Safflower polysaccharide inhibits the proliferation and metastasis of MCF-7 breast cancer cells

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Abstract. Breast cancer accounts for 22.9% of all types of cancer in females worldwide. Safflower polysaccharide (SPS) is an active fraction purified from safflower petals (*Carthamus tinctorius* L). The present study investigated the effects of safflower polysaccharide on the proliferation and metastasis of breast cancer cells. Cell viability was analyzed using an MTT assay following treatment of the MCF-7 cells with increasing concentrations of SPS. The results demonstrated that the SPS compound significantly inhibited the proliferation of the MCF-7 human breast cancer cell line and these inhibitory effects increased in a dose- and time-dependent manner. The half maximal inhibitory concentration (IC_{50}) value of SPS on breast cancer cells, following treatment for 72 h, was detected using an MTT assay and was calculated as 0.12 mg/ml. The apoptotic rate was detected using flow cytometry in the MCF-7 human breast cancer cell line and the results revealed that SPS induced cell apoptosis. The apoptotic rate of the MCF-7 cells treated with SPS was significantly higher compared with that of the untreated cells and increased in a dose-dependent manner. The expression of B-cell lymphoma 2 (Bcl-2) was downregulated and the expression of Bcl-2-associated X protein was upregulated in the MCF-7 cells treated with SPS in a time-dependent manner. Additionally, the expression of matrix metalloproteinase-9 was significantly reduced and the expression of tissue inhibitor of metalloproteinase-1 was increased in the MCF-7 human breast cancer cell treated with SPS. These results demonstrated that SPS inhibited the metastasis of MCF-7 breast cancer cells and understanding the underlying mechanisms may provide novel strategies in breast cancer therapy.

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Introduction

Cancer is one of the most life threatening diseases in humans worldwide. Breast cancer is the most frequent type of cancer affecting western females, accounting for 7-10% cases of systemic cancer (1,2). Breast cancer is predominantly a result of abnormal division and malignant proliferation of the breast duct or acinar cells (3,4). The incidence of breast cancer has increased in the majority of countries (5) and its metastasis to distant sites is the predominant cause of mortality (6). Currently, there remains no effective means to treat breast cancer. Inducing the apoptosis of tumor cells is an important strategy in cancer treatment (7-9). There are at least two pathways, which lead to cell apoptosis, an 'extrinsic' and an 'intrinsic' pathway, which are mediated by death receptors on the cell surface and by mitochondria, respectively (10-12). The intrinsic apoptotic pathway is characterized by the permeabilization of the mitochondria and the release of cytochrome *c* into the cytoplasm (13,14). Notably, the expression levels of the anti-apoptotic factor, B-cell lymphoma 2 (Bcl-2) and pro-apoptotic protein, Bcl-2-associated X protein (Bax), increase during the process of apoptosis, occurring in a mitochondria-mediated apoptotic signaling pathway (15,16).

The prognosis of patients with breast cancer is closely associated with the malignant invasion and early metastasis of the cancer cells (17,18). The involvement of matrix metalloproteinases (MMPs) in tumor invasion and metastases is an area of interest. The activation of MMP in the extracellular matrix degrade collagen and release growth factors and peptides (19,20). Under pathological conditions, the expression of MMPs increase significantly faster compared with that of the tissue inhibitors of metalloproteinase (TIMPs), which induce the activation of MMPs, alters the tumor microenvironment and activates cell surface receptors and downstream signaling pathways (21). MMP-9 and its specific inhibitor, TIMP-1, are closely correlated with tumor physiological and pathological processes (22). Activated MMP-9 and pro-MMP-9 bind to TIMP-1, and the activation of MMP-9 is often characterized by the degradation of TIMP-1 and the accumulation of extracellular matrix (23,24). Thus, MMP-9 is closely associated with

the occurrence and development of breast cancer. Therefore, it is important to identify potential effective therapeutic agents, which can effectively activate apoptosis-mediated cell death and inhibit the invasion and metastasis of breast cancer.

Safflower (*Carthamus tinctorius* L) is a traditional Chinese medicine. It is a herbaceous plant of the Asteraceae family and contains a variety of chemical constituents, including flavonoids, organic acids and polysaccharides (25). It has been reported that safflower polysaccharide (SPS) has an immune regulatory function, antitumor effect (25-27), may activate the phosphatidylinositide 3-kinase/Akt signaling pathways and regulate the cell cycle of cancer cells (28). However, the role of SPS on the proliferation and metastasis of breast cancer remains to be elucidated. The present study investigated the effects of SPS on the proliferation and metastasis of breast cancer cells to clarify the mechanisms underlying breast cancer and provide novel strategies for breast cancer therapy.

Materials and methods

Cells and reagents. The MCF-7 human breast cancer cell line (HTB-22™; American Type Culture Collection, Manassas, VA, USA) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (HyClone Laboratories, Inc., Logan, UT, USA). The MTT reagent was purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal Bcl-2 (cat. no. ab7973), rabbit polyclonal MMP9 (cat. no. ab38898) and mouse monoclonal TIMP1 (cat. no. ab1827) antibodies were purchased from Abcam (Cambridge, UK) and rabbit monoclonal Bax antibody (cat. no. AJ1079a) was obtained from Abgent (Suzhou, China).

SPS preparation. The dried red flower and leaves of the safflower (Tongrentang Co., Ltd., Beijing, China) were used to extract the polysaccharide. The dried red flower was fully impregnated into water, and was boiled four times for 1 h each time. The filtrate was combined and concentrated to a volume of 1/4 prior to adding 95% ethanol (Jinhua Chemical Reagent Co., Ltd., Guangzhou, China) to a volume of four-five times and incubated overnight at 4˚C. The SPS was precipitated by alcohol precipitation and collected by centrifugation at 200 x g/min for 5 min. The SPS was dissolved in deionized water and the SPS was precipitated twice using ethanol. The SPS content was determined using a sulfuric acid-phenol method, as described previously (29-32), and the polysaccharide purity was up to 76.05%.

MTT assay. Cell proliferation analysis was performed using an MTT assay. Briefly, the MCF-7 breast cancer cells were seeded into 96-well plates ($5x10^4$ cells/well), and following adherence, the cells were treated with SPS at concentrations of 0.04, 0.08, 0.17, 0.34, 0.68 or 1.36 mg/ml for 24, 48 and 72 h at 37˚C. A total of 20 μl MTT (5 mg/ml) was then added to the medium in each well for 4 h and 150 μl dimethylsulfoxide was added. The absorbance of the 96-well plates was read using a microplate reader (Ultraviolet Spectrophotometer AquaMate-Plus; Thermo Fisher Scientific, Waltham, MA, USA) at a test wavelength of 490 nm and a reference wavelength of 570 nm.

Western blot analysis. The breast cancer cells were seeded into 48-well plates ($5x10^5$ cells/well) for 8 h, prior to treatment with different concentrations of SPS (0.04, 0.20 and 0.60 mg/ml) for 24, 48 and 72 h. The cell lysates were prepared using sodium dodecyl sulfate (SDS) loading buffer. The lysates were separated using 10% SDS-PAGE gels and the proteins were transferred from the gel onto a nitrocellulose membrane (Biodee Corporation, Beijing, China) at 35 V for 3 h (constant voltage). The membrane was then blocked with TBST supplemented with 5% bovine serum albumin for 30 min prior to incubating the membrane with the specific antibodies in Tris-buffered saline with Tween-20 (TBST) containing 5% bovine serum albumin at 4˚C overnight. The membrane was washed three times with TBST and then incubated with the goat anti-mouse IgG (SN133) and goat anti-rabbit (BA1054) secondary antibodies [Sunshine Biotechnology (Nanjing) Co., Ltd., Nanjing, China] for 1 h at room temperature. The bands were detected in a dark room using an Immobilon Western Chemiluminescent kit (EMD Millipore, Billerica, MA, USA).

Flow cytometric analysis. The apoptotic rates of the MCF-7 breast cancer cells were determined using annexin V-propidium iodide (PI) staining, according to the manufacturer's instructions (Santa Cruz Biotechnolgy, Inc., Dallas, TX, USA). Briefly, the cells ($5x10^5$ cells/well) were seeded into 6-well plates for 6 h prior to treatment with SPS at the concentrations of 0.04 and 0.68 mg/ml for 48 h. The cells were then washed and resuspended in phosphate-buffered saline (HyClone Laboratories, Inc.). Annexin V (0.1 μg/µl) and PI (0.05 μg/μl) were added to the cells and incubated in the dark for 30 min on ice. The apoptotic rate was determined using fluorescent activated cell sorting (FACS) analysis (FACSCalibur Cell Sorting system; BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis. The data were analyzed using SPSS 11.5 statistical software (SPSS, Inc., Chicago, IL, USA) and the data are expressed as the mean ± standard error of the mean. One way analysis of variance and Student's t-test were used to analyze the results. $P<0.01$ was considered to indicate a statistically significant difference.

Results

SPS has an increasing antitumor effect on the MCF-7 breast cancer cell line in a time- and dose-dependent manner. In order to investigate the antitumor activity of SPS on breast cancer cells, the MCF-7 human breast cancer cell line was used and the antitumor effects of SPS were detected using an MTT assay. As shown in Fig. 1, the MCF-7 cells were treated by SPS at concentrations of 0.04, 0.08, 0.17, 0.34, 0.68 or 1.36 mg/ml. The results demonstrated that the inhibitory rates of SPS on the MCF-7 cells increased significantly as the concentration of SPS increased.

The MCF-7 cells were also treated with various concentrations of SPS for 24, 48 and 72 h, which revealed that the antitumor effects of SPS occurred in a time-dependent manner. As shown in Fig. 1, the inhibitory rate on the breast cancer cells treated with SPS for 72 h was significantly higher compared with those treated for 24 h ($P<0.01$) and 48 h ($P<0.05$). SPS had an effective antitumor effect at the concentration of

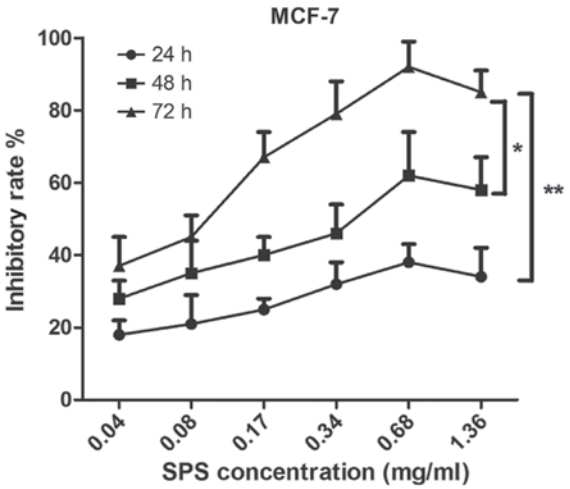

Figure 1. SPS has an increasing antitumor effect on MCF-7 breast cancer cells in a time- and dose-dependent manner. The inhibition rate of MCF-7 cells was determined using an MTT assay. The breast cancer cells ($2x10^4$ cells/well) were seeded into 96-well plates and incubated with various concentrations of SPS for 24, 48 or 72 h. Untreated MCF-7 cells were used as a negative control. The data are expressed as the mean ± standard error ($^*P<0.05$ and $^{**}P<0.01$, as compared with the control group). SPS, safflower polysaccharide.

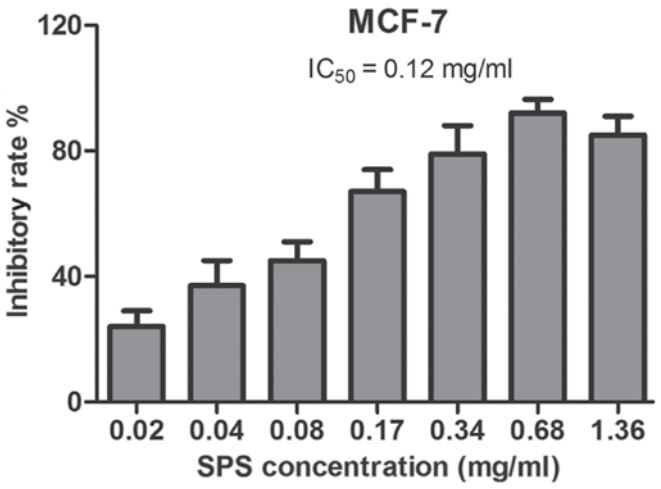

Figure 2. IC_{50} value of SPS on breast cancer cells treated for 72 h. The MCF-7 cells were incubated with various concentrations of SPS for 72 h and the inhibitory rate of the cells was measured using an MTT assay. Untreated cells were used as a negative control. The experiment was repeated three times and the samples were treated in duplicates. The data are expressed as the mean ± standard error. IC_{50}, half maximal inhibitory concentration; SPS, safflower polysaccharide. $^*P<0.05$, $^{**}P<0.01$, as compared with the control group.

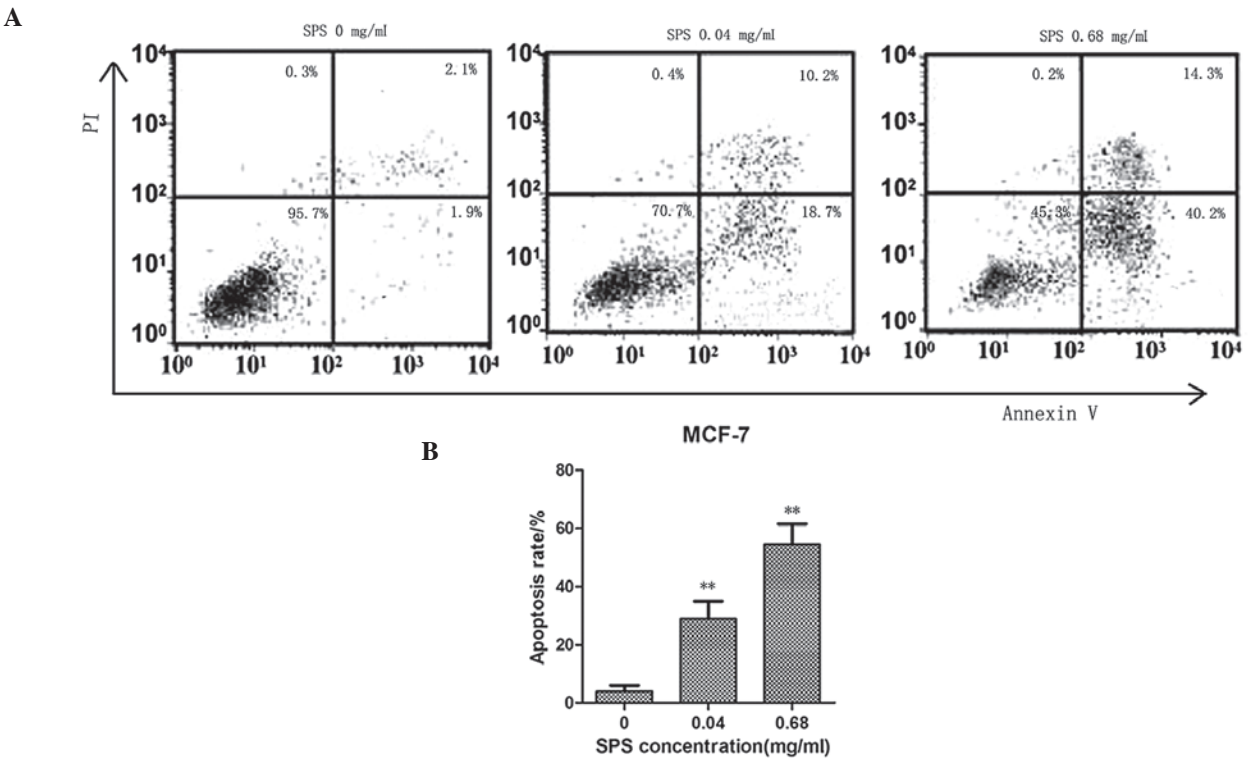

Figure 3. MCF-7 cell apoptotic rate increases with increasing concentrations of SPS. (A) Breast cancer MCF-7 cells ($2x10^5$ cells/well) were seeded into 6-well plates and treated with SPS (0, 0.04 or 0.68 mg/ml) following adherence. Untreated cells were used as negative controls. After 48 h, the apoptotic rates were determined by annexin V/fluorescein isothiocyanate and PI staining analysis. (B) Histogram of the percentage of apoptotic cells. The data represent three independent experiments. SPS, safflower polysaccharide, PI, propidium iodide. $^{**}P<0.01$, vs. the control group.

0.68 mg/ml at various time points. The inhibitory rate at the concentration of 1.36 mg/ml was lower compared with that at 0.68 mg/ml. These data revealed that SPS has an antitumor effect and that these inhibitory effects increased in a time- and dose-dependent manner.

Detection of the IC_{50} of SPS in breast cancer cells treated with SPS for 72 h. The duration of 72 h was selected as an appropriate time-point to treat the MCF-7 cells with SPS. This was repeated more than three times and all the samples were detected in duplicates. Untreated breast cancer cells were used

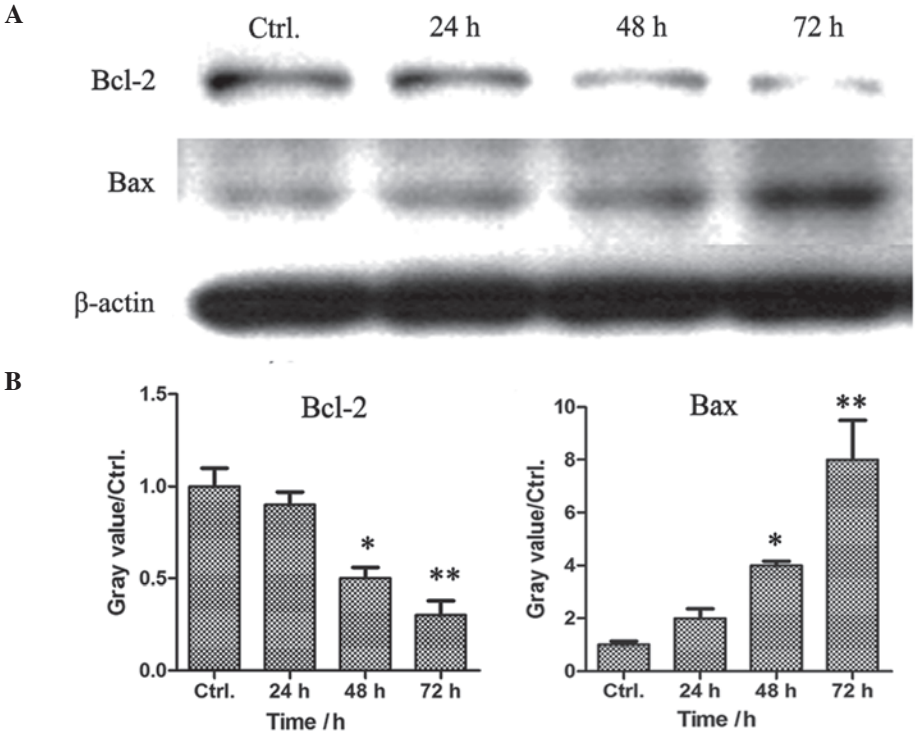

Figure 4. SPS downregulates the expression of Bcl-2 and upregulates the expression of Bax protein in the MCF-7 breast cancer cells. (A) MCF-7 breast cancer cells were seeded into 48-well plates. Following adherence, the cells were treated with SPS for 24, 48 or 72 h at a concentration of 0.68 mg/ml. Cell lysates were used to detect the expression levels of Bcl-2 and Bax by western blot analysis. β-actin was used as an internal reference. (B) Data represent the expression levels of Bcl-2 and Bax at 24, 48 and 72 h for at least three independent experiments. (*P<0.05 and **P<0.01, as compared with the control group). Ctrl, control; Bcl-2, B cell lymphoma-2; Bax, Bcl-2 associated X protein; SPS, safflower polysaccharide.

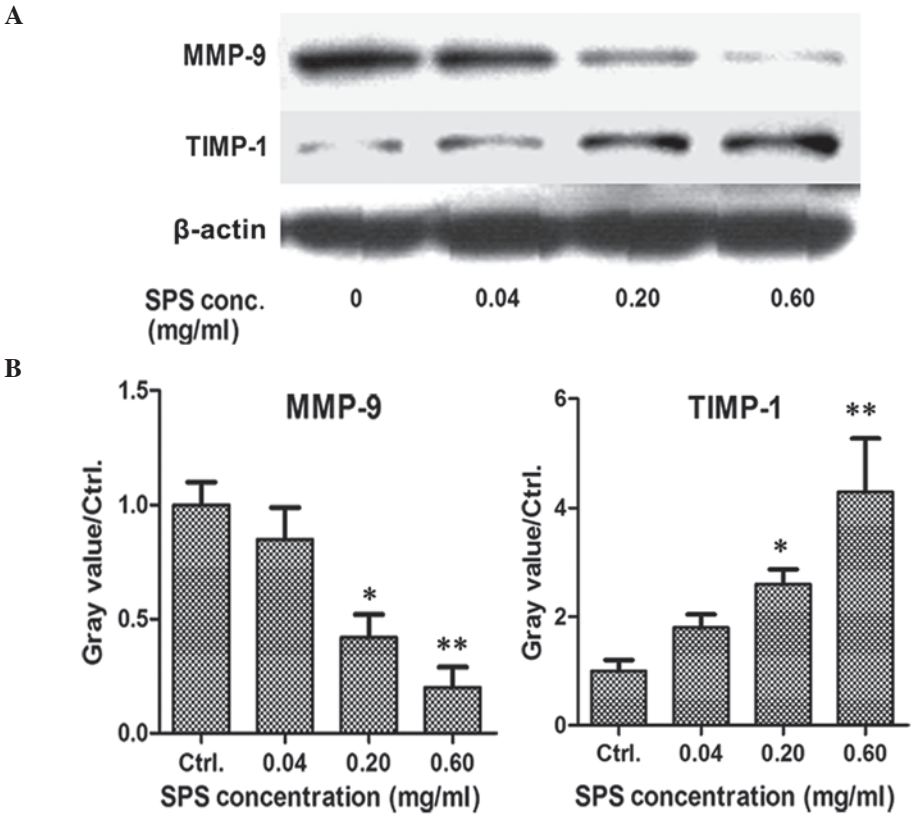

Figure 5. SPS inhibits the expression of MMP-9 and increases the expression of TIMP-1. (A) MCF-7 breast cancer cells were seeded into 48-well plates. Following adherence, the cells were treated with SPS for 48 h at concentrations of 0.04, 0.20 or 0.60 mg/ml. The expression levels of MMP-9 and its inhibitor, TIMP-1, were determined by western blot analysis. β-actin was used as an internal reference. (B) Data represent the expression levels of MMP-9 and TIMP-1 following treatment with various concentrations of SPS for at least two independent experiments, performed in duplicate (*P<0.05 and **P<0.01, as compared with the control group). MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; SPS, safflower polysaccharide; Ctrl, control.

as a negative control. As shown in Fig. 2, the IC_{50} value for 72 h treatment was calculated as 0.12 mg/ml.

MCF-7 apoptotic rate increases with increasing concentrations of SPS. The MCF-7 cell apoptotic rates were determined by FACS analysis. The MCF-7 breast cancer cell line was treated with various concentrations of SPS for 48 h. The lowest concentration of SPS was 0.04 mg/ml and the highest concentration of SPS was 0.68 mg/ml. As shown in Fig. 3, the apoptotic rates of the MCF-7 cells treated with SPS at the concentrations of 0.68 and 0.04 mg/ml were 54.5 and 28.9%, respectively. As expected, the apoptotic rates of the MCF-7 breast cancer cells treated with SPS were significantly higher compared with the untreated cells. The apoptotic rates also increased in a dose-dependent manner.

SPS downregulates the expression of Bcl-2 and upregulates the protein expression of Bax in MCF-7 breast cancer cells. Cell apoptosis is induced via the extrinsic pathway and the intrinsic pathway, which are mediated by death receptors and mitochondria, respectively. In the present study, FACS analysis demonstrated that SPS induced the apoptosis of the MCF-7 cells. Subsequent investigation aimed to determine whether the SPS-induced cell apoptosis was induced by the death receptor pathway or by the mitochondria-mediated signaling pathway. The MCF-7 breast cancer cells were treated with SPS at a concentration of 0.68 mg/ml for various time-periods. The cell lysates were prepared and the protein expression levels of Bcl-2 and Bax were detected by western blot analysis. As shown in Fig. 4, the expression of the apoptosis inhibitor, Bcl-2, reduced in a time-dependent manner, however, the expression of the apoptosis promoter, Bax, increased in the SPS-treated MCF-7 cells, suggesting that the SPS-induced cell apoptosis was dependent on the mitochondria-mediated signaling pathway.

SPS inhibits the expression of MMP-9 and increases the expression of TIMP-1. In order to investigate the role of SPS on tumor metastasis in breast cancer, the expression levels of MMP-9 and TIMP-1 were detected by western blot analysis. The MCF-7 breast cancer cells were treated with various concentrations of SPS for 72 h and the results demonstrated that the expression of MMP-9 was downregulated and the expression of TIMP-1 was upregulated in the SPS-treated cells at the concentration of 0.6 mg/ml (Fig. 5). All these results suggested that SPS inhibited breast cancer cell metastases.

Discussion

Breast cancer is the most common type of malignancy affecting females worldwide, with high incidence and mortality rates. Although the comprehensive treatment approach, combining surgery, radiotherapy and chemotherapy, can significantly prolong the survival rate of patients with breast cancer, it remains the most common cause of cancer-associated mortality in females (33). The recurrence and metastasis of breast cancer is the predominant cause of mortality (34,35), therefore, it is important to identify an effective therapeutic approach for the treatment of breast cancer. SPS is an active ingredient extracted from safflower, which is a traditional Chinese medicine that is commonly used clinically (25). SPS has been reported to exert a variety of pharmacological effects (36) and previous studies have demonstrated that SPS exhibits antitumor activity (27), although the underlying mechanism remained to be elucidated. The present study used the MCF-7 breast cancer cell line as a breast cancer model and investigated the effects of SPS on the proliferation and metastasis of the MCF-7 cells.

An MTT assay was used to detect whether SPS inhibited the proliferation of breast cancer cells. The results demonstrated that SPS suppressed the growth and proliferation rate of the MCF-7 cells in a dose- and time-dependent manner. SPS exhibited a potent antitumor effect at a concentration of 0.68 mg/ml for 24, 48 and 72 h. In addition, the rate of apoptosis was induced in the SPS-treated cells at a concentration of 0.68 mg/ml for 48 h and the expression levels of Bax and Bcl-2 were significantly increased and decreased, respectively, in the SPS treated MCF-7 cells. This suggested that SPS-induced cell apoptosis was dependent on the mitochondria-mediated signaling pathway. Tumor metastasis in breast cancer is the major cause of morality in patients with malignant tumors. The expression levels of MMP-9 and its inhibitor, TIMP-1, were also detected in the SPS-treated MCF-7 cells compared with the untreated cells. SPS significantly inhibited the expression of MMP-9 and increased the expression of TIMP-1, suggesting that SPS may suppress the invasion and metastasis of breast cancer cells.

The results of the present study may provide novel strategies for developing SPS-based therapies to treat breast cancer, which can be assisted by elucidating the mechanism underlying breast cancer.

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