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Antiproliferative effect of salvianolic acid A on rat hepatic stellate cells

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Abstract

Suppression of activation or proliferation, or induction of apoptosis in hepatic stellate cells (HSCs) have been proposed as therapeutic strategies against liver fibrosis. *Salvia miltiorrhiza* has been reported to exert antifibrotic effects in rats with hepatic fibrosis, but its mechanisms of action remain to be clarified. We have investigated the effects of salvianolic acid A (Sal A), an active principle from *S. miltiorrhiza*, on the proliferation-related biomarkers in a cell line of rat HSCs (HSC-T6) stimulated with platelet-derived growth factor-BB homodimer (PDGF-BB). DNA synthesis (bromodeoxyuridine (BrdU) incorporation), cell cycle related proteins and apoptosis markers were determined to evaluate the inhibitory effects of Sal A. The results showed that Sal A (1–10 μM) concentration-dependently attenuated PDGF-BB-stimulated proliferation (BrdU incorporation) in HSC-T6 cells. Sal A at 10 μM induced cell apoptosis in PDGF-BB-incubated HSCs, together with a reduction of Bcl-2 protein expression, induction of cell cycle inhibitory proteins p21 and p27, and down-regulation of cyclins D1 and E, suppression of Akt phosphorylation, reduction in PDGF receptor phosphorylation, and an increase in caspase-3 activity. Sal A exerted no direct cytotoxicity on primary hepatocytes and HSC-T6 cells under experimental concentrations. Our results suggested that Sal A inhibited PDGF-BB-activated HSC proliferation, partially through apoptosis induction.

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Introduction

Hepatic fibrosis is a wound-healing response of the liver to repeated injury (Friedman 2003; Bataller & Brenner 2005). Activation of hepatic stellate cells (HSCs) is a key feature in the progression of liver fibrosis (Friedman 2003; Bataller & Brenner 2005; Lotersztajn et al 2005). Activated HSCs are proliferative and fibrogenic, with accumulation of extracellular matrix (ECM), including α-smooth muscle actin (α-SMA) and type I collagen (Friedman 2003; Bataller & Brenner 2005; Lotersztajn et al 2005). Platelet-derived growth factor (PDGF) is reported to be the most potent mitogen for HSCs, and PDGF-BB homodimer is the most potent ligand (compared with PDGF-AA or PDGF-AB dimers) for the PDGF receptors to stimulate cell growth and proliferation (Pinzani & Marra 2001; Friedman 2003; Bataller & Brenner 2005). Suppression of activation or proliferation, or induction of apoptosis in HSCs have been proposed as therapeutic strategies against liver fibrosis (Iredale 2001; Friedman 2003; Bataller & Brenner 2005; Lotersztajn et al 2005).

Salvia miltiorrhiza extracts have been reported to exert antiproliferative and pro-apoptotic effects in HSCs (Chor et al 2005) and antifibrotic effects in rats with hepatic fibrosis (Hsu et al 2005). Salvianolic acid B, the major polyphenol of *S. miltiorrhiza*, has been demonstrated to exert protective effects against oxidative stress in hepatocytes and other cells (Kang et al 2004; Zhou et al 2005; Lin et al 2006b), and antifibrogenic effects in both HSCs (Lin et al 2006b) and patients with chronic hepatitis B (Liu et al 2002). Salvianolic acid A (Sal A, Figure 1) is an important bioactive polyphenol, next to salvianolic acid B in content, from *S. miltiorrhiza* (Li et al 1984). It has been shown to protect biomembranes and hepatocytes from lipid peroxidation (Liu et al 1992: Liu et al 2001). Sal A has been demonstrated to inhibit cell proliferation and collagen synthesis in NIH/3T3 fibroblasts and HSCs (Liu et al 2000a, b). In addition, Sal A has been reported to ameliorate oxidative stress in terms of malondialdehyde (MDA) production and glutathione depletion in hepatocytes injured by CCl_4 (Hu et al 2000).

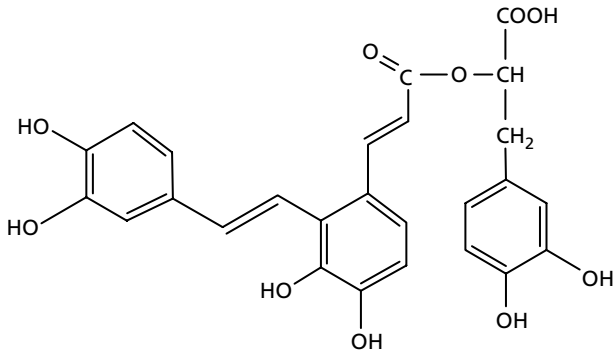

Figure 1 Chemical structure of salvianolic acid A (Sal A).

However, how Sal A contributes to the antifibrotic effects of *S. miltiorrhiza* remains unclear. This study aimed to investigate whether Sal A was effective against activation and proliferation of HSCs.

Materials and Methods

Cell culture and treatment

The HSC-T6 cell line, a generous gift from Professor S. L. Friedman of the Mount Sinai School of Medicine (NY), is immortalized rat HSCs transfected with lipofectamine containing a cDNA in which the expression of the large T-antigen of Simian virus 40 (SV40) is driven by the Rous sarcoma virus promoter (Vogel et al 2000). Bioassays of HSC-T6 activation had been established previously and reported by our group (Hsu et al 2005; Lin et al 2006a). HSC-T6 cells (1×10^5 cells/mL/well) were cultured in Waymouth's medium (containing 10% foetal bovine serum (FBS), pH 7.0) in 24 wells at 37°C in 5% CO_2. After 24 h, HSC-T6 cells were washed twice with PBS and the medium was replaced by serum-free medium. HSC-T6 cells (in serum-free medium) were exposed to PDGF-BB ($10\,ng\,mL^{-1}$) in the absence or presence of Sal A at the indicated concentrations ($1 \sim 10\,\mu M$) and incubated for 48 h to investigate cell proliferation. *N*-Acetylcysteine was used as a positive control of growth inhibitor (Takashima et al 2002).

Bromodeoxyuridine (BrdU) uptake

Pre-confluent HSC-T6 cells incubated in Waymouth's MB 752/1 medium without FBS were treated with Sal A at the indicated concentrations for 48 h. Two hours before harvesting cells, BrdU was added at a final concentration of 10 μM. Cells were fixed and stained following the protocols provided by the manufacturer and the method of Chen et al (2002).

Detection of apoptotic HSC-T6 cells by flow-cytometric analysis and terminal transferase deoxytidyl uridine end labelling (TUNEL)

HSC-T6 cells (2×10^6 cells) with or without Sal A treatment for 24 h were prepared as a single cell suspension in 200-μL phosphate-buffered saline (PBS) and fixed with 70% ethanol at −20°C overnight. Cells were harvested after centrifugation, 200 *g* for 10 min. The cell pellet was resuspended in PBS containing 0.1% Triton X-100 and propidium iodide (PI; $20\,\mu g\,mL^{-1}$) supplemented with RNase A ($200\,\mu g\,mL^{-1}$) at a concentration $\geq 1 \times 10^6$ cells mL^{-1}, and then incubated at 37°C for 30 min. The red fluorescence of individual cells was measured with CellQuest (CellQuest Inc., FL, USA) and the number of apoptotic cells was counted by ModFit.LT software (Verity Software House, ME, USA). For TUNEL stain, pre-confluent HSC-T6 cells cultured in Waymouth's MB 752/1 medium without FBS in slidewells were treated with PDGF-BB and Sal A (10 μM) for 24 h. Cells were washed twice with PBS before fixation. Apoptotic HSCs were detected using the DeadEnd fluorometric TUNEL System kit, following the protocols provided by the manufacturer and the method of Chen et al (2002).

Measurement of cellular activity of caspase-3

The activity of caspase-3, the main execution caspase, was measured by the colorimetric assay as previously reported, using acetyl-Asp-Glu-Val-Asp-ρNA (Ac-DEVD-ρNA) as the specific substrate (Wang et al 2001). HSC-T6 cells treated with PDGF-BB and Sal A were harvested in cell lysis buffer (50 mM HEPES, pH 7.4, 1.0 mM dithiothreitol, 0.1 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), and 0.1% Triton X-100). After centrifugation at 12 000 *g* for 15 min, the supernatants were collected and protein concentrations were determined by BCA (bicinchoninic acid) kit. Proteins (100 μg) were incubated for 30 min at 37°C with the reaction buffer (25 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 5 mM dithiothreitol, and 5 mM EDTA) in a total volume of 150 μL containing 25 mM of substrate Ac-DEVD-ρNA. Enzyme-catalysed release of ρNA was measured colorimetrically at 405 nm. Absorption units were converted to pmol 4-ρNA released using a standard curve generated with 4-ρNA.

Western blotting analyses

Whole cell protein extracts were prepared from HSC-T6 cells treated with or without Sal A. SDS-PAGE with 10% resolving gel (7% for PDGF receptor) was used to separate proteins ($30 \sim 50\,\mu g$/lane). The separated proteins were transferred onto Immobilon-PVDF (Millipore, Bedford, MA, USA) and detected using primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized on Kodax BioMax films (Eastman Kodak Company, Rochester, NY, USA) using ECL (enhanced chemiluminescence) detection reagent. To detect Bcl-2, p21 and p27, the protocols by Chen et al (2002) were followed. Tubulin was used as an internal control for equal protein loading. The protocols of Borkham-Kamphorst et al (2004) and Imanishi et al (2004) were followed to measure phosphorylation of PDGF receptors-β (PDGFR-β) and Akt.

Cytotoxicity assay

Hepatotoxicity assay was performed to exclude the possibility of cytotoxicity of Sal A to hepatocytes. Hepatocytes

were isolated using the two-step collagenase perfusion method mainly according to Cascales et al (1984). Briefly, the digested liver from male Sprague-Dawley rats (250–300 g) was cut into small pieces and the resulting suspension was filtered through 80- and 100-mesh screens, and centrifuged at 50 *g* for 2 min. After washing three times by resuspension in the Krebs–Henseleit bicarbonate buffer (in mM: NaCl 118, KH_2PO_4 1.19, KCl 4.76, $MgSO_4$ 1.19, $NaHCO_3$ 24.9) and re-centrifuged at 25°C, the hepatocytes were filtered through a 150-mesh screen and suspended in Leibovitz L-15 medium containing 0.2% bovine serum albumin (BSA), with approximately $5–10 \times 10^7$ hepatocytes obtained. Cell viability was above 85% as indicated by the trypan blue exclusion assay. The isolated hepatocytes were cultured in Leibovitz L-15 medium supplemented with 10% foetal calf serum, 45 $\mu g\,mL^{-1}$ benzylpenicillin and 50 $\mu g\,mL^{-1}$ streptomycin in collagen precoated 24-well plates (1×10^5 cells mL^{-1}) and incubated under 5% CO_2 in air at 37°C. After incubation with Sal A for 48 h, MTT assay was used to evaluate the cell viability of hepatocytes. During the last hour, the cells were incubated with minimum essential medium containing 0.1 mg mL^{-1} MTT. A_{540} absorption intensity was measured using enzyme-linked immunosorbent assay reader, according to the method of Hansen et al (1989). Relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The optical density of the formazan formed in the control cells was taken as 100% viability. Measurement of cytotoxicity to HSC-T6 was mainly similar to the assay for hepatocytes.

Plant material

Sal A (Figure 1) was isolated from *S. miltiorrhiza*. Briefly, the 80% aqueous ethanolic extract of the rhizoma of *S. miltiorrhiza* was concentrated with a vacuum rotary evaporator (Buchi, Postfach, Switzerland) under reduced pressure to yield the crude extract. For isolation and purification of Sal A, the crude extract was further separated by Diaion HP-20 and Sephadex LH-20 column chromatography sequentially. Repeated chromatography was required to obtain highly purified Sal A. The structure of Sal A was identified by spectra (IR, 1D- and 2D-NMR, and MS) analyses and also compared with an authentic sample (Lin et al 2003). The purity of Sal A was determined by reverse-phase high-pressure liquid chromatography (HPLC) with a phenyl column (250×4.6 mm i.d.). Linear gradient elution was conducted with a mobile phase consisting of acetonitile:methanol:20 mM monosodium phosphoric acid (1:3:6) with 0.1 mM octanesulfonic acid (pH 2.80) for 20 min and at a flow rate of 1 mL min^{-1}. The wavelength of the UV detector was set at 290 nm. The purity of Sal A used in this study was ≥95%. Sal A was dissolved in dimethyl sulfoxide (DMSO) and diluted with medium to give a final DMSO concentration under 0.1% in cultured medium.

Chemicals

Waymouth's MB 752/1 medium and foetal bovine serum (FBS) were from Gibco BRL (Gaithersburg, MD, USA). Recombinant PDGF-BB, 3-[4,5-dimethylthiazol-2–yl]-2,5-diphenyltetrazolium bromide (MTT), and trypan blue were purchased from Sigma Chemical (St Louis, MO, USA). The antibodies against cyclins D1 and E, p21, p27, Akt and PDGFR-β were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibodies against phospho-PDGFR and phospho-Akt were from Biosource Inc (Worcester, MA, USA). Western blotting detection reagents and anti-mouse IgG antibody conjugated with horseradish peroxidase and BrdU kit were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). DeadEnd fluorometric TUNEL System kit was purchased from Promega Co. (Madison, WI, USA). Protein assay kit was from Pierce Co. (Rockford, IL, USA). Ac-DEVD-ρNA was purchased from Calbiochem (Darmstadt, Germany). All other chemicals used were of analytical grade and purchased from commercial suppliers.

Statistical analysis

Data are presented as mean ± s.e.m. of three or more independent experiments. Inter-group comparisons were performed using non-parametric Kruskal–Wallis test, followed by post hoc Dunn procedure. Statistical significance was set at $P < 0.05$.

Results

Effect of Sal A on BrdU incorporation in HSC-T6 cells

PDGF-BB (5–20 ng mL^{-1}) stimulated BrdU incorporation in HSC-T6 cells, with a maximal level (168 ± 4% of controls) of stimulation at 10 ng mL^{-1}. We therefore chose 10 ng mL^{-1} PDGF-BB to study the inhibitory effects of Sal A further. As shown in Figure 2, Sal A at 1, 2, 5 and 10 μM reduced the PDGF-BB-stimulated ratio to 157 ± 4%, 142 ± 3%, 124 ± 7%, 99 ± 10%, respectively. The IC50 value of Sal A against PDGF-BB-stimulated BrdU incorporation was calculated to be 4.2 ± 0.3 μM.

Effects of Sal A on cell cycle distribution and apoptosis of HSCs

Propidium iodide labelling was carried out to analyse the effects of Sal A on cell cycle distribution. The percentages of cells in G0/G1, S and G2/M phases in the PDGF-treated HSC-T6 cells were altered by Sal A (10 μM) treatment. The percentage of DNA content in S phase at 24 h was 25 ± 3% in controls and was increased in cells treated with PDGF-BB (29 ± 4%) or PDGF-BB + Sal A (31 ± 3%). Moreover, the percentages of the sub-G0/G1 phase were 4 ± 1% and 3 ± 1% in controls and cells treated with PDGF-BB, respectively, whereas the percentage was significantly increased to 12 ± 4% in cells treated with PDGF-BB + Sal A. Significant increase of the sub-G0/G1 population suggested that Sal A might have induced apoptosis in PDGF-BB-treated HSCs. A similar increase

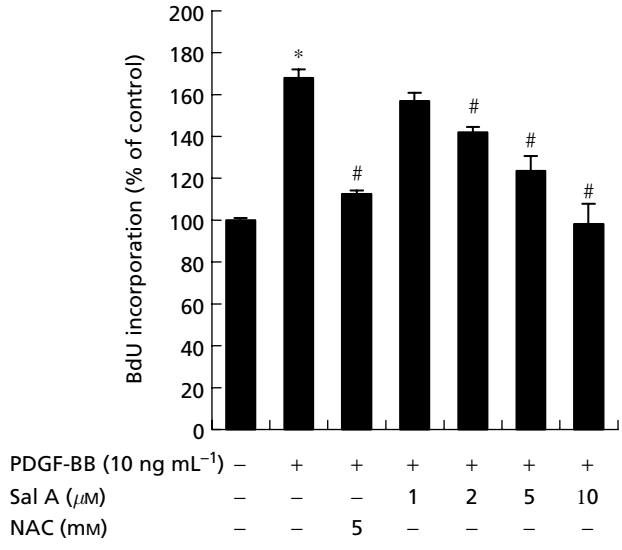

Figure 2 Inhibitory effects of Sal A on BrdU incorporation in HSC-T6 cells stimulated by PDGF-BB. Passaged HSC-T6 cells (in serum-free medium) were exposed to PDGF-BB in the absence or presence of Sal A (1–10 μM) and incubated for 48 h to investigate cell proliferation. Data are from three independent experiments and expressed as mean s.e.m. $^{*}P < 0.05$ for PDGF-BB group vs control group; $^{\#}P < 0.05$ vs PDGF-BB group. *N*-Acetylcysteine (NAC) was a positive control.

of the sub-G0/G1 population was observed for *N*-acetylcysteine in the PDGF-BB-treated HSC-T6 cells ($9 \pm 2\%$).

Morphological assessment by microscope as well as TUNEL staining is shown in Figure 3. Flow cytometric analysis showed that nuclear DNA was stained by 4′, 6-diamidino-2-phenylindole (DAPI) in all HSC-T6 cells, whereas PDGF-BB plus Sal A (10 μM) induced significant staining of apoptotic nuclei in HSC-T6 cells, as revealed in the TUNEL labelling. Moreover, PDGF-BB plus Sal A decreased the Bcl-2 protein expression in HSC-T6 cells (Figure 4), which might have contributed to induction of apoptosis.

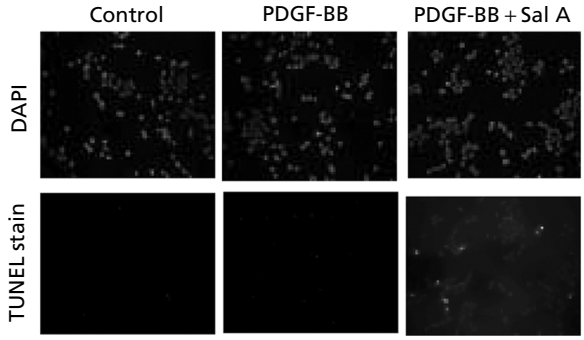

Figure 3 Representative TUNEL-stained HSC-T6 cells treated with solvent, PDGF-BB (10 ng mL^{-1}), or PDGF-BB + Sal A (10 μM). The green fluorescence, seen as bright spots in the lower panel, indicates apoptosis induced by PDGF-BB + Sal A. For comparison, representative 4′-6-diamidino-2-phenylindole (DAPI)-stained HSC-T6 cells treated with solvent, PDGF-BB (10 ng mL^{-1}), or PDGF-BB + Sal A (10 μM) are shown with bright spots in the upper panel due to blue fluorescence.

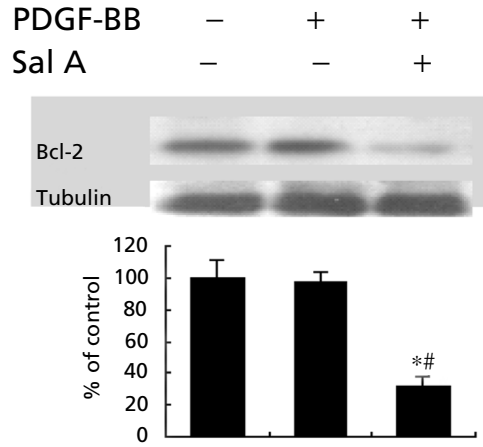

Figure 4 Effect of Sal A on the expression of Bcl-2 in HSC-T6 cells. Serum-starved HSC-T6 cells were stimulated with PDGF-BB (10 ng mL^{-1}) in the absence or presence of Sal A (10 μM) for 24 h. Bcl-2 protein levels were detected by immunoblotting and expressed as relative percentages of control. Data are from three independent experiments and expressed as mean ± s.e.m. Values are $^{*}P < 0.05$ vs control group; $^{\#}P < 0.05$ vs PDGF-BB group.

Effects of Sal A on the expression of cyclins D1 and E, and p21 and p27 proteins

Western blotting analyses demonstrated that PDGF-BB (10 ng mL^{-1}) significantly increased cell cycle stimulating proteins cyclins D1 and E by $279 \pm 35\%$ and $213 \pm 18\%$, respectively, in comparison with the controls, in HSC-T6 cells. But these ratios were reduced by Sal A (10 μM) to 153 ± 12 and $102 \pm 9\%$, respectively (Figure 5). In addition, PDGF-BB reduced the levels of cell cycle inhibitory proteins p21 and p27 in a time-dependent manner. The levels of p21 and p27 were significantly increased after Sal A treatment in PDGF-BB-stimulated HSC-T6 cells (Figure 6). For example, Sal A markedly increased the p21 and p27 expressions by 332 ± 23 and $223 \pm 9\%$, respectively, after 48-h treatment. These results indicated that Sal A modulated cell cycle regulatory proteins of activated HSC-T6 cells.

Effects of Sal A on the phosphorylation of Akt and PDGF receptor

Akt (protein kinase B (PKB)) is a serine-threonine kinase activated by phosphatidylinositol 3-kinase (PI3K), which is stimulated by growth factors such as PDGF (Bonner 2004). As shown in Figure 7A, phosphorylated Akt protein levels were increased by PDGF-BB ($182 \pm 5\%$ of controls). After addition of Sal A (10 μM), the levels were reduced to $133 \pm 10\%$ of controls. On the other hand, PDGF-BB induced the protein levels of phosphorylated PDGFR ($250 \pm 11\%$ of controls), which were reduced by the addition of Sal A (5 and 10 μM) (Figure 7B).

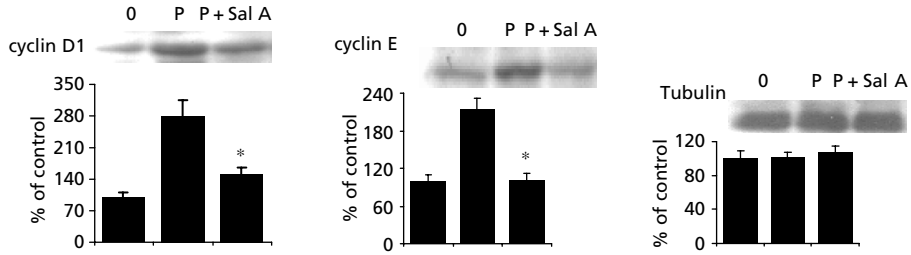

Figure 5 Expression of cell cycle regulatory proteins D1 and E in HSC-T6 cells treated with PDGF (P) or PDGF + Sal A (10 μM; P + Sal A). Whole cell protein extracts were prepared from HSC-T6 cells treated with PDGF-BB (10 ng mL^{-1}) or PDGF-BB + Sal A (10 μM) for 48 h. Cell cycle related proteins were detected by immunoblotting. Each result represented three independent experiments. Tubulin was used as an internal control for equal protein loading. *$P < 0.05$ vs PDGF-BB group.

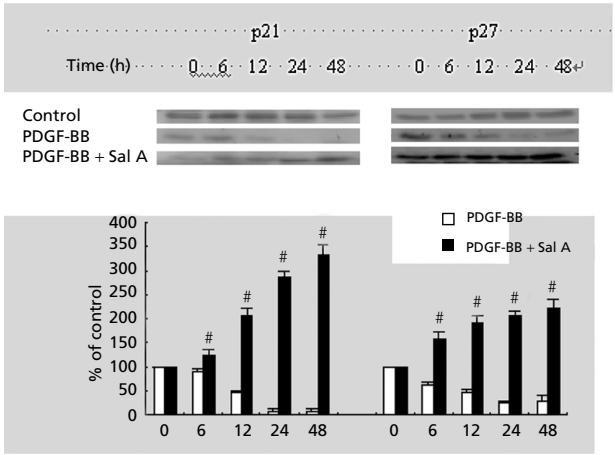

Figure 6 Expression of cell cycle regulatory proteins p21 and p27 in HSC-T6 cells treated with PDGF-BB or PDGF-BB + Sal A (10 μM). Whole cell protein extracts were prepared from HSC-T6 cells treated with PDGF-BB (10 ng mL^{-1}) or PDGF-BB + Sal A (10 μM) for different times. Cell cycle related proteins were detected by immunoblotting. Data are from three independent experiments. #$P < 0.05$ vs PDGF-BB group.

Effects of Sal A on the caspase-3 activity in HSC-T6 cells

Caspase-3 activity assay was measured by colorimetric assay using acetyl-Asp-Glu-Val-Asp-ρNA (Ac-DEVD-ρNA) as the specific substrate. As compared with the control group (183 ± 3 pmol ρNA min^{-1} (μg protein)$^{-1}$), PDGF-BB significantly decreased the percentage of caspase-3 activity (122 ± 5 pmol ρNA min^{-1} (μg protein)$^{-1}$) by 33 ± 3% in HSC-T6 cells. Sal A (10 μM) reversed the inhibitory effect of PDGF-BB, and significantly stimulated caspase 3 activity (350 ± 12 pmol ρNA min^{-1} (μg protein)$^{-1}$) by 187 ± 10% in HSC-T6 cells.

Cytotoxicity of Sal A on rat hepatocytes and HSC-T6 cells

The possibility of hepatocytotoxicity was checked by incubating primary cultured rat hepatocytes with different concentrations of Sal A for two days. The MTT assay showed that no significant cytotoxicity was observed with

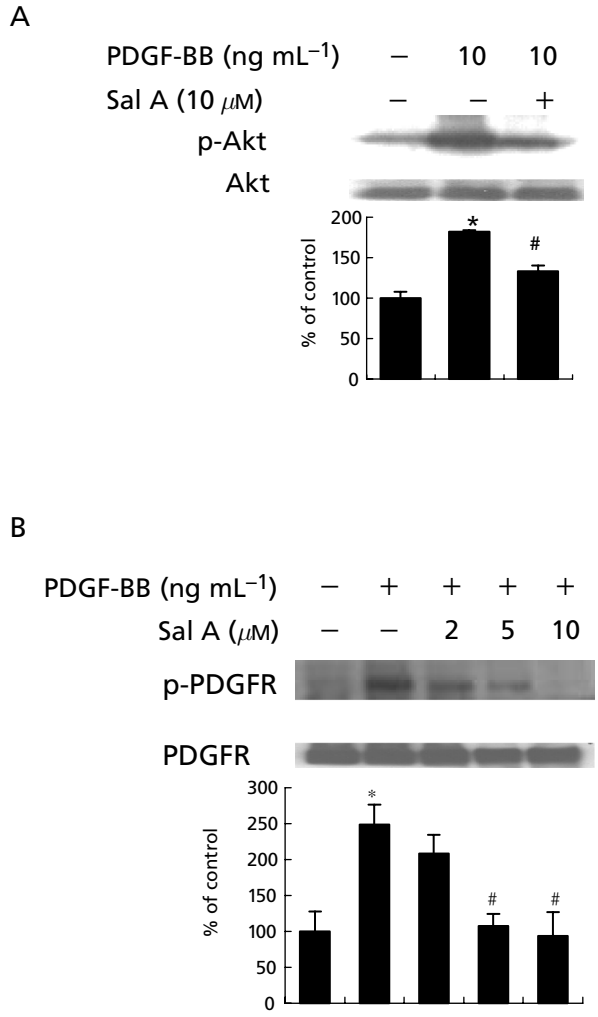

Figure 7 Phosphorylation of Akt and PDGF receptors (PDGFR) in HSC-T6 cells. Serum-starved HSC-T6 cells were stimulated with PDGF-BB (10 ng mL^{-1}) in the absence or presence of Sal A (10 μM). Phosphorylation of Akt (A) and PDGF receptors (B) were detected by immunoblotting. Results were obtained from three independent experiments and expressed as mean ± s.e.m. Values are expressed as relative percentages of control. *$P < 0.05$ for PDGF-BB group vs control group; #$P < 0.05$ vs PDGF-BB group.

Sal A (cell viability being $94 \pm 2\%$ of controls at $5\,\mu$M, $97 \pm 4\%$ at $10\,\mu$M and $96 \pm 2\%$ at $20\,\mu$M). Moreover, Sal A did not inadvertently affect HSC-T6 cell viability ($100 \pm 2\%$ of controls at $1\,\mu$M, $95 \pm 4\%$ at $2\,\mu$M, $93 \pm 3\%$ at $5\,\mu$M, and $91 \pm 6\%$ at $10\,\mu$M) over the range of concentrations tested.

Discussion

We have demonstrated that Sal A inhibited PDGF-BB-induced cell proliferation by reducing PDGFR and Akt phosphorylation, and inducing apoptosis with caspase-3 activation in HSC-T6 cells.

Although the antiproliferative effects of Sal A on HSCs have been reported (Liu et al 2000b), the molecular mechanism underlying these effects had not been clarified. In this study, we employed flow cytometry and measured cell cycle related proteins, PDGF-BB-related signalling molecules and apoptosis markers to provide some explanations for molecular mechanisms of Sal A. In addition to this study, there are other reports that herbal extracts such as Inchin-ko-to (TJ-135) and polyphenol epigallocatechin-3-gallate (EGCG) from green tea also inhibit PDGF-activated proliferation in HSCs (Imanishi et al 2004; Sakata et al 2004). Taken together, these studies indicated that the anti-PDGF-BB effects of herbal extracts were partially mediated through reducing PDGFR phosphorylation in HSCs. PDGF-BB is a cell mitogen that binds to the PDGF receptor (PDGFR) and triggers dimerization of PDGFRs, and induces receptor autophosphorylation on the tyrosine residue to initiate multiple intracellular signalling pathways in HSCs, including Ras-ERK (extracellular signal-regulated kinase) and PI3K-Akt pathways (Heldin & Westermark 1999; Pinzani & Marra 2001; Bonner 2004; Borkham-Kamphorst et al 2004). Our present results demonstrated that the amount of phosphorylated Akt in HSC-T6 cells was significantly enhanced by PDGF-BB, but such stimulation was attenuated by co-treatment with Sal A (Figure 7A).

Cyclins D1 and D2 are proposed to mediate crucially cell cycle progression at the G1 phase, and cyclins E and A are involved in cell cycle progression at the S phase. On the other hand, p21 and p27 are inhibitory proteins on cell cycle progression (Sherr 2000; Murray 2004). Kawada et al (1999) reported cyclins D1, D2 and E played a key role in the cell cycle transition of HSCs from G1 to S phase. In this study, we observed that Sal A not only reduced the expression of D1 and E, but also enhanced the expression of p21 and p27. Caspase-3 is a protease known to function as an executive enzyme at late stages of cell apoptosis (Bratton & Cohen 2001; Danial & Korsmeyer 2004). On the other hand, Bcl-2 is involved in preventing cell apoptosis (Danial & Korsmeyer 2004). Therefore, increased caspase-3 activity and decreased Bcl-2 expression by Sal A in PDGF-BB-activated HSCs might have contributed to the antiproliferative effects of Sal A with induction of apoptosis. It has been reported that an extract of Kangen-karyu – a Chinese prescription formula composed of six different herbs including *S. miltiorrhiza* – protected human fibroblasts from oxidative stress and dysfunction (Satoh et al 2005). In line with this finding, our previous studies showed that both Sal A and Sal B ameliorated oxidative stress in HSC-T6 cells (Lin et al 2006b; unpublished data). In this study, we have demonstrated that Sal A inhibited PDGF-BB-induced cell proliferation, together with reducing Bcl-2 expression and activating caspase-3. These results were consistent with Chor et al (2005), showing that an extract of *S. miltiorrhiza* exerted both antiproliferative and pro-apoptotic activity in HSC-T6 cells.

Recently, it has been observed that resolution of hepatic fibrosis in rats was associated with apoptosis of HSCs, and some antifibrotic chemicals such as gliotoxin have been proposed to act by the mechanism of inducing HSC apoptosis (Issa et al 2001; Wright et al 2001). In our study, Sal A alone at 1–10 μM did not induce apoptosis in HSC-T6 cells, but it could facilitate PDGF-BB-treated HSCs to undergo apoptosis, with increase in caspase-3 activity. Similarly, Xu et al (2003) reported that curcumin, a predominant yellow pigment isolated from dietary turmeric *Curcuma longa*, inhibited the proliferation of HSCs activated by 10% FBS, with induction of apoptosis and caspase-3 activity. Engulfment of apoptotic hepatocytes by HSCs and Kupffer cells has been shown to enhance expression of pro-fibrogenic genes and death ligands during liver fibrosis (Canbay et al 2004). Approaches to limiting apoptosis in hepatocytes but promoting apoptosis of HSCs have been proposed as therapeutic strategies against hepatic fibrosis (Iredale 2001; Canbay et al 2004). To rule out the possibility of hepatocytotoxicity due to Sal A, MTT assay and microscopic observations were conducted. It was found that Sal A (0–20 μM) did not significantly influence cell viability of primary hepatocytes. Moreover, Sal A (0–10 μM) alone was not cytotoxic to HSC-T6 cells.

Conclusions

Sal A inhibited PDGF-BB-induced cell proliferation in HSC T6 cells, together with: a reduction of Bcl-2 protein expression; induction of cell cycle inhibitory proteins p21 and p27, and down-regulation of cyclins D1 and E; suppression of Akt phosphorylation; reduction in PDGF receptor phosphorylation; and an increase in caspase-3 activity. Sal A exerted no direct cytotoxicity on primary hepatocytes and HSC-T6 cells under our experimental concentrations. The results suggested that Sal A inhibited PDGF-BB-activated HSC proliferation, partially through apoptosis induction.

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