Salvianolic acid B inhibit SDF-1α-stimulated cell proliferation and migration of vascular smooth muscle cells by suppressing CXCR4 receptor

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Abstract

The purposes of the present study were to investigate whether salvianolic acid B (Sal B) can inhibit stromal cell-derived factor-1α (SDF-1α)/CXCR4-mediated effects on the cell proliferation and migration of vascular smooth muscle cells (VSMCs) and to examine its possible molecular mechanisms. Under FBS-restricted condition (0.5% FBS), all of the cellular studies were investigated on VSMCs (A10 cells) stimulated with 10 ng/ml SDF-1α alone or co-treated with 0.075 mg/ml Sal B. Our results showed that SDF-1α markedly stimulated the cell growth and migration of A10 cells, whose effects can be significantly reversed by co-incubation of Sal B. Similarly, Sal B also obviously down-regulated the SDF-1α-stimulated up-regulation of CXCR4 (total and cell-surface level), Raf-1, MEK, ERK1/2, phospho-ERK1/2, FAK and phospho-FAK as well as an increase of the promoter activity of NF-κB. Besides, Sal B also effectively attenuated balloon angioplasty-induced neointimal hyperplasia. In conclusion, suppressing the expression levels of CXCR4 receptor and downstream molecules of SDF-1α/CXCR4 axis could possibly explain one of the pharmacological mechanisms of Sal B on prevention of cell proliferation, migration and subsequently neointimal hyperplasia.

Keywords: salvianolic acid B, stromal cell-derived factor-1α, vascular smooth muscle cells

1. Introduction

Angiographic restenosis is still a major limitation for the clinical application of percutaneous transluminal coronary angiography (PTCA) or intracoronary stent implantation. It has been well documented that restenosis, a healing response of injured vessels, is a complex and multifactorial process involving arterial remodeling and neointimal hyperplasia, which associated with cell proliferation and migration of vascular smooth muscle cells (VSMCs) (Gruntzig et al., 1979).

Stromal cell-derived factor-1α (SDF-1α) is an inflammation-activated small chemoattractant cytokine to exhibit its biological functions via a unique receptor, cysteine-x-cysteine chemokine receptor 4 (CXCR4) (Horuk, 2001). Both SDF-1α and CXCR4 proteins have also been recognized and expressed in VSMCs (Jie et al., 2010; Li et al., 2009; Schober et al., 2003). Numerous evidences noticed that expression level of SDF-1 correlated positively to balloon angioplasty-induced neointimal hyperplasia (Jorgensen et al., 2010; Li et al., 2007; Nuhrenberg et al., 2005), and neutralizing expression of SDF-1α would effectively reduce neointimal formation (Schober et al., 2003; Zernecke et al., 2005). Thereby, SDF-1α played a critical role to regulate the cell proliferation and migration of VSMCs in the progression of neointimal formation.

Salvianolic acid B (Sal B) is an active and a richest component isolated from the roots of Danshen (*Salvia miltiorrhiza*) and widely used to treat cardiovascular diseases (Zhong et al.,

2009; Zhou et al., 2005). It has been reported that Sal B has anti-migration properties on smooth muscle cells (Lin et al., 2007), and Sal B-rich extraction fraction of *Salvia miltiorrhiza* induced neointimal cell apoptosis in rabbit angioplasty model (Hung et al., 2001). To date, it remains unclear whether the inhibitory effect of Sal B on neointimal hyperplasia is involved in modulating SDF-1α/CXCR4 signaling pathway. Therefore, the purpose of this study was to examine whether Sal B can inhibit SDF-1α/CXCR4-mediated cell proliferation and migration on VSMCs and examine its possible molecular mechanisms.

2. Materials and methods

2.1. Chemicals

Anti-ERK1/2 (#sc-154), anti-phospho-ERK1/2 (#sc-7383), anti-FAK (#sc-1688), anti-MEK (#sc-6250), anti-Raf-1 (#sc-7262) antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies against mouse IgG (#sc-2005), and rabbit IgG (#sc-2004) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-CXCR4 (#PP-1615) antibody was purchased from Thermo Fisher Scientific (Fremont, CA, USA). Anti-β-actin (#ab8226) and anti-phospho-FAK (#ab4803) antibodies were purchased from Abcam (Cambridge, MA, USA). Recombinant mouse stromal cell-derived factor-1α (#PO-066) was purchased from Bioclone (San Diego, CA, USA). NF-κB firefly

luciferase reporter plasmid was given by Dr. Tin-Yun Ho (Graduate Institute of Chinese Medical Science of China Medical University, Taichung, Taiwan). Salvianolic acid B (Sal B) was kindly provided from Dr. Ming-Shi Shiao (Department of Life Science, Chang Gung University, Tao-Yuan, Taiwan). All other reagents were purchased from Sigma-Aldrich (Louis, MO, USA).

2.2. Cell culture

A10 cell line, the thoracic aortic smooth muscle cells of embryonic rat (BCRC number: 60127), were obtained from Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were cultured in GIBCO™ Dulbecco's modified Eagle's medium (#12800-017; Invitrogen, Carlsbad, CA, USA) containing 4 mM L-glutamine, 1.5 mg/ml sodium bicarbonate, 4.5 mg/ml glucose, 1.0 mM sodium pyruvate, 10% fetal bovine serum (FBS, #10099-141; Invitrogen), 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate. The cells were incubated in a humidified 5% CO_2 atmosphere at 37 °C and subcultured every 2 day. The cells were starved with 0.5% FBS medium for 24 h prior to experimental treatments. Each experiment was performed independently three times.

2.3. Protein extraction and Western blot

The A10 cells were washed with 1× phosphate buffered saline (PBS) and lysed by adding

appropriate volume of lysis buffer containing 6.25 mM Tri-HCl (pH=6.8), 20 mg/ml sodium dodecyl sulfate, 50 mM dithiothreitol (DTT). The cell lysate was then centrifuged at 13000 ×*g* at 4 °C for 10 min, and the supernatant was collected for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was measured by the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. Aliquots containing 30 μg protein were resolved on 10 % slab SDS-PAGE gels and then transferred to PVDF membranes (Immobilon-P™; Millipore, Bedford, MA, USA). Ponceau S was used to identify the successful transfer of proteins to the membrane. Briefly, nonspecific binding were blocked by incubating membranes in 5% non-fat milk. Primary antibodies against proteins were diluted as follows: 1:1000 for CXCR4, ERK1/2, phospho-ERK1/2, FAK, phospho-FAK, MEK, Raf-1 and β-actin. The secondary antibody was applied using a dilution of 1:2000. Substrates were visualized using Amersham ECL Plus™ Western Blotting Detection Reagents (GE Healthcare Bio-Sciences, Bucks, UK) and the luminescence signal acquired and analysis by Fujifilm LAS-3000 system (San Leandro, CA, USA). The results for each experiment were normalized to the band density of β-actin, and the relative protein expression was calculated according to the values of control group as 100 %.

2.4. Total RNA extraction

Total cellular RNA of cells was extracted as recommended by the manufacturer of TRIzol™ (GIBCO BRL, Rockville, MD). Briefly, the TRIzol method consists of the addition of 1 ml of the TRIzol reagent to the cells (about 3.5×10^5 cells). The cell lysate was vigorously agitated for 30 s and incubated at room temperature for 5 min. After this procedure, 200 μl chloroform was added to the tube, and the solution was centrifuged at 12000 ×*g* for 15 min. The aqueous phase was transferred to a clean tube, precipitated with 500 μL isopropyl alcohol, and centrifuged at 12000 ×*g* for 15 min. The resulting RNA pellet was then washed with 1 ml of 75% cold ethanol and centrifuged at 12000 ×*g* at 4 °C for 15 min. The pellet was dried at room temperature, resuspended in 20 μl of diethylpyrocarbonate (DEPC)-treated water, and stored at −80 °C. RNA was quantified by measuring absorbance at 260 nm and 280 nm and electrophoresed on a denaturing 1% agarose gel. The integrity and relative amounts of RNA were evaluated using ultraviolet visualization of ethidium bromide–stained RNA.

2.5. Real-time RT-PCR

For cDNA synthesis, 3 μg RNA was supplemented in a total reaction volume of 20 μl with ReverTra Ace set (#PU-TRT-200; TOYOBO, Osaka, Japan) composed of 1× RT buffer, 1 mM dNTPs, 0.5 nM oligo-dT, 40 U/μl RNase inhibitor, and 100 U/μl ReverTra Ace (reverse transcriptase). After incubation for 20 min at 42 °C, the mixture was incubated for

5 min at 99 °C to denature the products. The mixture was then chilled on ice for further use. Real-time PCR was performed using the ABI Prism 7900HT sequence detector (Applied Biosystems, Foster City, CA, USA). The reaction mixture contained 1 μl cDNA, 2 μl of each primer (10 μM), 10 μl of Smart Quant Green Master Mix with dUTP and ROX (#SA-SQGR-V2-1ml; Protech, Taipei, Taiwan) and 5 μl distilled water in a total volume of 20 μl. After hotstart activation for 15 min at 95 °C, we carried out 40 cycles, each consisting of 15 s at 95 °C, 15 s at 59 °C and 30 s at 72 °C. The dissociation curve for each amplification was analyzed to confirm that there were no nonspecific PCR products. The primer pairs used for Real-time PCR were: CXCR4, 5'- CGT CGT GCA CAA GTG GAT CT-3' (forward) and 5'- GTT CAG GCA ACA GTG GAA GAA G-3' (reverse). Beta-actin, 5'- GCT GTG TTG TCC CTG TAT-3' (forward) and 5'- GTG GTG GTG AAG CTG TAG-3' (reverse). The relative transcript expression of CXCR4 was calculated using equation $2^{-\Delta\Delta Ct}$ and presented as fold changes to control group.

2.6. Cell viability assay

A10 cells (8×10^3 cells/well) were seeded on 96-well plate and culture in growth medium for overnight. The cells were serum starved for 24 h and then treated with SDF-1α or Sal B in a total volume of 10% FBS-restricted culture medium. After 21 h of treatment, 10 μl of 1× PBS containing 5 mg/ml of MTT (3-[4,5-dimethylthiazol- 2-yl]-2,5-diphenyl tetrazolium

bromid) was added into each well. After 3 h of incubation, the cells were washed twice with iced 1× PBS and 100 µl of DMSO (dimethyl sulfoxide) was added to each well for dissolving completely fromazan crystals converted from MTT by mitochondrial redox activity of living cells. Absorbance values at 570 nm were determined for each well using 650 nm as the reference wavelength. The absorbance can be correlated to the percentage of vital cells, by comparing the data of the doped cells with those of the control group (0.5% FBS treated only). The percentage of cell viability was calculated according to the values of control group as 100 %.

2.7. Transwell migration assay

Cell migration was analyzed using a 24-well Transwell cell culture chambers with 8 µm pore size (Millicell® insert, Millipore) as described previously with minor modification (Min et al., 2004). The cells (2.5×10^4 cells/well) suspended in 0.5% FBS medium with the presence or absence of Sal B (0.075 mg/ml) was loaded into the upper wells. In all groups but control group, the 0.5% FBS medium containing 10 ng/ml SDF-1α was placed in the lower wells. After 24 h incubation, the cells were fixed with methanol and stained with Giemsa solution, and the cells attached onto the lower surface of the insert were counted under a microscopy at 200-fold magnification. Five randomly chosen fields were counted for each group.

2.8. Measurement of NF-κB promoter activity

The cells (3.5×10^5 cells/well) were seeded on 6-well plate and cultured in growth medium for overnight, and then cells were serum starved for 24 h. The cells were transfected using TransFast™ transfection reagent (Promega, Madison, WI, USA) and the promoter activities measured by Luciferase Assay System with lysis buffer (#E4030; Promega) and β-galactosidase Enzyme assay system (#E2000; Promega), according to the manufacturer's instructions. Briefly, cells were co-transfected with 100 ng of NF-κB firefly luciferase reporter plasmid and 2.6 μg β-galactosidase control vector (#E1081, Promega). After 24 h transfection, the cells were stimulated with 10 ng/ml SDF-1α or co-incubated with Sal B (0.075 mg/ml) under FBS-restricted condition (0.5% FBS). Firefly luciferase activity was normalized for transfection efficiency by the corresponding β-galactosidase activity. All transfection experiments were performed at least 3 times in duplicate. The results for each experiment were normalized to the activity of reference plasmid, and the relative promoter activity of NF-κB was calculated according to the values of control group (0.5% FBS treated only) as 100 %.

2.9. Measurement of cell-surface CXCR4 expression

Flow cytometry analysis was performed to evaluate the expression of cell-surface

CXCR4 on A10 cells. Briefly, the treated cells (3×10^5 cells) were trypsinized and resuspended in 100 µl of ice-cold PBS buffer and then incubated with 10 µg/ml anti-CXCR4 antibody on ice for 30 min. After washing twice with cold PBS buffer cells were further incubated with secondary antibody conjugated with fluorescent dye (Alexa Fluor 488; Invitrogen) in the dark for 30 min on ice. Finally, cells were washed twice and resuspended in 0.5 ml of PBS buffer for flow cytometry. Relative fluorescence intensity of cell samples was measured with a BD FACSCanto™ flow cytometer system.

2.10. Rat model of balloon angioplasty

Male Sprague Dawley rats (about 350~400 g) were purchased from BioLASCO (Taipei, Taiwan). Rats were housed in a 12 h light/dark cycles with free access to food and water. All animal care followed the institutional animal ethical guidelines of China Medical University. The balloon catheter (2F Fogarty) (Becton-Dickinson, Franklin Lakes, NJ, USA) was introduced through the right external carotid artery into the aorta, and the balloon was inflated at 1.3 kg/cm^2 using an inflation device. An inflated balloon was pushed and pulled through the lumen three times to damage the vessel. Two concentrations of Sal B (75 mg/ml and 100 mg/ml) suspended in 200 µl of 30% pluronic-F127 gel was coated onto arterial adventitia of balloon-injured carotid artery. Two weeks after balloon injury, rats were sacrificed. For morphological examination, right common carotid arteries were collected

and then fixed in 4% paraformaldehyde, embedded in Parafilm block. Embedded vessel tissues were cut into 10 μm-thick slices, and then, slices were stained with hematoxylin (Merck, Argentina, USA) and eosin Y (Merck). The manifestation of vessel restenosis was presented as the ratio of neointima to media area.

2.11. Statistics

All values are expressed as mean ± standard deviation (SD). Data were compared with one-way analysis of variance (ANOVA) with Bonferroni post-hoc test to evaluate differences among multiple groups. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Regulatory effects of SDF-1α and Sal B on cell growth of VSMCs

Our results demonstrated that the IC_{50} of Sal B is about 0.075 mg/ml in the VSMCs under 15% FBS medium for 24 h (Fig.1A). SDF-1α (10 ng/ml) significantly stimulates VSMC growth under 0.5% FBS medium for 24 h ($p < 0.01$; Fig. 1B). Thereby, the concentrations at 10 ng/ml for SDF-1α and 0.075 mg/ml for Sal B were used for the following studies.

3.2. Inhibitory effect of Sal B on CXCR4 receptor

Under 0.5% FBS medium, VSMCs (A10 cells) were treated with SDF-1α or co-incubated with Sal B for 24 hr to examine the transcript and protein level of CXCR4 receptor. Our data suggested that SDF-1α markedly increased transcript expression of CXCR4, whose stimulator effect can be significantly suppressed by Sal B co-treatment (Fig. 2A). Similarly, the experimental results showed that the expression level of total CXCR4 protein was markedly up-regulated in VSMCs stimulated with SDF-1α ($p < 0.05$; Fig. 2B), and this effect can be obviously down-regulated ($p < 0.05$) by Sal B co-treatment (Fig. 2B). The results of flow cytometry analysis also suggested that SDF-1α up-regulated the expression of cell-surface CXCR4 receptor as compared to that of control group, whose effect can be reversed by co-treatment of Sal B (Fig. 2C). The treatment of Sal B alone did not influence gene and protein expression of CXCR4 (data not shown).

3.3. Suppressive effect of Sal B on cell growth stimulated by SDF-1α

Under 0.5% FBS medium, the data showed that VSMC growth was markedly increased after 24 hr stimulation of SDF-1α ($p < 0.01$), whose effect can be evidently attenuated by Sal B co-treatment ($p < 0.01$; Fig. 3A). The cell morphology and amount of VSMCs were observed and photographed under same treatment condition (Fig. 3B), which revealed inhibitory capability of Sal B on SDF-1α-stimulated cell proliferation was similar to the result

from MTT cell proliferation assay (Fig. 3A).

3.4. Inhibitory effect of Sal B on SDF-1-triggered αcell migration

Under 0.5% FBS medium, cell migration of A10 cells was stimulated with SDF-1α to examine whether co-incubation of Sal B can suppress SDF-1α-triggered cell migration (Fig. 4A). The result showed that SDF-1α markedly induced VSMC migration as compared to that of control group, whereas Sal B can reduce SDF-1α-triggered cell migration in VSMC cells (Fig. 4B).

3.5. Down-regulatory effect of Sal B on ERK1/2-MAPK signaling activated by SDF-1α

The molecules of ERK1/2-MAPK pathway, a cell proliferation-associated signaling, were analyzed to investigate whether co-incubation of Sal B can reverse SDF-1α-stimulated activation of ERK1/2-MAPK pathway (Fig. 5). Under 0.5% FBS medium, the stimulation of SDF-1α significantly augmented the expression of Raf-1, MEK, total ERK1/2 proteins and activated ERK1/2 on cultured VSMCs ($p < 0.05$). These stimulatory effects of SDF-1α could be markedly attenuated by co-treatment of Sal B ($p < 0.05$; Table 1). The treatment of Sal B alone did not decrease the expression and activation of these proteins on cultured VSMCs (data not shown).

3.6. Down-regulatory effect of Sal B on FAK protein stimulated by SDF-1α

The FAK, a cell migration-associated molecule, was analyzed to investigate whether co-incubation of Sal B can reverse SDF-1α-stimulated up-regulation of FAK (Fig. 5). Under 0.5% FBS medium, SDF-1α significantly enhanced ($p < 0.05$) the protein expressions of total FAK and phospho-FAK on cultured VSMCs. Conversely, Sal B significantly attenuated the levels of FAK and phospho-FAK proteins augmented by SDF-1α ($p < 0.05$; Table 1). The treatment of Sal B alone did not decrease the expression and activation of FAK protein on cultured VSMCs (data not shown).

3.7. Suppressive effect of Sal B on promoter activity of NF-κB stimulated by SDF-1α

The promoter activity of NF-κB was measured by luciferase-based reporter gene assay in VSMCs stimulated with SDF-1α alone or co-incubated with Sal B. Under FBS-restricted condition, the result revealed that SDF-1α significantly increased promoter activity of NF-κB on A10 cells ($p < 0.05$), whereas Sal B could markedly inhibit SDF-1α-induced increase in promoter activity of NF-κB ($p < 0.01$; Fig. 6).

3.8. Preventive effect of Sal B on neointimal hyperplasia

The balloon angioplasty was applied to evaluate the clinical therapeutic potential of Sal B in the present study (Fig. 7). Our results showed that the balloon angioplasty can successful

induce neointimal formation of rat carotid artery and these thickening neointima could be attenuated by extra-arterial treatments of Sal B (75 and 100 mg/ml) as compared to those in the balloon-injury artery. Moreover, the area ratio of neointima to media (I/M ratio) of Sal B treatments was evidently decreased as compared to the balloon-injury group (Fig. 7E).

4. Discussion

To our knowledge, our studies demonstrate for the first time that Sal B can markedly attenuate the expressions of CXCR4 receptor stimulated by SDF-1α on VSMCs. Based on this result, Sal B might have potential application as an antagonist of CXCR4 receptor to block SDF-1α-induced cell proliferation and migration of VSMC and to reduce the development of neointimal hyperplasia.

It has been evidenced that SDF-1α were largely increased in the neointimal area to selectively recruited bone marrow-derived VSMC progenitor cells to the injury site and participate significantly in neointimal formation (Karshovska et al., 2007; Sata et al., 2002). Besides, Jie *et al*. also suggested that increased SDF-1α enhanced the up-regulation of CXCR4 receptor on VSMCs (Jie et al., 2010). In the present study, our results indicated that SDF-1α largely stimulate the cell proliferation and migration of VSMCs (Figs. 3 and 4), and the expression of CXCR4 could be up-regulated by SDF-1α stimulation on cultured VSMCs (Fig. 2).

SDF-1α/CXCR4 axis activates multiple signal transduction pathways including ERK1/2-MAPK, focal adhesion kinase (FAK), phosphoinositide-3 kinase (PI3K)/AKT/NF-κB (Ganju et al., 1998; Helbig et al., 2003; Kodali et al., 2006; Neuhaus et al., 2003; Tilton et al., 2000), which has been shown to widely exist in many cell types such as VSMCs. The ERK1/2-MAPK pathway generally participates in cell proliferation and

survival signaling, and it has been reported to be activated by SDF-1α in human arterial smooth muscle cells (Kodali et al., 2006). Our result showed that SDF-1α stimulated the activation of ERK1/2-MAPK cascade on cultured VSMCs, which can then be markedly attenuated by Sal B co-treatment (Fig. 5 and Table 1). On the other hand, FAK protein has also been indicated to promote several cellular responses such as cell proliferation, adhesion and migration (Parsons, 2003; Sieg et al., 1999; Xie et al., 2001). Li *et al*. mentioned that Administration of CXCR4 antagonist suppressed the cyclic stretch-induced expression and activation of FAK protein (Li et al., 2009). In the present study, FAK protein was obviously increased and activated on cultured VSMC stimulated with SDF-1α (Fig. 5 and Table 1), which could partially explain the cellular phenomenon of SDF-1α on cell proliferation and migration of VSMCs (Figs. 3 and 4). Additionally, PI3K/AKT/NF-κB axis is another pathway of the downstream signaling triggered by SDF-1α/CXCR4. NF-κB has been implicated in regulation of the cell motility, survival and apoptosis (Baichwal and Baeuerle, 1997; Helbig et al., 2003). Collins *et al.* mentioned that activation of the NF-κB has been associated with endothelial cells dysfunction and vascular inflammation (Collins, 1993). Applying CXCR4 antagonist, AMD300, has been evidenced to attenuate phosphorylation of AKT protein in primary cultured rat aortic VSMC, suggesting an association between SDF-1α/CXCR4 and PI3K/AKT signaling in VSMCs (Jie et al., 2010; Kodali et al., 2006). Likewise, our results also found that SDF-1α can significantly induced promoter activity of

NF-κB (Fig. 6) markedly decreased by Sal B, a potential blocker for CXCR4 receptor, which provided a beneficial effect to avoid the counteracting effects of inflammation on VSMCs.

The studies implicated that Sal B might inhibit cellular activations of SDF-1α through suppressing the expression of platelet-derived growth factor (PDGF) receptor to reduce PDGF-BB-mediated up-regulation of SDF-1α level (Song et al., 2009; Xue et al., 2006). Besides, Sal B has been reported to scavenge free radicals (Zhao et al., 2008), which might present a potential effect of Sal B to regulate expression level of SDF-1α *via* decreasing oxidative stress-induced up-regulation of SDF-1α.

The process of endothelialization is associated with intactness of the healing process after balloon angioplasty or stent implantation as well as negatively correlates with the risk of both thrombosis and restenosis (Bauter and Isner, 1997). Numerous studies suggested that SDF-1α/CXCR4-axis also involved in the process of endothelial repair. Chang et al. noticed that increased levels of serum SDF-1α were markedly correlated with an elevation of circulating endothelial progenitor cells (EPCs) (Chang et al., 2009). It has been demonstrated that the degree of neointimal hyperplasia can be reduced in a rodent model transfused with EPCs which are capable of trafficking into the vascular injury site (Werner et al., 2003). Thereby, the therapeutic strategy designed for inhibiting restenosis should be considered to avoid possible interference in the process of endothelialization. In the present study, we found that CXCR4 expression could be down-regulated by Sal B in VSMC cells,

which implicated the possible impacts of Sal B on the process of endothelialization in the injured artery. Therefore, pharmacological applications of Sal B should consider the several parameters, such as dosage, the time point of intervention and the duration of drug release, to find out a compromise between the prevention of neointimal hyperplasia and the process of re-endothelialization

5. Conclusion

Our experimental data suggested that Sal B effectively inhibited expression of cell-surface CXCR4 and then subsequently blocking SDF-1α/CXCR4-induced cellular responses including cell proliferation and migration on VSMC and attenuated neointimal formation in the rat model of balloon angioplasty (Fig. 8). All of the analyzed cellular mechanisms of Sal B provide valuable information for further therapy application to effectively prevent the neointimal hyperplasia after percutaneous coronary intervention.

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Figure captions

Fig. 1. Regulatory effects of SDF-1α and Sal B on cell growth of VSMCs. A10 cells were treated with serial concentration of SDF-1α in FBS-restricted medium (A) or stimulated with Sal.B in medium contained 15% FBS (B) for 24 h to analyze the cell viability by MTT assay. In panel A, * and ** indicate p <0.05 and p < 0.01 as compared with control group (15% FBS treated only), respectively. In panel B, ** indicates p < 0.01 as compared with control group (0.5% FBS treated only).

Fig. 2. Inhibitory effect of Sal B on CXCR4 receptor was analyzed by Real-time PCR (A), Western blot (B) and flow cytometry (C). * indicates p < 0.05 as compared with control group. † indicates p < 0.05 as compared with the group of SDF-1α treated only.

Fig. 3. Suppressive effect of Sal B on cell growth stimulated by SDF-1α was examined by MTT proliferation assay (A) and observed at 400 magnification by optical microscopy (B). ** indicates p < 0.01 as compared with control group, and ‡ indicates p < 0.01 as compared with the group of SDF-1α treated only.

Fig. 4. Inhibitory effect of Sal B on cell migration triggered by SDF-1α . Cell migration of cultured VSMCs was observed and cellular image acquired under optical microscopy at

200-fold magnifications (A). The relative amount of migrated cells was calculated according to the values of control group as 100 % (B). * indicates $p < 0.05$ as compared with control group (0.5% FBS treated only).

Fig. 5. Down-regulatory effects of Sal B on ERK1/2-MAPK signaling and FAK protein activated by SDF-1α. Under FBS-restricted condition, A10 cells were stimulated with SDF-1 alone or co-incubated with Sal B for 24 h to determine the protein expression of Raf-1, MEK, ERK1/2 and FAK, and for 15 min to examine the expression of phosphorylated proteins (ERK1/2 and FAK).

Fig. 6. Suppressive effect of Sal B on promoter activity of NF-κB stimulated by SDF-1α. ** indicates $p < 0.01$ as compared with control group. ‡ indicates $p < 0.01$ as compared with the group of SDF-1α treated only.

Fig. 7. Preventive effect of Sal B on neointimal hyperplasia. (A) Normal vessel without balloon injury. (B) Balloon injured vessel. (C) Balloon injured vessel treated with 75 mg/ml Sal B. (D) Balloon-injured vessel treated with 100 mg/ml Sal B (E). The manifestation of vessel restenosis was presented as the ratio of neointima to media area. Arrow indicates the neointimal layer from the internal elastic fiber. All graphs were taken at 40× magnifications.

** indicates $p < 0.01$ as compared with normal group. † indicates $p < 0.05$ as compared with the balloon-injury group.

Fig. 8. Schematic representation of effects of Sal B in the SDF-1α-mediated signaling pathway on VSMCs. CXCR4, cysteine-x-cysteine chemokine receptor 4; ERK1/2, extracellular signal-regulated kinases 1/2; FAK, Focal adhesion kinase; MEK, mitogen-activated/ERK kinase; NF-κB, nuclear factor-κB; Sal B, salvianolic acid B; SDF-1α, stromal cell-derived factor-1α.