Eriocalyxin B blocks human SW1116 colon cancer cell proliferation, migration, invasion, cell cycle progression and angiogenesis via the JAK2/STAT3 signaling pathway

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Abstract. Eriocalyxin B, a natural ent-kaurene diterpene compound, has been shown to prevent carcinogenesis and tumor development. However, little is known regarding the mechanism underlying the antitumor activity of Eriocalyxin B in human colon cancer. The aim of the present study was to examine the role of Eriocalyxin B in SW1116 cells, and to verify the hypothesis that the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling pathway may serve as a therapeutic target in human colon cancer treatment. Cell proliferation was measured with a Cell Counting kit-8 assay, and the cell cycle was assessed by flow cytometry. Cell migration and invasion were measured by Transwell analysis. In addition, western blot analysis was performed to detect the protein expression levels in SW1116 cells treated with various concentrations of Eriocalyxin B. The results demonstrated that 1 µmol/l Eriocalyxin B was effective at inhibiting JAK2 and STAT3 phosphorylation, followed by the downregulation of JAK2 and STAT3 downstream target expression, which resulted in the inhibition of cell proliferation, migration, invasion and angiogenesis. Eriocalyxin B also suppressed the expression of proliferation-associated protein (proliferating cell nuclear antigen) and angiogenesis-associated proteins (vascular endothelial growth factor and vascular endothelial growth factor receptor 2), as well as that of migration- and invasion-associated proteins (matrix metalloproteinase 2 and 9). These results suggested that Eriocalyxin B may suppress JAK2/STAT3 signaling, and thus act as a therapeutic or preventive agent in the treatment of human colon cancer.

Introduction

Signal transducer and activator of transcription 3 (STAT3) protein is a member of the STAT protein family. It acts as a cytoplasmic transcription factor and is phosphorylated by cytokines and growth factors, which results in its translocation from the cell surface to the nucleus. Ligand-bound cell surface receptors regulate tyrosine phosphorylation of the STAT3 protein through Janus kinase (JAK) and growth factor receptor tyrosine kinases (1). This process is rapid and transient during STAT3 protein activation in normal cells. However, abnormalities in the JAK/STAT signaling pathways have been demonstrated to be involved in the development and progression of several types of cancer (2,3).

STAT3 activation is observed in the majority of human colon cancer cell lines (4,5). However, the mechanisms underlying dysregulated STAT3 signaling initiation in the progression of human colon cancer have yet to be elucidated. STAT3 is known to promote the progression of cancer following activation by various pathways; however, a tumor suppressive role of STAT3 has recently been demonstrated, depending on the mutational background of the tumor (6,7). Colon cancer is the sixth most common cancer type and the fifth leading cause of cancer-associated mortality in China (8). Since dietary characteristics have changed in recent years, the mortality associated with colon cancer has been estimated to be 608,000 people per year worldwide (9). However, early screening, diagnosis and development of chemotherapy may not be feasible in elderly patients with human colon cancer with a poor prognosis, due to its high toxicity and adverse reactions. Therefore, it is necessary to identify novel treatment agents with less severe side effects.

Numerous drugs used worldwide are extracted from natural plant species, and are used for the clinical treatment of patients with cancer (10,11). Eriocalyxin B is a natural ent-kaurene diterpenoid compound extracted from Isodon eriocalyx var. laxiflora, a herb of the Labiatae family, distributed throughout Southwest China. It is reported to exhibit anti-inflammatory and antibacterial functions in local folk medicine, by modulating a variety of biological progresses via multiple signaling pathways (11,12). Furthermore, Eriocalyxin B inhibited proliferation and induced apoptosis in several types of cancer cells,
including ovarian cancer (13), pancreatic adenocarcinoma (14) and leukemia (15) cells. Eriocalyxin B was observed to arrest the cell cycle, induce apoptosis, and inhibit cancer invasion. However, the mechanisms underlying these anticancer effects remain under investigation. The present study aimed to investigate the molecular mechanisms underlying Eriocalyxin B induction of apoptosis and migration inhibition, as well as invasion in SW1116 human colon cancer cell lines.

Materials and methods

Reagents. Eriocalyxin B was purchased from BioBioPha Co., Ltd. (Kunming, China). Fetal bovine serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China), and Dulbecco's modified Eagle's medium and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, USA). Primary antibodies targeting STAT3 (rabbit polyclonal; cat. no. ab119352; 1:1,000), phosphorylated (p)-STAT3 (rabbit polyclonal; cat. no. ab76315; 1:1,000), vascular endothelial growth factor (VEGF; mouse polyclonal; cat. no. ab68334; 1:200), vascular endothelial growth factor receptor 2 (VEGFR2; rabbit monoclonal; cat. no. ab131441; 1:500), proliferating cell nuclear antigen (PCNA; mouse polyclonal; cat. no. ab92552; 1:5,000), matrix metalloproteinase (MMP)-2 (rabbit polyclonal; cat. no. ab110186; 1:1,000), and MMP-9 (mouse polyclonal; cat. no. ab119906; 1:500) were purchased from Abcam (Cambridge, MA, USA), and those targeting glyceraldehyde 3-phosphate dehydrogenase (GAPDH; rabbit monoclonal; cat. no. 5174; 1:1,500), JAK2 (rabbit monoclonal; cat. no. 3230; 1:1,000), and p-JAK2 (rabbit monoclonal; cat. no. 3776; 1:800), were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase-conjugated goat anti-rabbit/anti-mouse secondary antibodies (cat nos. A0208 and A0216; 1:1,000) were purchased from Beyotime Institute of Biotechnology (Haimen, China).

Cell culture. Human SW1116 colon cancer cell lines were obtained from the Academia Sinica Cell Bank (Shanghai, China), and cultured to 80% confluence in low-glucose DMEM supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin and 10 mg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cell cultures were incubated in an atmosphere containing 5% CO₂ at 37°C for 72 h.

Cell proliferation assay. The proliferative effects of Eriocalyxin B on the SW1116 cells were determined using a Cell Counting kit-8 assay (Dojindo Molecular Technologies, Inc., Rockville, MD, USA) as previously described (16). Briefly, the SW1116 cells in logarithmic growth-phase were harvested and cultured in 96-well plates in 100 µl volumes. The cells were then treated with various concentrations (0.2, 0.5 and 1 µmol/l) of Eriocalyxin B, and incubated for 0, 6, 12, 24 and 48 h at 37°C. Cells that were not treated with Eriocalyxin B served as a control group. Absorbance at a wavelength of 450 nm was measured using a Multiskan EX plate reader (Thermo Fisher Scientific, Inc.).

Cell cycle analysis by flow cytometry. For cell cycle analysis, the SW1116 cells were seeded in 12-well plates at a density of 1x10⁶ cells/well, prior to being treated with various concentrations of Eriocalyxin B (0.2, 0.5 and 1 µmol/l) for 48 h. Following treatment, the number of cells in the various phases of cell cycle was determined by PI staining, in order to ascertain the DNA content (17). Cells that were not treated with Eriocalyxin B served as a control group. Data acquisition was performed using an EPICSXL-MCL flow cytometer (Beckman Coulter, Inc. Brea, CA, USA) using Cell Quest software BD Accuri C6 version 1.0.264.21 (BD Biosciences, Franklin Lakes, NJ, USA).

Migration and invasion assay. The migration and invasion of the SW1116 cells were measured using a Transwell assay as previously described (18). Briefly, Transwell membranes (Corning Incorporated, Corning, NY, USA) coated with or without Matrigel (BD Biosciences) were used to measure cell migration and invasion ability. The SW1116 cells were treated with various concentrations of Eriocalyxin B (0.2, 0.5 and 1 µmol/l). The chambers were subsequently placed in a 37°C incubator for 48 h. The filters were fixed with 4% methanol and stained with 0.5% methylrosanilinum chloride solution (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The number of migratory and invasive cells was performed under a microscope (x200; CX41RF; Olympus Corporation, Tokyo, Japan).

Western blot analysis. Following treatment with Eriocalyxin B at the desired concentrations for the appropriate times, the expression levels of proliferation proteins (PCNA), migration and invasion proteins (MMP-2 and MMP-9) and angiogenesis-associated proteins (VEGF and VEGFR2) were detected by western blotting according to the manufacturer's instructions. Cells untreated with Eriocalyxin B served as a control group. GAPDH antibody was used as an internal control. The experiment was repeated three times independently. Briefly, the SW1116 cell lysates were harvested and protein was extracted using radioimmunoprecipitation buffer (JRDUN Biotechnology Co., Ltd., Shanghai, China) for 30 min. Untreated cells served as a control group. Evaluation of the number of migratory and invasive cells was performed under a microscope (x200; CX41RF; Olympus Corporation, Tokyo, Japan).

Figure 1. Eriocalyxin B inhibited the proliferation of SW1116 cells. The cells were treated with Eriocalyxin B (0.2, 0.5 and 1 µmol/l) for 0, 6, 12, 24 or 48 h, and cell proliferation was determined using a Cell Counting kit-8 assay. Each bar represents the mean ± standard deviation of three independent experiments. *P<0.05 or **P<0.01, compared control.
membranes were subsequently incubated with the primary antibodies for 2 h at 25°C, prior to being incubated with secondary antibodies for 1 h at 37°C, and were subsequently washed three times with Tris-buffered saline with Tween 20. The blots were visualized using enhanced chemiluminescence (EMD Millipore, Billerica, MA, USA), and the signals were quantified by densitometry using Quantity One software version 4.62 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. The data are presented as the mean ± standard deviation. One-way analysis of variance followed by Dunnett's test was used to analyze significant differences between results. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

**Eriocalyxin B inhibits the proliferation of SW1116 cells.** SW1116 cells were serum-starved overnight and subsequently cultured with or without various concentrations of Eriocalyxin B for 0, 6, 12, 24 and 48 h. Eriocalyxin B inhibited
the proliferation of SW1116 cells in a time- and dose-dependent manner. Following initial experimentation, Eriocalyxin B at dose of 1 µmol/l significantly inhibited cell proliferation, compared with Eriocalyxin B at dose of 0.2 µmol/l, and significant differences in cell proliferation occurred between 0.2, 0.5 and 1 µmol/l concentrations at 48 h (P<0.05 and P<0.01; Fig. 1).

Eriocalyxin B arrests the cell cycle of SW1116 cells. The cell cycle distribution of SW1116 cells was analyzed by flow cytometry following 48 h exposure to Eriocalyxin B at various concentrations (0.2, 0.5 and 1 µmol/l). The number of cells in the G<sub>0</sub>-G<sub>1</sub> phase following Eriocalyxin B treatment increased to 64.49% following 48 h of incubation. The number of cells in the S phase decreased to 12.13% following 48 h of incubation.

Figure 4. Eriocalyxin B inhibits SW1116 cell invasion, as demonstrated by a Matrigel invasion assay. SW1116 cells in serum-free medium with or without Eriocalyxin B were seeded into the upper chamber of the Transwell system. The bottom well was filled with Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Following 48 h incubation, the cells that had invaded through the Matrigel membrane were stained with 0.5% methylrosanilin chloride solution, and counted under a light microscope (x200). The experiments were performed three times in triplicate. Each bar represents the mean ± standard deviation of three independent experiments. *P<0.01, compared with control cells.

Figure 5. Effects of Eriocalyxin B treatment on the protein expression levels of p-JAK2, p-STAT3, MMP-2, MMP-9, PCNA, VEGF and VEGFR2. (A) The SW1116 cells were treated with various concentrations of Eriocalyxin B for 6 h and (B) 48 h. The protein expression levels were quantified by western blotting. Equal quantities of total cellular protein (20 µg) were separated by 10-15% SDS-PAGE and GAPDH was used as a loading control. Each bar represents the mean ± standard deviation of three independent experiments. *P<0.05 and *P<0.01, compared with control cells. p, phosphorylated; JAK2, Janus kinase 2; STAT3, signal transducer and activator of transcription 3; MMP, matrix metalloproteinase; PCNA, proliferating cell nuclear antigen; VEGF, vascular endothelial growth factor 2; VEGFR2, vascular endothelial growth factor receptor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
In the control group, the number of cells in the G₁-G₂ and S phase were 49.57 and 24.58%, respectively (Fig. 2). No significant difference was observed in the number of cells in the G₂-M phase.

Eriocalyxin B inhibits the migration of SW1116 cells. The migratory inhibition of SW1116 cells by Eriocalyxin B was analyzed using a Transwell assay, the results of which are presented in Fig. 3. Eriocalyxin B significantly inhibited SW1116 cell migration; however, this effect was not observed at 0.2 µmol/l Eriocalyxin B (P<0.01).

Eriocalyxin B inhibits the invasion of SW1116 cells. The invasion inhibition of the SW1116 cells by Eriocalyxin B was measured using a Matrigel-based Transwell assay, and the results are presented in Fig. 4. Eriocalyxin B significantly inhibited SW1116 cell invasion (P<0.01), however the effect of Eriocalyxin B at 0.2 µmol/l was not observed. These results clearly suggest that Eriocalyxin B targets SW1116 cells by exhibiting antimigratory and anti-invasive effects.

Eriocalyxin B effects the expression levels of SW1116 cell proteins. The effects of Eriocalyxin B on the JAK2/STAT3 signaling pathway and tumorigenesis-associated proteins in the SW1116 cells following treatment with Eriocalyxin B were determined by western blotting. Eriocalyxin B reduced the expression levels of p-JAK2 and p-STAT3 in SW1116 cells (P<0.05 and P<0.01; Fig. 5A), however the expression levels of JAK2 and STAT3 remained unaltered by Eriocalyxin B treatment. The expression levels of MMP9, MMP2, PCNA, VEGF and VEGFR2 were significantly decreased, as compared with the corresponding control groups (P<0.05 and P<0.01; Fig. 5B). These effects may result in the inhibition of proliferation, migration, invasion and angiogenesis in SW1116 cells following exposure to 1 µmol/l Eriocalyxin B.

Discussion

Human colon cancer is the fourth most common type of cancer in men and, the third most common in women worldwide, accounting for ~8% of all cancer-associated mortality (19,20). To date, to the best of our knowledge, no study has explored the effects of Eriocalyxin B on the development and progression of human colon cancer. Several studies have reported that modulation of constitutive JAK2/STAT3 activity via genetic and pharmacological approaches, demonstrated the role of abnormal activity of JAK2/STAT3 in malignant transformation and tumor progression, and thus suggested that JAK2/STAT3 may serve as a novel cancer drug target (21,22).

Eriocalyxin B suppressed cell proliferation, invasion, metastasis, and angiogenesis through a mechanism that has yet to be fully elucidated (23-25). Numerous genes regulated by signal transducers and transcription activators were involved in these processes, therefore it was hypothesized that Eriocalyxin B functions in human colon cancer by modulating JAK2/STAT3 activation. The present study demonstrated that Eriocalyxin B inhibited SW1116 cell proliferation by inhibiting the cell cycle at the G₁-G₂ phase and decreasing the number of cells in the S phase. In addition, no significant differences were observed in the number of cells in the G₂-M phases. Furthermore, the protein expression levels of PCNA were significantly downregulated following SW1116 cell treatment with various concentrations of Eriocalyxin B for 48 h. PCNA is overexpressed in numerous types of tumor regulated by STAT3, and is required for cells to proliferate (26).

The findings of the present study demonstrated that Eriocalyxin B inhibited SW1116 cell migratory and invasive abilities, predominantly via the downregulation of gene product expression, specifically of genes involved in cell migration, invasion and angiogenesis, such as MMP-2, MMP-9, VEGF and VEGFR2, which are regulated by STAT3. MMP-2 and MMP-9 have important roles in human colon cancer progression, tumor migration, invasion and angiogenesis (27). A previous study reported that the upregulation of MMP-2 and MMP-9 expression is associated with poor survival prognosis in patients with human colon cancer, which resulted from degradation of the extracellular matrix, and induction of cell metastasis (28). Angiogenesis is critical for cancer growth and progression. VEGF is a potent angiogenic growth factor in human colon cancer, and inhibition of VEGF resulted in inhibition of angiogenesis and cancer growth (29). VEGFR2 activation is closely associated with tumor- and vessel-mediated processes in human colon cancer, and is responsible for tumor survival, as well as tumor growth and metastasis (30).

To further investigate the mechanism underlying Eriocalyxin B-induced JAK2/STAT3 signaling inhibition in SW1116 cells, the present study further examined the proteins upstream of STAT3. JAK2 is considered to be associated with STAT3 activation. Phosphorylation of JAK2 was suppressed by treatment with Eriocalyxin B in the SW1116 cells. These results suggested that Eriocalyxin B exerts its inhibitory effects on STAT3 activation by inhibiting the activation of JAK2. Activation of the STAT3 signaling pathway has been observed in numerous patients with cancer (31). Upregulated levels of p-STAT3 expression is associated with poor survival rates in patients with colon (32), breast (33) and gastric cancer (34). A previous study reported a correlation between STAT3 pathway activation and high clinicopathological grade with advanced stage in a variety of cancer types (35). These results suggest that the JAK2/STAT3 signaling pathway may function as a therapeutic target and a prognostic marker in patients with colon cancer (36).

In conclusion, to the best of our knowledge, prior to the present study no research had been conducted on the role of Eriocalyxin B in SW1116 human colon cancer cells, nor its JAK2/STAT3 signaling protein targets. The present study therefore reports for the first time the anticancer mechanism underlying the effects of Eriocalyxin B in SW1116 cell tumorigenesis and colon cancer progression. It was demonstrated that this predominantly occurred by inhibiting the activation of the JAK2/STAT3 signaling pathway. Therefore, the JAK2/STAT3 signaling pathway has a significant role in human colon cancer oncogenesis, and may serve as a potential therapeutic target for human colon cancer treatment.

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References


