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ORIGINAL ARTICLE

Berberine Decreases Cell Growth but Increases the Side Population Fraction of H460 Lung Cancer Cells

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Abstract Berberine has been reported to inhibit tumor growth in lung cancer. Thus, the effects of berberine on cancer cells as well as the cancer stem cell (side population; SP) fraction were investigated in the H460 lung cancer cell line, and the effects of berberine treatment on cell growth, cell cycle, and cell death were evaluated. Changes in the SP fraction were examined after treatment with berberine, 5-fluorouracil (5-FU), and co-treatment. Berberine inhibited cancer cell growth in a dose-dependent manner. Treatment of the cells with berberine resulted in a 4% increase in cell death and an 8% increase in the number of cells of G_0/G_1 phase, compared to the untreated control. To examine the relationship between berberine and cancer stem cells, the SP fraction was analyzed. Surprisingly, the SP cell fraction was increased upon berberine treatment and further increased after co-treatment with 5-FU. These results are in contrast to the study of Kim et al. (2008) with MCF-7 breast cancer cells, in which berberine inhibited the growth of both cancer cells and the corresponding cancer stem cells. Results of the present study suggest that berberine should be used with caution in the treatment of various cancers, despite its positive effect on cancer cell growth inhibition.

Keywords berberine · cancer stem cells · cell growth · 5-fluorouracil · lung cancer · side population

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Introduction

Some plants are known to produce the secondary metabolites such as alkaloids, monoterpenes, and quinones (Lee et al., 2002). Specially, berberine is a nontoxic isoquinoline alkaloid compound, with a yellow color similar to curcumin when dissolved in distilled water (Liu et al., 2008). In Chinese medicine, berberine has been used as a protective agent against microbial infections (Liu et al., 2008) as well as for suppression of inflammation. Berberine has several physiological effects, including lowering of cholesterol levels and inhibition of insulin secretion (Leng et al., 2004). This compound is also known to be involved in the induction of cell cycle arrest and DNA breakage, inhibition of topoisomerase II activity, induction of programmed cell death through caspase activity (Jantova et al., 2006), and expression of the adenosine triphosphate-binding cassette membrane transporter (ABCG2) (Hirschmann-Jax et al., 2004). Importantly, berberine has anti-cancer effects, which are associated with the inhibition of tumor growth (Lin et al., 2006), metastatic ability (Peng et al., 2006), and drug-resistance (Kim et al., 2010) as well as inhibition of cell growth via cell cycle arrest, induction of apoptosis, and simultaneous induction of cell cycle arrest and apoptosis (Lin et al., 2006). Kim et al. (2008) reported that berberine inhibited tumor growth by targeting ABCG2 during metastasis in a breast cancer cell model. Ho et al. (2009) reported that berberine could inhibit tumor growth without loss of body weight, in comparison to doxorubicin, in a xenografted animal model. Anti-cancer effects of berberine have also been reported in lung cancer (Ho et al., 2009). Liu et al. (2008) demonstrated that berberine had protective effects against radiation-induced lung injury in lung cancer patients. Furthermore, Katiyar et al. (2009) reported that berberine

inhibited tumor growth in association with p53 in human lung cancer cells.

Side population (SP) cells are identified as cells effluxing the DNA-binding dye Hoechst 33342 (Goodell et al., 1996). SP cells have tumor-initiating properties and are generally drug-resistant (Hirschmann-Jax et al., 2004). Due to these features, SP cells have been used as a model system for studying cancer stem cells in several tumors, including neuroblastoma (Hirschmann-Jax et al., 2004), ovarian cancer (Szotek et al., 2006), glioma (Shen et al., 2008), breast cancer (Christgen et al., 2007), head and neck squamous carcinoma (Harper et al., 2007), esophageal carcinoma (Huang et al., 2009), urological cancer (Oates et al., 2009), prostate cancer (Mimeault and Batra, 2009), lung cancer (Meng et al., 2009), and Ewing's sarcoma (Yang et al., 2010). Interestingly, Kim et al. (2008) reported that berberine inhibited the SP cell fraction in a dose-dependent manner in breast cancer. To date, there are few reports on the relationship between SP cells and berberine in lung cancer. To test the effect of berberine on SP cells in lung cancer, the response of H460 lung cancer cells to berberine treatment was examined In terms of cell growth, proportions of cells in various stages of the cell cycle, cell death, and changes in the SP fraction following treatments with berberine, 5-fluorouracil (5-FU), as well as co-treatment with berberine and 5-FU. Berberine was found to decrease cell growth but increase the side population fraction in H460 lung cancer cells.

Materials and Methods

Cell culture. The H460 lung cancer cell line was obtained from the American Type Culture Collection (ATCC, USA). The cells were cultured in adherent conditions on tissue culture dishes (BD Biosciences, USA) in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (Invitrogen, USA), penicillin (100 U/mL), and streptomycin (100 mg/mL). The cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Treatment of cells with berberine and 5-FU. To observe the response of cells to berberine, H460 cells were harvested by treatment with trypsin-ethylenediaminetetraacetic acid (EDTA; Invitrogen). The cells were then seeded at a density of 5.0×10^5 cells in 100-mm culture dishes and grown for 72 h on media containing 0.1, 1, 5, and 10 μM of berberine and/or 0.1, 1, 5, and 10 μM of 5-FU. Cell viability was determined by cell counting using a Neubauer chamber following incubation with 0.4% trypan blue dye.

Analysis of H460 cells by flow cytometry. For cell cycle analysis, H460 cells were harvested by treatment with trypsin-EDTA as described above, and washed twice with phosphate buffered saline (PBS). Cells were fixed in 70% ethanol at −20°C for 2 h. After centrifugation at 500 × g for 5 min, the cells were washed with PBS and resuspended in PBS. A propidium iodide (PI) staining solution containing 50 μL/mL PI (Sigma-Aldrich, USA), 1 mg/mL RNase (Sigma-Aldrich), and 0.1% Triton X-100 in PBS was added to a fluorescence-activated cell sorting (FACS) tube in the dark at room temperature. The proportions of cells in different stages of the cell cycle were analyzed by flow cytometry using a FACSCalibur system (BD Biosciences) at excitation and emission wavelengths of 488 and 617 nm, respectively. All experiments were performed in triplicate.

Analysis of apoptosis. Analysis of apoptosis was performed using a commercially available annexin V staining kit (BD Biosciences). Briefly, the cells were harvested by treatment with trypsin-EDTA as described above, washed twice with PBS, and centrifuged. The resulting pellets were resuspended in 100 μL binding buffer, and then incubated with 5 μL annexin V-fluorescein isothiocyanate (FITC) and 5 μL PI for 15 min in the dark at room temperature. For the analysis of apoptosis, 400 μL of binding buffer was added to each mixture. Cells were analyzed by flow cytometry using a FACSCalibur system (BD Biosciences). All experiments were performed in triplicate.

Analysis of the side population fraction. SP cells were analyzed using methods described in a previous study (Kim et al., 2008). Briefly, cells were harvested by treatment with trypsin-EDTA as described above, washed twice with PBS, and centrifuged. The cells were counted and seeded at a density of 1.0×10^6 cells/mL on RPMI-1640 with 10% fetal bovine serum. Hoechst 33342 dye (5 μg/mL) was added to the FACS tube in the presence or absence of 50 μmol/L verapamil (Sigma-Aldrich), and the samples were incubated at 37°C in a humidified 5% CO_2 atmosphere with intermittent shaking. At the end of the incubation, the cells were centrifuged and washed with PBS. PI (1 μL/mL) was added to the tube to exclude non-viable cells. The side population was analyzed based on the fluorescence of the Hoechst 33342 dye. Hoechst 33342 dye fluorescence was measured with a 405/30 nm band pass filter (Hoechst Blue) and a 660 nm long pass filter (Hoechst Red). A second 488 nm argon laser was used to excite PI.

Statistical analyses. All experiments were repeated at least three times. Data were expressed as the mean ± standard deviation (SD). Comparisons of group means were performed using the Student's *t*-test, and $p<0.05$ was considered significant.

Results

Growth Inhibition after Berberine Treatment. The anti-cancer effects of berberine have been reported in many tumor cell types (Pandey et al., 2008). H460 lung cancer cell line was used to test the anti-cancer effects of berberine in lung cancer. To observe the effects of berberine on the growth rate of cells, 5.0×10^5 cells were seeded in 100-mm culture dishes. Cells were treated with increasing concentrations of berberine, and the number of viable cells was counted after 72 h. Berberine was found to inhibit the growth rate of cells in a dose-dependent manner. The half-maximal inhibitory concentration (IC_{50}) of berberine in H460 cells was calculated as 5 μM (Fig. 1). These results suggested that berberine had an anti-

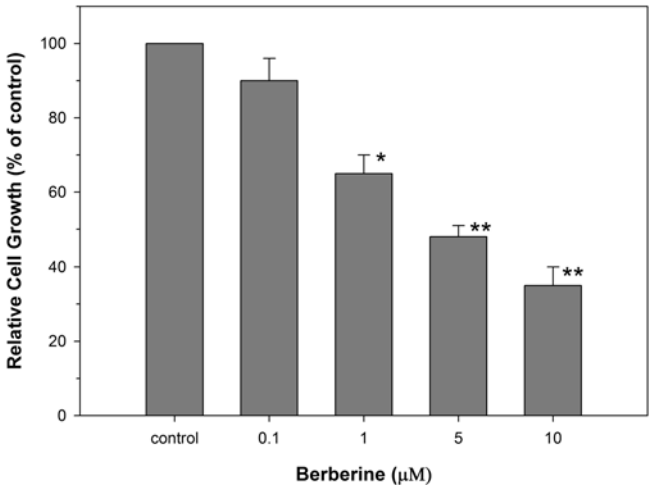

Fig. 1 Cell growth rate after treatment of H460 cells with berberine. H460 cells were treated with distilled water (control) or with 0.1, 1, 5, and 10 μM berberine for 72 h. The values shown represent the percentage of surviving cells following treatment with berberine, compared with the control. *p <0.05, **p <0.01.

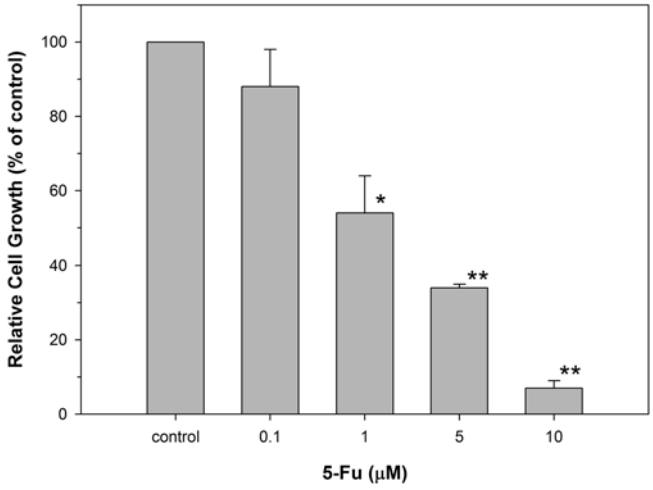

Fig. 3 Cell growth rate after treatment of H460 cells with 5-FU. H460 cells were treated with distilled water (control) or with 0.1, 1, 5, and 10 μM 5-FU for 72 h. The values shown represent the percentage of surviving cells after treatment with 5-FU, compared with the control. *p <0.05, **p <0.01.

cancer effect on the H460 lung cancer cell line.

Analysis of cell cycle and cell death following berberine treatment. The effect of berberine on the inhibition of cell growth was previously reported to be associated with cell cycle arrest (Lin et al., 2006). To examine the effect of berberine on cell cycle arrest, cells were treated with 5 μM berberine (the IC_{50} of berberine calculated in the previous section was chosen for use). Berberine was found to induce an 8% increase in the number of cells in G_0/G_1 phase cells compared to the control (Fig. 2A). These results suggest that the effect of berberine on growth inhibition is related to G_0/G_1 cell cycle arrest. Therefore, the effects of berberine on growth inhibition may result in the induction of cell death. annexin V and PI staining were used to examine the effects of berberine on cell death. Berberine (5 μM) was added to a cultured dish of H460 cells for 72 h, and the resulting staining pattern was analyzed. Berberine induced a 4% increase in cell death by apoptosis compared to the control (Fig. 2B). This finding suggests that berberine induces cell death as well as cell cycle arrest in the H460 lung cancer cell line.

Analysis of cell growth rates after 5-FU treatment. We then used another anti-cancer drug, 5-FU for co-treatment experiments. 5-FU has been used to treat a variety of cancers for many years (Sanua et al., 2005). 5-FU is known to induce cell cycle arrest by targeting the enzyme thymidylate synthase, resulting in cell cycle arrest in mainly the G_0/G_1 or S phases. To observe the effect of 5-FU on the cell growth rate, H460 cells were treated with

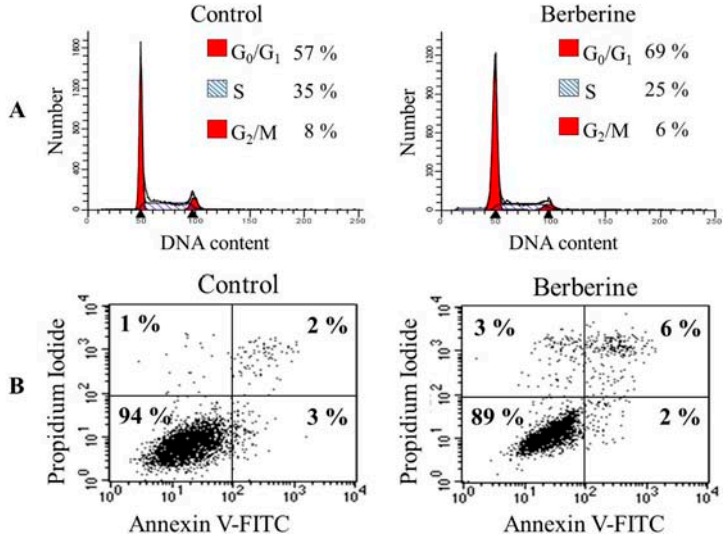

Fig. 2 Analysis of cell cycle and cell death following treatment of cells with berberine. H460 cells were treated with distilled water (control) or 5 μM berberine for 72 h. A: percentage of cells at each stage of the cell cycle was quantified after DNA staining with propidium iodide. B: all cells were stained with FITC-conjugated annexin V in a buffer containing propidium iodide, and analyzed by flow cytometry. The percentage of surviving cells is shown in the lower left part of the quadrant; in these cells, both annexin V and propidium iodide levels are low. Data from three representative experiments are shown.

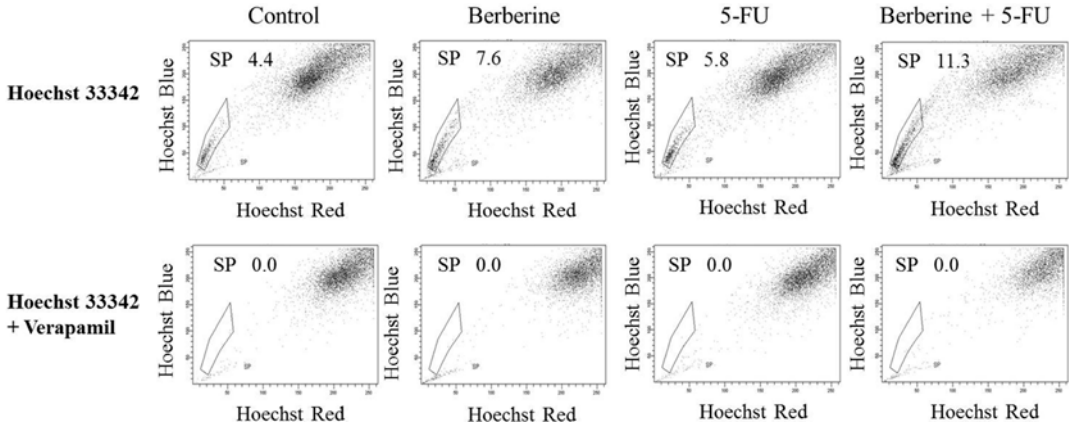

Fig. 4 Fraction of SP and non-SP cells following treatment of cells with berberine and/or 5-FU. H460 cells were treated with PBS (control), 5 μM berberine, and/or 1 μM 5-FU for 72 h. The fractions of SP and non-SP cells were analyzed in the presence of Hoechst 33342 (5 μg/mL) or with 50 μmol/L verapamil, an inhibitor of multidrug resistance protein, on a FACSAria cell sorter.

increasing concentrations of 5-FU for 72 h. We found that 5-FU inhibited the cell growth rate in a dose-dependent manner, with an IC_{50} of 1 μM (Fig. 3).

Changes in side population cells following treatment with berberine and 5-FU. Lung cancers have been previously reported to contain cancer stem cells (Meng et al., 2009). A recent study reported that berberine was effective at targeting the SP fraction of a breast cancer cell line (Kim et al., 2008), whereas another study reported a lower inhibitory effect of 5-FU on the SP fraction than on the non-SP fraction of a esophageal carcinoma cell line (EC9706 cells) (Huang et al., 2009). To examine the effect of berberine as a targeting agent for cancer stem cells, the effects of berberine and/or 5-FU treatment on H460 cells were observed by analysis of the SP fraction. As shown in Fig. 4, berberine, 5-FU, and co-treatment of cells with berberine and 5-FU increased the SP fraction in H460 cells by 7.6, 5.8, and 11.3%, respectively, compared with control cells (4.4%). Furthermore, the addition of verapamil, an inhibitor of the multidrug resistance protein, decreased the SP cell fraction significantly in all cases investigated as expected, confirming that the SP cells are cancer stem cells (Goodell et al., 1996). Although berberine inhibited the growth of H460 lung cancer cells, these results suggest that berberine cannot be used as a targeting agent to kill cancer stem cells when used alone or in combination with 5-FU, in contrast to previous reports.

Discussion

Berberine is an alkaloid extract derived from herbal plants, and has been shown to have anti-cancer effects in a variety of cancers. Anti-cancer effects of berberine have also been reported in lung cancer (Mitani et al., 2001). In the present study, the effects of berberine on cancer stem cells were evaluated. In particular, the effects of berberine on the growth rate of cells, the cell cycle, and cell death were examined. The SP fraction of cancer cells has been suggested as a model for studying cancer stem cells in a wide range of tumors (Christgen et al., 2007). Therefore, in the present study this subpopulation of cells following treatment with berberine, 5-FU, and after co-treatment was analyzed, and it was found that berberine inhibited the growth of these cells via the induction of G_0/G_1 cell cycle arrest and cell death. However, the number of cells in the SP fraction increased after treatment with berberine or 5-FU, and even further increased with co-treatment.

Berberine has been reported to be associated with the inhibition of metastasis and growth in lung cancer (Mitani et al., 2001; Peng et al., 2006). Katiyar et al. (2009) showed that berberine inhibited the growth of lung cancer cells through the induction of apoptosis, in association with p53. Luo et al. (2008) demonstrated various mechanisms of growth inhibition of berberine and showed that berberine inhibited the expression of cyclin D1 by downregulating the transcriptional activity of activating protein-1. On the other hand, results of the present study suggest that berberine inhibits the growth of cancer cells via the induction of G_0/G_1 arrest and cell death, as suggested in previous reports (Liu et al., 2008; Kim et al., 2010).

Cancer stem cells have been studied in a variety of tumors (Reya et al., 2001). Tumor initiating ability and drug-resistance have been reported as typical characteristics of cancer stem cells (Yajima et al., 2009). Interestingly, SP cells have been shown to possess the properties of cancer stem cells (Hirschmann-Jax et al., 2004; Meng et al., 2009). In the present study, the effects of berberine and 5-FU on the SP fraction were investigated to test if berberine could be used as a targeting agent for cancer stem cells or as a secondary drug following treatment with contemporary anti-cancer drugs. 5-FU has been reported to enhance drug-resistance by increasing the SP fraction in several cancer cells, including colon cancer (Chikazawa et al., 2010), human oral squamous cell carcinoma (Abe et al., 2009; Yajima et al., 2009), and liver cancer (Haraguchi et al., 2006). In contrast to 5-FU,

however, berberine has been shown to inhibit the SP fraction in breast cancer (Kim et al., 2008). In the present study, the number of cells in the SP fraction of a lung cancer cell line increased following berberine treatment, and further increased following co-treatment with 5-FU. These results suggest that the effect of berberine may vary depending on the origin of cancer.

In conclusion, our results suggest that, similar to other anti-cancer drugs, berberine inhibits the growth of H460 lung cancer cells. Furthermore, the treatment of cancer cells with berberine alone or in combination with 5-FU was also found to increase the survival of cancer stem cells, marked by an increase in the SP fraction. These results are in contrast to the study of Kim et al. (2008) using MCF-7 breast cancer cells where berberine inhibited both the growth of cancer cells and cancer stem cells. Therefore, our present results suggest that berberine should be used with caution in the treatment of various lung cancers, and illustrates a need to confirm the utility of berberine in the treatment of each type of cancer.

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