Original article

Crude extract of Rheum palmatum inhibits migration and invasion of U-2 OS human osteosarcoma cells by suppression of matrix metalloproteinase-2 and -9

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Abstract

Osteosarcoma is the most common primary bone malignancy and primarily occurs in adolescents and young adults. Crude extract of Rheum palmatum L. (CERP) has been used as a traditional Chinese medicine for different diseases and there is experimental evidence that it may have anti-cancer effects. However, there is no information showing that CERP can affect the mobility of human osteosarcoma cells. In this study we determined the effects of CERP on U-2 OS human osteosarcoma cells. We found that CERP significantly inhibited the migration and invasion of U-2 OS cells. CERP also reduced the activity of matrix metalloproteinase (MMP)-2 and MMP-9 and decreased expression levels of the proteins FAK, GRP78, PKC, HIF-1, SOS1, VEGF, PI3K, GRB2, Ras, p-ERK1/2, ERK1/2, p-p38, JNK1/2, p-JNK1/2, MEKK3, MKK7, PERK, p-PERK, iNOS, COX-2, NF-κB p65, IRE-1, UPA, and RhoA in U-2 OS cells. Confocal laser microscopy revealed that CERP decreased the expression of NF-κB p65, RhoA and Rock 1. These in vitro studies suggest that CERP may...
have novel anti-cancer actions in the treatment of osteosarcoma. Further studies including animal models of bone cancer are warranted.

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1. Introduction

Osteosarcoma is the most common bone tumor and occurs predominantly in children, adolescents, and young adults [1–4]. In children younger than 15 years, osteosarcoma is the sixth most common cancer and is associated with high pulmonary metastases [5]. Currently, patients with osteosarcoma who have lung metastasis have a 5-year survival rate no greater than 30% [6,7]. Advances in osteosarcoma therapy, including neoadjuvant and adjuvant chemotherapy, have enhanced patient outcomes [8], but the prognosis remains poor for most patients with metastatic or recurrent osteosarcoma [9].

Cancer cells from a primary site give rise to a metastatic tumor involving several regulatory pathways that can be used as biomarkers for predicting metastasis of cancer cells [10]. Matrix metalloproteinases (MMPs) play an initial and important role in cancer cell invasion and metastasis by controlling degradation of the extracellular matrix [11,12]. There is currently much effort focused on identifying MMP inhibitors [13–15].

In the Chinese population, traditional Chinese herbal medicines such as Rheum palmatum L. have been used for treating different diseases [16–18] including anti-inflammatory diseases [19]. Rheum undulatum L. components are used for treating chronic liver diseases [20,21]. Several studies have reported that major components of R. palmatum such as emodin and aloe-emodin induce apoptosis in different human cancer cell lines [22–26]. There are no reports on the effects of crude extract of R. palmatum (CERP) on the migration and invasion in human osteosarcoma cancer cells. Here we determined if water-soluble CERP can reduce the migration and invasion of U-2 OS human bone cancer cells. Our findings showed that CERP inhibits migration and invasion of U-2 OS cells by reducing protein expression of MMP-2 and -9 upregulation of NF-κB signaling.

2. Materials and methods

2.1. Chemicals and reagents

Propidium iodide (PI), Tris–HCl, trypsin, trypsin blue, and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), McCoy’s 5A medium, L-glutamine, penicillin–streptomycin, and trypsin in EDTA were purchased from Gibco BRL (Grand Island, NY, USA). CERP was kindly provided by Dr Chien-Chih Yu (School of Pharmacy, China Medical University, Taichung, Taiwan). All chemicals and reagents used were of analytical grade.

2.2. U-2 OS cell culture

The U-2 OS human osteosarcoma cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in 90% McCoy’s 5A medium with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin and maintained in 75-cm² tissue culture flasks at 37 °C under a humidified 5% CO₂ atmosphere as previously described [27,28].

2.3. Cell viability assay

U-2 OS cells at a density of 2 × 10⁵ cells/well were placed in 12-well plates for 24 hours. The cells were treated with 0, 50, 100, 250, 500 and 750 μg/mL CERP or 1× phosphate-buffered saline (PBS) as a control for 24 and 48 hours. Cells harvested from each treatment by centrifugation were stained with PI (5 μg/mL). The cells were analyzed using the PI exclusion method and flow cytometry (FACS Calibur; BD Biosciences, San Jose, CA, USA) as previously described [27,28].

2.4. Wound healing assay

Cell migration was determined using a wound healing assay. U-2 OS cells at a density of 5 × 10⁵ cells/well were maintained in 10-cm Petri dishes for 24 hours until they were completely confluent. The monolayer in each plate was scraped with a sterile yellow micropipette tip to remove cells to mimic a wound and the remaining cells were

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**Fig. 1 – CERP decreased the percentage of viable U-2 OS human osteosarcoma cells.** U-2 OS cells were incubated with 0, 100, 250, 500, 750, 1000 and 1500 μg/mL CERP for 48 hours. The percentage of viable cells was then determined by flow cytometry. * Significant difference compared to the control (p < 0.05, Student’s t test).
washed with PBS three times. Cells in each well were cultured in medium containing 0–500 μg/mL CERP for 24 hours. Then random fields were examined, selected, and photographed using an inverted microscope as previously described [29].

2.5. Cell invasion and migration assays

Cell mobility (migration and invasion) was evaluated using a Matrigel cell migration assay and invasion system as previously described [29,30]. The cell migration assay was performed using transwell (BD Biosciences, Franklin Lakes, NJ, USA) cell culture chambers (8-mm pore size; Millipore, Billerica, MA, USA). U-2 OS cells at a density of 5 × 10^5 cells/well were maintained in serum-free medium for 24 hours. Cells were then trypsinized and resuspended in serum-free McCoy’s 5A medium and placed in the upper chamber of the transwell insert and incubated with vehicle or CERP (50 and 75 μg/mL). Then 90% McCoy’s 5A medium containing 10% FBS was added to the lower chamber. Cells in both chambers were incubated for 24 or 48 hours before non-migrating cells in the upper chamber were removed by wiping with a cotton swab. Migrating cells on the lower surface of the filter were fixed with 4% formaldehyde in PBS. Cells in the lower chamber were stained with 2% crystal violet in 2% ethanol and were counted and photographed under a light microscope at 200x. The cell invasion assay was performed in the same way except that the filter membrane was coated with Matrigel from a BioCoat Matrigel invasion kit. Cells located on the underside of the filter were examined and counted under a light microscope at 200x as previously described [28,29].

2.6. Gelatin gel zymographic assay of MMP-2 and -9

Gelatin gel zymography was used to measure levels of MMP-2 and MMP-9 secreted from U-2 OS cells after exposure to CERP as previously described [28,29]. U-2 OS cells at a density of 5 × 10^5 cells/well were maintained in 12-well plates for 24 hours and then treated individually with CERP (50 and 75 μg/mL) for 24 and 48 hours. At the end of the incubation period, cells from each treatment were harvested. The supernatant from the control and CERP-treated groups was resuspended in none reducing loading buffer and incubated at 37 °C for 15 min. The samples were then applied to 10% SDS-PAGE gels cast with 0.1% gelatin and electrophoresed. The gels were then incubated in renaturing buffer (2.5% Triton X-100) for 30 minutes and then in developing buffer (50 mM Tris–HCl, pH 7.8, 10 mM CaCl₂, 150 mM NaCl) for 16 hours at 37 °C. Gels were stained with Coomassie brilliant blue R 250 and destained in 30% methanol/10% acetic acid to detect gelatinase secretion as previously described [28,29].

Fig. 2 – Wound healing assay to determine the effects of CERP on U-2 OS cell migration. Cells were maintained in 6-well dishes for 24 hours before a wound was introduced by scraping confluent cell layers with a pipette tip. CERP (0, 100 and 250 μg/mL) was added to each well and incubated for 24 and 48 hours. Representative images of treated and control cells are presented.
2.7. Western blot analysis of proteins associated with cell migration and invasion

U-2 OS cells at a density of $1 \times 10^6$ cells/well were placed in 6-well plates for 24 hours. Each well was incubated with or without CERP (250 µg/mL) at 37 °C for 0, 6, 12, 24 and 48 hours. After incubation, the cells were harvested and lysed with ice-cold 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM EDTA and 0.1% Triton X-100 by sonication and were then centrifuged at 13,000 g for 10 minutes at 4 °C [28,29]. The supernatant was collected and the total protein for each sample was measured using a Bio-Rad protein assay kit (Hercules, CA, USA) with bovine serum albumin (BSA) as the standard. Protein samples of 30 µg were loaded onto 12% SDS-PAGE gels and electrophoresed. The proteins were then electrotransferred to nitrocellulose membranes and blotted with appropriate primary antibodies (anti-FAK, GRP78, PKC, HIF-1, SOS1, VEGF, PI3K, GRB2, Ras, p-ERK1/2, ERK1/2, p-p38, JNK1/2, p-JNK1/2, MEKK3, MKK7, PERK, p-PERK, iNOS, COX-2, NF-κB p65, IRE-1, UPA, RhoA, MMP-2, and MMP-9; 1:100). The membranes were washed and stained with a secondary antibody (FITC-conjugated goat anti-mouse IgG at 1:100 dilution). An enhanced chemiluminescence reagent (Amer- sham Biosciences ECL) was used for detection. The bands were quantified using an NIH Image analyzer (NIH, Bethesda, MD, USA) [28,29].

2.8. Statistical analysis

Results are based on at least three independent experiments and expressed as mean ± SD. Differences between CERP-treated and control groups were analyzed using Student’s t test, with $p < 0.05$ considered significant.

3. Results

3.1. CERP alters the percentage of viable U-2 OS human osteosarcoma cells

To determine if CERP has cytotoxic effects, U-2 OS cells were treated with or without CERP (100, 250, 500, 750, 1000 and 1500 µg/mL) for 48 hours. The percentage of viable cells was quantified by flow cytometry. Fig. 1 shows that CERP did not
significantly decrease the percentage of viable cells at concentrations of 100–500 μg/mL. Higher CERP concentrations caused a significant reduction in cell viability.

3.2. CERP inhibits the migration and invasion of U-2 OS cells

U-2 OS cells were incubated with CERP (0, 100 and 250 μg/mL) for 24 and 48 hours and cell migration was evaluated using a wound healing assay. The results in Fig. 2 indicate that CERP inhibited the migration of U-2 OS cells in a dose- and time-dependent manner.

To further investigate the effects of CERP on cell migration and invasion, U-2 OS cells were treated with or without 100 and 250 μg/ml CERP for 24 and 48 hours and samples were seeded in Millicell chambers with an uncoated (for migration) or Matrigel-coated (for invasion) filter. Fig. 3 shows that CERP significantly inhibited migration of U-2 OS cells, in agreement with the data in Fig. 2. CERP significantly inhibited the invasion of U-2 OS cells in a time-dependent manner, as observed in Fig. 4.

Fig. 4 – CERP suppressed U-2 OS cell invasion in vitro. After penetration through Matrigel, U-2 OS cells on the lower surface of the filter were stained with crystal violet and photographed under a light microscope at 200×. Cells in the lower chamber were counted at 200×. Data represent the mean for three independent experiments. * Significant difference compared to the control (p < 0.05, Student’s t test).

Fig. 5 – CERP affected the activity of MMP-2 and MMP-9 in U-2 OS cells. Representative zymogram from three independent experiments was used to detect the activity of secreted MMP-2 and MMP-9 by using conditioned medium of U-2 OS cells culture with CERP. MMP-2 and MMP-9 activities were determined by densitometric analysis. Results are expressed as a percentage of the control (100%).
Fig. 6 — CERP affected NF-κB p65, RhoA, and ROCK-1 expression in U-2 OS cells. Cells were treated with 0, 100, and 250 μg/mL CERP for 24 hours. They were then fixed and stained using primary (A) anti-NF-κB p65, (B) RhoA, or (C) ROCK-1 antibodies overnight. Staining with a secondary antibody yielded green fluorescence and nuclear counterstaining with PI yielded red fluorescence. Photomicrographs were obtained using a Leica TCS SP2 confocal spectral microscope.
3.3. CERP inhibits MMP-2 and MMP-9 activity in U-2 OS cells

CERP inhibition of U-2 OS cell migration and invasion may be associated with effects on MMP-2 and -9 activity. The data in Fig. 5 show that U-2 OS cells constitutively secrete high levels of MMP-2 but low levels of MMP-9. However, after CERP treatment (50, 100, 250, 500 μg/mL) for 24 hours, levels of both MMP-2 and -9 levels were lower (Fig. 5).

3.4. CERP alters levels of proteins associated with migration and invasion

We examined the effects of CERP on levels of proteins associated with cell migration and invasion. CERP decreased cytosolic levels of NF-κB p65 (Fig. 6A), RhoA (Fig. 6B), and ROCK-1 (Fig. 6C). It also reduced protein levels of FAK, GRP78, PKC, HIF-1, and SOS1 (Fig. 7A), VEGF, PI3K, GRB2, and Ras (Fig. 7B), p-ERK1/2, ERK1/2, p-p38, JNK1/2, and p-JNK1/2 (Fig. 7C), MEKK3, MKK7, PERK, and p-PERK (Fig. 7D), iNOS, COX-2, NF-κB p65, and IRE-1a (Fig. 7E) and UPA, RhoA, MMP-2, and MMP-9 (Fig. 7F).

4. Discussion

Several studies have reported that R. palmatum has many biological activities and that components such as emodin and aloe-emodin have anti-cancer activity [31–33]. Migration and invasion are key functions in cancer dynamics. In the present study we investigated the effects of CERP on U-2 OS cell migration and invasion. CERP reduced cell migration and invasion and these effects were associated with decreases in MMP-2 and -9. It is well documented that both enzymes play important roles in cancer cell migration and invasion [34].

Numerous reports have shown that MMPs (a family of zinc-containing proteolytic enzymes) are associated with cancer cell migration and invasion [1,34]. We found that CERP reduced protein levels of MMP-2 and -9. CERP also decreased PI3K and inhibited the expression of FAK and the downstream kinases ERK1/2, JNK and p38 in U-2 OS cells. FAK/Src signaling plays an important role in tumor metastasis by increasing cell migration and invasiveness [35,36]. Activated FAK (Tyr397)/Src (Tyr416) may stimulate signaling through downstream cascade targets (PI-3K/AKT and Ras/ERK1/2) [37,38]. We found that CERP decreased levels of NF-κB p65 in U-2 OS cells. It was previously reported that NF-κB p65 alters downstream levels of MMP-2 and MMP-9 [39,40]. In the present study, CERP reduced protein levels of UPA and RhoA. UPA is involved in cancer cell migration and invasion [41,42]. Upregulation of UPA induces increases in cancer cell invasion and metastasis [43]. RhoA also stimulates metastasis of tumor cells [44,45]. On the basis of these observations, we propose decreases in MMP-2 and MMP-9 activity induced by CERP may involve ERK1/2MAPK, NF-κB and UPA signaling pathways.

CERP also reduced levels of Sos1, PKC, GRB2, and Ras proteins, which are associated with cell mobility [46–48]. It has been reported that protein kinase C epsilon (an oncogene) is overexpressed in several human cancers and is associated with cell proliferation, migration, invasion, and survival [49]. The FAK–Src complex allows Src to phosphorylate FAK and then to mediate its interaction with GRB2 to activate the Ras–ERK signaling pathway [50]. Aberrant regulation of RhoA
CERP affected the levels of proteins associated with cell migration and invasion. U-2 OS cells at the density of 1 \times 10^6 cells/dish were treated with 250 \mu g/mL CERP for 6, 12, 24, and 48 hours. Total protein extracts were prepared and protein expression was determined by Western blotting. (A) FAK, GRP78, PKC, HIF-1α, and SOS1. (B) VEGF, PI3K, GRB2, and Ras. (C) p-ERK, ERK1/2, p-p38, JNK1/2, and p-JNK1/2. (D) MEKK3, MKK7, PERK, and p-PERK. (E) iNOS, COX-2, NF-κB p65, and IRE-1. (F) UPA, RhoA, MMP-2, and MMP-9.

**Fig. 7** – CERP affected the levels of proteins associated with cell migration and invasion. U-2 OS cells at the density of 1 \times 10^6 cells/dish were treated with 250 \mu g/mL CERP for 6, 12, 24, and 48 hours. Total protein extracts were prepared and protein expression was determined by Western blotting. (A) FAK, GRP78, PKC, HIF-1α, and SOS1. (B) VEGF, PI3K, GRB2, and Ras. (C) p-ERK, ERK1/2, p-p38, JNK1/2, and p-JNK1/2. (D) MEKK3, MKK7, PERK, and p-PERK. (E) iNOS, COX-2, NF-κB p65, and IRE-1. (F) UPA, RhoA, MMP-2, and MMP-9.
proteins is associated with metastasis via promotion of tumor cell motility [51]. CERP may act as an effective inhibitor of ERK/Rho signaling in U-2 OS cells.

In conclusion, CERP effects on U-2 OS cancer cell migration and invasion may involve multiple signaling pathways, as hypothesized in Fig. 8. Our results show that CREP is effective in osteosarcoma cells. Future studies are required to determine if CERP reduces cell motility in other cancer types and if it can prevent metastasis in animal models of cancer.

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