RESEARCH ARTICLE

Suppression of Human Breast Cancer Cell Metastasis by Coptisine *in Vitro*

Jing Li*, Dong-Min Qiu, Shao-Hua Chen, Su-Ping Cao, Xue-Lan Xia

Abstract

Background: Coptisine, an isoquinoline alkaloid extracted from *Coptidis rhizoma*, has many biological activities such as antidiabetic, antimicrobial and antiviral actions. However, whether coptisine exerts anti-cancer metastasis effects remains unknown. Materials and Methods: Effects of coptisine on highly metastatic human breast cancer cell MDA-MB-231 proliferation were evaluated by trypan blue assay and on cell adhesion, migration and invasion by gelatin adhesion, wound-healing and matrigel invasion chamber assays, respectively. Expression of two matrix metalloproteinases (MMPs), MMP-9, MMP-2 and their specific inhibitors tissue inhibitor of metalloproteinase 1 (TIMP-1) and tissue inhibitor of metalloproteinase 2 (TIMP-2) were analyzed by RT-PCR. Results: Coptisine obviously inhibited adhesion to an ECM-coated substrate, wound healing migration, and invasion through the matrigel in MDA-MB-231 breast cancer cells. RT-PCR revealed that coptisine reduced the expression of the ECM degradation-associated gene MMP-9 at the mRNA level, and the expression of TIMP-1 was up-regulated in MDA-MB-231 cells, while the expression of MMP-2 and its specific inhibitor TIMP-2 was not affected. Conclusions: Taken together, our data showed that coptisine suppressed adhesion, migration and invasion of MDA-MB-231 breast cancer cells *in vitro*, the down-regulation of MMP-9 in combination with the increase of TIMP-1 possibly contributing to the anti-metastatic function. Coptisine might be a potential drug candidate for breast cancer therapy.

Keywords: Coptisine - migration - invasion - breast cancer - MMP-9 - TIMP-1

Asian Pac J Cancer Prev, **15 (14)**, 5747-5751

Introduction

Breast cancer is the most common cancer among women worldwide, and it ranks second as a cause of cancer death in women in the United States, exceeded only by lung cancer (Siegel et al., 2013). Although earlier detection and improved treatment have helped in the management of local-stage breast cancer, advanced metastatic cancer was rarely cured. Invasion and metastasis are the leading causes of mortality in patients with breast cancer.

Typically, cancer metastasis is divided into several continuous steps: malignant cancer cells firstly detached from the primary tumor, then migrated and invaded into other organs or blood or lymphatic vessels, which finally formed the second tumors in the lung, liver, brain and so on (Friedl and Wolf, 2003). Many reports have demonstrated that matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9, can degrade the components of extracellular matrix (ECM) in the surrounding tissues of the primary tumor, and then help tumor cells invade through the basement membrane of blood vessels, resulting in the movement of the cells to distant organs and the form of secondary tumors (Nelson et al., 2000; Khasigov et al., 2003; Lu et al., 2012). Therefore, to regulate the expression of ECM degradation enzymes, and then, to inhibit the metastasis of the tumor are considered as a target for therapeutic intervention (Yodkeeree et al., 2010).

Coptisine is a classic isoquinoline-type alkaloid, which is isolated and purified from the rhizome of a traditional Chinese herb Coptis chinensis Franch. In recent years, more and more biological effects of coptisine have been revealed, including antidiabetic (Jung et al., 2008), antimicrobial (Yan et al., 2008), antiviral (Li et al., 2008), anti-oxidative stress (Gong et al., 2012), and cardiovascular protection (Gong et al., 2012), etc. Most recently, the anti-tumor activity of coptisine has attracted much attention, and Lin et al showed that coptisine displayed obvious inhibition on the proliferation of both hepatoma and leukaemia cells (Lin et al., 2004). In 2014, Yu et al investigated the effect of coptisine on malignant osteosarcoma neovascurization and found that coptisine not only possessed obvious anti-osteosarcoma function *in vitro* and *in vivo* with low toxicity, but also inhibited tumor cells mediated vasculorization via down-regulating the expression of VE-cadherin and integrin β3 (Yu et al., 2014).

Considering the pivotal role of cancer migration and invasion in breast cancer progression, and it is unknown whether coptisine inhibits breast cancer cells migration and invasion. In this study, we investigated the effects

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of coptisine on the adhesion, invasion, and migration of MDA-MB-231 breast cancer cells. We also used RT-PCR to examine the impacts of coptisine on the gene transcription of ECM related genes such as MMP-2, MMP-9, TIMP-1, and TIMP-2 in order to demonstrate the potential mechanisms of coptisine on MDA-MB-231 cells.

Materials and Methods

Materials

Coptisine (purity≥98%) was purchased from Nature Standard (Shanghai, China). It was dissolved in dimethyl sulfoxide (DMSO) as a stock solution at -20°C, and diluted with medium before each experiment. The final DMSO concentration did not exceed 0.1% throughout the study. Dulbecco's Modified Eagle's Medium (DMEM) and trypsin-EDTA were purchased from Hyclone (Logan, Utah, USA). Fetal bovine serum (FBS) was purchased from Sijiqing (Hangzhou, China). MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was from Sigma (St. Louis, MO). Matrigel was purchased from Becton Dickinson (Bedford, MA, USA).

Cell culture

The human breast cancer cell MDA-MB-231 was purchased from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China. Cells were cultured in DMEM medium containing 10% FBS, 100U/ml penicillin, and 100ug/ml streptomycin, and cells were grown in a humidified atmosphere with 5% CO_2 at 37°C.

Cell growth inhibition assay

Cells in log phase were suspended and then seeded in T75 dishes supplemented with DMEM medium containing 10% FBS at a density of 1.0×10^6 cells/dish. Cells were treated with various concentrations of coptisine and incubated for 48h. Control experiments were performed under the same conditions but with the addition of DMSO instead of coptisine. Viable cell number was determined with the trypan blue exclusion assay.

Cell adhesion assay

Previously, the 96-well plates were coated with 0.1% gelatin overnight. After a pre-treatment with or without coptisine (16, 32, 64 uM) for 24h, MDA-MB-231 cells were trypsinized and suspended at a final concentration of 4.0×10^5 cells/ml in DMEM medium. 100 uL of cell suspension was seeded in each well and the cells were incubated at 37°C for 1h. After that, medium was carefully suctioned out from each well. Subsequently, each well was washed three times with DMEM medium in order to remove the non-adherent cells and then 200 uL of DMEM medium and 20 uL of 0.5% MTT solution was added, and the reaction was stopped by adding 0.1 mL of DMSO. At the end, absorbance was measured at 490nm by a Microplate Reader Elx800 (Bio-Tek Instruments Inc., Winooski, VT, USA).

Wound-healing assay

MDA-MB-231 cells were seeded in a six-well plate and cultured in medium with or without coptisine (16, 32 and 64 uM). When cells were grown to 80% confluency, the cell monolayers were wounded by white pipette tips and washed by serum-free medium to remove floating cells. Wounded monolayers were then incubated in fresh complete medium for 48h. Cell migration into the wound area and the average distance of migrated cells across the black lines was observed and counted under an inverted microscopy.

Cell invasion assay

The cell invasion assay was carried out using Transwell chambers (8um pore-size, Corning Costar, Cambridge, MA) coated with Matrigel. MDA-MB-231 cells (1.0×10^5 cells/chamber) were seeded in the upper chamber with or without the indicated doses of coptisine and incubated for 24h at 37°C , 5% CO_2. FBS (10%) acting as a chemoattractant was placed in the lower chambers. After incubation, all of the non-invaded cells on the upper surface were removed with a cotton swab; the invaded cells on the lower surface were fixed with 100% methanol and then stained with 1% crystal violet. The invaded cells were counted with a microscope and six randomly chosen fields were counted for each assay.

RT-PCR analysis

MDA-MB-231 cells were treated with coptisine for 48h. The mRNA levels of MMP-2, MMP-9, TIMP-1 and TIMP2 were determined using RT-PCR. The primer sets used in the PCR amplification were as follows: MMP-2 (forward: 5'-AACACCTTCTATGGCTGCCC-3', reverse: 5'-ACGAGCAAAGGCATCATCCA-3'); MMP-9 (forward: 5'-CGGTTTGGAAACGCAGATGG-3', reverse: 5'-AGAAGCCGAAGAGCTTGTCC-3'); TIMP-1 (forward: 5'-CTCGTCATCAGGGCCAAGTT-3', reverse: 5'-GTAGGTCTTGGTGAAGCCCC-3'); TIMP-2 (forward: 5'-TAGTGATCAGGGCCAAAGCG-3', reverse: 5'- AGGGCACGATGAAGTCACAG-3'); GAPDH (forward: 5'- GAGAAGGCTGGGGCTCATTT-3', reverse: 5'-GTCAGGTCCACCACTGACAC-3'). Total RNA was extracted with TRIzol (Invitrogen Life Technologies, Carlsbad CA, USA) according to the manufacturer's direction. RNA was converted to cDNA (Fermentas, Glen Burnie, MD, USA). Semi-quantitative RT-PCR analysis for MMP-2, MMP-9, TIMP-1, TIMP-2 and GAPDH were run with the following conditions: 94°C for 3min, followed by 25-32 cycles of 94°C for 30s, 52-58°C for 30s, and 72°C for 60s. The expression levels of MMP-2, MMP-9, TIMP-1 and TIMP-2 were normalized to the GAPDH internal control.

Statistical analysis

Data were subjected using SPSS version 13.0, and presented as the Mean±SD. Statistically significant differences between the control and experimental groups were calculated by Student's t test, and were indicated as $*p<0.05$ and $**p<0.01$.

Results

Effect of coptisine on the viability of MDA-MB-231 cells

The chemical structure of coptisine is shown in

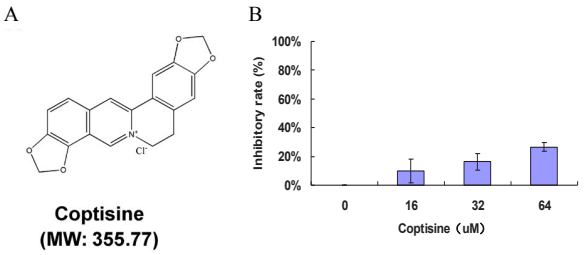

Figure 1. Effect of Coptisine on the Viability of MDA-MB-231 Cells. (A) Chemical structure of coptisine. (B) Coptisine shows no obvious effect on MDA-MB-231 cell viability

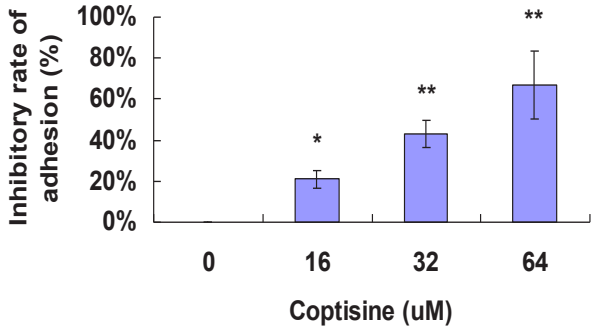

Figure 2. Effect of Coptisine on MDA-MB-231 Cell Adhesion *in vitro*. After cells were treated with 16, 32, and 64uM of coptisine for 24h, 100ul cell suspension was added to the wells pre-coated with gelatin. After 60 min of incubation, the numbers of adherent cells were determined by MTT assay. *p<0.05, **p<0.01.

(Figure 1A). The effect of coptisine on cell cytotoxicity was determined by Trypan blue exclusion assay. Results showed that the viability of MDA-MB-231 cells was little affected by a 48h treatment of coptisine at various concentrations (16, 32 and 64 uM), as shown in (Figure 1B). So, this concentration range was used in all subsequent experiments.

Effect of coptisine on MDA-MB-231 cell adhesion to gelatin

For the metastatic tumor cells, adhesion to extracellular matrices and basement membranes is considered to be the first step in its invasive process (Saiki et al., 1990). We evaluated the impact of coptisine on the adhesion of MDA-MB-231 cells to the substrates pre-coated with gelatin. After treatment with various concentration of coptisine (16, 32, 64 uM), the adhesion activity of MDA-MB-231 cells was decreased in a concentration-dependent manner, as shown in Fig.2. And the inhibition rate of 16, 32 and 64uM coptisine was 20.89%±4.40%, 43.09%±6.43% and 67.00%±16.59%, respectively compared with control.

Effect of coptisine on MDA-MB-231 cell migration activity

In addition to cell adhesion ability, cell motility was an important characteristic of tumor metastasis. We tested the migration activity of cells treated with or without coptisine by the wound healing assay. As shown in (Figure 3), coptisine significantly suppressed the migration of MDA-MB-231 cells across the wounded space, and

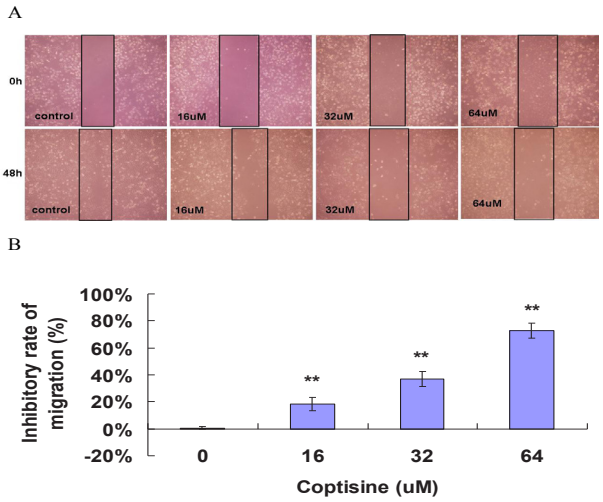

Figure 3. Effect of Coptisine on MDA-MB-231 Cell migration activity *in vitro*. The cells were plated in a six-well plate and cultured in medium with or without coptisine. When cells were grown to 80% confluency, the cell monolayers were wounded by white pipette tips and then incubated in fresh complete medium for 48 h. Migration was assessed by inverted microscope. Black lines indicate the wound edge. Migrated cells across the black lines were counted in six random fields for each treatment. **p<0.01.

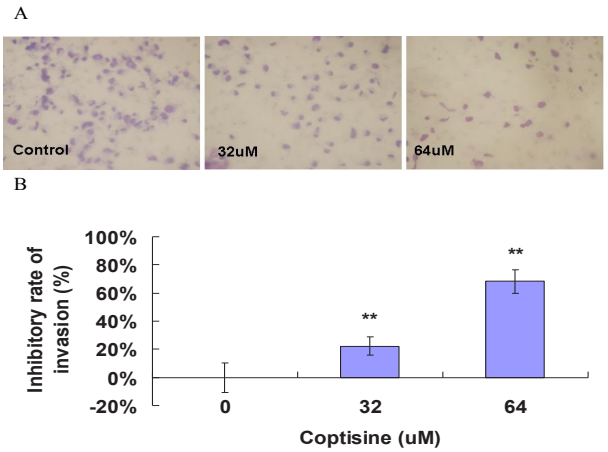

Figure 4. Effect of Coptisine on MDA-MB-231 Cell invasion *in vitro*. The cells were treated with the indicated dosages of coptisine for 24h. The invading cells on the lower insert membranes were stained with crystal violet and observed under a microscope (400×). The number of invading cells was calculated as a percentage of invasion. **p<0.01.

the inhibition rate was 18.33%±5.13%, 37.00%±5.57% and 72.67%±5.69% with 16, 32 and 64uM coptisine, respectively.

Effect of coptisine on MDA-MB-231 cell invasion

To further examine the effect of coptisine on the invasion of MDA-MB-231 cells, the Transwell chamber assay was performed. Results showed that coptisine dramatically reduced the number of invaded cells with the concentration of 32 and 64uM, and the inhibition rate was 22.34%±6.71% and 68.36%±8.36%, respectively (Figure 4).

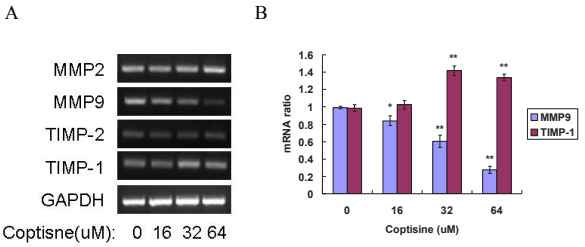

Figure 5. Effect of Coptisine on the Expression of MMPs and TIMPs in MDA-MB-231 Cells. The cells were incubated with indicated concentrations of coptisine. The mRNA expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 was analyzed by RT-PCR. The expression of GAPDH was included as an internal control. The PCR products were quantified by densitometric analysis with that of the untreated group being 100%. The values are expressed as mean±SD of three independent experiments. *p<0.05 compared with control; **p<0.01 compared with control

Effect of coptisine on the expression of MMPs and TIMPs

The increasing data displayed that MMPs, especially MMP-9 and MMP-2, could degrade the components of the basement membrane and promote the invasion and metastasis of malignant tumors (Yahayo et al., 2013; Yadav et al., 2014). The extent of matrix degradation is determined by the relative activities of MMPs and their natural inhibitors (TIMPs). It is well established that the activation and function of MMP-9 and MMP-2 could be controlled by TIMP-1 and TIMP-2, respectively (Lee et al., 2004; Li et al., 2012).

The mRNA levels of MMP-2, MMP-9, TIMP-1 and TIMP-2 were further elucidated by RT-PCR after MDA-MB-231 cells were treated with coptisine (16, 32 and 64 uM) for 48h. Fig. 5A indicated that coptisine treatment significantly decreased MMP-9 expression in a dose-dependent manner. On the contrary, the expression of TIMP-1, a specific inhibitor of MMP-9 was up-regulated by coptisine. The expression of MMP-9 and TIMP-1 mRNAs was shown in Fig. 5B. However, no statistically influence on the expression of MMP-2 and TIMP-2 was observed by either concentration of coptisine (Fig. 5A). So, our data indicated that the anti-metastatic activity of coptisine was due to the modulation of MMP-9 and TIMP-1 expression.

Discussion

Many women with breast cancer can be cured with good prognosis and appropriate treatment in the early state of the disease. Mortality from breast cancer most results from metastatic disease and metastasis is the greatest barrier to cancer cure. Therefore, the inhibition of invasion and metastasis is one of the most significant issues in breast cancer research. In view of this, the new drug or phytochemical compound which possesses anti-metastatic capability has attracted a lot of interest, especially those substances producing non-serious side effect (Supabphol et al., 2009). Active monomers from traditional Chinese medicine pool are a rich source of anti-breast cancer invasion and metastasis drug discovery. In recent years, more and more active compounds from traditional Chinese medicine such as oroxylin A, baicalein, platycodin D, curcumin, Momordica cochinchinensis seed extracts are being widely studied for their anti-cancer metastatic properties (Wang et al., 2010; Hassan and Daghestani, 2012; Lu et al., 2012; Chun and Kim, 2013; Zheng et al., 2014). Oroxylin A, a bioactive flavonoid extracted from Scutellaria radix, could block carcinoma invasion and metastasis *in vitro* and *in vivo* through down-regulation of MMP-2/MMP-9, up-regulating TIMP-2 and suppressing the ERK1/2 signaling pathway, therefore oroxylin A might be developed as a therapeutic potential candidate for the treatment of cancer metastasis (Lu et al., 2012). Baicalein, a flavonoid from Chinese herbs, has been found to suppress the adhesion, migration and invasion of MDA-MB-231 cancer cells by decreasing the expression of MMP-2/9 involved mitogen-activated protein kinases (MAPK) signaling pathway (Wang et al., 2010). In this study, we examined the anti-cancer invasion potential of coptisine against breast cancer cells *in vitro* and the mechanism underlying the effects.

Our data revealed that coptisine significantly inhibited the adhesion, migration and invasion of human breast cancer cell MDA-MB-231. Prior studies have shown that two gene families, MMPs and TIMPs are involved in a wide range of proteolytic events, including tumor growth, migration, metastasis and angiogenesis (Hahn-Dantona et al., 1999; Khuri et al., 2001; Hassan et al., 2012; Sharma et al., 2012). To further illustrate the invasion inhibitory effect of coptisine, we examined the expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 on treatment of MDA-MB-231 cells with coptisine. Results showed that coptisine efficiently down-regulated MMP-9 at the mRNA level in human MDA-MB-231 cells, while the expression of TIMP-1 mRNA was increased. But the expression levels of MMP-2 and TIMP-2 were not apparently affected. It suggested that coptisine had potential anti-invasive properties by regulating the expression of MMP-9 and TIMP-1. Our results are in line with the previous studies which also have demonstrated that MMP-2 is not often responsive to exogenous factors such as growth factors, chemical sustances in MDA-MB-231 cells although MMP-2 is similar to MMP-9 structurally and catalytically (Hahn-Dantona et al., 1999; Chun and Kim, 2013).

In conclusion, our data indicated that coptisine efficiently suppressed the adhesion, migration and invasion of human breast cancer MDA-MB-231 cells accompanied with the interference to the expression of MMP-9 and its specific inhibitor TIMP-1. These findings provide a theoretical foundation for the possibility of coptisine as a potential therapeutic candidate against tumor invasion. In order to explore this possibility, further studies on more detailed mechanisms and functions of coptisine are required.

Acknowledgement

The study was supported by grant from Wuxi Higher Health Vocational Technology School.

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