Abstract. Improvements in skin cancer treatment are likely to derive from novel agents targeting the molecular pathways that promote tumor cell growth and survival. Icariside II (IS) is a metabolite of icariin, which is derived from Herba Epimedii. The aim of the present study was to evaluate the antitumor effects of IS and to determine the mechanism of apoptosis in A431 human epidermoid carcinoma cells. A431 cells were treated with IS (0-100 µM) for 24 or 48 h and cell viability was detected using the WST-8 assay. Apoptosis was measured by the Annexin-V/propidium iodide (PI) flow cytometric assay. Western blot analysis was used to measure the expression of cleaved caspase-9, cleaved poly ADP ribose polymerase (PARP), phosphorylated EGFR receptor (P-EGFR), and phosphorylated ERK (P-ERK) and AKT. A431 cells were also pretreated with IS (0-100 µM) 2 h prior to treatment with epidermal growth factor (EGF; 100 ng/ml) for 10 min. Phosphorylated EGFR receptor (P-EGFR), P-STAT3, P-ERK and P-AKT were detected by western blot analysis. The results demonstrated that IS inhibited the cell viability of the A431 cells in a dose-dependent manner. Pretreatment with LY294002, a phosphatidylinositol 3-kinase (PI3K) inhibitor, EGF (an EGFR agonist) and AG1478 (an EGFR inhibitor) partially reversed IS-induced decreases in cell viability. Treatment with 50 µM IS resulted in an increased number of apoptotic cells mirrored by increases in cleaved caspase-9 and cleaved PARP. In addition, treatment with 50 µM IS significantly inhibited the activation of the Janus kinase (JAK)-STAT3 and mitogen-activated protein kinase (MAPK)-ERK pathways, but promoted the activation of the PI3K-AKT pathway. Furthermore, IS effectively inhibited the EGF-induced activation of the EGFR pathways. In conclusion, IS inhibited the cell viability of the A431 cells through the regulation of apoptosis. These effects were mediated, at least in part, by inhibiting the activation of the EGFR pathways.

Introduction

Non-melanoma skin cancer (NMSC), comprising predominantly of basal cell carcinoma and squamous cell carcinoma (also termed epidermoid carcinoma), is one of the most common malignancies in the United States, with more than two million novel cases annually (1). Although skin cancer may often be treated with surgery, chemotheraphy or radiation therapy, the risk of recurrence and metastasis remain a concern.

Improvements in skin cancer treatment are likely to derive from novel agents targeting the molecular pathways that promote tumor cell growth and survival. The epidermal growth factor receptor (EGFR) is an 170-kDa glycoprotein consisting of an extracellular ligand-binding domain, a transmembrane domain containing a single hydrophobic anchor sequence and an intracellular domain with tyrosine kinase activity (2). Following ligand binding, tyrosine-phosphorylated EGFR initiates the activation of downstream pathways, including Janus kinase (JAK)/signal transducer and activator of transcription (STAT), phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK) cascades (3). Activation of the EGFR downstream pathways results in cell proliferation, migration, adhesion, anti-apoptosis, angiogenesis and metastasis (4). Previously, novel therapeutic approaches targeting the EGFR superfamily and their downstream pathways were generated (4).

Herba Epimedii has been used traditionally as a medicinal herb in East Asia, including in Korea, China and Japan, to treat conditions such as hypertension, coronary heart disease, osteoporosis, menopausal syndrome, rheumatism, neurasthenia, bronchitis and hypogonadism. Icariin is the active ingredient of Herba Epimedii (5) and icariside II (IS) is a metabolite of icariin (6). A previous study demonstrated that IS was a novel...
anticancer agent that induced apoptosis in certain tumor cell lines, including PC-3 prostate cancer cells (7), U266 multiple myeloma cells (8), U937 acute myeloid leukemia cells (8,9) and A549 lung cancer cells (10) in vitro. IS has been demonstrated to inhibit the activation of the JAK-STAT3 signaling pathway in U266 and U937 cells (9). As EGFR is one of the upstream modulators of JAK-STAT, it was hypothesized that IS inhibits the activation of the EGFR signaling pathways. A431 cells are an established epidermoid carcinoma cell line that overexpresses EGFR. In the present study, we evaluated the antitumor effects of IS on A431 cells in vitro and demonstrated the possible mechanism involved in IS-induced apoptosis.

Materials and methods

Reagents and cell culture. IS (purity, >98%; Fig. 1A) was isolated from the enzymatic hydrolysis of icariin, as previously described (6). The A431 human epidermoid carcinoma cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 4 mM L-glutamine, 3.7 g/l sodium bicarbonate, 4.5 g/l glucose and 10% fetal bovine serum. Cells were maintained in a 5% CO₂ humidified incubator at 37°C. WST-8 was obtained from Dojindo Laboratories (Kumamoto, Japan); fluorescein isothiocyanate (FITC)-conjugated Annexin V was supplied by R&D Systems (Minneapolis, MN, USA); and antibodies against caspase-9, cleaved caspase-9, cleaved poly ADP ribose polymerase (PARP), cleaved PARP, EGFR, phosphorylated (P)-EGFR (Tyr1068), STAT3, P-STAT3, extracellular signal-related kinases (ERK), P-ERK, AKT, P-AKT and β-actin were obtained from Cell Signaling Technology, Inc., (Beverly, MA, USA). Human EGF was also obtained from Cell Signaling Technology, Inc. The EGFR inhibitor (AGFI478) and the PI3K inhibitor (LY294002) were supplied by Sigma-Aldrich (St. Louis, MO, USA).

Cell viability assays. IS [dissolved in dimethyl sulfoxide (DMSO)] was used for the treatment of cells. The final concentration of DMSO was <0.1% (v/v). Cell viability was measured by the WST-8 assay (Dojindo Laboratories) according to optimized manufacturer’s instructions. Briefly, A431 cells were seeded at a density of 4,000 cells/ml in 96-well culture plates in DMEM, then incubated in a humidified incubator at 37°C overnight prior to treatment with different concentrations of IS (0, 10, 25, 50 and 100 µM). Following 24 or 48 h of incubation post-treatment, 10 µl WST-8 was added to each well for 1 h. Subsequently, the optical density (OD) was measured at 450 nm. The percentage of viable cells was determined using the formula: Ratio (%) = (OD [IS] - OD (blank))/OD (control) x 100. The cell viability data were the averages of three independent experiments, each containing three replicates.

Flow cytometric analysis. Following the treatment of A431 cells with IS (0 and 50 µM) for 24 h, 1x10⁶ cells were harvested and washed once with binding buffer [Hepes buffer: 10 mM HEPES/NaOH (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂]. Following aspiration of the supernatant, the cells were resuspended in 100 µl binding buffer containing 1 µl FITC-conjugated Annexin V antibody and 5 µl PI for exactly 5 min in the dark at room temperature. The cells were then analyzed on a FACSCalibur cytometer (BD Biosciences, San José, CA, USA). The data were analyzed using FlowJo software v6.0 (Tree Star, Inc., Ashland, OR, USA).

Western blot analysis. A431 cells were treated with different concentrations of IS (0, 10, 25 and 50 µM) for 24 h, or pretreated with IS at various concentrations (0, 25, 50 and 100 µM) for 2 h prior to the treatment with EGF (100 ng/ml) for 10 min. The cells were then resuspended in lysis buffer [150 mmol/l NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mmol/l Tris-Cl (pH 8.0), 2 µg/ml aprotinin, 2 µg/ml leupeptin, 40 mg/ml phenylmethylsulfonyl fluoride and 2 mmol/l dithiothreitol] and centrifuged at 12,000 x g for 15 min to remove the nuclei and cell debris. The supernatants were immediately frozen at -80°C until use. The protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA, USA) and 30 µg cellular proteins were electrophoresed onto a polyvinylidene difluoride (PVDF) membrane following separation by 10% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated for 1 h with 5% skim milk at room temperature, followed by overnight incubation at 4°C at a 1:1,000 dilution of primary antibody against caspase-9, cleaved caspase-9, PARP, cleaved PARP, EGFR, P-EGFR (Tyr1068), STAT3, P-STAT3, ERK, P-ERK, AKT, P-AKT or β-actin. Blots were washed twice with Tris-buffered saline and Tween-20 (TBST) prior to the addition of a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Blots were washed again with TBST prior to development by enhanced chemiluminescence using Supersignal West Femto chemiluminescent substrate (Pierce, Rockford, IL, USA). Band intensities were quantified using UN-SCAN-IT gel analysis software (version 6; Silk Scientific, Orem, Utah, USA). The optical density for target protein is shown as a proportion of the β-actin optical density. The western blot analysis was repeated three times.

Statistical analysis. Data are presented as the mean ± standard deviation. Data analysis was performed by one-way analysis of variance. For the comparison of two groups, a Student's t-test was used. P<0.05 was considered to indicate a statistically significant difference.

Results

IS is cytotoxic to A431 cells in vitro. The viability of A431 cells following treatment with increasing concentrations of IS (0, 10, 25, 50 and 100 µM) for 24 or 48 h was investigated. As demonstrated by the WST-8 assay, treatment with IS resulted in significantly decreased cell viability in a dose-dependent manner (Fig. 1B). For example, treatment with 10 µM IS for 24 h did not decrease the cell viability. However, treatment with 10 µM IS for 48 h significantly decreased the cell viability compared with that at 24 h (83.3%). In addition, treatment with 100 µM IS for 24 h markedly decreased the cell viability (to 23%), while treatment with 100 µM IS for 48 h resulted in a further decrease (to 8.9%).
IS increases the apoptosis of A431 cells. To determine whether the cytotoxicity of IS occurred by apoptosis, the percentage of Annexin V-positive and PI-negative A431 cells was measured following treatment with increasing concentrations of IS (0-50 µM, Fig. 1C). Treatment with 50 µM IS for 24 h resulted in an increased number of apoptotic cells (26.2%) compared with that of the medium control group (5.3%).

IS induces apoptosis by increasing the levels of cleaved caspase-9 and cleaved PARP in the A431 cells. PARP and caspase-9 are terminal pro-apoptotic proteins (11), and are cleaved to produce the active forms. The effect of IS on the expression of PARP (Fig. 2A and B) and caspase-9 (Fig. 2C and D) was determined. Protein expression in A431 cells treated with various concentrations of IS (0, 10, 25 and 50 µM) for 24 h was measured by western blot analysis. As predicted, treatment with 50 µM IS significantly increased the expression of cleaved caspase-9 and cleaved PARP and significantly decreased the expression of caspase-9 and PARP. However, treatment with 10 and 25 µM IS for 24 h did not increase the expression of cleaved caspase-9 and cleaved PARP.

IS inhibits the activation of STAT3 and ERK but promotes the activation of AKT in A431 cells. STAT3, ERK and AKT have been demonstrated to be constitutively active in numerous types of tumors and to promote tumorigenesis by preventing apoptosis (4). Therefore, western blot analysis was conducted to determine the expression levels of STAT3, ERK and AKT and their activated (phosphorylated) counterparts. It was demonstrated that IS (at 10, 25 and 50 µM) significantly reduced the phosphorylated forms of STAT3 and that IS (at 25 and 50 µM) significantly reduced the phosphorylated forms of ERK. However, treatment with 50 µM IS significantly promoted the activation of AKT (Fig. 2E and F).

IS inhibits the EGF-induced activation of EGFR signaling pathways in A431 cells. A431 cells are an established epidermoid carcinoma cell line, that overexpresses EGFR. EGF activates EGFR signaling pathways, including JAK-STAT and MAPK-ERK. Western blot analyses demonstrated that EGF (100 ng/ml, 10 min) induced significant increases in the expression of P-EGFR, P-STAT3 and P-ERK in A431 cells, compared with that of the medium control. IS (50 and 100 µM) pretreatment for 2 h resulted in significant decreases in the expression of P-EGFR (Fig. 3A and B) and P-STAT3 (Fig. 3C and D), compared with that of the EGF alone group. Only pretreatment with 100 µM IS inhibited the EGF-induced activation of ERK (Fig. 3C and D). In addition, IS (at 50 and 100 µM) pretreatment significantly inhibited the EGF-induced decreases in EGFR.

IS-induced decreases in cell viability are partially reversed by LY294002, EGF and AG1478. As demonstrated by the WST-8
Figure 3. Icariside II (IS) induces apoptosis via inhibition of EGFR in A431 cells.

**Figure 2.** Icariside II (IS) promotes the activation of cleaved poly ADP-ribose polymerase (PARP) and caspase-9, and inhibits the activation of signal transducer and activator of transcription 3 (STAT3) and extracellular signal-regulated kinase (ERK) in the A431 cells. Western blot analysis was performed for (A and B) PARP and cleaved PARP; (C and D) caspase-9 and cleaved caspase-9; and (E and F) AKT, STAT3 and ERK, and their phosphorylated (active) counterparts (P-AKT, P-STAT and P-ERK, respectively) to measure the changes in their expression in the A431 cells, following treatment with various concentrations of IS (0, 10, 25 and 50 µM) for 24 h. All lanes were loaded with 30 µg protein and β-actin was used as the loading control. Band intensities were quantified as described in Materials and methods. The optical density (OD) for target protein was demonstrated as a proportion of the β-actin OD. "**"P<0.01 and "*"P<0.05, compared with the medium control group.

**Figure 3.** Icariside II (IS) inhibits epidermal growth factor (EGF) induced activation of the EGF receptor (EGFR) signaling pathways in A431 cells. The A431 cells were pretreated with various concentrations of IS (0, 25, 50 and 100 µM) for 2 h, followed by treatment with EGF (100 ng/ml) for 10 min. The EGFR inhibitor (AG1478, 20 µM) was used as a positive drug control. Protein expression levels of (A and B) EGFR, phosphorylated (P)-EGFR; and (C and D) signal transducer and activator of transcription (STAT3), P-STAT3, extracellular signal-regulated kinase (ERK), and P-ERK were detected by western blot analysis. All lanes were loaded with 30 µg protein and β-actin was used as the loading control. Band intensities were quantified as described in Materials and methods. The optical density (OD) of the target protein is shown as a proportion of the β-actin OD. **"P<0.01 and "*"P<0.05, compared with the EGF alone group.
Recently, novel therapeutic approaches demonstrated that IS activated the PI3K-AKT signaling pathway in A431 cells. Constitutive activation of PI3K-AKT is required for IS to induce apoptosis. In addition, this study demonstrated that the apoptotic effect of IS was dependent on JNK2 and JNK3 and p38 MAPK. A previous study has demonstrated that IS inhibits the activation of the EGFR signaling pathways. The present study demonstrated that IS inhibited the EGF-induced activation of JAK-STAT and MAPK-ERK, it was hypothesized that IS may have partially reversed the IS-induced inactivation of JAK-STAT and MAPK-ERK (10). In the present study, it was demonstrated that IS induced apoptosis via the activation of caspase-9 and PARP. In conclusion, IS significantly inhibited the cell viability of A431 cells (P<0.01), compared with the medium control. P<0.05, compared with the 50 µM IS alone group.

Discussion

IS is obtained from *Epimedium* plants. This flavonol glycoside has been demonstrated to have apoptotic potential against PC-3 prostate cancer cells (7), U266 multiple myeloma cells (8), U937 acute myeloid leukemia cells (8,9) and A549 lung cancer cells (10) *in vitro*. In the present study, similar results with A431 epidermoid carcinoma cells were demonstrated, suggesting that IS induces apoptosis in tumor cells in general. In addition, IS-induced apoptosis via the activation of caspase-9 and PARP in A431 cells was also observed in this study.

Constitutive activation of several signaling pathways, such as JAK-STAT, MAPK, PI3K-AKT and nuclear factor-κB (NF-κB) have been determined to confer survival and proliferative advantages to tumor cells (12,13). IS has been demonstrated to inhibit the activation of the JAK-STAT3 signaling pathway in U266 and U937 cells (9). Similar results with A431 epidermoid carcinoma cells were observed in the present study. The MAPK family consists of three predominant members: ERKs (ERK1 and ERK2), c-Jun NH2-terminal kinases (JNK1, JNK2 and JNK3) and p38 MAPK (14). A previous study has demonstrated that the apoptotic effect of IS was dependent on the activation of JNK and p38 MAPK in A549 cells, which was almost completely inhibited by SB203580 (an inhibitor of p38 MAPK) and SP600125 (an inhibitor of JNK) (10). In the present study, it was demonstrated that IS induced apoptosis via the inactivation of MAPK-ERK. Thus, the activation of JNK and p38, as well as the inactivation of ERK, may be required for IS to induce apoptosis. In addition, this study demonstrated that IS activated the PI3K-AKT signaling pathway in A431 cells. Constitutive activation of PI3K-AKT is involved in the survival of tumors; therefore, the upregulation of PI3K-AKT may not be beneficial in the induction of tumor apoptosis (15). However, the present study demonstrated that pretreatment with LY294002, a PI3K inhibitor, partially reversed IS-induced decreases in cell viability. A previous study indicated that the PI3K-AKT pathway negatively regulates STAT-transcriptional activities in tumor cells, suggesting that a mechanism for the effects of IS may be AKT activation, which induces JAK-STAT signaling (16).

EGFR is closely associated with various tumors of epithelial origin, including breast (17), colon (18), ovarian (19) and lung (20) cancer. Recently, novel therapeutic approaches targeting the EGFR and its downstream pathways have been demonstrated. In addition, certain natural products, including rhein (21), magnolol (22), vicenin (23) and shikonin (24), have been shown to induce tumor apoptosis via inhibition of the EGFR signaling pathways. IS is a metabolite of icarin, which is derived from Herba Epimedii (6), and has been demonstrated to inhibit the activation of JAK-STAT and MAPK-ERK signaling pathways. As EGFR is one of the upstream modulators of JAK-STAT and MAPK-ERK, it was hypothesized that IS inhibits the activation of the EGFR signaling pathways. The present study demonstrated that IS inhibited the EGFR-induced activation of the EGFR, JAK-STAT and MAPK-ERK pathways in A431 cells.

It was also determined that pretreatment with EGF (20 ng/ml) and AG1478 (1 µM) partially reversed the IS-induced decreases in cell viability. Therefore, pretreatment with EGF may have partially reversed the IS-induced inactivation of EGFR and its downstream survival pathways. Alternatively, IS may have a lower binding affinity for EGFR than that of AG1478. Thus, pretreatment with AG1478 decreased the level of IS available to bind to EGFR.

In conclusion, IS significantly inhibited the cell viability of A431 cells *in vitro* through the regulation of apoptosis. These effects were mediated, at least in part, by inhibiting the activation of the EGFR signal transduction pathways.

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References


