Salvia miltiorrhiza Inhibits Tumor Cell Growth in Association with Rb Dephosphorylation through Up-regulation of p21 Via a p53-dependent Pathway

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ABSTRACT

Background: *Salvia miltiorrhiza* (SM), a traditional oriental medicine, has been reported to have anti-tumor properties, but its exact mechanism remains to be elucidated. In this study, we investigated several of the molecular events that occur in human breast carcinoma MCF-7 cells and human pulmonary adenocarcinoma A549 cells. **Methods:** For this purpose, we evaluated the growth-inhibitory effect of SM in association with the expressions of p53, p21, cyclin D1, and pRb, which are known to be involved in cell cycle arrest. The extent of thymidine incorporation was also examined to assess G1/S phase cell cycle arrest in both cells by ^{3}H-thymidine incorporation. **Results:** Our results show that SM inhibits the growth and the proliferation of MCF-7 and A549 cells. Furthermore, we also observed increased expression of p21 via a p53-dependent pathway in both cell lines after treating with SM. In addition, treatment with SM for 24 hours caused the suppression of hyperphosphorylated retinoblastoma protein (pRb) expression and the dephosphorylation of pRb. **Conclusion:** These findings suggest that the growth inhibitory and the anti-proliferation effects of SM on MCF-7 cells and A549 cells are mediated via the decreased expression and dephosphorylation of pRB by p21 up-regulation in a p53-dependent manner. To the best of our knowledge, this study is the first to report upon the molecular mechanisms involved in SM-induced tumor cell growth inhibition. **(Immune Network 2002;2(1):19-24)**

Key Words: *Salvia miltiorrhiza*, p53, p21, pRb, MCF-7, A549

Introduction

Salvia miltiorrhiza (SM) is a herbal plant that has been widely used in traditional oriental medicine for the treatment of cardiovascular disorders and liver diseases. Moreover, it has been reported that the root extract of SM has pharmacological actions, which include, antibacterial, antioxidant, antimutagenic, anti-inflammatory, and anti-platelet aggregation activities (1-3). Recent studies have also shown that SM has anti-tumor potential (4-6), for example, it was reported that SM crude extract markedly prolonged the survival period of Ehrlich ascites carcinoma-bearing mice. Moreover, tanshione II-A sodium sulfonate, a compound isolated from this plant, was found to potentiate the cytotoxic action of hydroxycamptothecine against Ehrlich ascites carcinoma (7). Two further components from SM, przemaquinone A and B, were also found to have anti-tumor activity in mice (8). In addition, several studies have concerned the action of SM extract on transformed cells *in vitro*. In humans, the methanolic extract of SM exhibited a significant anti-proliferative effect against cultured human cancer cell lines (4,5), and more recently SM was reported to inhibit cell growth and to induce apoptosis in human hepatoma cells (9). However, the molecular mechanism of SM in the inhibition of tumor cell growth has not been previously reported.

The tumor suppressor gene, p53, is involved in cell cycle regulation (10-12). One of the most important functions of wild-type p53 is to cause cell cycle arrest in conjunction with p21 (13). p21 is a cyclin-depen-

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dent kinase inhibitor (CKI), which prevents the phosphorylation of the retinoblastoma proteins (pRb) and induces the release of E2F, thereby preventing cells from entering the S phase of the cell cycle.

The present study was designed to investigate the molecular mechanism of the anti-proliferative effect of SM on human breast carcinoma MCF-7 cells and human pulmonary carcinoma A549 cells *in vitro*. In this study, we evaluated the growth-inhibitory effect of SM in association with the expressions of p53, p21, cyclin D1, and pRb, which are involved in cell cycle arrest. The extent of thymidine incorporation was determined to assess G1/S cell cycle arrest in both cell types.

Materials and Methods

Cell culture. MCF-7 cells and A549 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 g/ml of streptomycin.

Preparation of soluble extract from SM. SM root was obtained from an oriental herbal medicine company in Korea. Aqueous extract was prepared as reported previously (14). Briefly, after the dried root was cut into 0.5 cm lengths, and the water-soluble extract so obtained was filtered and stored at -70°C until required. The concentrations quoted are based on the dried extract weight (g/ml).

Cell proliferation assay. DNA synthesis was examined by measuring ^{3}H-thymidine incorporation into the cellular DNA, as previously described (15). Briefly, the cells were plated in 96-well plates at 2×10^5 cells/well in 100μl of DMEM for 24 hours. For SM treatment, cells were cultured with 50, 100, 250, and 500μg/mL of the water-soluble extract for 3 days. ^{3}H-thymidine (0.5 uCi, Amersham Pharmacia Biotech) was added 16 hours before culture termination, and cells were harvested and processed for radioactivity measurement in a Beckman Scintillation Counter (Packard, Tri-Carb 2100 TR).

Western blot analysis. Cells were seeded and cultured for 24 hours in 6-well culture plates (6×10^5 cells/well), and then treated with an aqueous extract of SM to a final concentration of 500μg/ml. After further incubations for 6 and 24 hours, whole cell extracts were prepared in 50μl of lysis buffer (40 mM Tris, 10 mM EDTA, 120 mM Nacl, 0.1% NP-40, pH 7.5). The protein concentration was determined using BCA (Bicinchoninic acid) reagent (Pierce, Rockford, IL). Equal amounts of proteins were run on SDS-polyacrylamide gels and transferred to PVDF membranes (Bio-rad, CA) by electroblotting. Proteins were visualized by enhanced chemiluminescence (ECL) (Amersham Life Science, Arlington Heights, IL). Purified mouse monoclonal antibodies to human p53 and p21 were purchased from Transduction Lab. (Lexington, KY), and monoclonal antibodies to cyclin D1 and pRb were obtained from Santa Cruz biotechnology, Inc. (Santa Cruz, CA).

Statistical analysis. Results are shown as the means of three individual experiments and are presented as means±standard deviation (SD). In the cases of the MTT and ^{3}H-thymidine incorporation assays, values are expressed as percentages of the control value. Data were analyzed by Student's t-test. A *P* value of less than 0.05 was considered statistically significant.

Results

Effect of SM on tumor cell proliferation. Fig. 1. illustrates the effect of SM on A549 and MCF-7 cell proliferation as measured by the ^{3}H-thymidine incorporation assay. After incubation with cells for 3 days SM caused a dose-dependent reduction in cell proliferation. At 500μg/ml, A549 and MCF-7 cells in the SM-treated groups only proliferated by 58% and 39.3%, respectively. We also examined the cytotoxic effect of SM MCF7 cells and A549 cells by using the MTT assay. However, we did not obtain any evidence supporting the direct toxicity of SM on either cancer cell type in the concentration range 1 to 1,000μg/ml (data not shown).

SM up-regulates p21 via a p53-dependent pathway. It has been shown that p53, a tumor suppressor protein and a transcription factor, regulates a DNA damage-triggered G1 checkpoint (16) by transcriptionally activating p21 (16-18), which in turn inhibits cyclin-

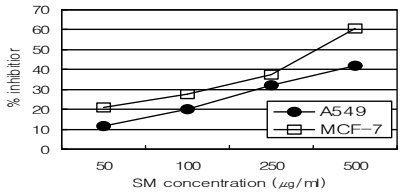

Figure 1. The effect of SM on cell growth in A549 and MCF-7 cells measured by ^{3}H-thymidine incorporation. Tumor cells were incubated with SM (50～500μg/ml) for 3 days. Data are presented as means and are representative of three experiments. % inhibition is defined as the CPM of the experimental group expressed as a percentage of the CPM of the normal control group (cultured in media only).

dependent kinase (Cdk)-mediated Rb phosphorylation (16-18). To study the possibility that the SM treatment of MCF-7 cells might induce p53 and consequently p21, which is then responsible for Rb, we treated MCF-7 cells and A549 cells with SM (500μg/ml), and then determined the p53 and p21 levels. As shown in Fig. 2, treatment with SM for 6 and 24 hours caused a significant increase in the level of p53 protein in both cell types. Moreover, the cellular expression levels of p21, an important downstream target of p53 (19), also increased in response to the addition of SM in both cells, and p21 expression increased dramatically after 6 hours-exposure and remained high at 24 hours in both cell lines (Fig. 3). To elucidate the effects of SM on the level of expression of G1 phase cyclin, we analyzed the protein level of cyclin D1 by Western blotting. However, no significant change in the expression level cyclin D1 were observed after incubation with SM for 6 h and 24 h in either cell line (Fig. 4).

These results indicate that treatment of MCF-7 cells and A549 cells with SM causes the up-regulation of p21 in a p53 dependent pathway, but that treatment has no effect on cyclin D1 expression.

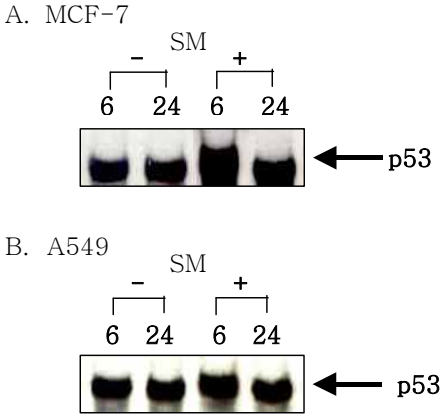

Figure 2. Effect on p53 expression in MCF-7 and A549 cells. 0.5 mg/ml of SM was added to cells for 6 and 24 hours, and total extracts were prepared. The protein lysates loaded into each lane (30μg) were electrophoresed on 10% SDS polyacrylamide gels. Proteins were then transferred onto (a?) PVDF membrane. Western blotting was performed using mouse monoclonal p53 antibody.

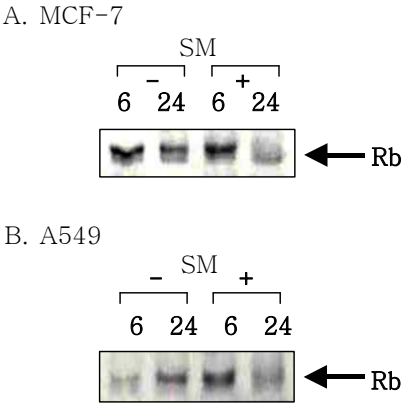

Figure 4. Effect of SM on pRb expression and phosphorylation in MCF-7 and A549 cells. Protein extracts from both cells, were treated with 0.5 mg/ml of SM for 6 and 24 h. Western blotting was conducted using 50μg of total protein and pRb was detected using mouse monoclonal pRb antibody.

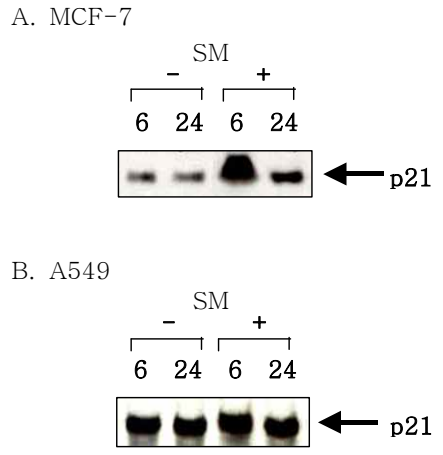

Figure 3. Effect of SM on p21 expression in MCF-7 and A549 cells. Protein extracts from both cells were treated with 0.5 mg/ml of SM for 6 and 24 h. Western blotting was conducted using 30μg of total protein, and p21 was detected using of mouse monoclonal p21 antibody.

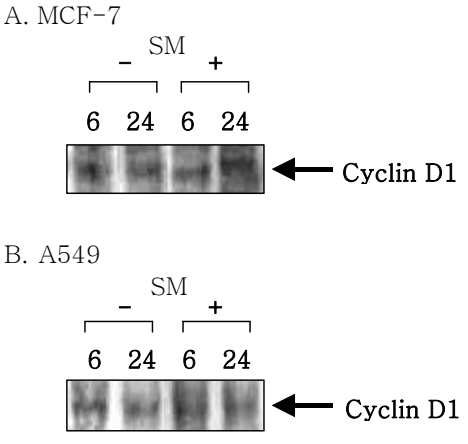

Figure 5. Effect of SM on cyclin D1 expression in MCF-7 and A549 cells. Protein extracts from both cells were treated with 500 μg/ml of SM for 6 and 24 h. 30μg of total protein was then Western blotted and detected using mouse monoclonal cyclin D1 antibody.

SM inhibits the phosphorylation and expression of pRb. The pRb, Rb gene product, is an important checkpoint protein in the G1/S phase transition of the cell cycle and an important downstream target of p21. To determine whether the increased expression of p21 is related to the dephosphorylation of Rb, cells were treated with or without SM (0.5 mg/ml) for 6 and 24 hours. As shown in Fig. 5, the expression levels of Rb and pRb decreased in both MCF-7 and A549 cells that had been treated with SM for 24 hours.

These findings indicate that SM inhibited the phosphorylation and expression of pRb, and suggest that this might be mediated by the induction of p21 in a p53-dependent manner.

Discussion

This study demonstrates that the treatment of two different human carcinoma cell lines, i.e., breast carcinoma MCF-7 cells and pulmonary carcinoma A549 cells, with SM, significantly inhibits cellular growth in a dose-dependent manner as compared to a control group. Furthermore, the results obtained suggest a mechanism for this inhibition.

To assess the inhibition of DNA synthesis by SM, we measured the incorporation of ^{3}H-thymidine into the DNA of MCF-7 and A549 cells, and found a decreased level of ^{3}H-thymidine incorporation into the DNA of both cell lines after SM exposure (Fig. 1). However, SM was not found to be toxic to either cell line (data not shown). These results indicate that SM reduces DNA synthesis by blocking the entry of cells into the S phase, thus inhibiting cell proliferation.

Recently, it was reported that SM exhibited antitumor properties, based on its induction of apoptosis in cultured tumor cell lines (4,5,9,20). Ryu et al. (4) found that tanshinones extracted from SM were able to inhibit the proliferation of KB, Hela, colo-225 and $HepG_2$ cells, which were derived from human carcinomas. Wu et al. (5) also reported that substances purified from SM extract showed a significant cytotoxic effect on various human cancer cell lines. Liu et al. (21) studied the effects of SM in rats and reported upon the protective effect of SM against aflatoxin-B1-induced hepatocarcinogenesis. However, the mechanistic involvement of SM in carcinogenesis has not been elucidated.

p53 gene expression inhibits cellular proliferation by arresting the G1 phase, and the inactivation of the p53 tumor suppression gene is a common finding in diverse types of human cancers (22,23). After exposure to DNA-damaging agents, increased p53 concentrations induce transient alterations in cell cycle progression, and permits optimal damage repair before DNA synthesis is initiated (G1 arrest) and/or mitosis (G2 arrest), thus avoiding the propagation of mutagenic lesions and possible neoplastic transformation (24,25). One way in which wild-type p53 regulates cycle progression involves the induction of its downstream target gene *Waf1/CIP* (26). The $p21^{waf/cip1}$ coded by the *Waf1/CIP* gene is a well documented mediator of p53-dependent G1 cell cycle arrest following DNA damage (27) and a potent inhibitor of Cdk, which is responsible for inactivating the pRb, thus $p21^{waf/cip1}$ inhibits cell cycle progression at both G1 and G2 checkpoints (27-29).

Our results demonstrate that SM increases the expressions of p53 and p21, and thus inhibits cell proliferation. Although p21 is considered a critical downstream effector of the p53 pathway in mammalian cells, recent evidence has shown that p21 can also be induced by p53 independent pathways (30). In the present study, increased p21 expression was coincident with that of p53, thus suggesting that SM induces cell cycle arrest by activating the p53 pathway (Fig. 2, 3).

G1/S phase cell cycle arrest occurs by the binding of p21 to Cdks responsible for the phosphorylation of cell cycle proteins, like pRb, which provide for entry into the S-phase (31). As shown in Fig. 6, the expression levels of Rb and its phosphorylated form pRb, were reduced in SM-treated MCF-7 and A549 cells. This suggests that SM induces cell cycle arrest by the dephosphorylation of Rb via the activation of the Cdk inhibitor, p21.

Cell cycle progression is also controlled by a protein complex composed of cyclins and Cdks, in which the cyclin acts as the regulatory molecule and Cdk as the catalytic subunit (32-34). Thus, we investigated the effect of SM on the expression of cyclin D1, and found that cyclin D1 levels were unaffected. When all of these findings are considered together, it appears that the inhibition of pRb phosphorylation by SM may be the result of the up-regulation of the Cdk inhibitor p21 and not due to **D-type cyclin** inhibition.

In summary, this study demonstrates that the soluble extract of SM is capable of inhibiting cell proliferation and inducing G1/S arrest in MCF-7 and A549 cells. Moreover, the study also suggests that SM-induced growth inhibition and G1/S arrest might be associated with the dephosphorylation of pRb through the up-regulation of p21 via a p53 dependent pathway. To our knowledge, these results are the first concerning cell-cycle protein mechanisms in SM-induced tumor cell growth inhibition.

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