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Effect of the Chinese herb Mesima Reishi UE-1 on invasion of human ovarian cancer cells in vitro

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To study the effects of Mesima Reishi UE-1 on the metastatic phenotype of the human ovarian cancer cell line HO8910. HO8910 cells were pretreated with different concentration of Mesima Reishi UE-1 in vitro. Using cell proliferation assay, spreading and adhesion assay, cell migration and invasion assay, zymography, immunocytochemical staining and RT-PCR, we examined the effects of Mesima Reishi UE-1 on cell proliferation, motility, adhesion and expression of MMP-2, 9 in vitro. Mesima Reishi UE-1 directly inhibited HO8910 cell proliferation in vitro. And pretreated with different concentrations of Mesima Reishi UE-1, HO8910 cells motility, adhesion and invasion were inhibited significantly. The activity of MMP-9 was inhibited in a dose-dependent manner. The protein and mRNA expression of MMP-2, 9 were also inhibited. Mesima Reishi UE-1 suppresses invasion of human ovarian cancer cells in vitro.

Key words: Mesima Reishi UE-1, polysaccharides, human ovarian cancer, tumor invasion, matrix metalloproteinase.

INTRODUCTION

Human ovarian cancer, which occurs mainly in post-menopausal women, is the leading cause of death from gynecological malignancy (http://www.ovariancancer.org/about-ovarian-cancer/statistics/accessed 10/19/09). Since ovarian cancer often remains clinically silent, the majority of patients with ovarian carcinoma have advanced intraperitoneal metastatic disease at diagnosis, resulting in a poor prognosis. About 80% of ovarian cancer cases are diagnosed at an advanced stage after metastasis has occurred (http://www.ovariancancer.org/about-ovarian-cancer/statistics/accessed 10/19/09; Roomi et al., 2010). There are more than 200,000 women worldwide who die of ovarian cancer every year. Developing and exploiting Chinese herbal medicines to treat cancer has become a hot spot in new anti-cancer agents’ research.

Mesima Reishi UE-1 is a compound preparation which is extracted from Phellinus linteus and Ganoderma lucidum and composed of polysaccharides. P. linteus and G. lucidum were mixed in equal proportion (1:1) to produce Mesima Reishi UE-1. The anti-tumor function of P. linteus was first discovered by Japanese scholars (Ikekawa et al., 1968). Since the 1970s, a series of studies has shown that G. lucidum can suppress the growth of transplanted tumors in mice (Li et al., 2008; Sanodiya et al., 2009; Weng, 2007). The chemical components of G. lucidum are complicated: They involve polysaccharides, flavonoids and alkaloids, amino acids, steroids, oligosaccharides, proteins, mannitol, etc (Zjawiony et al., 2004). Among these components, G. lucidum polysaccharide (GLPS) has been identified as one of its major bioactive components, showing multiple pharmacological effects, such as immunomodulation, anti-oxidation, hepatoprotection, anti-proliferation and anti-angiogenesis and also decrease tumor weigh, and
prolong patients’ survival time (Xu et al., 2011; Hu et al., 2002). The mechanism is thought to be due to the inhibition of vascular endothelial proliferation, as a consequence of reduction of vascular endothelial growth factor (VEGF) produced by the tumor cells, and the induction of tumor cell apoptosis (Shao et al., 2004). The polysaccharides were consisted of D-rhamnose, D-xylene, D-fructose, D-galactose and D-glucose and linked together by β-glycosidic linkages (Cao and Lin, 2004). The main medicinal components were polysaccharides including intracellular polysaccharide (IPS) and exopolysaccharide (EPS) (Guo et al., 2009). The structure of a portion of water-soluble mycelial polysaccharide from P. linteus was a core (1-3)-linked glucan heavily substituted via (1-6) links with (1-3) linked mannose chains (Baker et al., 2008). During the past few decades, it has been found that polysaccharides isolated from P. linteus also have antitumor and immune-modulating activities (Moradali et al., 2007; Zhang et al., 2007; Li et al., 2004; Kim et al., 2006; D Sliva et al., 2008).

Tumor invasion and metastasis are the most important characteristics of malignancy, which is the consequence of biological behaviors, including enhanced tumor cell adhesion, proliferation, and enzymatic degradation of matrix (Leber and Efferth, 2009; Coussens and Werb, 2002; Zhang et al., 2005; Chambers et al., 2002). Matrix metalloproteinase (MMP) expression has been shown to be linked to tumor invasion in many different tumors (Roomi et al., 2010; Hojjilla et al., 2003; Deng et al., 2010; Strongin, 2006) and matrix metalloproteinases (MMPs) play essential roles in the whole process of tumor invasion and metastasis (Hojilla et al., 2003; Deng et al., 2010; Strongin, 2006; Van Hinsbergh and Koolwijk 2008; Rundhaug, 2005). Clinical studies have marked the association of MMP expression with progression of ovarian cancer (Roomi et al., 2010; Lopata et al., 2003; Torng, 2004). During this process, MMP-2 and MMP-9 play a critical role in tumor invasion and metastasis (Li et al., 2008; Overall and Klefeld, 2006; Duffy et al., 2008). Patients with metastatic cancers can no longer be cured by local therapy alone and usually die after painful chemotherapy. Thus, control of cancer metastasis is an important issue in tumor treatment (Chia et al., 2010; Malemud, 2006).

In this study, we examined the effect of UE-1 on invasion of human ovarian cancer cells by investigating the proliferation, adhesion, motility, the activity and expression of MMPs. The results show that UE-1 can suppress the invasion of human ovarian cancer cells in vitro.

MATERIALS AND METHODS

Cell culture

HO8910 cells (human ovarian cancer cell line) were obtained from the Key Laboratory of Pathobiology, Ministry of Education, Norman Bethune College of Medicine. Cells were cultured in high glucose-Dulbecco modified eagle medium (H-DMEM; Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, USA) in humidified 5% CO₂ atmosphere at 37°C.

Drugs and reagents

Mesima Reishi UE-1 was kindly provided by Nikkei Edible Fungus Research Institute. The ratio of polysaccharides of G. lucidum is 45.5 and 38.9% of P. linteus. They were then divided into a high dose group (200 mg/l) and a low dose group (100 mg/l) by diluted to required concentration with H-DMEM supplemented with 10% FBS (Zhang et al., 2010). H-DMEM supplemented with 10% FBS was employed as a control.

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Cell proliferation assay

HO8910 cells were harvested by trypsinization when grown until 80% confluence and resuspended in 10% FBS H-DMEM. Cells were seeded onto 96-well culture plates at a density of 2000 cells/well and incubated at 37°C for 24 h. Then cells were washed with phosphate-buffered saline (PBS) 3 times and cultured in H-DMEM containing Mesima Reishi UE-1 at concentration of 0, 100 and 200 mg/l. Then cells in each group were seeded onto 12 replicate 96-well culture plates with H-DMEM containing Mesima Reishi UE-1 0, 100 and 200 mg/l. After 48 h, H-DMEM supplemented with 10% FBS H-DMEM with Mesima Reishi UE-1 was kindly provided by Nikkei Edible Fungus Research Institute. They were then divided into a high dose group (200 mg/l) and a low dose group (100 mg/l) by diluted to required concentration with H-DMEM supplemented with 10% FBS (Zhang et al., 2010). H-DMEM supplemented with 10% FBS was employed as a control.

Cell spreading assay

HO8910 cells were harvested by trypsinization when grown until 80% confluence and resuspended in 10% FBS H-DMEM. Cells were seeded onto 96-well culture plates at a density of 2000 cells/well and incubated at 37°C for 24 h. Cells were cultured in high glucose-Dulbecco modified eagle medium (H-DMEM; Hyclone, USA) containing 0, 100 and 200 mg/l Mesima Reishi UE-1 0, 100 and 200 mg/l, respectively. 96-well culture plates were precoated with 5 µg/cm² fibronectin (FN; BD Bioscience, USA). Then cells in each group were seeded into 6 replicate wells at 1x10⁴ cells/well and incubated at 37°C for 60 min. They were then washed with serum-free medium. The shape of the cells was photographed under a microscope (Olympus, Japan) at 0.5, 3 and 24 h. Cells with round shape were defined as not spreading and those with protuberance and extension had spread.

Cell adhesion assay

96-well culture plates were precoated with Fibronectin as in the cell spreading assay. Serum-free medium (50 µl), supplemented with 10 g/l Bovine serum albumin (BSA), was added to each plate to block nonspecific adhesion. HO8910 cells were cultured for 48 h in 10% FBS H-DMEM containing Mesima Reishi UE-1 0, 100 and 200 mg/l. HO8910 cells were then divided into 12 replicate wells at 1x10⁴ cells/well and allowed to attach for 2 h at 37°C. The cells were washed with PBS 3 times to remove non-bound cells. Cell counting reagent (CCK-8; Sigma, US) (10 µl) were added to
each well. After incubation for 60 min at 37°C, absorbance was measured at 450 nm. The absorbance value of the washed plates was A; the value of unwashed plates was B, the adherence ratio A/B×100%. Results are expressed as mean (±SD).

Cell migration assay (wound healing assay)

5x10^5 cells were seeded onto a 6-well culture plate and then grown until monolayer confluence. A cell wound was produced with a 100 µl micropipette tip and gently washed with serum-free medium to remove the detached cells. Monolayers with cell wounds were cultured in 10% FBS H-DMEM containing 0, 100 and 200 mg/l Mesima Reishi UE-1. The wound area was observed by microscope (Olympus, Japan) with a 100 x magnification at 24, 48, 72 and 96 h.

Cell invasion assay (Boyden chamber invasion assays)

NIH3T3 mouse fibroblast cells were cultured until confluence in RPMI1640 supplemented with 10% FBS. Then cells were starved for 24 h in serum-free medium and the supernatant was collected as conditioned medium. Cell invasion assays were performed using Boyden chambers with 8 µm pores. Boyden chambers which contain polycarbonate membrane were precoated with 60 µl Matrigel (1:4 dilution) and incubated at 37°C for 30 min. HO8910 cells were cultured for 48 h in 10% FBS H-DMEM containing 0, 100 and 200 mg/l Mesima Reishi UE-1. Cells were harvested and resuspended in serum-free medium. 1x10^5 cells in 100 µl medium were seeded into each of the chambers. 400 µl conditioned medium was added into the lower chamber to create a chemotactic gradient. After 4 h incubation at 37°C, the membranes were fixed with 4% paraformaldehyde for 30 min and stained by hematoxylin-eosin staining. Ten microscopic fields from the under surface were randomly chosen for analysis. Results are expressed as mean standard deviation (±SD).

Protein expression of MMP-2, 9 (immunocytochemistry)

HO8910 cells were plated onto chamber glass slides and grown until 80% confluence. The cells were then cultured in 10% FBS H-DMEM containing Mesima Reishi UE-1 0, 100 and 200 mg/l for 48 h and then fixed with 4% paraformaldehyde for 30 min at room temperature. Reaction products were visualized by the SP immunohistochemistry kit (SP kit; Maixin, China) using diaminobenzidine tetrahydrochloride (DAB; Maixin, China) as the developer. Those cells which cytoplasm appeared brown granules and color stronger than the background were positive. Twenty visual fields were chosen on each slide. The gray-scale value (IOD) was analyzed with image-Pro plus 6.0. Results are expressed as the mean (±SD).

mRNA expression of MMP-2, 9 (reverse transcriptase-polymerase chain reaction (RT-PCR) analysis)

HO8910 cells were cultured for 48 h in 10% FBS H-DMEM containing Mesima Reishi UE-1 0, 100 and 200 mg/l, respectively. Cells were harvested by trypsinization. Total RNA was extracted from the experimental or control cells by TRizol (Invitrogen, USA) according to the manufacturer’s protocol.

5 µg RNA was transcribed into cDNA by Moloney murine leukemia virus (M-MLV) reverse transcriptase and oligonucleotide (T) 18 primer. 1 µl cDNA was added into the PCR system (Takara, Dalian, China) for 35 cycles at an annealing temperature of 55°C. MMP-2, 9 and β-actin were amplified using the following primer pairs: 5’-AAGGATGGTCTACTGGCA-3’ (MMP-2 forward), 5’-AGAGATTCTCAGGGTC-3’ (MMP-2 reverse), 5’-TGCCGAGATGC GTGGAGA-3’ (MMP-9 forward), 5’-GCGAATCTCCGAGTATTTT-3’ (MMP-9 reverse), 5’-TGGAACTCTGTCGGATCCATGAAAC-3’ (β-actin forward), 5’-TAAAGCAGCTGCTAAGCTGCC-3’ (β-actin reverse).

Amplification of β-actin was used to estimate the efficiency of cDNA synthesis and equal loading. 5 µg amplified products were analyzed by electrophoresis on 1% agarose gel and photographed by gel Doc 2000 photography system (Bio-Rad, USA). The β-actin of each sample was calculated for the relative expression intensity to quantify and analyze the bands density of the products on the gel. Results are expressed as the mean (±SD) of the band optical density.

RESULTS

Effects of UE-1 on proliferation of HO8910 cells in vitro

HO8910 cells were cultured in 10% FBS H-DMEM containing Mesima Reishi UE-1 0, 100 and 200 mg/l for 48 h. There was a significant (p < 0.01) inhibition of HO8910 cell proliferation in vitro (Figure 1). Inhibition ratio of the experimental groups= [(OD value of the control- OD value of experimental groups)/ OD value of the control]×100% were 48.28 and 55.17%, respectively.

Effects of UE-1 on spreading of HO8910 cells in vitro

There was no difference in cell shape between the experimental groups and the control at 30 min. By 3 h, a few of the control cells had spreading features, while the experimental groups were still a round shape. The experimental groups had no change compared with their 30 min morphology. The spreading of cells in the control was significantly more than that of the two experimental groups by 12 h. At 24 h, cells of the control had fully spread, while only a few cells of the experimental groups
Figure 1. The effect of UE-1 (mg/L) on proliferation of HO8910 cells in vitro (n = 6, ±s).

Figure 2. The effect of UE-1 (mg/L) on the spreading of HO8910 cells. 1x10^4 cells were seeded into each well of 96-well plates precoated with fibronectin. Cells were washed three times and photographed after incubation for 3 h (upper panel), 12 h (middle panel), 24 h (lower panel). Photographed cells were adherent to the fibronectin ×100.

had spreading features. There were many dead cells in the experimental groups (Figure 2).

Effects of UE-1 on adherence behavior of HO8910 cells in vitro

The adherence assay showed that after 2 h co-culture, the cells attached to fibronectin in the experimental groups were fewer than in the control. This effect was significant (p < 0.05) in the 200 mg/ml UE-1 group (Figure 3).

Effects of UE-1 on migration behavior of HO8910 cells in vitro

The migration assay (Figure 4) showed that migration capability of the experimental groups was limited, while the cells of the control group were growing actively, fully spread with high migration capability. The motility and migration speed of the cells which pretreated with UE-1 were decreased significantly. By 96 h, the wounds of the
control culture had healed, while those of the experimental groups were still unhealed. Adherence of the experimental group cells was decreased and there were many floating dead cells.

**Effects of UE-1 on invasion of HO8910 cells in vitro**

After 6 h, cells in all groups had invaded through Matrigel in the Boyden chamber assay (Figure 5). The cell number of the control which migrated through the gel and invaded to the lower surface of the chamber membrane with spreading features was considerable. In the experiment groups, fewer cells had invaded and few of them were spread. All these shows that UE-1 significantly \((p < 0.01)\) decreased the invasive ability of the cells when compared with the control cells \((n = 6, \bar{x} \pm s)\).

**Effects of UE-1 on protein expression of MMP-2, 9**

We used immunocytochemistry to examine protein
He et al.         9893

*P < 0.01 versus control

**Figure 5.** The effect of UE-1 (mg/L) on the invasion of HO8910 cells *in vitro*. Results describe the number of cells which had invaded to the lower surface of the chamber membrane. Invasive cell number was quantified by counting the invasive cells as described at a magnification of ×400.

expression of MMP-2 and MMP-9 in each group. There was strong positive expression of MMP-2 and MMP-9 in the control cells whereas expression was low in the experiment groups’ cells. The control group cells were triangular, round or polygonal, had high adherence and refraction while the cell number in the experimental groups was decreased accompanied by with low refraction and particles in the cytoplasm. Inhibition of protein expression of MMP-2 and MMP-9 was in a dose-dependent manner (Figure 6 and Table 1).

**Effects of UE-1 on mRNA expression of MMP-2, 9**

HO8910 cells were cultured for 48 h in 10% FBS HDMEM containing Mesima Reishi UE-1 0, 100 and 200
Table 1. Effect of UE-1 (mg/L) on the expression of MMP-2 and MMP-9 protein of HO8910 cells.

<table>
<thead>
<tr>
<th>Group (mg/l)</th>
<th>MMP-2 expression (IOD value)</th>
<th>MMP-9 expression (IOD value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UE-1 0</td>
<td>184.17±652.39</td>
<td>157.80±280.93</td>
</tr>
<tr>
<td>100</td>
<td>129.06±847.21*</td>
<td>109.26±371.98*</td>
</tr>
<tr>
<td>200</td>
<td>63.98±161.09**</td>
<td>52.12±197.37**</td>
</tr>
</tbody>
</table>

(n= 20, \( \bar{x} \pm SD \)). Twenty visual fields were counted at a magnification of x200. IOD Value refers to gray-scale value that showed protein expression quantity of MMP-2, 9. **P < 0.01; *P < 0.05.

Figure 7. (A) Effect of UE-1 (mg/L) on the mRNA expression of MMP-2, 9 of HO8910 cells in vitro. Amplification of β-actin was used to ascertain RNA integrity and equal loading. MMP-2, 442 bp; MMP-9, 229 bp; β-actin, 362 bp. Lane 1, Marker, DL2000; lane 2, 0 (mg/L the following to be same) MMP-9; lane 3, 100 MMP-9; lane 4, 200 MMP-9; lane 5, 0 MMP-2; lane 6, 100 MMP-2; lane 7, 200 MMP-2; lane 8, 0 β-actin; lane 9, 100β-actin; lane 10, 200 β-actin; (B) effect of UE-1 (mg/L) on the mRNA expression of MMP-2 and MMP-9 of HO8910 cells in vitro. The intensity showed mRNA expression quantity of MMP-2 and MMP-9 (n = 6, ±s).

Effects of UE-1 on activity of MMP-9

HO8910 cells were cultured for 48 h in 10% FBS H-DMEM containing Mesima Reishi UE-1 0, 100 and 200 mg/l, respectively. Zymography was used to test the activity of MMP-9. The result shows that the activity of MMP-9 was inhibited significantly in a dose-dependent manner. The activity inhibition ratio of 100 and 200 mg/l
DISCUSSION

Cancer is a multifactorial disease in which cumulative genetic and epigenetic alterations affect multiple distinct regulatory circuits within cells. To treat cancer, there is a growing belief that combination therapy using multiple drugs targeting various cellular pathways would yield better outcomes than monotherapies (Chia et al., 2010). Tumor invasion and metastasis are the most distinctive characteristics of malignant neoplasms and important indicators of the tumor patients' prognosis. These are complicated processes, accompanied by extracellular matrix degradation and changes in cell surface receptor expression (Leber and Efferth, 2009; Coussens and Werb, 2002). In this respect, the Chinese herb Mesima Reishi UE-1, which contains various phytochemicals that target multiple dysregulated pathways in cancer cells may provide a complementary way to treat cancers. Then effect on the phenotypic of the ovarian cancer line HO8910 were than studied. Our results demonstrate that the Mesima Reishi UE-1 not only inhibited the proliferation, migration and invasive ability of the cell line, but also inhibited the activity and expression of MMP-2 and MMP-9.

Inhibiting tumor cell proliferation is the most basic means of treating carcinoma in situ and invasive cancer (Zhang et al., 2005). Timely control of the proliferation of cancer cells has significant meaning for cancer therapy. This study demonstrates that low concentrations of UE-1 inhibited HO8910 cell growth, but that inhibition was out of a dose-effect relationship when the concentration exceeded a certain range.

Adhesion of cells is dependent on their surface
receptor expression, which also regulate cell shape maintenance, cell division and cell movement. Adhesion is the initiation step in cancer invasion. The capacity of highly invasive tumor cells adhere to extracellular matrix components is usually great (Curran and Murray, 2000). Consequently, inhibiting the adhesion of tumor cells can depress tumor invasion and prevent tumor metastasis (Coussens and Werb, 2002). In this study, after pretreatment with different concentrations of Mesima Reishi UE-1, adhesion and spreading of HO8910 cells were inhibited significantly. When the control cells had already spread, only a few experimental cells were protuberation and extension. There were also a certain amount of dead cells. All of these objectives imply that EU-1 has the effect of inhibiting cell adhesion.

Migration is an indispensable step in tumor invasion and metastasis. Highly invasive tumor cells typically have higher migration capacity (Coussens and Werb, 2002). Our study shows that after pretreated with different concentrations of Mesima Reishi UE-1, HO8910 cell motility was inhibited significantly, and the speed of migration was significantly slower than in the control group. At 96 h, the wound of the control group had healed, while the experimental group’s wound still existed. Furthermore, during co-cultivation, the wound of the high concentration group was shorted slightly, both experimental groups showed inhibited adhesion of cells and some cells were floating. All of these observations expressly showed that the adhesion and proliferation were inhibited. In the cell invasion assay, compared with the control group, the experimental group had many fewer cells which migrated through Matrigel and the cells which had migrate through Matrigel were not fully spread. These show that the invasion of HO8910 was significantly inhibited by UE-1.

Matrix metalloproteases play the most essential roles in tumor invasion and metastasis. They can help tumor cells to invade into blood vessel and the lymphatic system, in order to promote tumor proliferation and create a path for tumor cell spreading (Chambers et al., 2002; Hojilla et al., 2003). Thus, knowledge of matrix metalloprotease regulation is of great importance for developing therapeutic strategies (Roomi et al., 2010). Matrix metalloprotease (MMP) is one of the most important proteases which degrade ECM in vivo by degrading extracellular matrix and disrupting the basement membrane. A number of extracellular factors, including cytokines, growth factors, cell contact with ECM and inducers and inhibitors, have been implicated in the regulation of MMP expression in different types of tumor cells (Roomi et al., 2010; Deng et al., 2010; Curran and Murray, 2000; Fortunato and Menon, 2002). Consequently, there is evidence that MMPs can further promote tumor invasion through tissue barriers. During this process, degradation of the extracellular matrix and components of the basement membrane by proteases, such as matrix metalloproteinase (MMP)-2, MMP-9, plays a critical role in tumor invasion and metastasis (Li et al., 2008; Overall and Kleifeld, 2006; Duffy et al., 2008; Chia et al., 2010). Thus, activity of MMPs is one of the most important facts tumor invasion and metastasis (Leber and Effertth, 2009; Chen et al., 2008). Measuring the effects of tumor cell metalloproteinase secretion is an important index for assessing the anti-invasive effects of a drug. While at least 20 members in the MMP family, mainly secreted by tumor cells and mesenchymal cells, MMP-2 and MMP-9 are the enzyme which has the closest relation to tumor invasion process (Bachmeier et al., 2005). In recent years, much effort has been taken to develop drugs that can inhibit metastasis. However, till now promising antimetastatic agents are still lacking (Duffy et al., 2008; Chia et al., 2010). In this study, the results show that pretreated with different concentrations of Mesima Reishi UE-1, the protein and mRNA expression of MMP-2 and MMP-9 in HO8910 cells were inhibited significantly. The activity of MMP-9 was also inhibited in a dose-dependent manner.

In this study, we have demonstrated that Mesima Reishi UE-1 can directly inhibit proliferation, motility, adhesion, MMP-2 and MMP-9 activity, protein and mRNA expression of HO8910 cells. In addition to this study, it has been shown that Mesima Reishi UE-1 can also inhibit chick chorioallantoic membrane (CAM) angiogenesis (Zhang et al., 2005). To our knowledge, no prior research has examined the effects of Mesima Reishi UE-1 on human ovarian cancer in vitro. Given the well-evidence that of Mesima Reishi UE-1 can suppress human ovarian cancer cells invasion in vitro, the results from this study lay an experimental foundation for the future development of new anti-tumor agents and clinical application of traditional Chinese medicine.

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