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Cepharanthine induces autophagy in colorectal cancer cells

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Abstract

Cepharanthine (CEP), an alkaloid derived from *Stephania cepharantha Hayata*, exhibits anti-cancer activities in various types of cancer. Colorectal cancer ranks as the third most common cancer worldwide and the third leading cause of cancer-related deaths among both men and women. Previous studies have reported that autophagic cell death serves as an alternative target for overcoming tumor resistance. This study aims to evaluate the anti-cancer effects of CEP on autophagy in colorectal cancer cells. The results showed that CEP significantly inhibited the growth of colorectal cancer cells (with an IC50 of 43.82 μM in HCT-116 cells and 12.08 μM in HT-29 cells at 24 hours). CEP induced autophagy by enhancing autophagic flux and the expression of autophagic-related proteins, including LC3-II, ATG5, and beclin-1. These changes correlate with an elevated expression of AMP-activated protein kinase (AMPK), an upstream activator of the autophagy pathway, in both cell lines. Our findings suggest that CEP could be a potential adjuvant chemotherapy treatment for colorectal cancer. These data support further in vivo investigations to establish the potential clinical applicability of CEP.

Keywords: Colorectal cancer; Autophagy; Cepharanthine

Introduction

Colorectal cancer (CRC) is the most common gastrointestinal cancer (1). As of 2023, the American Cancer Society reports that colorectal cancer ranks as the third most common cancer worldwide and is the third leading cause of cancer-related deaths among both men and women (2). Common treatments for colorectal cancer include surgery, radiation, chemotherapy, and targeted therapy. Although these treatments are effective, some patients do not benefit optimally due to drug resistance, side effects, and recurrence. Consequently, there remains a need for the development of novel anticancer agents to increase treatment options for colorectal cancer patients.

Autophagy, a cellular self-degradation process, serves to eliminate aggregated proteins and damaged organelles (3). This mechanism plays important roles in various physiological and pathological processes, including cellular homeostasis, apoptosis, and cancer development (4). It has been reported that induction of autophagy-associated cell death may be beneficial in cancer therapy (5), particularly in combating

chemotherapeutic-resistant cancer cells (6). In addition, autophagy is closely related to apoptosis (7). Previous studies have reported that epigallocatechin-3-gallate increases the sensitivity of colorectal cancer cells to radiation by inhibiting cell proliferation and inducing autophagy (8).

Cepharanthine (CEP), a natural compound, extracted from the roots of the *Stephania cepharantha Hayata* plant, has demonstrated anti-cancer effects in various cancers such as hepatocellular carcinoma, myeloma, oral squamous cell carcinoma, and lung cancer. These effects are attributed to its ability to inhibit cell proliferation and induce both apoptotic and autophagic cell death (9-13). Previous studies have reported that CEP inhibits the growth of human colorectal cancer cells via cell cycle arrest and apoptosis by increasing ROS generation and decreasing anti-apoptotic Bcl-2 protein expression (14). It has been reported that combined treatment of CEP and 5-FU inhibits the growth of p-53 mutant human colorectal cancer cells by apoptosis (15). In addition, CEP induces autophagy through the upregulation of LC3-II protein, resulting in the inhibition of cell proliferation and migration in lung adenocarcinoma cells (16) and breast cancer cells (17).

Although apoptosis-inducing effects of CEP have been extensively studied in various types of cancer, the effects of CEP on autophagy have not been thoroughly studied in colorectal cancer cells. The present study aims to elucidate the effects of CEP on autophagy in human colorectal cancer cells as well as its underlying mechanisms of action.

Materials and Methods

Materials and Reagents

Cepharanthine (CEP) was obtained from Abcam (Cambridge, UK). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), Roswell Park Memorial Institute (RPMI) 1640 medium, and penicillin-streptomycin were obtained from Gibco (New York, USA). Dimethyl sulfoxide (DMSO) and Thiazolyl blue tetrazolium bromide (MTT) were obtained from Sigma (Missouri, USA). CYTO-ID® Autophagy detection kit was obtained from Enzo Life Sciences (New York, USA).

Cell culture

Human colorectal cancer cell lines, HCT-116 and HT-29, were obtained from the American Type Culture Collection (Manassas, VA, USA). HT-29 cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin. HCT-116 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin. Cultures were maintained at 37°C in a humidified chamber with 5% CO_2.

Cell viability assay

Cell viability was determined using a 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. Initially, cells were seeded into a 96-well plate at a density of $5x10^3$ cells/well and incubated overnight. Subsequently, the cells were treated with various concentrations of cepharanthine (5, 10, 20, 40, and 80 μM) for 24 hours. After incubation, MTT solution was added to each well and incubated for an additional 4 hours. Then, the supernatants were carefully aspirated and 50 μl of DMSO was added to dissolve the purple formazan crystals. The absorbance was measured at wavelengths of 570 and 650 nm. using a microplate reader (Bio Tek, USA). Half maximal inhibitory concentration (IC_{50}) value was calculated by GraphPad Prism (GraphPad Software, USA; Version 9.0)

Autophagic flux detection

Autophagic flux, representing the entire process of autophagy, includes autophagosome formation, maturation, and fusion with lysosomes. Following CEP treatment, cells were stained with CYTO-ID® Green autophagy detection kit (Enzo Life Sciences) according to the manufacturer's protocol. Briefly, cells were washed twice with 1x Phosphate buffer saline (PBS) and stained with CYTO-ID® Green dye, diluted in 1x assay buffer (1:1000) at room temperature for 30 minutes in the dark. Following the incubation, cells were washed twice with assay buffer and an additional 500 μl of 1x assay buffer was added. A total of 10,000 events was immediately analyzed per sample and mean fluorescence was recorded at FL1 peak emission values at wavelength 480 nm. using a BD LSR II flow cytometer (BD Biosciences, USA).

Western blot analysis

Following CEP treatment, cells were washed with 1x PBS and lysed with lysis buffer (150 mM NaCl, 50 mM Tris-base, 0.5% NP-40). After centrifugation, protein samples in the lysate supernatants were collected, and measured the concentration by DC protein assay (Bio-Rad, USA). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After that, the membrane was blocked with 5% non-fat milk for 2 hours prior to incubation of specific primary antibodies to LC3-I/LC3-II, ATG5, beclin-1, and AMPK (Cell signaling, Technology, USA) overnight at $4^{\circ}C$, following by incubating in an appropriate HRP-linked secondary antibody (Cell signaling, Technology, USA) for 1 hour at room temperature. Protein bands were detected using Clarity Western ECL substrate (Bio-Rad, USA) and analyzed using a C-Digit Blot Scanner (LI-COR, USA). GAPDH was used as the loading control for protein normalization.

Statistical analysis

All data are expressed as mean ± standard error of mean (SEM) and are representative of three independent experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Turkey's post-hoc test. A p-value less than 0.05 was considered to indicate a statistically significant difference. Data was analyzed using GraphPad Prism (GraphPad Software, USA; Version 9.0).

Results

CEP reduced the viability of colorectal cancer cells

The cytotoxic effects of CEP were examined on human colorectal cancer cell lines, HCT-116 and HT-29 using MTT assay. Cells were treated with various concentrations of CEP (5, 10, 20, 40, and 80 μM) for 24 hours. As shown in Figure 1. CEP significantly reduced the cell viability of both cell lines in a concentration-dependent manner. The IC_{50} values at 24 hours were 43.82 μM in HCT-116 and 12.08 μM in HT-29. These results highlight that CEP inhibited the growth of CRC cells.

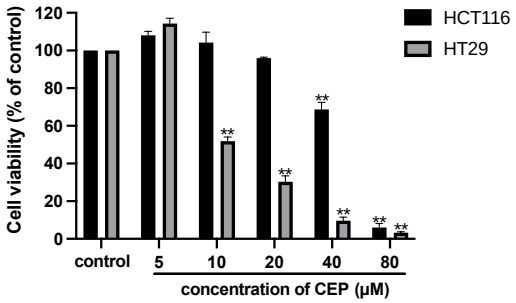

Figure 1. Cytotoxicity effects of CEP on colorectal cancer cells. HCT-116 and HT-29 cells were treated with various concentrations of CEP for 24 hours and cell viability was analyzed by MTT assay. **p ≤ 0.01 vs. control.

CEP induced autophagy in colorectal cancer cells

Autophagy is the process of maintaining cell metabolism. Numerous studies reported that autophagy can act to suppress tumor growth in several types of cancers including colorectal cancer (18). Previous studies have reported that CEP induces autophagy in lung cancer cells and breast cancer cells (16, 17). The conversion of LC3-I to LC3-II is an important process of autophagy and the increase of LC3-II is a key marker of autophagic activity. Beclin-1 and ATG5 are also autophagy-related proteins involved in forming the autophagosome (19). We therefore determined the effect of CEP on autophagy in colorectal cancer cells by measuring autophagic flux and the expression of autophagy-related proteins. The results demonstrated that CEP, at a concentration of 40 μM, significantly increased autophagic flux (Figure 2A), accompanied by an increase

in LC3-II and ATG5 protein expression (Figure 2B) in HCT-116 cells. Similarly, CEP at a concentration of 10 μM significantly increased autophagic flux (Figure 3A), accompanied by an increase of LC3-II and beclin-1 protein expression (Figure 3B) in HT-29 cells. Together, these findings underscore that CEP induced autophagy in colorectal cancer cells through the upregulation of autophagic-related protein expression.

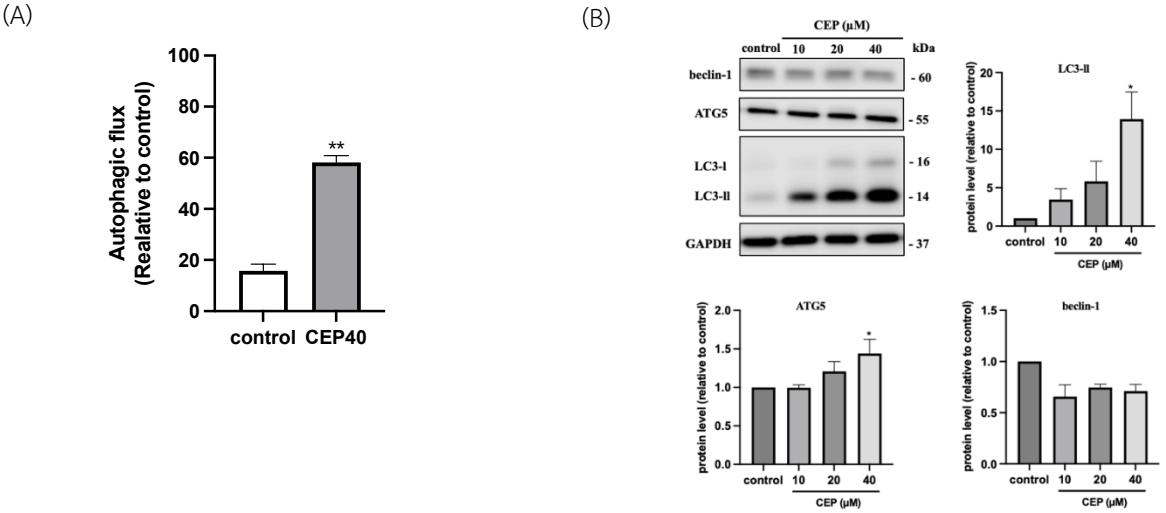

Figure 2. CEP effectively induced autophagy in HCT-116 cells. HCT-116 cells were treated with CEP at 10, 20, and 40 μM for 24 hours and autophagic flux was assessed by Flow cytometry (A) and the expression of LC3-II and ATG5 proteins were measured using Western blot analysis (B). *p≤ 0.05 and **p ≤ 0.01 vs. control.

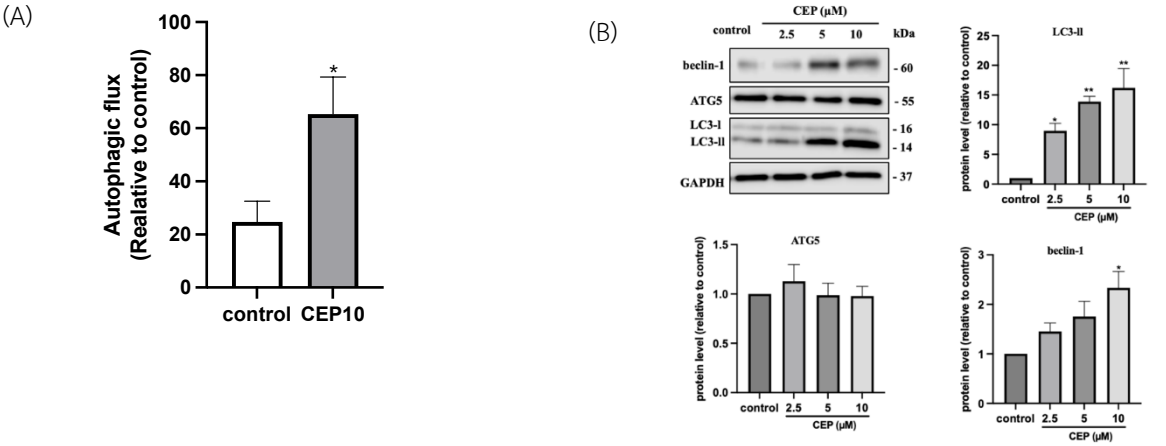

Figure 3. CEP induced autophagy in HT-29 cells. HT-29 cells were treated with CEP at 2.5, 5, and 10 μM for 24 hours and autophagic flux was determined by Flow cytometry (A) and the expression of LC3-II and beclin-1 proteins were measured by Western blot analysis (B) * p ≤ 0.05 and ** p ≤ 0.01 vs. control.

CEP induced autophagy by activating the AMPK pathway

The adenosine monophosphate-activated protein kinase (AMPK) pathway plays a pivotal role in maintaining the cellular homeostasis (20) and regulating autophagy (21). We therefore investigate the activation status of the AMPK pathway in colorectal cancer cells following CEP treatment. As shown in Figure 4, the phosphorylation of AMPK significantly increased after CEP treatment in both cell lines. These results strongly suggest that CEP induces autophagy by activating the AMPK pathway in colorectal cancer cells.

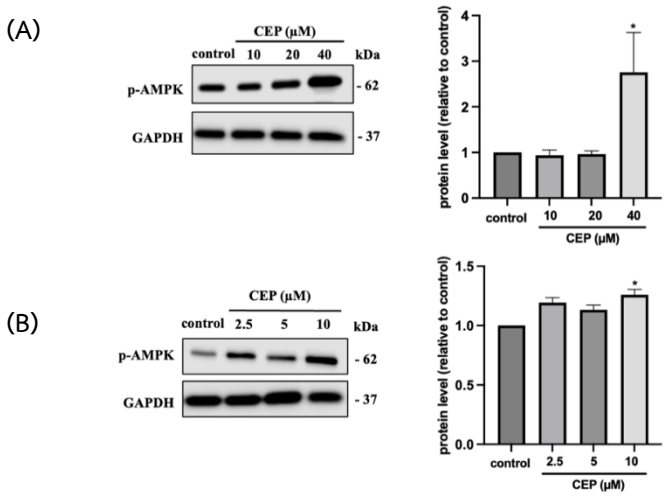

Figure 4. CEP induced autophagy through the AMPK activation in HCT-116 and HT-29 cells. HCT-116 cell was treated with CEP at 10, 20, and 40 μM for 24 hours. HT-29 cells were treated with CEP at 2.5, 5, and 10 μM for 24 hours. The expression of phospho-AMPK proteins in the presence of 40 μM CEP in HCT-116 cells (A) and in the presence of 10 μM CEP in HT-29 cells (B) was measured by Western blot analysis. * $p \leq 0.05$ vs. control.

Discussions

Cepharanthine (CEP), derived from the *Stephania cepharantha Hayata,* is approved in Japan for treating various diseases such as radiation leukopenia, alopecia aerates, and xerostomia. CEP has multiple pharmacological effects including, anti-inflammatory, anti-oxidant, anti-viral, and anti-cancer properties (22). CEP exhibits efficacy against various cancers, including lung cancer, breast cancer, ovarian cancer, and colorectal cancer (17, 23, 24). Previous studies have reported that CEP could inhibit the growth of breast cancer cells containing p53 mutant in MDA-MB-231 cells more than p53 wild-type in MCF-7 cells (17). We also found that CEP showed more cytotoxicity in p53-mutant HT-29 cells than in p53 wild-type HCT-116 cells. Taken together, these findings suggest that CEP exhibits varying degrees of cytotoxicity, depending on p53 status.

Autophagy is known as programmed cell death Type II (25). Autophagy plays a role in various physiological and pathological processes including, cellular homeostasis, apoptosis, and cancer (4). However, the role of autophagy in cancer seems to be more complex, depending on tumor type and stages (26). Autophagy serves as an alternative cell death mechanism and holds promise as a target for anticancer therapy (27), particularly in overcoming chemotherapy-resistant cancer cells (28). Previous studies have reported that CEP inhibits the growth of breast cancer and lung cancer cells via autophagy by increasing the LC3-II expression (16, 17). Similar to previous studies, CEP induced autophagy by increasing the expression of autophagic-related proteins, including LC3-II, ATG5, and beclin-1 in CRC cells.

Adenosine monophosphate-activated protein kinase (AMPK) pathway plays crucial roles in maintaining cellular energy homeostasis and regulating autophagy (29). AMPK activates autophagy through ULK1 phosphorylation, initiating the autophagy process (30). Previous studies have reported that isoanguastone A inhibits the growth of CRC cells via autophagy through the activation of the AMPK signaling pathway (31). Similarly, we found that CEP increases the phosphorylation of AMPK proteins in HCT-116 and HT-29 cells, suggesting that CEP-induced autophagy may be mediated by the AMPK pathway in colorectal cancer cells.

Conclusions

In summary, our study demonstrated that CEP significantly inhibited the growth of human colorectal cancer cells. CEP induced autophagic cell death by increasing the expression of autophagic-related proteins, including LC3-II, ATG5, and beclin-1 via the activation of the AMPK pathway. Our findings collectively suggest that CEP holds promise as an alternative adjuvant chemotherapy for the treatment of colorectal cancer. However, it is crucial to acknowledge the limitations inherent in cell culture studies, as cellular behavior *in vitro* may not precisely mimic the complexities of the *in vivo* environment. Consequently, further *in vivo* investigations are necessary to enhance our understanding and establish the potential clinical applicability of CEP.

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