RESEARCH ARTICLE

Cepharanthine Suppresses Metastatic Potential of Human Cholangiocarcinoma Cell Lines

Kwuntida Uthaisar[1,3,4], Wunchana Seubwai[2,3], Pattravedee Srikoon[4], Kulthida Vaeteewoottacharn[1,3], Kanlayanee Sawanyawisuth[1,3], Seiji Okada[4]*, Sopit Wongkham[1,3]*

Abstract

Cholangiocarcinoma (CCA) is a highly metastatic tumor with poor responses to traditional chemotherapeutic agents. We have focused on new drugs which can exert effects either alone or in combination with available agents for a better treatment of CCA and recently reported efficacy of cepharanthine (CEP), a natural biscoclaurine alkaloid extract with anti-proliferative activity against human CCA cell lines. CEP treatment effectively suppressed tumor growth in CCA-inoculated mice without serious side-effects and also increased cell apoptosis in primary histocultures of CCA patient tissues, suggesting therapeutic potential of CEP against human CCA. In the present study, we further showed anti-metastatic effects of CEP on migration and invasion of human CCA cell lines. CEP at 10 μg/mL effectively suppressed migration activity of KKU-M213 and KKU-M214 as shown by wound healing and Boyden chamber assays. Similar suppressive effects were observed in invasion assays. The molecular mechanism underlying CEP actions on cell motility and invasion could be shown to involve suppression of ICAM-1 and MMP-2. Our results suggest CEP as a potential therapeutic agent for treating metastatic CCA.

Keywords: NF-kB - metastasis - migration - invasion - bile duct cancer

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Introduction

Cepharanthine (CEP), a naturally occurring biscoclaurine alkaloid extracted from Stephania cepharantha Hayata, has been widely used in Japan for treatment of many acute and chronic diseases (Furusawa and Wu, 2007). Various biological activities of CEP in medicine have been reported, e.g., reduction of multi-drug resistance (Abe et al., 1995; Nakajima et al., 2004), potentiation of chemotherapy (Kato and Suzumura, 1987; Hibasami et al., 1991), induction and suppression of apoptosis (Kaufmann and Earnshaw, 2000; Wu et al., 2001), inhibition of allergies and histamine release (Kohno et al., 1986; Kohno et al., 1987), inhibition of platelet aggregation (Kometani et al., 1985; Hashizume et al., 1991), anti-HIV (Okamoto et al., 1998; 1999), anti-malaria (Tamez et al., 2005), and antitumor activity and reduction of metastasis (Ebina et al., 1990; Yasukawa et al., 1991; Okada et al., 1999).

Cholangiocarcinoma (CCA) is a commonly known leading cause of death in the northeast of Thailand where the prevalence is highest in the world (Sripa et al., 2007). It is an assertive and mortal cancer due to its high metastasis and resistance to traditional chemotherapy. It is our attempt to search for a new drug which can exert its effect either alone or in combination with the present chemotherapy for a better treatment of CCA. Recently, we have reported the efficacy of CEP on anti-tumor activity of CCA cell lines (Seubwai et al., 2010). CEP significantly inhibited growth of human CCA cell lines in a dose- and time-dependent manner, regardless to the histologic type of tumor origin. We found that CEP controlled the growth of CCA cells through NF-kB inactivation. Moreover, CEP treatment effectively reduced tumor size in CCA-inoculated mice without serious side-effects and also increased cell apoptosis in primary histocultures of CCA patients' tissues. Our results suggest the therapeutic potential of CEP against human CCA. In the present study, we showed further the anti-metastatic effect of CEP on migration and invasion of human CCA cell lines. The downstream signaling related to NF-kB that are associated with cell migration and invasion namely E-cadherin, intercellular adhesion molecule 1 (ICAM-1) and matrix metalloproteinase (MMP)-2 were investigated to elucidate the possible underlined mechanisms.

Materials and Methods

Cell lines

Human CCA cell lines, KKU-M213 and KKU-M214,

*[1]Department of Biochemistry, [2]Department of Forensic Medicine, [3]Liver fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand, [4]Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan *For correspondence: sopit@kku.ac.th, okadas@kumamoto-u.ac.jp*

Table 1. Sequence of Primers used in the study

Gene	Forward Primer 5'-3 '	Reverse Primer 5'-3 '	Size (bp)
E-cadherin	GAACAGCACGTACACAGCCCT	GCAGAAGTGTCCCTGTTCCAG	77
ICAM-1	GGCTGGAGCTGTTTGAGAAC	ACTGTGGGGTTCAACCTCTG	202
MMP-2	GGTTCATTTGGCGGACTG	AGGCTGGTCAGTGGCTTG	205
B2M	AAGATGAGTATGCCTGCCG	CGGCATCTTCAAACCTCC	236

were kindly provided by Associate Prof. B. Sripa, the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand. CCA cells were cultured in Dulbecco modified Eagle medium (DMEM) media with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

Cepharanthine

CEP, an extracted from Stephania cepharantha Hayata, was a product of Kaken Shoyaku Co., Ltd., Tokyo, Japan. CEP was dissolved in PBS and diluted to 10 μg/mL in DMEM with 1% fetal bovine serum for wound healing assay or in serum free media for migration and invasion assay.

Viable cell assay

Viability of cells was determined using MTT assay (Invitrogen, Eugene, Oregon). Briefly, 1.2x10^4 CCA cells in complete media without or with various concentration of CEP were seeded in a 96 well culture plate and incubated at 37°C, 5% CO_2 for 12 h. Subsequently, 10 μL of MTT solution (final concentration, 0.5 mg/mL) was added to each well and incubated at 37°C for 4 h. To dissolve the crystals, 100 μL of 0.01 N HCl in isopropanol was added, and the absorbance was measured at 570 nm with an automatic ELISA plate reader (Magellan; Tecan Trading AG, Switzerland). Numbers of cells were evaluated as % of control without CEP.

Wound healing assay

CCA cell lines, KKU-M213 (5.2x10^5) and KKU-M214 (4x10^5), were seeded into a 12-well plate and incubated overnight in DMEM with 10% FBS. The monolayer cells were scratched with a sterile 200 μL pipette tip, washed twice with PBS to remove detached cells. The medium was replaced with DMEM with 1% FBS and further cultured at 37°C, 5% CO_2. Pictures of the scratched wound were taken at the indicated time point until 12 h. The relative migrating distance was calculated by subtracting the distance between the edges at indicated time point from the distance measured at the starting point divided by the distance measured at the starting point.

Migration and invasion assays

Cell invasion assay was performed using a Boyden chamber. Briefly, the polycarbonate membranes of 8 μm pore size-transwell inserts were coated with 0.5 mg/mL Matrigel (BD Biosciences, MA, USA) overnight. The membranes were rehydrated and 4x10^4 of CCA cells were placed onto a pre-coated insert of the transwell unit. Media with 10% FBS was used as a chemoattractant in the bottom chamber. Cells were allowed to invade the pre-coated membrane at 37°C/5% CO_2, 10 h for KKU-M213 and 15 h for KKU-M214. Cells on the upper side were gently wiped off with a cotton tip applicator. The invaded cells were fixed with 4% paraformaldehyde and stained with 0.4% w/v Sulphorhodamine-B. The migration assay was performed as indicated for invasion assay except cells were seeded on an uncoated insert. The numbers of migrating or invading cells were counted at least for 9 low power fields (100x magnification) and expressed as percentage of the control. Duplicate assays in each experiment and two independent experiments were performed.

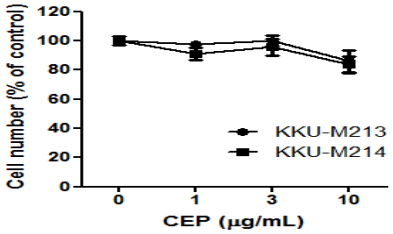

Figure 1. Effect of CEP on Cell Viability. CEP (1-10 μg/mL) has no effect on growth of CCA cell lines, KKU-M213 and KKU-M214. Cells were treated with various concentration of CEP for 12 h and the cell numbers were determined by MTT assay. The numbers of cells are presented as % of control (CEP non-treated group). The data are the means±S.D. averaged from 3 independent experiments

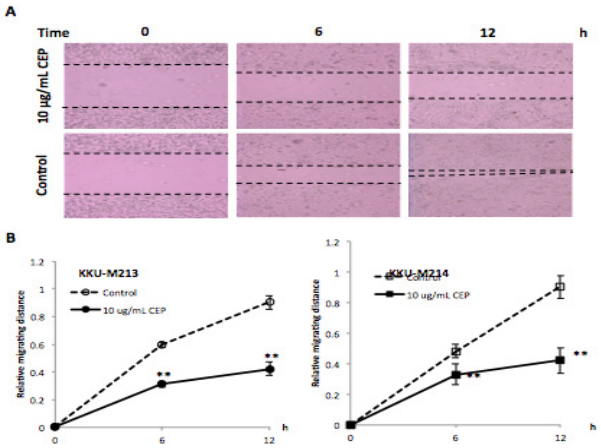

Figure 2. CEP Suppressed Migration Activity of CCA Cells. CEP significantly decreased the migrating ability of CCA cells in the wound healing assay. Scratched Wounds were incubated in the absence or presence of 10 μg/mL CEP for 24 h. (A) The diameters of the wound were recorded at 6, 9, and 12 h. (B) The relatively migrating distances are compared between the CEP-treated and non-treated groups in both of the cell lines. The data are means±S.D. averaged from triplicates of one experiment. Two independent experiments were performed. *P<0.05, **P<0.001

Real time RT-PCR

Total RNA was isolated from cells with Trizol® reagent

(Invitrogen, Carlsbad, CA) and 2 μg of RNA was reverse transcribed in to cDNA using High-capacity cDNA Reverse Transcription kits (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed in the LightCycler® 480 systems. The reaction mixture (20 μL) contained template DNA, forwarded and reversed primers of E-cadherin, ICAM-1 and MMP-2 (Table 1) and LightCycler® 480 SYBR Green I Master (Rox) (Roche Diagnostics, Mannheim, Germany). The PCR protocol consisted of an initial denaturation step of 95°C for 5 min, followed by 50 amplification cycles with 10 sec at 95°C, 10 sec at annealing temperature; 60°C and 3 sec at 72°C. The expression levels of the target genes were normalized with reference to β2-microglobulin (β2M) and values were expressed as fold change ($2^{-\Delta\Delta cp}$), where ΔΔcp=[cp target gene-cp β2M (tested cell)]/[cp target gene-cp β2M(control cells)].

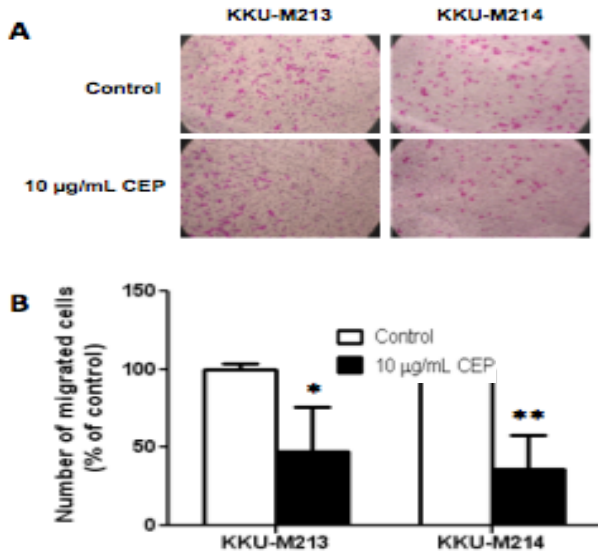

Figure 3. CEP Decreased Cell Migration. The effect of CEP on cell migration of KKU-M213 and KKU-M214 were determined using Boyden chamber assays. Cell migrations were assessed in the absence (control) or presence of 10 μg/mL CEP, 10 h for KKU-M213 and 15 h for KKU-M214. The data are means±S.D. of duplicates from one experiment. Two independent experiments were performed. *P<0.05, **P<0.001

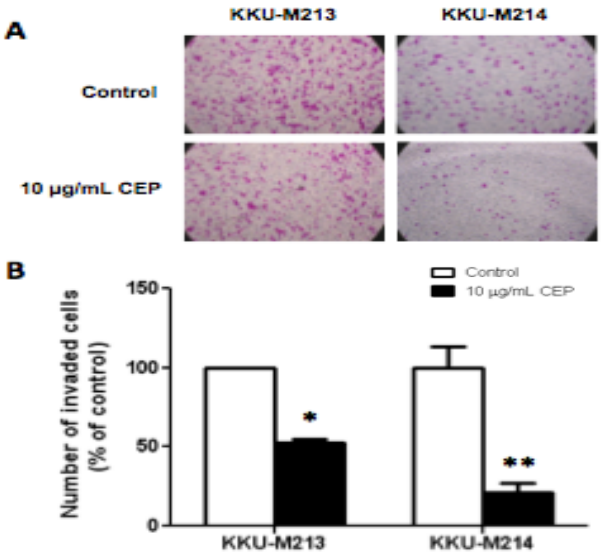

Figure 4. CEP Decreased Cell Invasion. The effects of CEP on cell invasion of KKU-M213 and KKU-M214 were determined using Boyden chamber assays. Cell invasions were assessed in the absence (control) or presence of 10 μg/mL CEP, 10 h for KKU-M213 and 15 h for KKU-M214. The data are means±S.D. of duplicates from one experiment. Two independent experiments were performed. * P <0.05, ** P <0.001

Statistical analysis

Statistical analysis was performed using Sigma stat3.1 (Systat Software Inc., San Jose, CA, USA). The significant difference between two means was determined using the Student's t-test and P<0.05 was required for statistical significance.

Results

CEP suppressed migration of CCA cell lines in wound healing assay

We determined the growth inhibitory effect of CEP by culturing cells in the presence of various concentration of CEP for 12 h and the cell numbers were determined by MTT assay. There was no significant difference between the cell numbers of KKU-M213 cultured in the absence and presence of CEP at 2.5-10 μg/mL (Figure 1A). Similar observation was also obtained for KKU-M214 (Figure 1B).

The effect of CEP on cell migration was investigated using wound healing assay. Scratched wounds were performed in the monolayer cells of KKU-M213 and KKU-M214 in the presence or absence of 10 μg/mL of CEP for 12 h. Comparing to the control, CEP significantly inhibited migration ability of KKU-M213 at every observed time point (Figure 2A). The relatively migrating distances compared between cells with and without CEP treatment indicated that CEP effectively reduced migrating distance of both KKU-M213 and KKU-M214 as early as 6 h after treatment (P<0.001) (Figure 2B).

CEP decreased migration and invasion ability of CCA cells

The effects of CEP on cell migration and invasion of CCA were determined using Boyden chamber assays. The numbers of migrating and invading cells were drastically decreased in the presence of 10 μg/mL CEP. CEP could reduce cell migration and invasion of KKU-M213 to less than 50% of the controls (P <0.05) (Figures 3A, 4A) and of KKU-M214 to less than 40% (P<0.001) (Figures 3B, 4B).

CEP mediated anti-migration and anti-invasion via suppression of ICAM-1 and MMP-2

In this study, the downstream molecules of NF-kB associated with cell migration and invasion, e.g., E-cadherin, ICAM-1 and MMP-2 in CEP treated cells were investigated in comparison with those without CEP treatment using real time-PCR. As shown in Figure 5, ICAM-1 and MMP-2 expressions were significantly decreased in the CEP treated KKU-M213 and KKU-M214 cells.

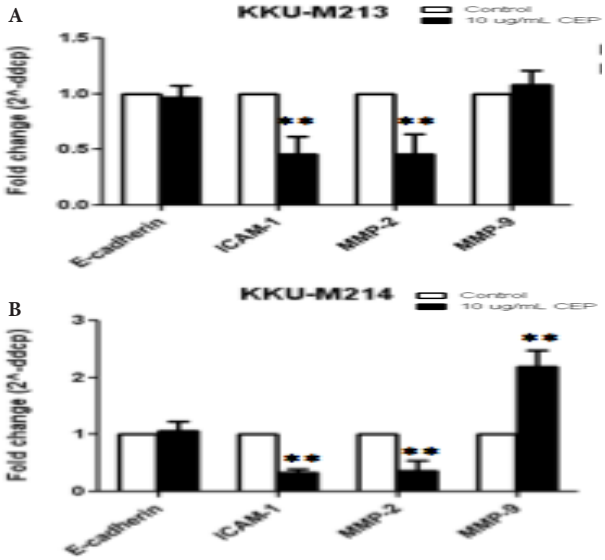

Figure 5. CEP Significantly Altered Downstream Signals of NF-kB. Expression levels of downstream signals of NF-kB (E-cadherin, ICAM-1 and MMP-2) which are associated with migration and invasion were determined by the real-time RT PCR. Expressions of the genes are shown as relatively fold change of the controls. The data are means±SD from at least two separated experiments. **P<0.001

Discussion

CEP, the root extract of *Stephania cepharantha* Hayata, is widely used for treatment of acute and chronic diseases in Japan (Furusawa and Wu, 2007). It has been reported recently that CEP inhibited tumor growth of several cancers (Ebina et al., 1990; Yasukawa et al., 1991; Harada et al., 2001) including CCA (Seubwai et al., 2010). CEP also inhibited lung metastases of Lewis lung carcinoma and rat osteosarcoma in animal models (Ito et al., 1991; Okada et al., 1999).

We have recently reported the anti-tumor activity of CEP on CCA cell lines (Seubwai et al., 2010). The growth inhibitory effect of CEP was proven in CCA-inoculated mice and the primary histocultures of CCA patients' tissues. The anti-tumor activity of CEP was shown to be through NF-kB inactivation. In the present study, we showed further the effect of CEP on migration and invasion of CCA cells. Treatment with CEP in KKU-M213 and KKU-M214 significantly decreased migrating distance, the number of migrating and invading cells. Up to our search, this is the first report on the action of CEP on migration and invasion activities of cancer cells, in vitro.

Inactivation of NF-kB was shown to be the key mechanism by which CEP exerted its action (Harada et al., 2009; Takahashi-Makise et al., 2009; Seubwai et al., 2010). In this study, the downstream signaling molecules of NF-kB that are associated with cell migration and invasion namely E-cadherin, ICAM-1, and MMP-2, were investigated. E-cadherin is a transmembrane protein. Loss of E-cadherin function or expression has been implicated in cancer progression and metastasis. In the present study, treated cell with CEP did not affect the E-cadherin expression of both CCA cell lines, and hence E-cadherin may not involve in inhibitory action of CEP on CCA cell migration and invasion.

ICAM-1, also known as CD54 (Cluster of Differentiation 54) is a type of intercellular adhesion molecule which is typically expressed on endothelial cells and cells of the immune system. Beyond its classical functions as an adhesion and viral entry molecule, ICAM-1 has now been characterized to possess a role in proinflammatory pathways. The tumor cells of metastatic lesions of rat squamous cell carcinomas showed weak or negative expression of ICAM-1 (Li et al., 1997), whereas increased expression of ICAM-1 was shown to enhance metastasis of liver and pancreatic cancers (Sun et al., 1999; Sawada et al., 2006). In our study, ICAM-1 was suppressed in either CEP treated KKU-M213 or KKU-M214 and related to the decreasing of cell migration and invasion. Anti-ICAM-1 antibody had significant inhibitory effect on cell invasion of lung cancer both *in vitro* and *in vivo* (Lin et al., 2006). Inhibition of NF-kB translocation and binding to the ICAM-1 promoter significantly reduced ICAM-1 expression. NF-kB is the positive regulator of ICAM-1 and hence suppression of NF-kB action could reduce expression of ICAM-1 as supported in our study.

Tumor invasion and metastasis is a multistep process that involves proteolytic degradation of the extra cellular matrix (ECM), alteration of the cell–cell and cell–ECM interactions, and migration of the cancer cell through the basement membrane (Kleiner and Stetler-Stevenson, 1999). MMPs can markedly increase the invasive behavior of tumor cells and their ability to metastasize in experimental animal models (Bernhard et al., 1994; Tsunezuka et al., 1996). Although some of these activities can be attributed to the matrix-degrading abilities of the MMPs, the majority of these biologic effects are mediated primarily by the proteolytic activity of MMPs on several non-matrix substrates such as chemokines, adhesion molecules, growth factors, growth factor receptors, pro-apoptotic and anti-apoptotic molecules (McQuibban et al., 2001; 2002; Hemers et al., 2005). MMP-2 (gelatinase A), which is able to degrade type IV collagen and promotes angiogenesis and mitogenesis (Stetler-Stevenson, 1994; Overall and Kleifeld, 2006), is over-expressed in many human malignancies.

Currently, MMP-3, MMP-8, MMP-9, MMP-12 and MMP-14 have been documented to exert anti-cancer effects –MMP-3 and MMP-9 exert both tumor-promoting and anti-cancer effects, MMP-8, MMP-12 and MMP-14 exert only anticancer effects – while MMP-1, MMP-2 and MMP-7 exert primarily cancer-promoting effects (Konstantinopoulos, 2008). Upon CEP action, CEP possibly suppressed the cancer-promoting effects of MMP-2 directly or indirectly via suppression of NF-kB action resulting in reducing of MMP-2 expression in both CCA cell lines.

The anti-tumor activities of CEP on tumor growth, apoptosis and metastasis via NF-kB activation have been shown in many cancers (Harada et al., 2009; Takahashi-Makise et al., 2009) including CCA (Seubwai et al., 2010). In the current study, we showed for the first time that CEP did exert its activity on anti-metastatic activities namely cell migration and invasion of CCA cell lines. As it has been known that CEP exerts its activity via NF-kB, therefore NF-kB is the ideal molecular target

for treating CCA. The long history of CEP for treatment in human without serious side effects, together with the accumulation of studies indicating the anti-proliferative and pro-apoptotic effects against a diverse range of tumors both *in vitro* and *in vivo* (Furusawa et al., 1998; Harada et al., 2001; Wu et al., 2001; Biswas et al., 2006; Tamatani et al., 2007), raise the possibility of CEP to be a unique therapeutic agent in many cancers including CCA.

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