Saikosaponin a and Saikosaponin d Inhibit Proliferation and Migratory Activity of Rat HSC-T6 Cells

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ABSTRACT The proliferation and migration of hepatic stellate cells (HSCs) profoundly impact the pathogenesis of liver inflammation and fibrogenesis. As a perennial herb native to China, *Bupleurum falcatum* is administered for its anti-inflammatory, antipyretic, and antihepatotoxic effects. Saikosaponin a (SSa) and Saikosaponin d (SSd) are the major active components of triterpene saponins in *Bupleurum falcatum*. This study analyzes how SSa and SSd affect rat HSC-T6 cell line proliferation and migration. Experimental results indicate that, in addition to suppressing HSC-T6 proliferation, wound healing activity and cell migration in a time- and dose-dependent manner, SSa and SSd significantly induce apoptosis. Additionally, SSa and SSd decreased the expressions of extracellular matrix-regulated kinase 1/2 (ERK1/2), platelet-derived growth factor receptor 1 (PDGFR1), and subsequently transforming growth factor- β 1 receptor (TGF- β 1R), α -smooth muscle actin, TGF- β 1 and connective tissue growth factor. They also decreased phosphorylation of p38 (p-p38) and ERK1/2 (p-ERK1/2) of HSC-T6. Furthermore, both SSa and SSd can block PDGF-BB and TGF- β 1-induced cell proliferation and migration of HSC-T6. These results suggest that SSa and SSd may inhibit proliferation and activation of HSC-T6, and the modulated mechanisms warrant further study.

KEY WORDS: • PDGF • rat hepatic stellate cells • saikosaponin • TGF- β 1

INTRODUCTION

AT THE FINAL PHASE of virtually all acute or chronic liver diseases, hepatic fibrosis accumulates extracellular matrix (ECM) and activated hepatic stellate cells (HSCs). After liver injury, quiescent HSCs undergo a step of activation (*i.e.*, phenotypical transformation) and develop a myofibroblast-like phenotype.^{1,2} Activated HSCs, which are proliferative and fibrogenic, are identified by high levels of expression of α -smooth muscle actin (α -SMA) and ECM. Additionally, activated HSCs can secrete cytokines and growth factors, as well as migrate and contract. These cells may be the major targets for antifibrotic therapy.

Several cytokines and growth factors secreted by HSCs are essential to the fibrotic process, including platelet-derived growth factor (PDGF) and transforming growth factor- β 1 (TGF- β 1). PDGF is the most potent proliferative cytokine for HSCs, while TGF- β 1 profoundly impacts cell migration and ECM production.^{2–5} Notably, the expressions of PDGF and PDGF receptor (PDGFR) are increased in both experimental fibrosis in rats and fibrotic liver in humans. Additionally, liver fibrosis is induced when PDGF is genetically overexpressed in mice. In addition to functioning in many roles in mammalian cells, TGF- β 1 is also widely considered to be a potent chemoattractant of HSCs. Liver injury increases the levels of expression of TGF- β 1, and TGF- β 1 has been implicated as a mediator of fibrosis in many liver diseases. Besides transforming HSCs into myofibroblasts and stimulating the synthesis of ECM, TGF- β 1 is the most potent stimulus to liver fibrogenesis. Moreover, the antagonist in experimental models can block its stimulation.⁶

Bupleurum falcatum is administered in traditional Chinese medicine to treat liver injury. As the major active components of triterpene saponins in *Bupleurum falcatum*,

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Saikosaponin a (SSa) and Saikosaponin d (SSd) have a common steroid-like structure and are reported to have some steroid-related pharmacological activities, including exert analgesic, anti-inflammatory, immunomodulatory, antiviral, and hepatoprotective activities.^{7–11} According to recent studies, both SSa and SSd induce cell cycle arrest and apoptosis in many cancer cells.^{12–16} In particular, SSa and SSd have been shown to be liver protective agents. Some studies demonstrated that SSa and SSd can protect liver from injury in rat models induced by CCl₄ and dimethylnitrosamine.^{9,17,18} However, exactly how SSa and SSd inhibit liver fibrosis remains unclear. This study elucidates how SSa and SSd affect PDGF-BB and TGF- β 1-stimulated rat HSC-T6 cells. The possible mechanisms underlying proliferative and migratory activities of HSC-T6 are examined as well.

MATERIALS AND METHODS

SSa and SSd

The SSa and SSd used in this study were purchased from Wako Pure Chemical Industries, Ltd. The product numbers of SSa and SSd are 194-10401 and 198-10421, respectively. In addition, the purity of SSa is 90%; the purity of SSd is 90%. SSa and SSd were dissolved with dimethyl sulfoxide (DMSO) and stored at -80°C .

Cell culture

The HSC-T6 cells, immortalized rat HSCs transfected with the large T antigen of SV40 vector containing a Rous sarcoma virus promoter, were cultured at 37°C in a 5% CO₂ atmosphere and in Dulbecco's-modified Eagle's medium (DMEM; Gibco) supplemented with 100 U/mL penicillin, 100 g/mL streptomycin, and 5% heat-inactivated fetal bovine serum (FBS; Gibco).

Cell proliferation detection

HSC-T6 was seeded in 96 well plates at a density of 2000 cells/well in DMEM supplemented with 5% FBS. After 18-h incubation at 37°C in a 5% CO₂ atmosphere, the old medium was discarded and replaced with fresh DMEM/1% FBS containing either SSa (at working concentrations of 0, 0.5, 1, 2, 5, 7.5, and 10 μM) or SSd (at working concentrations of 0, 0.01, 0.1, 0.5, 1, 2, and 3 μM). SSa and SSd were dissolved in DMSO (Sigma-Aldrich) to yield a maximum final concentration of 0.5% in a treated well. After 0, 4, 8, 12, 24, 48, and 72 h of incubation, 3-(4,5-methyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Invitrogen) was added at 5 mg/mL for 3 h. Formazan products were then solubilized with 50 μL of DMSO, and optical density was determined at 650 nm using an ELISA reader (infinite M200PRO; TECAN). Additionally, the survival and proliferation of living cells were confirmed using a real-time cell analyzer instrument (xCELLigence system; Roche Applied Science) for a period of 72 h. The proliferative effect of PDGF-BB on HSC-T6 was assayed by seeding cells into a 96-well plate, followed by discarding and replacing the old medium with fresh DMEM/0.1% FBS containing

10 ng/mL PDGF-BB (R&D Systems). MTT assay was also performed to detect cell proliferation as mentioned earlier. Experiments were performed in triplicate at least three times.

Detection of apoptosis

Specific apoptosis was evaluated by treating HSC-T6 with SSa (10 μM) and SSd (1 μM) for 72 h. Slight amounts of cell debris were gathered and fixed in 4% paraformaldehyde. The cell nuclei were stained with haematoxylin (Merck) and inspected using an optical microscope. Most of the cell debris and all living cells attached on the culture plate surface were collected, and then fixed in 70% ethanol/PBS, pelleted, and resuspended in a buffer solution containing 200 $\mu\text{g/mL}$ RNase A and 0.01 mg/mL propidium iodide (PI). Sub-G1 phase of cell cycle was determined by flow cytometry analysis. For the DNA fragmentation assay, HSC-T6 was treated with SSa (10 μM) and SSd (1 μM) for 0, 24, 48, and 72 h. Finally, the extracted DNA was separated by electrophoresis in a 1.5% agarose gel stained with ethidium bromide.

Western blotting

HSC-T6 was seeded in 12-well plates at a density of 2×10^5 cells/well. After 16 h of incubation, HSC-T6 was treated with SSa (10 μM) or SSd (1 μM) for a series of time. All cell debris and living cells were then lysed in a RIPA buffer containing protease inhibitors (Roche). The total protein concentration was quantified using a protein assay kit (Bio-Rad Laboratories). Next, 25 μg of total extract proteins were separated by SDS-PAGE, and then transferred to methanol-activated PVDF membranes. Additionally, PVDF membranes were incubated for 1 h at room temperature with primary antibodies at a dilution of 1:1000 to detect p38 α , phosphorylation of p38 (p-p38), extracellular matrix-regulated kinase 1/2 (ERK1/2), p-ERK1/2 (Santa Cruz), TGF- β 1R (Cell Signaling), PDGFR1, connective tissue growth factor (CTGF), proliferating cell nuclear antigen, TGF- β 1 (GeneTex), and α -SMA (abcam). The hybridization signals were detected using a Vilber Lourmat Fusion-SL-3500 WL (PeqLab). Finally, fold changes in protein expression were expressed as ratios calculated by dividing specific protein band densities by the β -actin band density, and then normalizing to the control group.

Wound healing assay

Wound healing assay was performed by seeding HSC-T6 in 24 well culture plates at 2×10^5 cells/well in DMEM/5% FBS, followed by incubation for 48 h at 37°C in a 5% CO₂ atmosphere. The cell monolayer was longitudinally scratched by a sterile 10- μL pipette tip to form a 1 mm width stripe. The culture medium was then replaced with 1 mL of fresh DMEM/1% FBS containing different concentrations of SSa (0, 5, and 10 μM) or SSd (0, 0.5, and 1 μM). Next, the cells that migrated into the wounded area or protruded from the border of the wound were visualized and photographed using an inverted microscope (ZEISS) at intervals of 0, 12, and 24 h. Additionally, the effects of SSa and SSd on

FIG. 1. SSA and SSd inhibited the proliferation of HSC-T6 cells. HSC-T6 cells were treated with serial concentrations of SSA and SSd within 72 h. The cell proliferation of HSC-T6 was detected by MTT assay (A, B) and xCELLigence system (C, D). Data are means $\pm$ SD from three independent experiments. HSC, hepatic stellate cell; SSA, Saikosaponin a; SSd, Saikosaponin d.

TGF- β 1-induced wound healing activity were assayed by pretreating HSC-T6 with SSA (10 μ M) and SSd (1 μ M) for 24 h. Also, a 1 mm width stripe was scratched as mentioned earlier. The fresh DMEM/0.1% FBS containing TGF- β 1 (10 ng/mL) with/without SSA (10 μ M) or SSd (0.1 μ M) was replaced. After 24 h of incubation at 37°C in a 5% CO₂ atmosphere, the wound was observed and photographed by an inverted microscope (ZEISS). Each experiment was repeated three times.

Cell migration assay

HSC-T6 migratory ability was evaluated using millicell cell culture insert (Millipore) containing PET membrane with a pore size of 8 μ m. Before cell migration assay, 2×10^5 HSC-T6 was pretreated with SSA (0, 5, and 10 μ M) or SSd (0, 0.5, and 1 μ M) for 24 h. The cells were then suspended with the Millicell

cell culture inserts containing 100 μ L cell suspension of HSC-T6 (10,000 cells) were then inserted into the plate wells. In this experimental system, 10% FBS in the well of 24-well plate functioned as the chemoattractant. The cells were allowed to migrate for 12 h. The HSC-T6 that migrated through the pores and adhered onto the outer side of PET membrane was stained with giemsa solution (Sigma) and counted under the microscope (ZEISS). Totally, 10 fields were counted for each PET membrane. HSC-T6 migratory activity was also monitored by the xCELLigence system (Roche Applied Science).

Statistical analyses

All data are presented as means of three independent experiments (mean $\pm$ SD). Statistical analysis was performed using the unpaired Student's *t*-test, with *P* < .05 considered significant.

FIG. 2. SSa and SSd induced apoptosis in HSC-T6 cells. HSC-T6 cells were treated with SSa (10 μ M) and SSd (1 μ M) for 0, 4, 8, 12, 24, 48, and 72 h, and cell morphology was observed by an optical microscope. Magnification, $\times 200$ (A). Slight amounts of death debris accumulated after 72 h for nuclear staining with haematoxylin to detect apoptotic effects. The apoptotic bodies (indicated by the arrows) were visualized using an optical microscope at $\times 200$ (B). Apoptotic cells of HSC-T6 were determined by flow cytometry after PI staining and the sub-G1 phase of cell cycle was significantly increased (C). SSa and SSd induced DNA fragmentation of all test cells post-treatment for 0, 24, 48 and 72 h. M, size marker (D). PI, propidium iodide.

RESULTS

SSa and SSd significantly inhibited proliferation and induced apoptosis in HSC-T6 cells

Whether SSa or SSd is cytotoxic to HSC-T6 was determined by treating the cells with SSa and SSd for 0, 4, 8, 12, 24, 48, and 72 h. Experimental results indicated that both SSa and SSd decreased cell proliferation of HSC-T6 in a time- and dose-dependant manner, as evidenced by MTT assays and xCELLigence system (Fig. 1). Additionally, after treatment with SSa (10 μ M) or SSd (1 μ M), the amount of cell debris increased over time (Fig. 2A), demonstrating that of SSa and SSd increase cell death. We attempted to determine the mode of cell death induced by SSa and SSd by studying nuclear staining, DNA fragmentation and cell cycle distribution after SSa

and SSd treatments. After incubation with SSa (10 μ M) or SSd (1 μ M) for 0, 4, 8, 12, 24, 48, and 72 h, the amount of cell death debris increased, as observed by an optical microscope (Fig. 2A). Slight amounts of death debris were then gathered after 72 h for nuclear staining with haematoxylin, followed by harvesting of other cell debris and living cells for PI staining. Figure 2B shows typical nuclear condensation and apoptotic bodies of HSC-T6. Additionally, both early apoptotic and late apoptotic cells were determined by flow cytometry after PI staining, in which the sub-G1 phase of cell cycle was significantly increased (Fig. 2C). Furthermore, 10 μ M SSa induced DNA fragmentation in HSC-T6, the inducing effect of SSd was more significant at a concentration of 1 μ M than SSa (Fig. 2D). Above results suggest that apoptosis is involved in the cytotoxicity caused by SSa and SSd in HSC-T6.

FIG. 3. SSa and SSd inhibited p-38 and ERK1/2 phosphorylation; PDGFR1 and TGF-β1R expressions. HSC-T6 cells were treated with or without SSa and SSd for 0, 4, 8, 12, 24, 48 and 72 h. Total extracted proteins were subjected to Western blot analyses to detect the expressions of p-38α, ERK1/2, PDGFR1 and TGF-β1R, and the levels of phosphorylation of p-38 (p-p38) and ERK1/2 (p-ERK1/2). ERK1/2, extracellular matrix-regulated kinase 1/2; PDGFR1, platelet-derived growth factor receptor 1; TGF-β1R, transforming growth factor-β1 receptor.

SSa and SSd inhibited p-38 and ERK1/2 phosphorylation; PDGFR1 and TGF-β1R expressions

Whether SSa and SSd affect mitogen-activated protein kinase (MAPKs) activities was investigated by performing Western blotting of activated forms of ERK1/2 and p38 in cell extracts of HSC-T6. According to Figure 3, p-p38 was

significantly decreased after SSa (10 μM) and SSd (1 μM) treatment for 4 h and sustained for 72 h, whereas the amount of p38α protein in each sample was not different. This figure also revealed a decrease in the amount of ERK1/2 protein and the levels of phosphorylation after treatment of SSd. Similar results were also observed within 24 h of SSa treatment. However, the amounts of ERK1/2 protein and

FIG. 4. SSa and SSd inhibited wound healing and cell migratory activity in HSC-T6 cells. HSC-T6 cells were pretreated with or without SSa (5 and 10 μM) and SSd (0.5 and 1 μM) for 24 h, and the cell monolayer was longitudinally scratched to form a 1 mm width stripe. After 24, 48, and 72 h incubations, the cells migrated into the wounded area (A). The migration of HSC-T6 was determined using a millicell cell culture insert. After pretreatment with SSa (10 μM) and SSd (1 μM) for 24 h, HSC-T6 migrated through pores of the membrane for 12 h, and then HSC-T6 was stained with giemsa solution (B). The total protein of HSC-T6 was extracted after SSa and SSd treatment, and Western blotting was performed to detect the protein expressions of α-SMA, TGF-β1, CTGF, and PCNA. **P* < .05, ***P* < .01 versus control (C). CTGF, connective tissue growth factor; α-SMA, α-smooth muscle actin; PCNA, proliferating cell nuclear antigen.

phosphorylated levels of ERK1/2 increased to basal level at 72 h. Additionally, after SSa and SSd treatment, the expressions of PDGFR1 and TGF- β R1 were also decreased. The above results demonstrate that the proliferative inhibition by SSa and SSd on HSC-T6 may be MAPK dependent, and SSa and SSd may affect the signaling functions induced by PDGF or TGF- β 1.

SSa and SSd inhibited migratory activity of HSC-T6

According to Figure 4A, HSC-T6 markedly migrated to the wounded area, with closure 72 h after wounding. However, in the presence of SSa (5 and 10 μ M) and SSd (0.5 and 1 μ M), the cell migratory activity was inhibited significantly. Additionally, to the extent to which SSa and SSd affected cell migration was further confirmed by performing the transwell assay. The cell migration of HSC-T6 was blocked significantly by SSa and SSd ($P < .01$; Fig. 4B), indicating that the HSC-T6 migratory activity markedly decreases after SSa and SSd treatment.

SSa and SSd decreased the expressions of α -SMA, TGF- β 1 and CTGF

This study also evaluated the protein expression levels of α -SMA, TGF- β 1, CTGF, and proliferating cell nuclear antigen (PCNA) by performing Western blotting analysis. In contrast to untreated control cells, 10 μ M SSa decreased the

protein expression levels of α -SMA, TGF- β 1, and CTGF by about 30%, 60%, and 27%, respectively. Additionally, greater suppression was observed when HSC-T6 cells were treated with 1 μ M SSd, and the decreased protein expression levels of α -SMA, TGF- β 1, and CTGF were about 39%, 83%, and 82%, respectively. However, the SSa- and SSd-treated groups, as well as the control group did not differ in the amount of PCNA protein (Fig. 4C).

SSa and SSd inhibited PDGF-BB and TGF- β 1-induced chemotactic migration and proliferation

Cell migratory activity was also determined by the xCELLigence system. According to Figure 5A and B, chemotactic stimulation of PDGF-BB enhanced the cell migration of HSC-T6 at a concentration of 10 ng/mL within 12 h. Conversely, pretreatment with SSa (5 and 10 μ M) or SSd (0.5 and 1 μ M) for 24 h not only inhibited the cell migratory activity of HSC-T6, but also significantly inhibited the chemotactic effects of PDGF-BB. In addition, PDGF-BB markedly increased HSC-T6 proliferation in this study, with SSa (5 μ M) and SSd (0.5 μ M), the inducing effects of PDGF-BB were blocked significantly (Fig. 5C). Moreover, the effects of SSa and SSd on TGF- β 1-induced wound healing were determined by pretreating HSC-T6 with SSa (10 μ M) and SSd (1 μ M) for 24 h. Wounded HSC-T6 monolayer was incubated in DMEM/0.1%FBS containing TGF- β 1 (10 ng/mL) with/without SSa or SSd. According to Figure 5D,

FIG. 5. SSa and SSd blocked PDGF-BB and TGF- β 1-induced proliferative and migratory activities. The cell migratory activity of HSC-T6 was enhanced by PDGF-BB; however, the enhancing effects were blocked by pretreatment of SSa (A) and SSd (B). SSa and SSd also blocked PDGF-BB-induced proliferative effects (C) and TGF- β 1-induced wound healing activity (D) in HSC-T6 cells.

TGF- β 1 triggered HSC-T6 migration leading to wound closure 24 h after wounding. However, with SSa and SSd, TGF- β 1-induced wound healing activity was suppressed.

DISCUSSION

Bupleurum falcatum, a perennial herb native to China, has been administered for medicinal purposes in China for over two millennia. Scientific studies in the past two decades have elucidated the physiological effects of *Bupleurum radix* *in vitro* and *in vivo*.^{19–21} SSa and SSd are the major bioactive compounds in *Bupleurum* species. This study examines the time-course and dose-dependent manner of SSa- and SSd-induced cellular death and proliferative inhibition in HSC-T6 cells. SSa and SSd significantly inhibited cell proliferation and induced cell death by apoptosis with an ED₅₀ of $\sim 10 \mu\text{M}$ for SSa. At this concentration, SSa triggers the apoptotic effects of HSC-T6 cells significantly. Similar results were observed in HSC-T6 cell with SSd treatment ($1 \mu\text{M}$; one tenth of the concentration of SSa). Thus, we used SSa ($10 \mu\text{M}$) and SSd ($1 \mu\text{M}$) to perform our study in this manuscript. The antiproliferative and apoptotic activities of SSd, which appears to be much more effective than the effects of SSa, and the distinctive mechanisms involved with SSa and SSd, warrant further study.

MAPK signal transduction pathways are involved in cell growth, differentiation, and migration. Related studies have indicated that these pathways are activated in liver fibrosis and cirrhosis.^{22,23} As is well known, ERK1/2 and p38 are the main members of MAPKs. Among the many different growth factors, the activities of these two protein kinases include PDGF and TGF- β 1. PDGF plays a major role in regulating the gene expressions of fibrosis-related proteins, and the activated ERK1/2 is also associated with HSCs proliferation and migration.²⁴ TGF- β 1 is a potent and crucial mediator in chronic liver injury. TGF- β 1 triggers the transmission of intracellular signaling via the Smad2/3 protein complex, upregulates CTGF expression, and regulates cell proliferation, migration, and ECM synthesis.⁴ According to a previous study, as the major regulator of ECM production, CTGF plays important roles in hepatic fibrosis.²⁵ Moreover, previous studies suggest that TGF- β 1 may transmit signals through non-Smad pathways to mediate cellular effects. These non-Smad cellular effects can be achieved by activating ERK, PI3, and p38 via TGF- β 1.^{26,27} In this study, SSa and SSd down-regulate p-p38, whereas the amount of p38 α protein of each sample is not different. Despite decreasing the expression and phosphorylation levels of ERK1/2, there are increases in the basal levels after treatment with SSa for 72 h. However, in the SSd-treated group, both protein expression and phosphorylation levels of ERK1/2 fail to recover to the basal level. Additionally, although SSa decreases the expressions of PDGFR1 and TGF- β 1 after 72 h of treatment, the expressions of these two receptor proteins are decreased significantly after 12 h of treatment in the SSd treated group (Fig. 3). These results might explain partially why SSd inhibits proliferation better than SSa.

As mentioned earlier, the expressions of PDGFR1, TGF- β 1R, α -SMA, CTGF, and TGF- β 1 significantly influence proliferation, differentiation and activity of HSCs. This study also found that both SSa and SSd significantly decreased wound healing and migratory activity of HSC-T6 cells (Fig. 4A, B). Additionally, SSa and SSd decreased the expressions of α -SMA, CTGF, and TGF- β 1 (Fig. 4C). Moreover, PDGF-BB can enhance the proliferative and migrated activity of HSC-T6, whereas TGF- β 1 can increase wound healing activity. However, SSa and SSd blocked these promoting effects of PDGF-BB and TGF- β 1 (Fig. 5). These results suggest that SSa and SSd may decrease PDGFR1, TGF- β 1R, α -SMA, CTGF, and TGF- β 1 expressions of HSC-T6, ultimately decreasing HSC-T6 activities. This preliminary study on SSa and SSd demonstrates that they inhibit proliferation and induction of apoptosis, as well as down-regulate migratory activity of HSC-T6. However, the more detailed mechanisms of the modulation by SSa and SSd are still unknown, and efforts are already underway in our laboratory for further investigation.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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