Inhibitory effect of emodin on migration, invasion and metastasis of human breast cancer MDA-MB-231 cells in vitro and in vivo

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Abstract. In breast cancer, metastasis is the main reason for patient mortality. In the present study, we used breast cancer MDA-MB-231 cells and a mouse xenograft model to demonstrate the effect of emodin on the migration, invasion and metastasis of human breast cancer MDA-MB-231 cells and the related mechanisms. In vitro, wound healing and Transwell assays showed that emodin dose-dependently inhibited the migration and invasion of MDA-MB-231 cells. Enzyme-linked immunosorbent assay (ELISA) showed that emodin decreased the secretion of MMP-2 and MMP-9. Western blot analysis showed that emodin downregulated the expression levels of MMP-2, MMP-9, uPA and uPAR as well as p38 inhibitor SB203580 and ERK inhibitor PD98059, even though TIMP-1 and TIMP-2 were not obviously changed in the MDA-MB-231 cells. Furthermore, emodin inhibited the activity of p38 and ERK1/2 in the MDA-MB-231 cells. In vivo, emodin inhibited lung metastasis in mice bearing the breast cancer MDA-MB-231 cells. These results indicated that emodin inhibited the lung metastasis of human breast cancer in a mouse xenograft model, and inhibited the invasion of MDA-MB-231 cells associated with the downregulation of MMP-2, MMP-9, uPA and uPAR expression as well as decreased activity of p38 and ERK.

Introduction

Breast cancer is the most common malignant tumor in women worldwide and most patients with advanced breast cancer have serious systemic metastasis, which is associated with a high death rate (1). Chemotherapy is commonly used in the treatment of metastatic breast cancer. However, this treatment remains ineffective due to its toxicity to normal cells (2). Natural medicine has been widely used for treating malignant tumors for thousands of years (3,4). There is great potential in identifying drugs from natural medicine for the treatment of metastatic breast cancer due to their wide range of biological activities and low toxicity.

Emodin (6-methyl-1,3,8-trihydroxyanthraquinone) (Fig. 1A), an active monomer extracted from Rheum, Polygonum, Buckthorn and Semen, has been shown to be active against pancreatic (5,6), lung (7,8), breast (9,10), liver (11), prostate (12,13), ovarian (14), cervical (15), colon (16) and human tongue cancer SCC-4 cells (18,19). Moreover, it has been reported that emodin induces apoptosis of human breast cancer MCF-7 (20) and MDA-MB-453 cells (21). However, there are no reports on the effect of emodin on breast cancer MDA-MB-231 cells.

Proteolytic enzymes play important roles in the process of cancer development and metastasis. Its members such as MMP-2, MMP-9 and uPA play important roles in breast cancer metastasis, by degrading the extracellular matrix (ECM) and basement membrane (BM) to facilitate invasion and migration of cancer cells (22-24).

Previous studies have shown that the mitogen-activated protein kinase (MAPK) signaling pathway is associated with the invasion and migration of breast cancer cells through degradation of ECM (25,26). In the present study, we demonstrated the efficacy of emodin treatment on breast cancer cell migration, invasion and metastasis, and confirmed that emodin inhibits the invasion, migration and metastasis of MDA-MB-231 cells in vivo and in vitro. Emodin may function as an inhibitor of p38 and ERK in the regulation of MAPK signaling pathways through downregulation of MMP-2, MMP-9, uPA and uPAR expression.

Materials and methods

Materials. Emodin was obtained from the Shanghai Standardization for the Traditional Research Center and was dissolved in dimethyl sulfoxide (DMSO). Matrigel was purchased from Sigma (St. Louis, MO, USA) and
3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) was purchased from Promega (Madison, WI, USA). SB203580 and PD98059 were obtained from Biomol (Plymouth, PA, USA). The antibodies used in the present study were MMP-9, MMP-2, TIMP-1, TIMP-2, uPA and uPAR and were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); p38, p-p38, ERK2 and p-ERK1/2 were from Cell Signaling Technology (Boston, MA, USA). IRDye™ fluorescence antibodies were obtained from LI-COR Biosciences (Lincoln, NE, USA).

Cell culture. Human breast cancer MDA-MB-231 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 0.01 mg/ml insulin, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO₂ in a humidified atmosphere.

Cell viability assay. Cell viability was determined by MTS assay. Briefly, MDA-MB-231 cells (5x10⁴ cells/well) were seeded in 96-well culture plates. After overnight incubation, various concentrations of emodin were added to the cells for varying times followed by the addition of 20 μl MTS at 37°C for 4 h. Optical density (OD) was measured at 490 nm using an ELISA plate reader (BioTek, Winooski, VT, USA).

Wound healing assay. MDA-MB-231 cells were seeded at a density of 1-5x10⁴ cells/well in 12-well culture plates and allowed to form a confluent monolayer. The layer of cells was scraped with a 20-200 μl micropipette tip to create a wound of ~1 mm width. Cells were washed twice with PBS and replaced with serum-free medium containing various concentrations of emodin. At 0, 24, 48 and 72 h, cells were washed with PBS and then fixed with 4% paraformaldehyde followed by staining with 0.5% Coomassie brilliant blue. Images of the wounds were monitored under a phase-contrast microscope at x100 magnification.

Migration and invasion assays. Cell migration was analyzed with the aid of a Transwell (Corning Incorporated, Corning, NY, USA). To analyze cell invasion, the upper surface of a filter membrane in the upper compartment of a Transwell was coated with 30 μg Matrigel. Cells (1x10⁵) suspended in 200 μl of serum-free medium were seeded onto the upper compartment of the Transwell and the lower chambers were filled with medium containing 10% FCS and various concentrations of emodin. After 24 h, the cells remaining in the upper chamber were removed and cells on the underside of the filters were fixed with 70% ethanol followed by staining with 0.5% Coomassie brilliant blue for 10 min. The migrated or invaded cells were visualized and counted from six randomly selected fields (magnification, x100) under a phase-contrast microscope.

Effects of emodin on breast cancer MDA-MB-231 xenografts. Six-week-old female athymic nude mice were obtained from the Laboratory Animal Center at the Shanghai University of Traditional Chinese Medicine and were housed in a pathogen-free condition throughout the experimental duration. All procedures conformed to the consideration of animal welfare and were approved by the Ethical Committee of Shanghai Traditional Chinese Medicine. Briefly, mice were injected with 3x10⁶ MDA-MB-231 cells (suspected in Matrigel). One day after tumor cell inoculation, the mice received either 1% DMSO/10% Tween-80 in PBS (sham-treated group, n=8) or 40 mg/kg emodin (emodin-treated group, n=8) every two days through intraperitoneal injection. Mice were closely monitored, their bodies were weighed and tumor weights were measured weekly. Eight weeks after tumor cell inoculation, the mice were sacrificed and tumors were excised. Lungs were also collected from the sacrificed animals, sectioned and stained with hematoxylin and eosin (H&E). Representative fields for each group were photographed, and the metastatic nodules were counted.

Enzyme-linked immunosorbent assay (ELISA). To detect the effect of emodin on the secretion of MMP-2 and MMP-9 from MDA-MB-231 cells, the cells were treated with 20, 40 and 80 μM emodin for 24 h. Supernatants were then collected and analyzed by ELISA using human MMP-9 (R&D Systems, Minneapolis, MN, USA) and human MMP-2 ELISA kits (Ray Biotech, Norcross, GA, USA) according to the manufacturer's instructions.

Western blot analysis. Whole cell lysates were electrophoresed on 8 or 10% SDS-PAGE. The levels of MMP-2, MMP-9, TIMP-1, TIMP-2, uPA, uPAR, p38, p-p38, ERK2, p-ERK1/2 and GAPDH were detected by first incubating with the respective primary antibodies (1:1,000-5,000) and visualized by IRDye™700DX (red) or IRDye™800DX (green) conjugated secondary antibodies (1:10,000-20,000). Images were generated using the Odyssey Infrared Imaging system (LI-COR Biosciences). Quantitative analyses of western blots were performed using the AlphaEase FC (FluorChem FC2) software. Relative protein expression was standardized to the level of GAPDH.

Statistical analyses. All data are the means ± SD. Comparisons between groups were analyzed by the Student’s t-test and one-way analysis of variance (ANOVA). P<0.05 was considered to indicate a statistically significant result.

Results

Emodin inhibits breast cancer cell migration. To determine the general effect of emodin on breast cancer cell survival, varying concentrations of emodin were added to the MDA-MB-231 cell culture for up to 3 days. MTS assay showed that cell survival was significantly decreased after cells were treated with emodin at concentrations of 20, 40 and 80 μM for 48 and 72 h (vs. the untreated cells, P<0.05) (Fig. 1B). Subsequently, the effect of emodin on cell migra-
tion was examined by performing a wound healing assay with non-lethal concentrations and incubation periods (≤80 µM, ≤72 h). As shown in Fig. 2A, the wound gaps in the 10, 20, 40 and 80 µM emodin-treated groups were significantly wider than those of the untreated groups at 24, 48 and 72 h. The cell migration was further analyzed by Transwell assay. Similar to the results of the wound healing assay, emodin effectively inhibited cell migration in a dose-dependent manner, and an ~7, 30, 60 and 75% reduction could be observed in the MDA-MB-231 cells treated with 10, 20, 40 and 80 µM emodin, respectively (Fig. 2B and C). The results indicated that emodin can effectively inhibit the motility of MDA-MB-231 cells, since emodin shows a small effect on cell viability at these concentrations.

**Emodin inhibits breast cancer cell invasion.** To determine the effect of emodin on cell invasion, MDA-MB-231 cells were treated with 10, 20, 40 and 80 µM emodin followed by allowing cells to invade in Matrigel-coated Transwells for 24 h. The number of cells that invaded was reduced by emodin in a dose-dependent manner (Fig. 3). Compared to the untreated group, emodin at 40 and 80 µM suppressed invasion by ~57 and 44%, respectively. These data clearly showed that emodin is a strong suppressor of breast cancer cell invasion.

**Emodin reduces MMP-2 and MMP-9 secretion from MDA-MB-231 cells.** MMP-2 and MMP-9 are known to play an essential role in cancer cell invasion and metastasis by facilitating the degradation of ECM and BM. Amounts
Figure 3. Effect of emodin on MDA-MB-231 cell invasion. (A) A Transwell chamber was used for the invasion assay and images were captured (magnification, x200). The filter membranes were coated with Matrigel. MDA-MB-231 cells were treated with (a) 0, (b) 10, (c) 20, (d) 40 or (e) 80 µM of emodin for 24 h. No cells were seeded in (a). (B) Results are presented as means ± SD of three independent experiments; SD is denoted by the error bars. *P<0.01, vs. untreated control.

Figure 4. Emodin reduces the extracellular secretion of MMP-2 and MMP-9 in MDA-MB-231 cells. MDA-MB-231 cells were treated for 24 h with the indicated concentrations of emodin. Then each cell culture medium was collected and analyzed by ELISA using (A) human MMP-2 and (B) MMP-9 ELISA kits. ELISA was conducted according to the manufacturer's instructions. Each experiment was repeated 3 times. *P<0.05 and **P<0.01, vs. untreated control.

Figure 5. Effects of emodin on proteolytic enzymes and MAPK in MDA-MB-231 cells. Cells were treated with or without 10, 20, 40 and 80 µM emodin for 24 h. The proteins were analyzed by western blotting. The density ratio of proteins to emodin PDH is shown as relative expression. (A) uPA, uPAR, MMP-2 and MMP-9, TIMP-1 and TIMP-2 expression. (B) p38, p-p38, ERK1/2 and p-ERK expression. Values are expressed as means ± SD. Experiments were repeated 3 times. *P<0.05 and **P<0.01, vs. untreated cells.
of MMP-2 and MMP-9 secreted from MDA-MB-231 cells were measured with or without emodin treatment. As shown in Fig. 4A and B, emodin greatly decreased the secretion of MMP-2 and MMP-9 from MDA-MB-231 cells (P<0.01).

Emodin inhibits the expression of MMP-2, MMP-9, uPA, uPAR, p38 and ERK. To determine whether the expression of proteolytic enzymes, p38 and ERK were affected by emodin, the levels of MMP-2, MMP-9, uPA, uPAR, TIMP-1 and TIMP-2, p38, p-p38, p-ERK1/2 and ERK2 were evaluated in MDA-MB-231 cells treated with or without emodin. Western blotting with the respective antibodies showed that the expression levels of uPA, uPAR, MMP-2 and MMP-9 were concentration-dependently decreased by emodin treatment (P<0.05 or P<0.01; Fig. 5A). The levels of TIMP-1 and TIMP-2 were increased, but they were not significantly altered by emodin treatment (P>0.05; Fig. 5A). Moreover, emodin also reduced the expression levels of p38, p-p38 and p-ERK1/2 without altering the levels of ERK2 expression (P>0.05 or P<0.01; Fig. 5B). These results indicate that emodin is most likely to inhibit MDA-MB-231 cell migration, invasion and metastasis by downregulating the levels of uPA, uPAR, MMP-2 and MMP-9 proteolytic enzymes, and reducing activities of p38 and ERK.

Emodin, SB203580 and PD98059 inhibit cell invasion by regulating the expression of MMP-2, MMP-9, uPA and uPAR. To investigate the potential functional link between the expression of proteolytic enzymes, ERK and p38, we treated MDA-MB-231 cells with the p38-specific inhibitor SB203580 and ERK-specific inhibitor PD98059 in the presence or absence of emodin followed by analysis of cell invasion and proteolytic protein expression. Both the invasive ability of MDA-MB-231 cells and the expression levels of MMP-2, MMP-9, uPA and uPAR were significantly suppressed by 15 µM SB203580 (Fig. 6), 25 µM PD98059 (Fig. 7) and 80-µM emodin treatment (P<0.05 or P<0.01; Figs. 6 and 7), respectively. However, the treatment of SB203580 or PD98059 and combination with emodin did not exhibit a significantly greater inhibitory effect (P>0.05; Figs. 6 and 7). These results indicate that emodin is most likely to decrease MMP-2, MMP-9, uPA and uPAR expression and in vitro invasion as well as p38- and ERK-specific inhibitors.

Effects of emodin on breast cancer MDA-MB-231 cell xenografts. The ability of emodin to potently block in vitro invasion prompted us to investigate its effectiveness to inhibit in vivo metastasis. After athymic nude mice were injected with MDA-MB-231 cells for one day followed by (every two days)
Figure 7. Effect of emodin, PD98059 and their combination on MDA-MB-231 cell invasion and MMP-2, MMP-9, uPA and uPAR expression. (A) A Transwell chamber was used for the invasion assay (magnification, x100). The filter membranes were coated with Matrigel. Cells were untreated (b) or treated with 80 µM emodin (c), 25 µM PD98059 (d) or 80 µM emodin plus 25 µM PD98059 (e) for 24 h. (a) Blank (cells were not added). (B) MDA-MB-231 cells were treated with or without 80 µM emodin, 25 µM PD98059 and 80 µM emodin plus 25 µM PD98059 for 24 h. The proteins were analyzed by western blotting. The density ratio of proteins to GAPDH is shown as relative expression. Values are expressed as means ± SD. Experiments were repeated three times. *P<0.05, **P<0.01, ***P<0.001 vs. untreated cells.

Figure 8. Emodin inhibits tumor growth and pulmonary metastasis in mice bearing breast cancer MDA-MB-231 cell xenografts. Mice received 40 mg/kg emodin (i.p.) for 8 weeks (n=8). Lung tissues were removed and fixed with Bouin's solution for 24 h. The number of metastatic nodules in the lungs was counted under a dissecting microscope (magnification, x100). (A) Histological appearance of representative lungs from sham- and emodin-treated mice. (B) The quantification of metastatic lung nodules. (C) Tumor weight in the sham- and emodin-treated mice. **P<0.01 vs. sham-treated group.
intraperitoneal injection of 40 mg/kg emodin and vehicle (sham-treated) for 2 months, the metastatic nodules in lungs were evaluated by H&E-stained sections. The average number of tumor nodules in the sham-treated group was 21.60±3.92 while this number was 8.6±1.51 in the emodin-treated group (P<0.01; Fig. 8B), suggesting that emodin effectively decreases tumor cell colonization to the lung. Another noticeable difference between the two groups was that the sizes of these nodules were significantly larger in the sham-treated group than those in the emodin-treated group (Fig. 8A). Moreover, the average tumor weight was 0.46±0.07 g in the sham-treated group while the average tumor weight was 0.10±0.05 g in the emodin-treated group (Fig. 8C), representing over 78% of inhibition in tumor outgrowth. Together, these results suggest that emodin can block breast tumor outgrowth and metastasis.

**Discussion**

Throughout medical history, natural plant products have proven to be valuable sources of novel anticancer drugs, including vincristine, camptothecin, taxel and their derivatives (27,28). Although these natural products have been shown to be highly effective in clinical trials (29), some drugs such as vinblastine and paclitaxel can produce drug resistance (30), and other drugs such as vincristine, produce a strong cytotoxic effect (31). It has also been reported that treatment with topotecan, a water-soluble derivative of camptothecin, caused immunologic suppression in breast cancer (LM2-4) xenografts (32). Therefore, the identification of safer and more effective drugs from natural medical plants for breast cancer metastasis is crucial.

Emodin is a laxative ingredient derived from plants such as Rheum, Polygonum, Buckthorn and Senna (33). Because of its low toxicity, it can be used for medical treatment, as well as for health care and daily supplements such as it is used in hair care and skin care products. It can also be added to natural pigment and laxatives (34). Previous studies have shown that emodin not only has anticancer activity in breast cancer (9,10), but also did not exhibit drug resistance and other side-effects (33). A previous study (13) reported that 40 mg/kg dosage of emodin suppresses cancer cell growth in vivo, and we found that emodin at this dosage inhibited both tumor outgrowth and spontaneous lung metastasis of breast cancer (Fig. 8), without an obvious change in body weight, liver and
kidney functions in the mouse MDA-MB-231 cell xenograft model (Fig. 9). Moreover, emodin effectively reduced the invasion and the migration of breast cancer cells (Figs. 2 and 3). These findings indicate that emodin may be a potential safe and effective agent to suppress breast cancer metastasis.

Invasion and migration of cancer cells is an important step in cancer metastasis (35). In advanced breast cancer with poor prognosis, proteolytic enzymes such as MMP-2, MMP-9 and uPA are overexpressed (36), and they play a very important role in ECM and BM degradation (37). Moreover, ECM degradation promotes cell invasion and migration (38,39). Previous studies have confirmed that certain natural products can inhibit cancer metastasis by inhibition of ECM degradation through inhibition of proteolytic enzymes (40). In the present study, we found that emodin not only reduced the invasion and the migration of breast cancer cells, but also significantly inhibited the expression of MMP-2, MMP-9, uPA and uPAR, and the secretion of MMP-2 and MMP-9, even though TIMP-1 and TIMP-2 were not obviously changed in the breast cancer cells (Figs. 4 and 5), which indicated that emodin inhibited the invasion and the migration of breast cancer cells through downregulation of the expression of MMP-2, MMP-9 and uPA, and reduction in the secretion of MMP-2 and MMP-9.

MAPK is an important signaling pathway, which is critically involved in the process of cancer progression and metastasis by regulating cell proliferation, differentiation and apoptosis, angiogenesis, invasion and tumor metastasis (41,42). Both ERK and p38 are important members of the MAPK pathways for cancer invasion and metastasis, which are involved in the regulation of MMP-2, MMP-9 and uPA in breast cancer (43). Previous research has found that certain natural products can inhibit breast cancer metastasis by downregulated the expression of MMP-2, MMP-9 and uPA through inhibition of the p38 signaling pathway (44). In the present study, emodin as well as SB203580, a p38-specific inhibitor and PD98059, an ERK specific inhibitor, decreased the activation of p38 and ERK1/2 and the expression levels of MMP-2, MMP-9 and uPA, but the combination treatment of emodin and SB203580 or PD98059 did not obviously alter these inhibitory effects in MDA-MB-231 cells (Figs. 6 and 7). Together, these findings indicate that emodin inhibits breast cancer cell invasion through the downregulation of MMP-2, MMP-9, uPA and uPAR expression and as an inhibitor of p38 and ERK.

Many reports suggest that, emodin, as an inhibitor, can inhibit a variety of protein kinases such as MAPK and NF-κB and proteolytic enzymes such as MMPs and uPA, but also inhibits cancer cell invasion and metastasis (45). There is previous evidence that emodin inactivated ERK1/2 in lung cancer H1703 or A549 cells and reduced the phosphorylation of the p38 MAPK in atherosclerosis (46). In breast cancer, emodin suppressed HER-2/neu tyrosine kinase activity in MDA-MB-435 cells, which overexpressed HER-2/neu and thereby suppressing the proliferation of these cells (47). In the present study, we found that emodin inhibited migration and invasion of MDA-MB-231 cells, and also inhibited the expression levels of p-p38 and p-ERK1/2, MMP-2, MMP-9 and uPA. Collectively, the present study suggests that emodin is a non-specific protein kinase inhibitor affecting p38, ERK and proteolytic enzymes, MMPs and uPA in inhibiting invasion of breast cells.

In conclusion, emodin inhibited lung metastasis in mice bearing human breast cancer MDA-MB-231 xenografts in a nude mouse xenograft model. Emodin also inhibited the invasion and migration of MDA-MB-231 cells by downregulating the expression of MMP-2, MMP-9, uPA, p-p38 and p-ERK1/2 and decreasing the activity of p38 and ERK. Furthermore, emodin inhibited the invasion of MDA-MB-231 cells by downregulating the expression of MMP-2, MMP-9, uPA and uPAR as well as decreasing the activity of p38 and ERK.

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