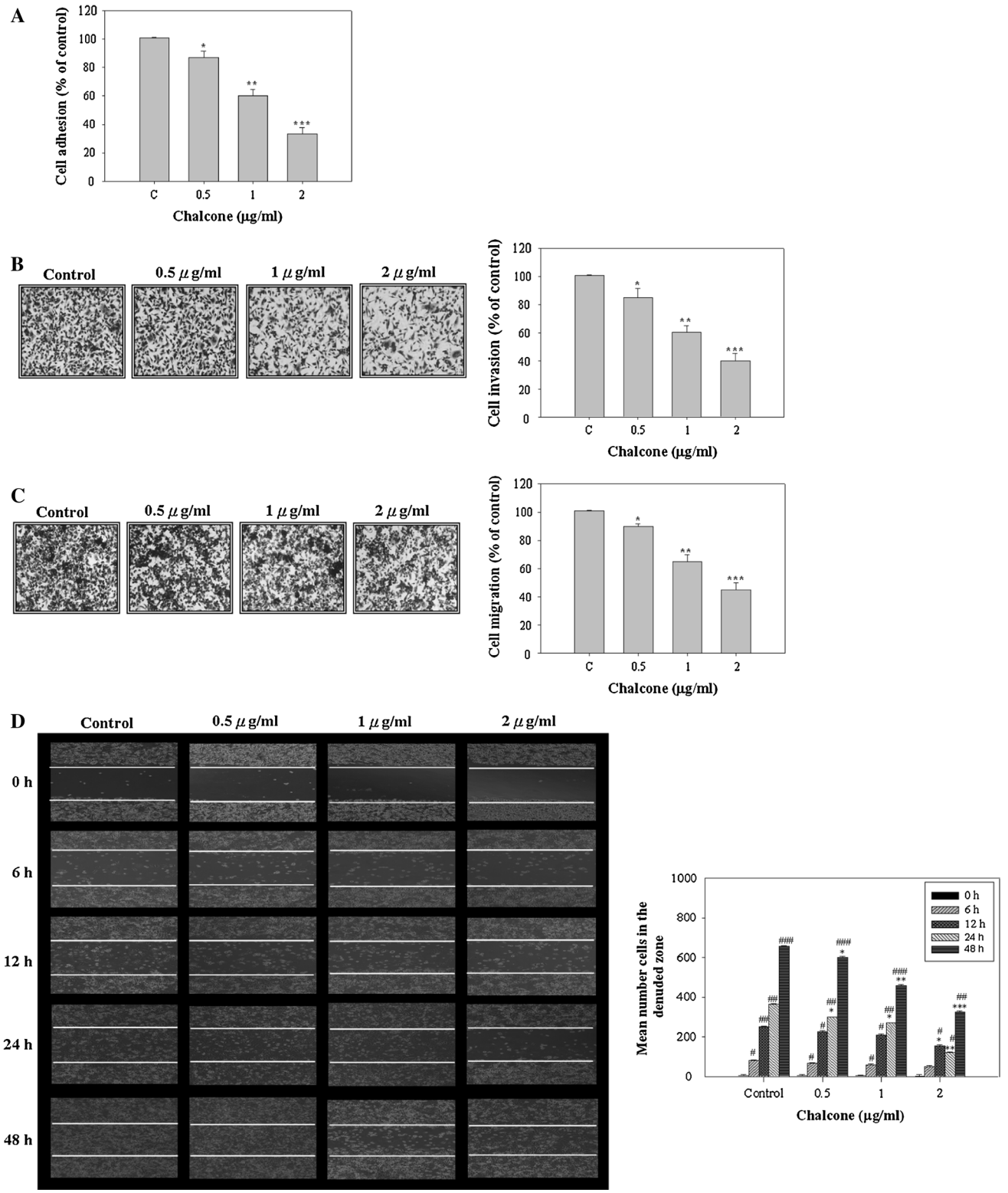

Fig. 2 The effects of chalcone on the cell–matrix adhesion, invasion, and migration in AGS cells. Cells were treated with various concentrations (0, 0.5, 1, and 2 μg/ml) of chalcone and were then subjected to analyses for **a** cell–matrix adhesion, **b** invasion, and **c** migration as described in "Materials and methods section." Values are expressed as mean ± SD of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the untreated control (dose 0); $\#P < 0.05$, $\#\#P < 0.01$, $\#\#P < 0.001$ compared with the 0 h-treated time

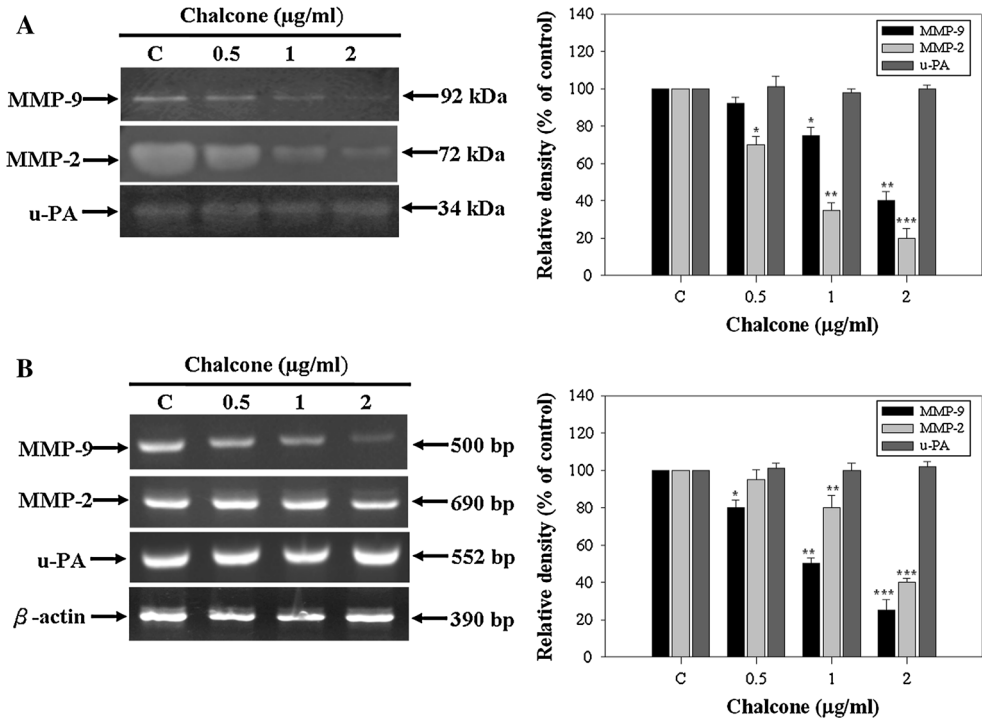

Fig. 3 Effect of chalcone on MMP-2, MMP-9, and u-PA expressions in AGS cells. **a** Cells were treated with various concentrations (0, 0.5, 1, and 2 μg/ml) of chalcone for 24 h. The conditioned media were collected, and MMP-2, MMP-9, and u-PA activities were determined by gelatin or casein-plasminogen zymography. MMP-2, MMP-9, and u-PA activities were quantified by densitomeric analysis. **b** Cells were treated with various concentrations of chalcone for 6 h. And then, RNA samples were extracted and subjected to a semi-quantitative RT-PCR for MMP-2, MMP-9, and u-PA with β-actin being an internal control. The PCR products were quantitated by densitometric analysis with that of the untreated group being 100 %. Values are expressed as mean ± SD of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the untreated control (dose 0)

the contribution of MMP-2/-9 and u-PA in the inhibitory effect of chalcone on the metastatic ability of AGS cells. As shown in Fig. 3a, treatment with chalcone for 24 h dramatically reduced MMP-2 and MMP-9 activity in a dose-dependent manner in the concentration range of 0.5-2 μg/ml, while it had no significant effect on u-PA activity. We next used a RT-PCR assay to investigate the inhibitory effect of chalcone on the mRNA levels of MMP-2/-9 and u-PA. The results of RT-PCR showed that chalcone could reduce the mRNA expression of MMP-2 and MMP-9, but not u-PA; the decrease in mRNA was particularly strong when the chalcone concentration reached 2 μg/ml (Fig. 3b). These results reveal that chalcone might regulate MMP-2 and MMP-9 expression at transcription level. Furthermore, these results also suggested that the anti-metastatic effect of chalcone was related to the inhibition of enzymatically degradative processes of tumor metastasis.

Chalcone suppresses the FAK/JNK signaling pathway in AGS cells

As we have shown that treatment of chalcone to AGS cells inhibited the cell invasion, migration, and activity of MMP-2 and MMP-9, the underlying mechanisms were further investigated by studying the effect of chalcone on the constitutive activation status of the three major mammalian MAPKs and Akt. Figure 4a showed that the treatment of chalcone reduced the phospho-FAK, phospho-JNK1/2 levels at 6 h after incubation, whereas it had no significant effect on phosphorylated form of FAK, ERK1/2, p38, and Akt. Moreover, no significant change in the total amount of FAK, JNK1/2, ERK1/2, p38, and Akt proteins was observed.

To further investigate whether the inhibition of chalcone was mainly occurring through inhibition of the FAK/JNK signaling pathway and MMP-2/-9 activity, the experiment is divided into two sections, dealing firstly with FAK siRNA and secondly with specific JNK inhibitor (SP600125). Firstly, AGS cells were transiently transfected with FAK siRNA. Subsequently, the siRNA-transfected cells were exposed to the presence or absence of chalcone (0.5 μg/ml) for 6 h. Then, the phosphorylation of JNK1 and JNK2 was determined by Western blot assay and the activity of MMP-2/-9 was determined by gelatin zymography assay. Figure 4b showed that a sole treatment of chalcone (0.5 μg/ml) or FAK siRNA, respectively, reduced the expressions of JNK1 and JNK2 by 25.3 and 30 % or

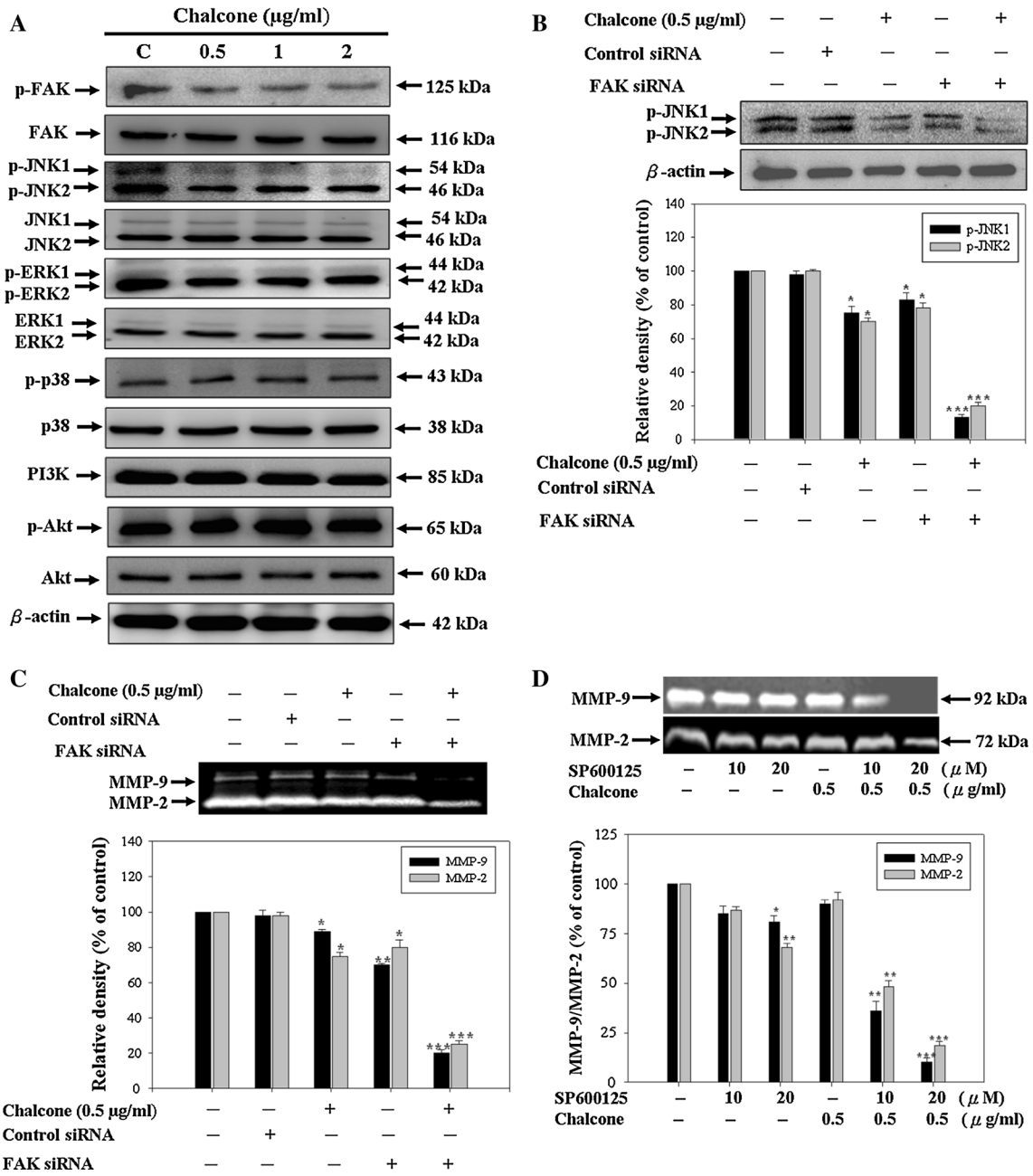

Fig. 4 Effect of chalcone on the phosphorylation of FAK and JNK in AGS cells. **a** AGS cells were treated with various concentrations (0, 0.5, 1, and 2 μg/ml) of chalcone for 6 h. The phosphorylation of FAK, JNK, ERK, p38, and Akt and the expression of PI3K were analyzed by Western blotting. The relative density of phosphorylated forms of FAK, JNK, ERK, p38, and Akt was normalized to total values of FAK, JNK, ERK, p38, and Akt, which were determined by densitometric analysis. **b** For the determination of the effects of FAK siRNA and chalcone on the phosphorylation of JNK1/2, cells were plated in culture dishes and pretreated with FAK siRNA for 12 h and then incubated in the presence or absence of chalcone (0.5 μg/ml) for 6 h. Afterward, the cell lysates were subjected to Western blotting assay. **c** For the determination of the effects of FAK siRNA and chalcone on the activity of MMP-2/-9, cells were plated in culture dishes and pretreated with FAK siRNA for 12 h and then incubated in the presence or absence of chalcone (0.5 μg/ml) for 6 h. Afterward, the conditioned medium was subjected to gelatin zymography assay. **d** Cells were pretreated with a JNK inhibitor (SP600125; 10 or 20 μM) for 1 h and then treated with or without chalcone (0.5 μg/ml) for 24 h, and the enzyme activity levels of MMP-9 and MMP-2 were determined by gelatin zymography assay. Values represent mean ± SD of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the untreated control (dose 0)

17.5 and 22.2 % and that the combination treatment (FAK siRNA + 0.5 μg/ml chalcone) could reduce phosphorylated JNK1 and JNK2 proteins even more dramatically, by 87.4 and 80.1 %. Figure 4c showed that a sole treatment of chalcone (0.5 μg/ml) or FAK siRNA, respectively, reduced the activity of MMP-9 and MMP-2 by 11.4 and 25.1 % or

30.2 and 20 % and that the combination treatment (FAK siRNA + 0.5 μg/ml chalcone) could reduce MMP-9 and MMP-2 activity even more dramatically, by 80.1 and 74.8 %. Secondly, AGS cells were pretreated with a JNK inhibitor (SP600125; 10 or 20 μM) for 1 h and then incubated with or without chalcone (0.5 μg/ml) for 24 h. The result of gelatin zymography assay showed that a sole treatment of SP600125 (10 or 20 μM) or chalcone (0.5 μg/ml) separately reduced the expressions of MMP-9 and MMP-2 by 15.2, 19.1, and 10.4 % and 13.5, 31.9, and 8.2 %, respectively, and the combination treatment could reduce the activity of MMP-9 and MMP-2 by 64.2 and 51.8 % (10 μM SP600125 + 0.5 μg/ml chalcone) and 89.8 and 81.5 % (20 μM SP600125 + 0.5 μg/ml chalcone) (Fig. 4d).

The data finding revealed that chalcone inhibited the expressions of MMP-2 and MMP-9 on AGS cells which could partly occur through JNK1/2 inactivation. Indeed, the above-mentioned results showed that chalcone could inhibit the phosphorylation of JNK1/2 and the activity of MMP-2/-9 which was further supported by the use of the FAK siRNA and JNK inhibitor in our experimental model.

Chalcone suppresses the DNA-binding activity of NF-κB and the degradation and phosphorylation of IκBα in AGS cells

It was known that the MMP-2/-9 promoter has several transcription factor-response elements, including binding sequences for NF-κB and AP-1 [21, 32]. Also, NF-κB and AP-1 family of transcriptional factors have been known to translocate to the nucleus and regulate the expressions of multiple genes involved in MMP-2/-9 secretion. AGS cells were treated with various concentrations of chalcone (0, 0.5, 1, and 2 μg/ml) for 12 h, and nuclear extracts were analyzed by the EMSA for NF-κB and AP-1 DNA-binding activities. Figure 5a has shown a decrease in the DNA-binding activity of NF-κB. By contrast, AP-1 binding activity was not affected by chalcone. Further, the expressions of NF-κB, c-Fos, and c-Jun in nuclear extracts were analyzed by Western blotting assay to assess the possible inhibitory effect of chalcone. Figure 5b showed that the nuclear level of NF-κB (p65) was gradually diminished at doses of 0.5, 1, and 2 μg/ml chalcone when compared to the 0 μg/ml after treatment for 12 h. However, there was no noticeable change in the translocation of c-Fos and c-Jun under the same treatment condition. Also, the activation of NF-κB is through the phosphorylation of IκBα to release the NF-κB for nuclear translocation [24, 25] and for binding to the promoter sites of target genes. To examine the effect of chalcone on IκBα regulation, we investigated whether chalcone has inhibitory effects on IκBα degradation and phosphorylation. As shown in Fig. 5c, chalcone blocked IκBα degradation through inhibiting phosphorylation of IκBα. Also, the intensity of Western blotting reflected that the chalcone at a concentration >0.5 μg/ml could enhance IκBα protein expression.

Discussion

Tumor metastasis is complex processes which is highly selective and consists of a series of sequential and interrelated steps, including changes (1) damaging intercellular interaction, (2) increasing cancer cells and extracellular matrix (ECM) interaction, (3) damaging ECM components, and (4) increasing invasion and migration of cancer cells [33]. Cancer occurs after a single cell in a tissue is progressively damaged to produce cells with uncontrolled proliferation. The uncontrolled proliferation and mitosis produce a primary tumor. Besides, cell invasion and migration are complex processes involving many types of intracellular and extracellular components and are associated with signaling pathways.

Since invasion and migration are a critical event in cancer progression and especially in metastasis, the inhibitory effect of chalcone on cell invasion and migration was evaluated. We found that chalcone significantly inhibited the invasion and migration of human gastric adenocarcinoma AGS cells. Invasive cancer cells through a coated membrane involve not only ECM degradation, but also the formation of adhesive interactions between cells and the matrix. Therefore, the cell adhesion assay was carried out and the result showed that chalcone caused a reduction in cell adhesion aspect. Furthermore, the obvious reduction in cell adhesion may be correlated with the significant decreases in migration and invasion by chalcone treatment. Consequently, chalcone significantly inhibited the invasion (assessed using the Boyden chamber assay) and migration (examined by both Boyden chamber and wound-healing assays) of AGS cells. Based on the above observation, we used the wound-healing assay in vitro to observe the effect of chalcone on cellular migration. Chalcone potently retarded the migration of cells toward the wounded area. The results of this study demonstrated that chalcone inhibited cell migration which is vital during the early phase of wound healing.

During cancer progression, some tumor cells become motile and gain the capacity to attack the host tissue leading to metastasis. FAK can be activated in response to diverse stimuli and plays an important role in the proliferation and metastasis of cells [34]. The overexpression and phosphorylation of FAK correlate with the increase of cell motility and invasion and alteration in the cytoskeleton [35]. Adhesion and spreading of cells on a variety of ECM proteins, including collagen type IV, lead to an increase in tyrosine phosphorylation and activation of FAK [36]. In

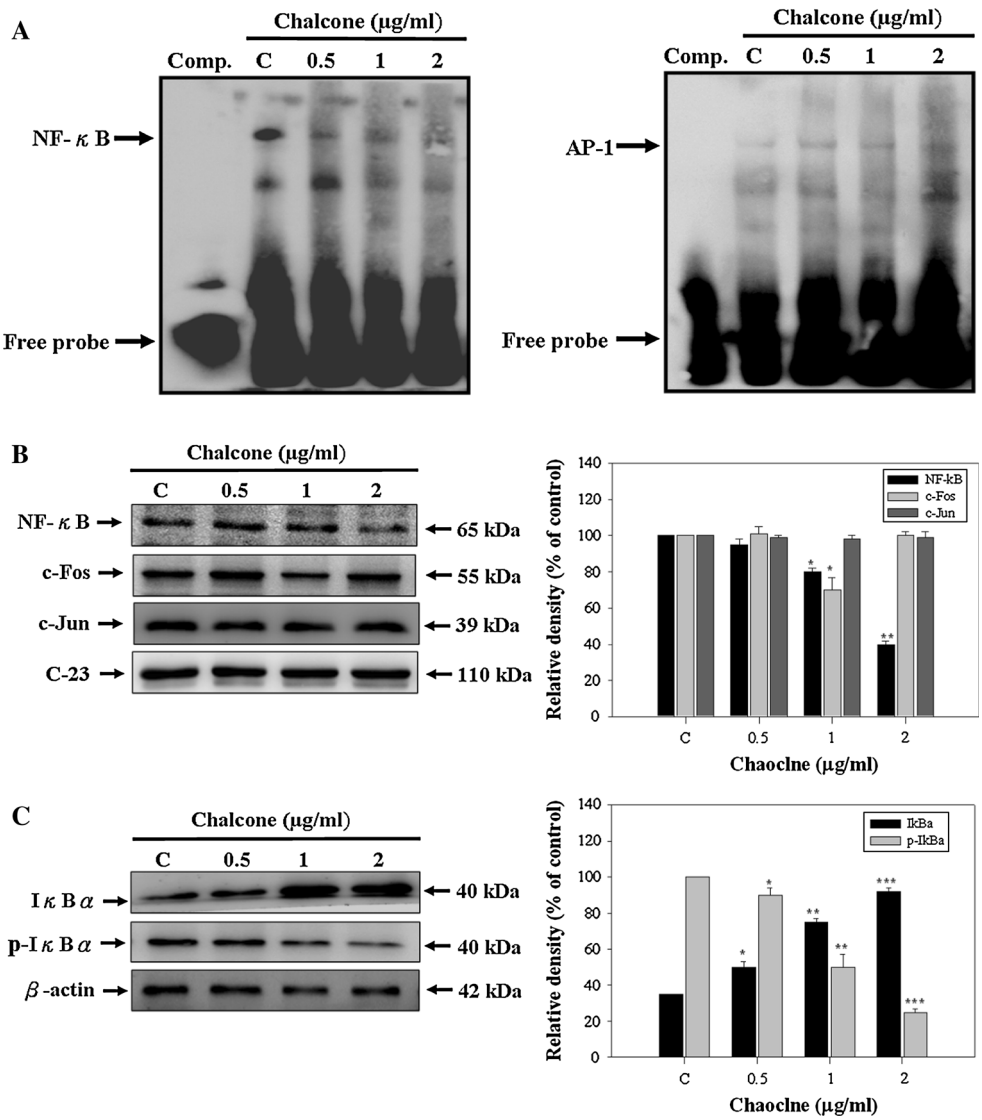

Fig. 5 Effect of chalcone on the DNA-binding activities of NF-κB and AP-1 in AGS cells. **a** Cells were treated with various concentrations (0, 0.5, 1, and 2 μg/ml) of chalcone for 12 h, and then, nuclear extracts were prepared and analyzed for a NF-κB and AP-1 DNA-binding activity using biotin-labeled consensus NF-κB- and AP-1-specific oligonucleotide. Then, EMSA assay was performed as described in ''Materials and methods'' section. Lane 1: nuclear extracts incubated with 100-fold excess unlabeled consensus oligonucleotide (comp.) to confirm the binding specificity. Excess free probe is indicated at the bottom. **b** Nuclear extracts were also analyzed by Western blotting with anti-NF-κB, c-Fos, and c-Jun antibodies. C23 was a nucleus protein loading control. **c** Equal amounts of cytoplasmic proteins were resolved by SDS-PAGE, transferred to PVDF membrane, and probed with specific antibodies (anti-p-IκBα and anti-IκBα). β-Actin was used as loading controls. Results from three repeated and separated experiments were similar

melanoma cells, the increased expression of FAK correlates with increased cell motility [37]. Overexpression of FAK has also been reported in breast cancer and sarcoma [38, 39]. Also, FAK is at the crossroad of several signaling pathways, including PI3K/Akt and MAPK pathways [40, 41]. Our study showed that chalcone caused a dose-dependent decrease in cellular level of phospho-FAK and phospho-JNK. Nevertheless, there was no significant change in total and phosphorylated levels of ERK, p38, and Akt at the same dosage.

The expression level of MMPs in various cancer cells directly associates with invasion and metastasis. MMPs, belonging to a family of Zn-dependent endopeptidases, are the major proteases in angiogenesis and are closely correlated with invasive and metastatic potentials of cancer cells [42]. They are secreted as inactive proenzymes and are activated by partial proteolytic cleavage. MMP-2, MMP-9, and dominant MMPs are released by most endothelial cells and appear to play important roles in the degradation of type VI collage, a major constituent of basement membrane, in cancer invasion and metastasis [43]. In this study, we observed that chalcone led to a significant reduction of MMP-2 and MMP-9 at the enzyme activity and mRNA levels. These results demonstrated that chalcone could inhibit invasion and metastasis in AGS cell through down-regulation of MMP-2 and MMP-9 expression. It is of importance to note that MMP-2 and MMP-9 play roles in tumor progression, as over-expression of the MMP-2/-9 genes in nude mice led to enhanced tumorigenesis in a melanoma cancer model [44]. We have demonstrated that reduction of proteolytically active MMP-2/-9 is involved in chalcone-mediated cell invasion and migration. In addition, the transcription of MMP-2/-9 gene is regulated by upstream sequences, including motifs corresponding to

NF-κB and AP-1 binding elements [21, 22]. Here, we have also found that chalcone inhibited MMP-2/-9 expression through preventing IκBα being phosphorylated and enhancing IκBα protein expression, both leading to inactivation of NF-κB DNA-binding activity. Under normal condition, NF-κB is maintained in the cytoplasm through interactions with an inhibitor of NF-κB (IκB), but upon dissociation, moves into the nucleus and promotes cancer cells' proliferation, angiogenesis, and metastasis. A comprehensive analysis of MMP-2-/-9 expression in AGS cells will be necessary to fully understand the biological properties of AGS cells and to promote the development of new therapeutic strategies.

In summary, we explored the antimetastatic effects and mechanistic actions of chalcone in human gastric adenocarcinoma AGS cells. These results imply that the antimetastatic effect of the chalcone on AGS cells might be through inactivating FAK/JNK signaling, as well as enhancing IκBα protein expression to reduce NF-κB DNA-binding activity, leading to the downregulation of MMP-2/-9 expressions, thus reducing cell invasion and migration in vitro. The present study suggests that chalcone may be developed into a promising agent for cancer therapy.

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