

Maslinic acid suppresses the growth of human gastric cells by inducing apoptosis via inhibition of the interleukin-6 mediated Janus kinase/signal transducer and activator of transcription 3 signaling pathway

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Abstract. The present study aimed to determine whether maslinic acid effectively inhibits the proliferation of MKN28 cells, and to investigate the mechanisms underlying its antitumor functions. MKN28 cell viability was evaluated using a Cell Counting Kit-8, cell proliferation was analyzed by a colony formation assay and flow cytometry was used to investigate the rate of apoptosis. Western blot analysis was performed in order to determine the differential expression levels of Janus kinase (JAK), signal transducer and activator of transcription 3 (STAT3) and apoptosis associated proteins B-cell lymphoma 2 (Bcl-2), Bcl-2 associated X protein (Bax) and Bcl-2 associated agonist of cell death (Bad). Interleukin-6 (IL-6) concentration was evaluated using ELISA. IL-6 and anti-IL-6 antibodies were used to investigate the role of IL-6 in MKN28 cells treated with maslinic acid proliferation, and the STAT3 phosphorylation rates. The results demonstrated that maslinic acid treatment significantly reduced cell proliferation, induced apoptosis and was accompanied by a significant decrease in Bcl-2, Bax and Bad expression levels. Maslinic acid treatment also resulted in the downregulation of phosphorylated-STAT3 and JAK2, and significantly inhibited the protein expression of IL-6. Maslinic acid is able to inhibit MKN28 cell proliferation and the phosphorylation of STAT3 by down-regulating the expression of IL-6. These results suggest that maslinic acid suppresses the growth of MKN28 cells by

inducing apoptosis via its inhibition of the IL-6/JAK/STAT3 signaling cascade.

Introduction

Maslinic acid is a natural triterpene from *Olea europaea* L. (1), which acts as an antitumor, antibacterial (2) and anti-HIV substance (3), and exhibits antiallodynic and analgesic properties by regulating cell metabolism and immune function (4). Maslinic acid has been revealed to exert therapeutic effects on a variety of solid tumors, including in bladder, prostate, colon, esophageal, colorectal, cervical and ovarian cancer (5-8). However, the molecular mechanisms underlying the antitumor functions of maslinic acid remain elusive.

Interleukin-6 (IL-6) is a pleiotropic cytokine, which serves an important role in cell proliferation, differentiation, apoptosis and metastasis by participating in tumor pathogenesis (9-11). IL-6 may activate the Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) signaling pathway and the Ras/mitogen-activated protein kinase (MAPK) signaling pathway (12,13). It has previously been demonstrated that STAT3 is constitutively phosphorylated in MKN28 cells, and it has been revealed that the inhibition of IL-6 by IL-6 receptor (R) antagonists, JAK inhibitors or the expression of a dominant-negative STAT3 mutant, can induce apoptosis in MKN28 cell lines *in vitro* (14-16). Therefore, it has been suggested that the IL-6/JAK/STAT3 signaling pathway provides an important antiapoptotic signal in tumor cells, and may be a promising target for the development of novel therapeutic strategies for gastric cancer. To the best of our knowledge, no previous studies have provided data investigating the effect and underlying mechanisms of maslinic acid in gastric cancer.

The present study demonstrated that maslinic acid inhibits the proliferation and induces apoptosis of MKN28 cells. These findings were associated with the downregulation of phosphorylated (p)-STAT3 protein and its upstream kinase, JAK. Inhibition of IL-6 production in MKN28 cells may account for the inhibition of STAT3 mediated by maslinic acid. Overall, the results of the present study provided evidence for the potential clinical application of maslinic acid as a novel therapeutic agent against gastric cancer.

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Materials and methods

Cell culture and reagents. The MNK28 human gastric cancer cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified incubator with 5% CO₂.

Maslinic acid was obtained from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany) and stock solution was prepared in dimethyl sulfoxide at 1 mM. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). The bicinchoninic acid assay (BCA) kit (71285-3) was purchased from Beyotime Institute of Biotechnology (Haimen, China). Antibodies against STAT3 (cat. no. ab119352), p-STAT3 (Tyr705; cat. no. ab76315), JAK2 (cat. no. ab108596) and p-JAK2 (cat. no. ab32101) were purchased from Abcam (Cambridge, UK). B-cell lymphoma 2 (Bcl-2; cat. no. 2870), Bcl-2 associated agonist of cell death (Bad; cat. no. 9292), Bcl-2 associated X protein (Bax; cat. no. 2772) and β -actin (cat. no. 3700) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Enhanced chemiluminescence (ECL) reagent was purchased from EMD Millipore (Billerica, MA, USA). A Human IL-6 Quantikine ELISA kit (D6050), recombinant human IL-6 protein (cat. no. 206-IL-050/CF) and human IL-6 antibody (cat. no. MAB206-100) were obtained from R&D Systems, Inc. (Minneapolis, MN, USA).

Cytotoxicity assay. MKN28 cells were seeded into 24-well plates at a density of 1×10^4 cells/well and treated with various concentrations of maslinic acid (0, 0.1, 1 or 10 μ M) at 37°C for 24 h; subsequently CCK-8 reagent was added for a further 2-h incubation at 37°C. Optical density was evaluated at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell number was determined using the trypan blue dye exclusion method (17).

Apoptosis analysis by Annexin V-propidium iodide (PI) double staining. The apoptotic rate of MNK28 cells was evaluated by flow cytometry using the Annexin V-fluorescein isothiocyanate (FITC)/PI double staining method. MNK28 cells were seeded in 6-well plates and treated with maslinic acid, as described above. Cells were trypsinized with 0.25% EDTA-free trypsin, then washed with PBS and centrifuged at 300 x g for 3 min, prior to incubation with 1 μ g/ml Annexin V-FITC and 10 μ g/ml PI for 15 min at room temperature in the dark. Samples were analyzed using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and presented as two parameter dot-plots.

Clone formation assay. For each treatment group, $\sim 1 \times 10^2$ cells were seeded into each well of a 6-well plate. Following incubation with 0, 0.1, 1 and 10 μ M of maslinic acid at 37°C for 12 days, the cells were washed with PBS and images of each clone were captured under a light microscope.

Western blotting. Following treatment with maslinic acid for 24 h, MKN28 cells were collected and lysed in

radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM Na₃VO₄, 1 mM NaF and a protease inhibitor cocktail). The extracts were incubated on ice for 30 min and supernatants were collected by centrifugation at 13,400 x g for 10 min at 4°C. Subsequently, protein concentrations were determined by BCA assay, 30 μ g protein were separated by electrophoresis on 10% SDS-PAGE gel and electro-transferred onto a polyvinylidene fluoride membrane with transfer buffer (25 mM Tris, 250 mM glycine and 20% methanol) at 100 V for 2 h. The membrane was blocked in 5% nonfat skimmed milk and probed with the corresponding primary antibodies at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated goat-anti mouse (1:5,000; cat. no., sc-2005) and mouse-anti rabbit (1:5,000; cat. no. sc-2357) secondary antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Primary antibodies included anti- β -actin (1:5,000), rabbit anti-human anti-STAT3 (1:1,000), rabbit anti-human anti-p-STAT3 (1:1,000), rabbit anti-human anti-Bad (1:1,000), rabbit anti-human anti-Bcl-2 (1:1,000), rabbit anti-human anti-Bax (1:1,000), rabbit anti-human anti-JAK2 (1:1,000) and rabbit anti-human anti-p-JAK2 (1:1,000). Protein expression was detected using an ECL system (GE Healthcare Biosciences, Pittsburgh, PA, USA). For western blotting, band density was determined with Quantity One 1-D software (Bio-Rad Laboratories, Inc.). Western blotting was performed in triplicate.

IL-6 analysis by ELISA. The concentration of IL-6 in the cell culture supernatants was evaluated using the ELISA method. Briefly, MKN28 cells were plated in 24-well plates. Following treatment with maslinic acid for 24 h, the supernatant was harvested at 13,400 x g for 10 min at 4°C for analysis. Supernatants were analyzed using an IL-6 ELISA protein assay kit, according to the manufacturer's instructions. Color development was determined using a microplate reader (MK3; Thermo Fisher Scientific, Inc.) set to 450 nm. A standard curve was plotted using data generated by evaluation of the absorbance of recombinant-IL-6 serial dilutions.

IL-6 and anti-IL-6 antibody treatment. Following serum starvation for 4 h, 10 μ g/ml maslinic acid, 10 ng/ml recombinant IL-6 protein or 10 μ g/ml anti-IL-6 antibody were added to the medium for 24 h of treatment at 37°C. Cell viability was determined using CCK-8 and the phosphorylation status of STAT3 was evaluated by western blot analysis as previously described.

Statistical analysis. Data are expressed as the mean \pm standard deviation of the mean of separate experiments ($n \geq 3$). Student's t-test was performed for comparison of the means between the two groups, and one-way analysis of variance was used for analyzing the means of multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Maslinic acid inhibits cell viability and proliferation and induces apoptosis in MKN28 cells. In order to investigate the inhibitory effects of maslinic acid on MKN28 cell viability

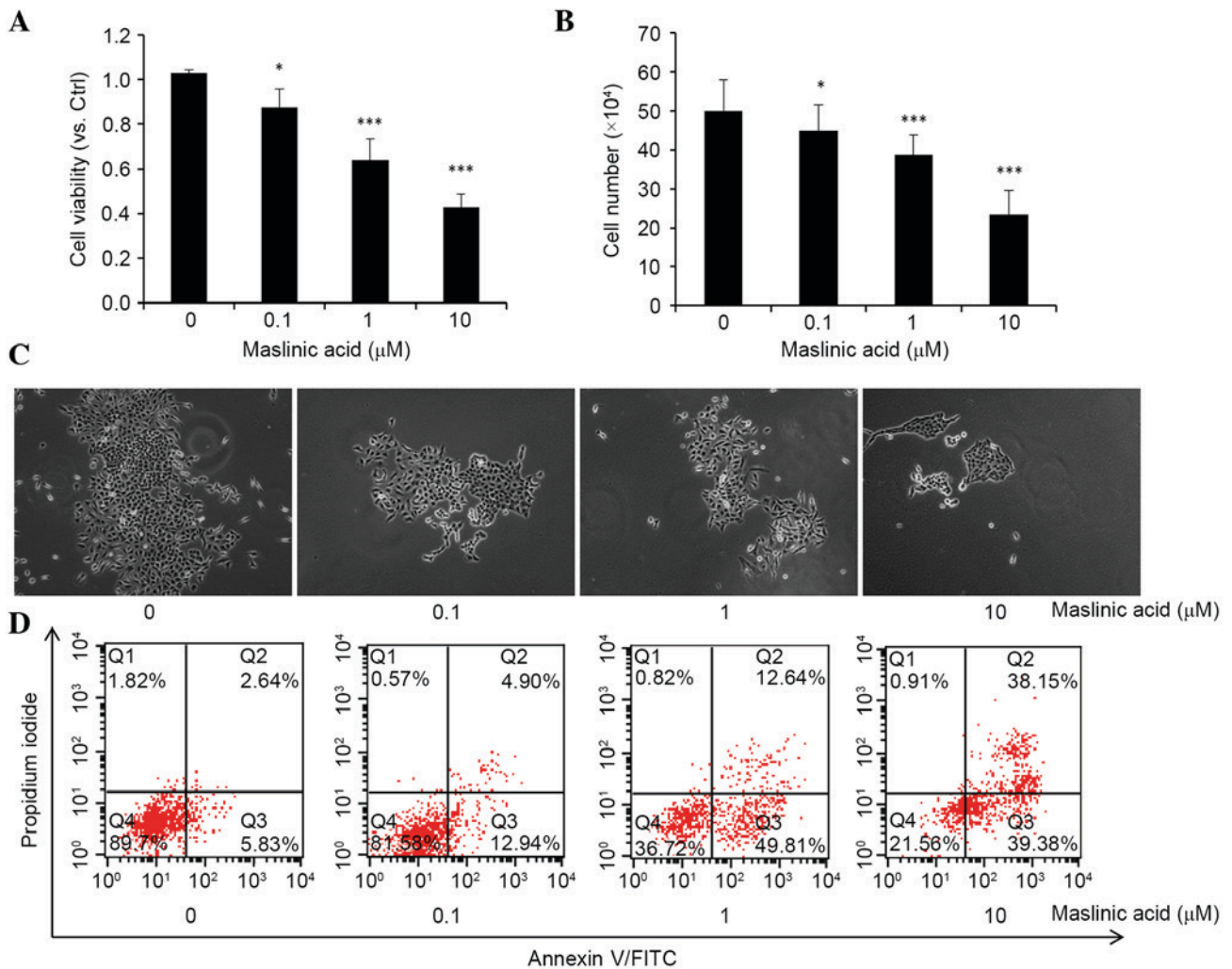


Figure 1. Maslinic acid inhibits the growth and induces apoptosis of MKN28 cells. (A) Cell viability of MKN28 cells and (B) cell number was analyzed by CCK-8 and trypan blue staining following treatment with maslinic acid at various concentrations (0, 0.1, 1.0 and 10.0 μM). (C) Monolayer colony formation assays suggested that maslinic acid reduced monolayer colony formation in MKN28 cells. (D) The apoptosis of MKN28 cells treated with various concentrations of maslinic acid (0, 0.1, 1.0 and 10.0 μM) for 24 h was evaluated by Annexin V-FITC/PI double staining. Results are presented as the mean \pm standard deviation of experiments conducted in triplicate. Statistical significance is indicated as ** $P < 0.01$ and *** $P < 0.001$. Ctrl, control; CCK-8, Cell Counting Kit-8; FITC, fluorescein isothiocyanate; PI, propidium iodide.

and proliferation, the present study evaluated the cell number using trypan blue dye exclusion, and determined cell viability using the CCK-8. As presented in Fig. 1A and B, maslinic acid significantly reduced cell viability and proliferation in a dose-dependent manner with a half-maximal inhibitory concentration of 8.45 μM , $P = 0.032$, 0.00092 and 0.000036, respectively. Colony formation assays also demonstrated a significant reduction in the size and number of colonies that received treatment with maslinic acid, $P = 0.047$, 0.00083 and 0.00006, respectively (Fig. 1C). The results revealed that maslinic acid has an inhibitory effect on the viability and proliferation of MKN28 cells.

Subsequently, the present study examined the apoptosis rate of MKN28 cells following treatment with maslinic acid for 24 h (Fig. 1D). A significantly dose-dependent increase in the percentage of early apoptotic (Annexin V⁺/PI⁻) and late apoptotic/necrotic (Annexin V⁺/PI⁺) cells was observed following culturing of MKN28 cells with maslinic acid for 24 h, $P = 0.048$, 0.0082 and 0.00074, respectively. Totals of 5.83% for early apoptotic and 2.64% for late apoptotic/necrotic

in the control group, 12.94% for early apoptotic and 4.90% for late apoptotic/necrotic in the 0.1 μM maslinic acid treatment group, 49.81% for early apoptotic and 12.64% for late apoptotic/necrotic in the 1 μM maslinic acid treatment group and 39.38% for early apoptotic and 38.15% for late apoptotic/necrotic in the 10 μM maslinic acid treatment group, were observed. These results indicate that maslinic acid inhibits MKN28 cell viability by inducing apoptosis.

Maslinic acid inhibits the JAK/STAT3 pathway. JAK/STAT signaling is involved in oncogenesis and cancer progression by upregulation of anti-apoptotic genes (18). To explore the mechanisms underlying maslinic acid induced apoptosis, the Bcl-2 protein family expression was analyzed by western blotting. Maslinic acid treatment significantly increased the expression level of Bad compared with DMSO group, $P = 0.0089$, 0.00035 and 0.00001, respectively. Bcl-2/Bax expression level was significantly decreased in MKN28 cells treated with maslinic acid for 24 h compared with the DMSO group, $P = 0.0049$, 0.00088 and 0.000053, respectively (Fig. 2A). These results

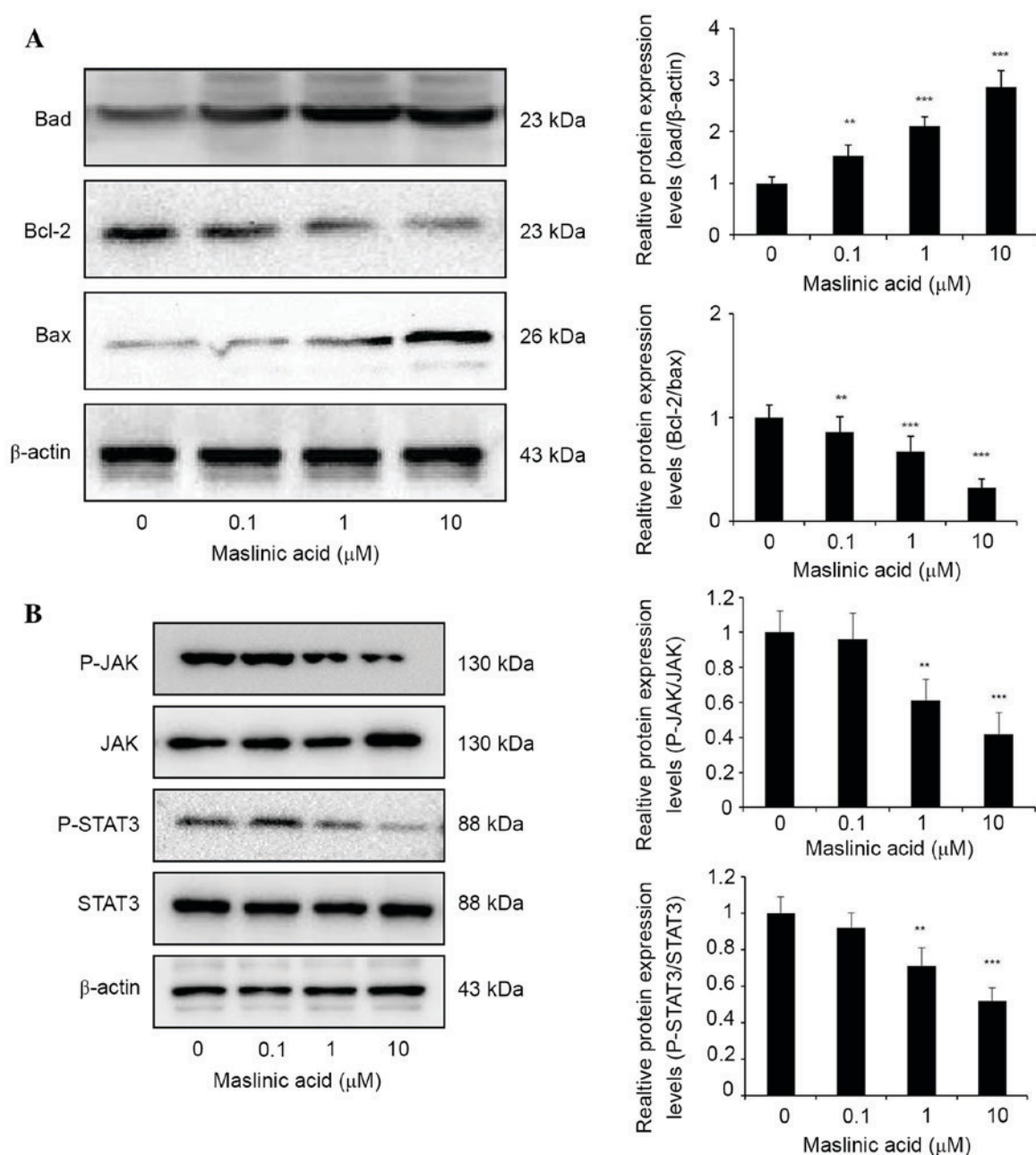


Figure 2. Maslinic acid treatment of MKN28 cells leads to an increased expression level of apoptosis associated protein and reduced JAK/STAT3 phosphorylation activity. MKN28 cells were treated with various concentrations (0, 0.1, 1.0 and 10.0 μ M) of maslinic acid for 24 h; subsequently, (A) the expression levels of Bcl-2 family proteins and (B) the phosphorylation of JAK/STAT3 were evaluated by western blotting. β -actin and unphosphorylated JAK or STAT3 served as a loading control. Experiments were conducted in triplicate. Statistical significance is reported as ** $P < 0.01$ and *** $P < 0.001$. JAK, Janus kinase; STAT3, signal transducer and activator of transcription 3; Bad, Bcl-2 associated agonist of cell death; Bax, Bcl-2 associated X protein; p, phosphorylated.

indicated that the inhibition of proliferation due to maslinic acid treatment may result from significantly attenuated expression levels of Bcl-2 protein family products, including Bcl-2, Bax and Bad.

JAK/STAT3 signaling regulates gene products involved in various cellular processes including survival, proliferation and cell cycle progression (19). Western blot analysis revealed that maslinic acid treatment resulted in a marked downregulation of STAT3 phosphorylation levels without observable significant effects on the total STAT3 protein level in MKN28 cells. Inhibition of JAK2 phosphorylation at a concentration of 1 and 10 μ M maslinic acid was observed, with negligible effects on total JAK2 protein levels, suggesting that the inhibition of

p-JAK2 may contribute to the inhibition of STAT3 activity induced by maslinic acid (Fig. 2B).

Maslinic acid inhibits IL-6-mediated STAT3 activation in MKN28 cells. IL-6 is one of the most prevalent cytokines that mediates its effects via the phosphorylation of STAT3 (20). As JAK/STAT3 activation is known to be involved in hematologic malignancies (21), it was imperative to determine whether maslinic acid had deleterious effects on the production of IL-6. The present study investigated the production of IL-6 in MKN28 cells using ELISA prior to and following maslinic acid treatment. A decrease of IL-6 protein in the medium following maslinic acid treatment was revealed (Fig. 3A).

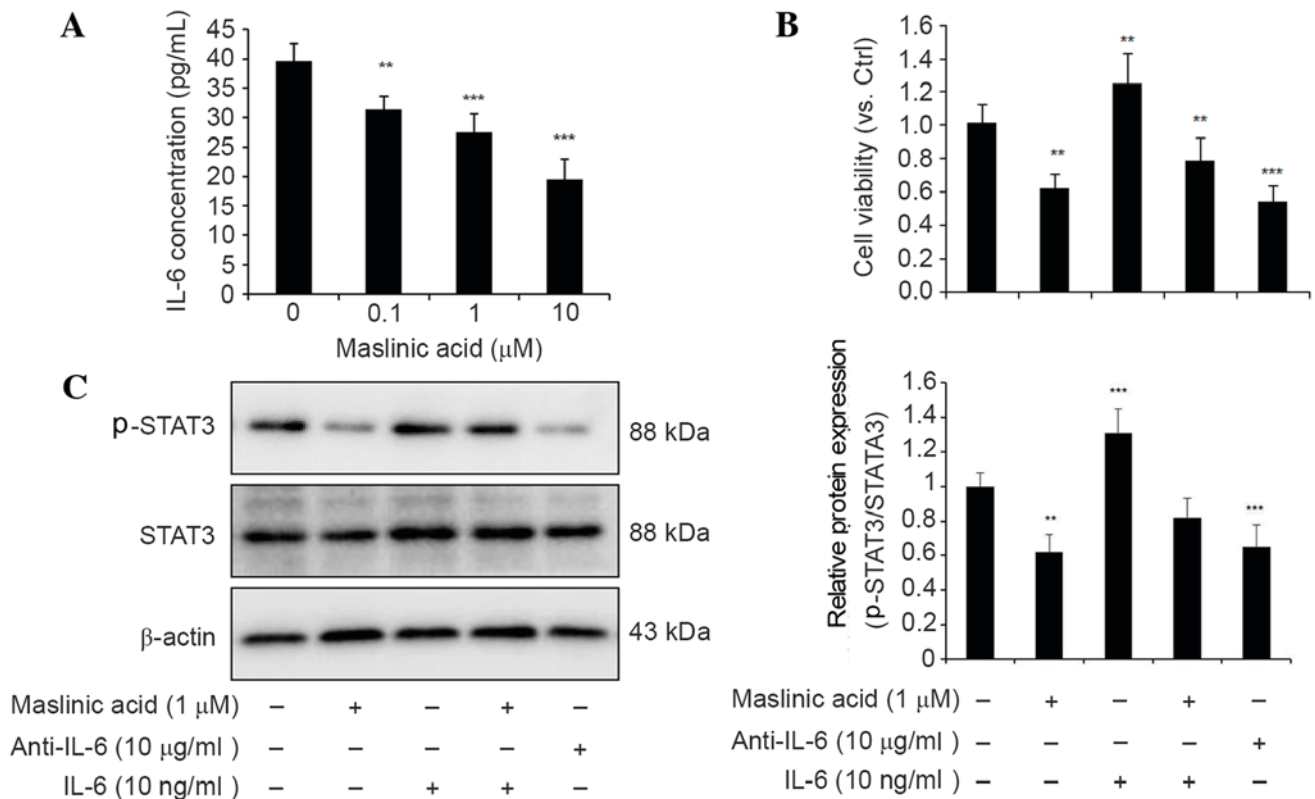


Figure 3. Effect of treatment with various concentrations of maslinic acid on the phosphorylation of STAT3 protein in MKN28 cells. (A) The concentration of IL-6 in the medium following treatment with maslinic acid was detected using an ELISA. (B) CCK-8 was used to analyze the viability of MKN28; cells were harvested for protein extraction and subjected to (C) western blot analysis with an anti-p-STAT3 antibody to determine the phosphorylation status of STAT3 protein. Total STAT3 and β -actin were used as the controls. Experiments were conducted in triplicate. Statistical significance is reported as ** $P < 0.01$ and *** $P < 0.001$. STAT3, signal transducer and activator of transcription 3; IL-6, interleukin-6; CCK-8, Cell Counting Kit-8; p, phosphorylated; ctrl, control.

Therefore, it is likely that suppression of the JAK/STAT3 signaling pathway following maslinic acid treatment may be induced by the upstream inhibition of IL-6 expression.

Pretreatment with the anti-IL-6 antibody for 24 h also resulted in the inhibition of cell viability and phosphorylation of STAT3. The addition of recombinant IL-6 to the medium resulted in an increase in cell viability and the reactivation of STAT3 phosphorylation, suggesting that IL-6 is responsible for STAT3 activation (Fig. 3B and C). Taken together, these findings suggest that maslinic acid serves an important role in the inhibition of IL-6-mediated STAT3 activation in the MKN28 gastric cancer cell line.

Discussion

Maslinic acid is a natural triterpene from *Olea europaea* L. (1). Accumulating evidence exists that maslinic acid may inhibit the growth of various human tumor cell lines *in vitro* (1,22,23). *In vivo* studies also demonstrated that maslinic acid inhibits the growth of various types of cancer cells in mice, including the HT29 human colon-cancer cell line (24), Panc-28 pancreatic cell line, Panc-1, BxPC-3, AsPC-1 (22) and Raji B lymphoma cell line (25). Although the antitumor effects of maslinic acid have been demonstrated *in vitro* and *in vivo* for various types of cancer (22-25), little is known about its effect and underlying mechanisms in gastric cancer.

In the present study, maslinic acid was demonstrated to inhibit MKN28 cell viability in a dose-dependent manner

using CCK-8, and to induce cell apoptosis as determined using flow cytometry. The western blot analysis results revealed that maslinic acid increased the protein level of Bad and inhibited the Bcl-2/Bax ratio, which indicated the cell survival or apoptosis grade. These results confirmed that maslinic acid exhibits a significant anticancer effect on MKN28 cells. Subsequently, the present study focussed on the molecular mechanisms underlying the maslinic acid-mediated inhibition of MKN28 cell proliferation.

Inflammation is recognized to serve an important role in the pathogenesis of numerous types of tumors, and is a critical component of tumor progression (26). The production and release of various survival factors, including IL-6, a major mediator of inflammation, serves to block apoptosis in cells during the inflammatory process, keeping them alive in toxic environments (27). IL-6 binds to the soluble IL-6R (glycoprotein (gp)80, present either on the cell surface or in solution), which then induces dimerization of gp130 chains resulting in activation of the associated JAKs (28). JAKs phosphorylate gp130, leading to the recruitment and activation of STAT3 transcription factors, as well as other molecules (29). STAT3 is constitutively activated in various gastric cancer cells, and is often associated with cell survival, proliferation and transformation (30).

The present study revealed that maslinic acid treatment inhibits IL-6 protein secretion and results in loss of STAT3 phosphorylation, accounting for the inhibition of the IL-6/STAT3 signaling pathway. Western blot analysis

was used for evaluation of the expression levels of JAK2, an upstream protein tyrosine kinase, which serves an important role in STAT3 homo-dimer formation and activation (31). The results demonstrated that maslinic acid treatment of MKN28 cells results in a significantly diminished expression level of p-JAK2 protein. Taken together, these results suggest that the inactivation of upstream p-JAK2 is involved in the inhibition of the IL-6-JAK/STAT3 signaling pathway in MKN28 cells.

A number of previous studies have indicated that constitutive activation of STAT3 is mediated via autocrine/paracrine stimulation by cytokines, including the IL-6 family of cytokines, involved in hematopoietic development (32-35). In order to further explore the mechanisms underlying inhibition of IL-6 induced by maslinic acid, the present study used an anti-IL-6 antibody to block the endogenous IL-6 or recombinant IL-6 protein, in order to restore STAT3 phosphorylation in MKN28 cells, thus suggesting that IL-6 was responsible for STAT3 activation. The results confirmed that the overexpression of IL-6 promoted tumor growth and STAT3 phosphorylation, and the inhibition of IL-6 production may decrease cell proliferation and phosphorylation of STAT3. In addition, maslinic acid may decrease IL-6 protein levels in the culture medium of MKN28 cells. Thus, the anticancer properties of maslinic acid may result from its inhibition of IL-6 expression and subsequent inhibition of downstream JAK2/STAT3 signaling.

To the best of our knowledge, no previous studies have investigated the effect of maslinic acid on the IL-6-JAK/STAT3 signaling cascade in gastric cancer cells. The results of the present study have demonstrated for the first time that the underlying mechanism of maslinic acid anti-gastric tumor activity is the inhibition of IL-6 expression and subsequent downregulation of the JAK/STAT3 signaling pathway. It was revealed that maslinic acid inhibited the generation and secretion of IL-6 in MKN28 cells, induced JAK and STAT3 phosphorylation and downregulated the expression levels of STAT3-mediated proteins involved in apoptosis and proliferation (Bad, Bcl-2 and Bax). However, further investigation is required in order to elucidate the direct molecular mechanisms underlying the maslinic acid inhibition of IL-6. It is possible that maslinic acid may be useful as a therapeutic treatment for gastric cancer.

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