Rosmarinic Acid Inhibits Proliferation and Induces Apoptosis of Hepatic Stellate Cells

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Hepatic stellate cells (HSCs), activated during liver injury, are defined as the most important target in the therapy of hepatic fibrosis. In the present study, we evaluated the effect of Rosmarinic acid (RosA) on the proliferation and apoptosis in activated hepatic stellate cells (HSC-T6), which is useful to decrease this cell population.

The proliferation of HSC-T6 was significantly inhibited after treated with various concentrations of RosA for different times. Flow cytometric analyses and transmission electron microscope (TEM) observations revealed that HSC-T6 treated with RosA underwent apoptosis in a time dependent manner and displayed typical apoptotic features in the cells. The phosphorylation in signal transducer and activator of transcription protein-3 (STAT3), which regulates cell survival, proliferation and differentiation in a variety of tissues, was markedly decreased as the result of Western blot assay and correlated with downregulation of CyclinD1 and B cell lymphoma/leukemia-2 (Bcl-2). In conclusion, these results suggested that RosA was able to inhibit proliferation and induce apoptosis in HSC-T6, partly due to the inhibition of phosphorylation in STAT3, which contributed to the reversal of hepatic fibrosis.

Key words hepatic fibrosis; hepatic stellate cell; Rosmarinic acid

Hepatic fibrosis is the result of wound-healing response to repeated liver injury1) due to various etiologies such as parasitic disease, chronic viral infection, immunologic attack, chronic alcohol abuse, toxic damage etc.2) Associated with hepatic fibrosis, excessive extracellular matrix (ECM) proteins are deposited in the liver3) which may cause portal hypertension and progress to cirrhosis or hepatocellular carcinoma. As a result, hepatic fibrosis is a severe disease with high morbidity and mortality, representing a serious worldwide healthcare problem4) and effective antifibrotic treatments are urgently needed.

Numerous studies indicate that following liver injury, irrespective of etiology, quiescent hepatic stellate cells (HSCs) undergo significant morphological and functional changes,5) known as “activation,” and transdifferentiate into proliferative, fibrogenic, contractile and chemotactic myofibroblast-like cells, which are considered to be the main ECM-producing cells undergoing pathogenic fibrosis.6) HSCs are thus considered to be the primary therapeutic target for the treatment of hepatic fibrosis.

Although removal of the causative agent has been shown to effectively intervene the pathological process,7) approaches to removing fibrogenic cells are being evaluated such as development of drug delivery systems that target activated HSCs. It appears most promising to inhibit proliferation and induce apoptosis of HSCs, accompanied by eliminating the major source of both collagen and tissue inhibitor of metalloproteinase-1, as well as the inhibitor of matrix metalloproteinases, and thereby facilitating net matrix degradation.8) Signal transducer and activator of transcription protein-3 (STAT3), a member of the STAT protein family, mediates the growth promoting activity of angiogenic growth factors as well as other cytokines including interleukin (IL-6, 10, 17) and leptin by stimulating the expression of survival and proliferation genes.9) Among those factors, some play a great role in the development of hepatic fibrosis, therefore STAT3 may be a good target for pharmacologic modulation of this condition.

Rosmarinic acid (α-o-caffeoyl-3,4-dihydroxyphenyl lactic acid; RosA) is a naturally occurring hydroxylated phenolic compound which is mainly found in species of Boraginaceae and the subfamily Nepetoideae of the Lamiaceae.10) Some authors have found that it has many interesting biological activities: adstringent, antioxidative, antiinflammatory, antimutagen, antibacterial, antiviral, antitumor, antihepatitis and protecting the liver,11) inhibiting blood clots and enhancing fibrinolysis. Hence, the presence of RosA in medical plants, herbs and spices has beneficial and health promoting effects.

Our previous studies had demonstrated that RosA had better antifibrotic function than Silymarin and fufangbiejiuaranganpian which are used clinically. Moreover, RosA is well absorbed from the gastrointestinal tract and skin, and is rapidly eliminated from the blood circulation after intravenous administration (t1/2=9 min) showing a very low toxicity with a LD50 of 561 mg/kg in mice.11) In the present work, we report on the antiproliferative and proapoptotic effects of RosA in HSC-T6 and its possible signaling pathways.

MATERIALS AND METHODS

Reagents Sulforhodamine B (SRB) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); Newborn calf serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co. (Hangzhou, China); Dulbecco’s modified Eagle’s medium (DMEM) was produced by Hyclone Co. (Logan City, UT, U.S.A.); Cell lysis buffer for Western blot was purchased from Beyotime Institute of Biotechnology (Qingdao, China). RosA (formula: C18H16O8, molecule weight: 360) was obtained from Shandong Engineering Research Center for Nature Drug (Yantai, P. R. China), the purity of which is 99.5% according to HPLC and the chemical structure was shown in Fig. 1. Other chemicals were at least of analytical grade.

Cell Culture The rat hepatic stellate cell line (HSC-T6),

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with SV40 transfection showing an activated phenotype, was purchased from Hepatopahy Research Institute, Shanghai University of Traditional Chinese Medicine (Shanghai, China) and CCC-HEL was donated by Yantai University (Yantai, China). Those two cells were maintained in DMEM plus 10% newborn calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C under a humidified atmosphere of 5% CO₂ and 95% air. RosA was dissolved in DMEM, then filtered through 0.22 μm membrane and diluted to different concentrations.

**SRB Assay** The protein-staining SRB assay was used to measure the cell proliferation. Briefly, cells at the logarithmic growth phase were digested with 0.25% trypsin and seeded into 96-well culture plates at a density of 5 × 10³ (HSC-T6) and 1 × 10⁴ (CCC-HEL) cells/well. After overnight incubation, the cells were incubated with medium containing different concentrations of RosA for another 12, 24 and 48 h. Then 50 μl of trichloroacetic acid (TCA) was added to each well for additional 1 h at 4 °C. The plate was washed five times with water and dried under room temperature. The TCA-fixed cells were stained with 100 μl of 4 g/l SRB for 10 min, then the plate was washed five times with 1% acetate and air-dried overnight. The resulting crystals formed were dissolved with 150 μl of 10 mmol/l Tris (hydroxymethyl) aminomethane hydrochloride (Tris–HCl). Absorbance was measured with an Elisa Reader at 540 nm reference wavelength. The antiproliferative activity of the RosA was expressed in terms of the IC₅₀ value.

**Assessment of Apoptosis by Annexin V-Propidium Iodide (PI) Assay** When treated with or without RosA for 12, 24 and 48 h, apoptotic and necrotic cells were determined by annexin V-propidium iodide (PI) assay. Briefly, the culture supernatant was collected, and the adherent cells were digested with 0.25% trypsin and washed with the collected culture supernatant. Then the cell pellet was resuspended in phosphate-buffered solution (PBS). Approximately 5—10 × 10⁵ cells were stained with annexin V-FITC and PI according to the manufacturer’s instructions before analysis by flow cytometry.

**Observation of the Morphology under Transmission Electron Microscope (TEM)** Cells were treated with RosA for 24 and 48 h in 6-well plates. Culture supernatant to which 3% glutaraldehyde had been added, was centrifuged with 1.5 × 10³ rpm at 4 °C for 5 min, and cells were resuspended with 3% glutaraldehyde again. Adherent cells were fixed with 3% glutaraldehyde for about 10 min and then obtained with cell scraper. The cells in culture supernatant and adherent cells were transferred into one tube and again centrifuged with 1.5 × 10³ rpm at 4 °C for 5 min. Cells were then fixed in fresh 3% glutaraldehyde for at least 4 h at 4 °C, post-fixed in 1% osmium tetroxide for 1.5 h, dehydrated in a gradient ethanol series, infiltrated with Epon812, embedded and cured at 37 °C (24 h), 45 °C (24 h) and 60 °C (24 h). Ultrathin sections were ultracut with ultracut E ultramicrotome and stained with uranyl acetate and lead citrate prior to observation under JEM-1400 TEM (Jeol Ltd., Japan).

**Protein Extraction and Western Bolt Analysis** After RosA treatment for the indicated times, the cells in the supernatant were collected by centrifuging and attached cells were washed with PBS. They were lysed for 60 min at 4 °C with cell lysis buffer [20 mm Tris–HCl (pH 7.5), 150 mm NaCl, 1% Triton X-100, sodium pyrophosphate, β-glycerophosphate, ethylenediaminetetraacetic acid (EDTA), Na₂VO₃, leupeptin]. Protein concentration was quantified by bicinchoninic acid (BCA) protein assay kit and boiled with sample buffer in a water bath for 5 min. Protein samples were separated with 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels for 2 h, transferred onto a polyvinylidene difluoride (PVDF) membrane, which was then blocked in 5% non-fat milk for 1.5 h. Blots were incubated with specific primary antibodies overnight at 4 °C, followed by species-specific secondary antibodies conjugated with immunoglobulin G (IgG) horseradish peroxidase, incubated for 1 h at room temperature. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL)-assay kit.

**Statistical Analysis** All data are presented as the mean ± standard deviation (S.D.). Differences between groups were evaluated by a two-tailed t-test.

**RESULTS**

**Effects of RosA on Cell Proliferation** We were interested in learning the effect of RosA on cell proliferation. As shown in Fig. 2A, HSC-T6 treated with RosA grew significantly slower in a dose and time dependent manner compared to non-treated cells with an IC₅₀ value of 7.1 μg/ml, and we took 8 μg/ml RosA as the most appropriate concentration for the following study. The expression of CyclinD1 in the cells was assayed by Western blot, and the result showed that the content in cells cultured with 8 μg/ml RosA for 12, 24 and 48 h was markedly decreased (Fig. 6). These data suggest that RosA has an inhibitory effect on HSC-T6 proliferation which may be associated with G1/S cell cycle arrest.

It is exciting to target activated HSCs without adverse-function on liver hepatocytes. We also examined the effect of RosA on the growth of CCC-HEL in order to learn whether RosA has selective growth inhibition on abnormal cells. The growth curve, shown in Fig. 2B, indicated that RosA did not inhibit the growth of CCC-HEL in the range of 1—50 μg/ml with an IC₅₀ of 130 μg/ml (much larger than that of HSC-T6).

To explore whether the effect was due to cytotoxicity, we looked at the activity of lactate dehydrogenase (LDH) in HSC-T6 culture supernatant treated with different concentrations of RosA for 48 h. No significant difference was found among all the groups (data not shown). Taken together, these results lead to the conclusion that RosA actually inhibited proliferation of HSC-T6 without significant toxicity and evident adverse function on normal cells.

**Effects of RosA on Apoptosis** There were morphologic...
changes and death in cells treated with RosA (Fig. 3), which suggest that cells might undergo apoptosis. Therefore, we studied the ability of RosA to induce apoptosis in HSC-T6. Annexin V-FITC/PI analysis was used to evaluate the degree of apoptosis: early apoptotic cells were in the annexin V\^+/PI\^- area, whereas damaged cells appeared in the annexin V\^-/PI\^+ fraction, but late apoptosis or necrotic cells were seen in the annexin V\^-/PI\^- fraction. As shown in Fig. 4, after exposure to RosA for 12, 24 and 48 h, the percentage of apoptotic cells increased from 8.7±0.9% representing the basal levels of apoptosis to 15.8±1.3%, 23.8±1.5% and 37.9±2.5% (mean±S.D., n=3 for each time point). Among the proteins that play a key role in the regulation of apoptosis affected by kinds of apoptosis-inducing agents are members of the B cell lymphoma/leukemia-2 (Bcl-2) family. We also examined the levels of Bcl-2 and the results showed that RosA inhibited the expression of Bcl-2 as time increased (Fig. 6).

It is also necessary to observe the ultrastructural changes of HSC-T6 under TEM, which may aid in determining the function of organelles in cell progress. Results in Fig. 5 showed that treated cells underwent serial changes, such as autophagosome, enlarged rough endoplasmic reticulum (RER), porphyritic chromatin and lower density of mitochondrial matrix, which are characteristic of apoptosis in cells.

The Mechanisms of RosA Inhibiting Proliferation and Inducing Apoptosis  STAT3 is a transcription factor participating in many biological processes, especially those of cell survival and proliferation. We wanted to know whether the effect of RosA on HSC-T6 was partly due to the Janus kinase (JAK)/STAT3 signal pathway. Western blot was used for the detection of levels of STAT3 and phosphorylated STAT3 (p-STAT3). As demonstrated in Fig. 6, compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein level, the expression of p-STAT3 took a high degree of correlation in the expression pattern of Bcl-2 and CyclinD1 protein in HSC-T6 cells treated with 8 µg/ml RosA, however, the level of STAT3 protein showed no significant changes.

DISCUSSION

Liver diseases may be divided into four stages: acute liver injury, chronic liver injury, hepatic fibrosis and liver cancer. Recent evidence indicates that even advanced fibrosis but not cirrhosis (the end stage consequence of fibrosis) is reversible. As is known, activated HSCs play a key role in the pathogenesis of hepatic fibrosis and their population most likely affect the degree of fibrogenesis in liver diseases. Inhibition of HSCs activation, proliferation and induction of apoptosis has become potentially important for the prevention or treatment of hepatic fibrosis.

Our previous work showed that RosA had greater impact on immunological, chemical and alcoholic hepatic fibrosis
than that on other organ diseases. As reported, RosA had a potentially antifibrogenic effect and its mechanism might involve the inhibition of tumor growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF) expression and inhibition of activation of HSC\(^{18}\). However, this work was to explore whether RosA could inhibit proliferation and induce apoptosis for the first time.

Results from the current work showed that RosA inhibited the proliferation of HSC-T6 in a dose and time-dependent manner without significantly affecting the normal liver cell line at the same dose. The IC\(_{50}\) of RosA on these two types of cells were 7.1 µg/ml and 130 µg/ml, and may confirm the selective growth inhibition of RosA on abnormal cells, making possible its clinical use. Moreover, similar levels of LDH in cell supernatant among control and RosA treated groups indicated that the effect of inhibition was not due to cytotoxicity. We also found that RosA could downregulate the translation of CyclinD1 in HSC-T6 co-cultured with 8 µg/ml RosA for 12, 24 and 48 h using flow cytometry, TEM and Western blot assay. It was clear that RosA prevented fibrosis progression in liver partly due to its activity of anti-proliferation and pro-apoptosis.

To clarify the possible mechanism responsible for the anti-proliferation and pro-apoptosis of RosA, we analyzed the levels of CyclinD1, Bcl-2, STAT3 and p-STAT3. STAT3 is a transcription factor that participates in many biological processes and plays a very important role in the survival and proliferation of HSCs.\(^{19,20}\) Responding to the stimulation of many cytokines, STAT3 could be activated through phosphorylation and translocate to the nucleus where they bind to specific DNA responsive elements within the promoters of target genes and thus regulate the transcription of associated genes.\(^{21}\)

CyclinD1, a positive cell cycle regulator, forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S. Our studies found that RosA could downregulate the translation of CyclinD1 in HSC-T6. There is accumulating evidence that STAT3 promotes G1 cell-cycle progression and, interestingly, CyclinD1 promoter was transcriptionally induced by v-Src in a STAT3-dependent manner.\(^{22}\) It also has been shown that blocking serine phosphorylation of STAT3 in fibroblasts transformed by the Src oncprotein inhibits their growth in
Therefore, the inhibition of proliferation and G1 arrest induced by RosA might be correlated with downregulation of CyclinD1 partly through the STAT3 signaling pathway.

JAK/STAT signaling is associated with regulation of target genes including Bcl-2 family proteins (Bcl-2, Bcl-xL, Mcl-1 and survivin), which regulate the cytosolic efflux of cytochrome c from mitochondria. Other researchers have found that RosA promotes apoptosis in human Jurkat T lymphoma cells via a mitochondrial pathway. Moreover, the apoptosis of activated T cells from rheumatoid arthritis patients also can be induced by RosA treatment. Inhibition of STAT3 expression and activation were able to suppress growth and induce differentiation and apoptosis in cancer stem cells of glioblastoma. Consistent with these studies, we found the level of Bcl-2 in treated cells was significantly decreased in consistent with that of p-STAT3. Cytokines exert their biological functions through the JAK/STAT pathway, which is implicated in a variety of immune and inflammatory diseases. And numerous cytokines including IL-6, leptin and interferon γ (IFNγ) are associated with HSCs activation and play an important role in maintaining the activated phenotype. The research of A.M. Lakner indicated that IL6/STAT3 contributed to the initiation of HSCs transdifferentiation. Others found that STAT plays critical roles in the liver innate system during antiviral defense, acute phase response, hepatic injury and regeneration. In phagocytosing HSCs, there were different anti-apoptotic pathways, including the (JAK1/STAT3-dependent pathway and NADPH oxidase-dependent PI3K/Akt/nuclear factor-κB (NF-κB) induction pathways. Many studies in non-neuronal cells, such as astrocytes, fibroblasts and lymphocytes, showed that STAT3 signaling is enhanced by oxidative stress in a cytokine-independent manner. However, studies had shown that JAK/STAT signaling was the primary pathway for upregulation of pro-inflammatory mediators genes during acute phase response II, the body’s innate immune response provoked as a result of liver injury. Because RosA has many biological functions, further study is needed on how it affects the level of p-STAT3.

In summary, we have provided evidence that RosA can inhibit proliferation and induce apoptosis in HSC-T6, perhaps partly by the down-regulation of CyclinD1 partly through the STAT3 signaling pathway.

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REFERENCES
2) Trappoliere M., Caligiuri A., Schmid M., Bertolani C., Failli P., Vizzutti F., Novo E., di Manzano C., Marra F., Loguerchio C., Pinzani M.,