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Original Article

Anti-fibrotic Effects of *Salvia miltiorrhiza* and Ligustrazine Injection on LX-2 Cells Involved with Increased N-myc Downstream-Regulated Gene 2 Expression*

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ABSTRACT **Objective:** To investigate the effects of *Salvia miltiorrhiza* and Ligustrazine Injection (SML) on proliferation and apoptosis of human hepatic stellate cell LX-2 and the expression of N-myc downstream-regulated gene 2 (NDRG2, a tumor suppressor gene). **Methods:** HSCs from the LX-2 cell line were cultured *in vitro*. The proliferative state of different initial LX-2 cell numbers was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. LX-2 cells were plated in 96-well plates at an approximate density of 2.50×10^4 cells/mL and cultured for 24 h followed by the application of different concentrations of SML (1, 2, 4 and 8 μL/mL). Cell proliferation was measured using the MTT assay at 24 and 48 h. Apoptosis was detected by flow cytometry at 24 h. LX-2 cells were treated with different concentrations of SML and extracted with protein lysis buffer. The levels of NDRG2 and β-catenin were measured by Western blot. **Results:** With the exception of the 1 and 2 μL/mL concentrations, 4 and 8 μL/mL SML inhibited cell proliferation in a concentration-dependent manner at 24 and 48 h ($P<0.05$). With the exception of the 1 and 2 μL/mL concentrations, the NDRG2 expression level was greatly increased in a concentration-dependent manner. However, the level of β-catenin was unaffected. **Conclusion:** SML inhibit LX-2 cell proliferation in a concentration-dependent manner, and the mechanism may be associated with NDRG2 over-expression.

KEYWORDS *Salvia miltiorrhiza* and Ligustrazine Injection, N-myc downstream-regulated gene 2, hepatic stellate cell, proliferation, apoptosis

It has been reported that fatty liver, liver fibrosis (LF), and liver cirrhosis (LC) may be caused by alcoholism, hepatitis virus, metabolic disorders, etc., which is harmful to human health and eventually leads to liver cancer.[1] LF is one of the key links to liver cancer. Previously, LF was considered to be an irreversible pathological process. However, with the rapid development of scientific medicine, the pathogenesis of LF has been reported and LF was found to be a reversible injury-repair response. Recently, more and more studies have focused on Chinese medicine used for the treatment of LF.[2] *Salvia miltiorrhiza* and Ligustrazine Injection (SML), composed by salvianic acid A and ligustrazine (tetramethylpyrazine, TMP), is commonly used with the function of "activation blood to resolve stasis", which have been reported to effectively reduce myocardial damage from severe burn[3] and produce obvious therapeutic effects on pregnancy-induced hypertension (PIH).[4] Salvianic acid A is one of the important components in the medicine. It was reported that salvianic acid A can repress the proliferation and activation of hepatic stellate cell (HSC)-T6,[5] inhibit the synthesis and secretion of type Ⅰ and Ⅲ collagen by down-regulating the expression of transforming growth factor-beta 1 (TGF-β1),[6] and potentially via the repression of C-Jun N-terminal kinase (JNK) signal transduction.[7] What is more, salvianic acid A could decrease the extracellular matrix by down-regulation of tissue inhibitor of metalloproteinase-1

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(TIMP-1)/matrix metallopeptidase-1 (MMP-1).[6] However, there are few researches on SML.

LX-2 cell line, a stable and sustainable source of human HSC, has been widely characterized as an advisable cell-based model for studies of LF.[8] In the present study, LX-2 cells could be regarded as partially activated based on the fact that they expressed α-smooth muscle actin (α-SMA) under all culture conditions.[9] LX-2 can undergo further activation by cultured on uncoated plastic. LX-2 is well-established for studying hepatic fibrogenesis at the cellular level.

Nowadays, it has been reported that the expression of N-myc downstream-regulated gene 2 (NDRG2), which is a potential tumor suppressor gene, was decreased during the activation of HSCs,[10] and NDRG2 over-expression could inhibit the activation of HSCs, which may be involved in the LF injury repair process. This experiment focused on the effect of SML on the proliferation activity of LX-2 cells and the NDRG2 expression in LX-2 cells.

METHODS

Drugs and Reagents

SML was produced from Baite Pharmaceutical Co. (Guizhou, China). The specification of SML is 5 mL. 3-(4,5-Dimethylthiazol2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Amresco (USA). LX-2 cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Trypsin and antibodies directed against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Biosynthesis Biotechnology (Beijing, China). Antibodies directed against NDRG2 were purchased from Abcam (Cantab, UK). Antibodies directed against-catenin were purchased from Sigma (USA). Phenylmethylsulfonylfluoride (PMSF), sodium fluoride, sodium chloride, and sodium phosphate were purchased from the Beyotime Institute of Biotechnology (China). The BD FACSAria flow cytometer was purchased from the Becton Dickinson Company (USA).

Cell Culture

LX-2 cells were obtained from the Cell Engineering Research Center, Fourth Military Medical University (China), and were maintained in high glucose DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin and grown at 37 ℃ in a humidified atmosphere with 5% CO_2. The culture medium was changed once a day. When the cell density reached 80%–90%, the cells were treated with 0.25% trypsin for approximately 2–3 min and passaged.

Proliferation of Different Initial LX-2 Cell Numbers

LX-2 cells in the logarithmic growth phase were centrifuged at 1,000 r/min for 5 min, and the cell density was then adjusted to the following concentrations: 0.75×10^4, 1.0×10^4, 1.25×10^4, 1.50×10^4, 1.75×10^4, 2.00×10^4, 2.25×10^4, and 2.50×10^4 cells/mL. The cells were plated in 96-well plates at the above-mentioned concentrations. A 100 μL of cell suspension was added to each well (the number of cells per well was 750, 1000, 1250, 1500, 1750, 2000, 2250, and 2500), and DMEM containing 10% FBS was used to bring the total volume to 200 μL. Every concentration had 4 replicates. The proliferation activity of the LX-2 cells was measured using the MTT colorimetric assay every 24 h for a total of 144 h. A cell proliferation curve drawn after the completion of the experiment was then statistically analyzed.

Concentration of Drug

The extracellular fluid in a person 50 kg in weight and 170 cm in height accounts for 20% of their weight, i.e., 10 L. In humans, the administration and dose of SML is 10 mL once per day. Therefore, the drug concentration in the body is 1 μL/mL; thus, the following concentration gradient for SML were used: 1, 2, 4 and 8 μL/mL.

MTT Proliferation Assay

LX-2 cells in the logarithmic growth phase were centrifuged at 1,000 r/min for 5 min, and the cells were then plated in 96-well plates at approximately 2.50×10^4 cells/mL and cultured for 72 h. A total of 100 μL of cell suspension was added to each well. Then, the medium was changed, and SML was added to each well at a final concentration of 1, 2, 4 or 8 μL/mL. Each concentration had 4 replicates plus a negative control. After the cells were treated for 24 and 48 h, 5 mg/mL MTT was added to each well, and the compound was incubated at 37 ℃ for 4 h. To dissolve the formazan crystals, the culture medium was replaced with 180 μL of dimethyl sulfoxide. After shaking the 96-well plate at room temperature for 10 min, the absorbance (OD) of each well was determined at

490 nm. Each experiment was repeated 3 times, and the proliferation inhibition rate was calculated by taking the mean for each group. The proliferation inhibition rate (%) = (1–the absorbance of the experimental group/ the absorbance of the control group) × 100%.

Apoptosis by Flow Cytometry

Phosphatidylserine (PS) is an active substance located in the inner cell membrane, and this phospholipid can be flipped from the internal layer to the surface of the cell membrane. Annexin-V is a calcium-dependent phospholipid binding protein that can specifically bind to PS. Apoptosis can be detected via a flow cytometry procedure in which Annexin-V is marked by fluorescein isothiocyanate (FITC).

LX-2 cells were plated in 6-well plates at approximately 5×10^6 cells/mL. When the cells grew to 80% confluence, the medium was changed, and SML was added to each well at a final concentration of 1, 2, 4 or 8 μL/mL. After 24 h, apoptosis was measured using a FACSAria flow cytometer following the manufacturer's instructions.

Western Blot Analysis

LX-2 cells were extracted with radio-immunoprecipitation assay (RIPA) buffer [Tris-HCl, NaCl, ethylene diamine tetraacetic acid (EDTA), Triton X-100, sodium deoxycholate, sodium dodecyl sulfate (SDS) supplemented with protease inhibitors (1% aprotinin, leupeptin, 1% activated Na_3VO_4, and PMSF)]. The protein concentration was determined using a microplate reader (BioRad, USA). The proteins were separated using a 10% SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane. The anti-NDRG2, anti-β-catenin, and anti-GAPDH antibodies were used to detect the expressions of NDRG2, β-catenin and GAPDH, respectively, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. The protein bands were detected by ChemiDOTMXRS and analyzed using Quantity One software.

Statistical Analysis

All values were presented as mean ± standard deviation ($\bar{x} \pm s$). The mean values were analyzed with SPSS software (version 19.0) using analysis of variance (ANOVA) and least significant difference Duncan. $P<0.05$ was considered statistically significant.

RESULTS

Proliferation State of LX-2 Cells Seeded at Different Initial Densities

As observed in Figure 1, LX-2 cells seeded at different initial densities exhibit different growth rates. With increased initial cell numbers, there was a higher rate of cell proliferation; it appeared that 72 h is a dividing line. Cell proliferation was slow for the first 3 days, and it was significantly elevated after 72 h. Considering these differences, the density of 2.50×10^4 cells/mL was chosen as the initial cell density and 72 h as the measuring time.

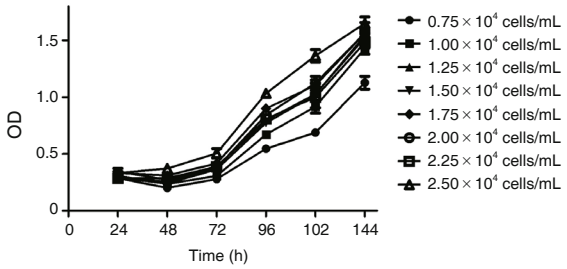

Figure 1. Proliferation State of LX-2 Cells Seeded at Different Initial Densities

Note: The legend indicates the number of cells per well

Proliferation of LX-2 Cells Treated with SML

As shown in Figure 2, when the drug concentration was greater than or equal to 4 μL/mL, the inhibitory rate at 24 and 48 h demonstrated a concentration-dependent increase ($P<0.05$). However, 1 and 2 μL/mL concentrations resulted in no inhibition, which may be related to the drug concentration.

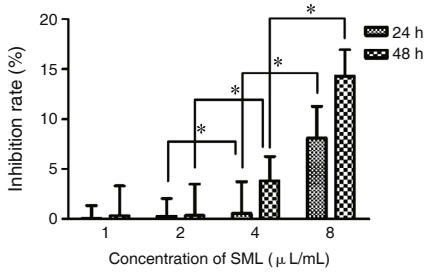

Figure 2. Proliferation of LX-2 Cells Treated with SML at 24 and 48 h

Note: *$P<0.05$

Apoptosis of LX-2 Cells Treated with SML

As shown in Figure 3, the results demonstrated that SML can induce apoptosis in a dose-dependent manner in LX-2 cells after 24 h. The number of cell death arising from apoptosis and necrosis was significantly increased in the treated cells with

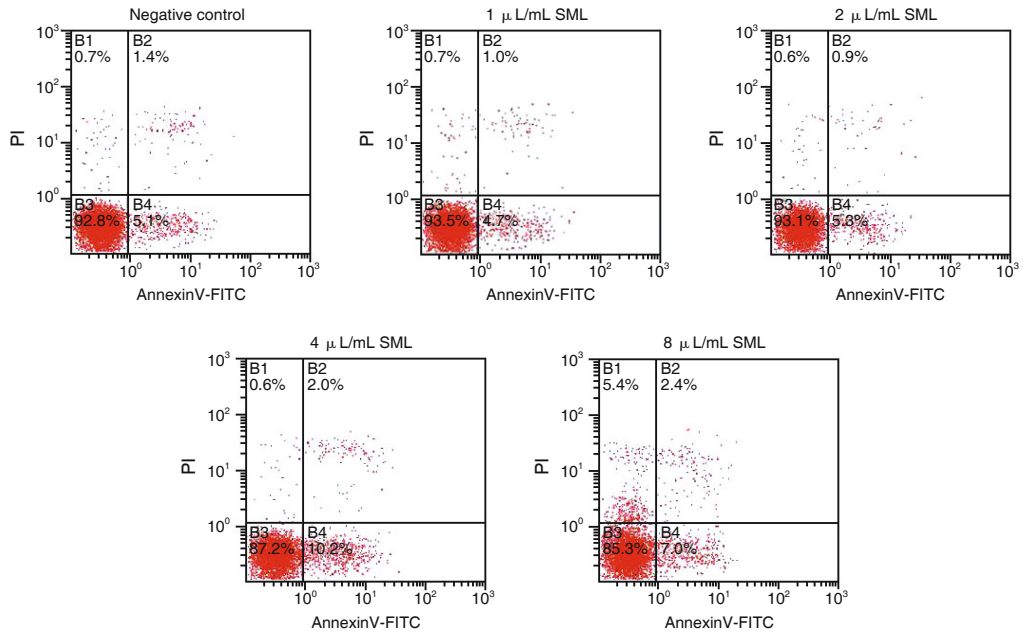

Figure 3. Apoptosis of LX-2 Cells Treated with SML at 24 h

Notes: In each plot, B1 represents necrotic cells, B2 represents later apoptosis cells or necrotic cells, B3 represents viable cells, B4 represents early apoptosis cells

SML compared with the control cells. Respective percentages of these cells (B2+B4+B1) after treatment with SML (1, 2, 4, and 8 μL/mL) for 24 h were 6.4%, 6.8%, 12.8%, and 14.7%.

NDRG2 and β-Catenin Expression in LX-2 Cells Treated with SML

With the exception of 1 and 2 μL/mL concentrations of SML, the level of NDRG2 was greatly increased in a concentration-dependent manner, while the level of β-catenin was unaffected (Figure 4).

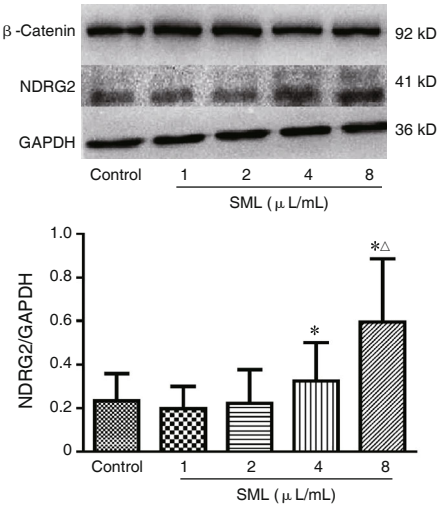

Figure 4. Western Blot Analysis of NDRG2 and β-Catenin Expression in LX-2 Cells Treated with SML

Notes: *P<0.05, compared with the control group; △P<0.05, compared with the 4 μL/mL SML

DISCUSSION

LF is a dynamic result of the liver injury repair process, the activation of which can be caused by viral infections, autoimmune diseases, drug injury, etc. The activation of HSCs plays an important role in the occurrence and progression of LF. Normally, HSCs are in a quiescent state and the main function is to store vitamin A which is essential in the regulation of retinoic acid homeostasis.[11] However, the factors that lead to liver injury (alcohol, hepatitis virus, etc.) can stimulate the activation and proliferation of HSCs and result in these cells exhibiting a myofibroblast (MFB) phenotype.[12] MFB can synthesize a large quantity of extracellular matrix (ECM). The synthesis and degradation of the ECM is regulated by multiple signaling pathways, cytokines, and growth factors, including the TGF-β/Smad signaling pathway,[13] the MMP/TIMP system,[14] and platelet-derived growth factor (PDGF).[15] The sustained activation of HSCs leads to the deposition of a large quantity of liver extracellular matrix, which eventually leads to LF.

Studies have shown that NDRG2 expression gradually decreases during the activation of HSCs,[10] and over-expression of NDRG2 could inhibit the activation of HSCs and reduce the expression of α-SMA and CD44.[16] NDRG2 was considered a

potential tumor suppressor gene that could induce apoptosis in multiple tumor cells.[17-20] Previous studies indicated that the modulation of NDRG2 is a promising strategy for the treatment of liver fibrosis.[21] In our study, we found that with the increment of LX-2, the expression of NDRG2 also increased, that is, the mechanism that SML inhibiting LX-2 cell proliferation may be associated with NDRG2 over-expression.

Certain components of Chinese medicine have satisfactory curative effects for hepatic fibrosis. The main components of SML include tanshinol and ligustrazine hydrochloride. Animal experiments have showed that ligustrazine could significantly reduce the level of alanine aminotransferase (ALT) and malondialdehyde and improve the activity of liver superoxide dismutase in rats with carbon tetrachloride-induced liver fibrosis.[22,23] Tanshinol had a significant inhibitory effect on LX-2 cells[24] and could reduce the level of liver hydroxyproline as well as the mRNA levels of collagen Ⅰ and Ⅲ.[25]

In our study, we investigated the effects of different concentrations of SML on LX-2 cells using the MTT assay. It was found that in a short time, i.e., 24 and 48 h, certain concentrations of SML (1 and 2 μL/ mL) had no obvious inhibitory effects on LX-2 cells. However, with an increase in dose and action time, the drugs had inhibitory effects on LX-2 cells that were concentration-dependent at 24 and 48 h, indicating that we must consider the concentration and action time of the drugs during clinical applications. In addition, we examined the apoptosis rate of LX-2 cells using flow cytometry at 24 h. We found that there was no significant difference although LX-2 cell apoptosis was observed in a short time frame, which may be related to the duration of drug action; in other words, taking the medicine could result in better effects over a long period of time. Finally, the effects of SML on the expression of NDRG2 and β-catenin were measured by Western blot. We found that SML could increase the expression of NDRG2, which may be one of the anti-hepatic fibrotic mechanisms mediated by SML. In other words, increased NDRG2 expression is involved in the anti-fibrotic effects of SML on LX-2 cells. However, these drugs had no obvious effects on the expression of β-catenin.

In conclusion, the results of this study show that SML inhibits LX-2 cell proliferation in a concentration-dependent manner, and the possible underlying mechanism may be associated with the over-expression of NDRG2. These results provide evidence that the further study of this mechanism may be able to guide clinical treatment. In addition, NDRG2 may serve as a potential therapeutic target for the treatment of LF.

Conflict of Interest

The authors claimed no potential conflicts of interest relevant to this article.

Author Contributions

Zheng J and Yang MH conceived and designed the experiments; Ma LT, Ren QY, Hu Y, Bian H, Zhang Y and Zhou YC performed the experiments; Ma LT and Bai Y analyzed the data; Zheng J wrote the paper.

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