Hepatocellular carcinoma HepG2 cell apoptosis and caspase-8 and Bcl-2 expression induced by injectable seed extract of Coix lacryma-jobi

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BACKGROUND: Many Chinese herbs, especially herbal injections, have been shown to have anti-tumor effects in recent years. However, since most reports focus on the clinical effectiveness of these herbs, their mechanisms of action are not well understood. In this study, we assessed apoptosis in the hepatocellular carcinoma (HCC) cell line HepG2 induced by an injectable extract from the seed of Coix lacryma-jobi (Semen coicis, SC), and monitored the expression of Bcl-2 and caspase-8.

METHODS: Injectable SC was applied to HepG2 cells at different concentrations and the cells were collected 12, 24 and 48 hours later. 5-fluorouracil was used as a positive control group, and fluorescence-activated cell-sorting cytometry was used to measure the apoptosis rate of HepG2 cells and the expression of Bcl-2 and caspase-8 proteins.

RESULTS: SC induced apoptosis in HepG2 cells in a concentration- and time-dependent manner, and the expression of caspase-8 was elevated and prolonged. However, it did not significantly influence the expression of Bcl-2.

CONCLUSION: Injectable SC may induce apoptosis in HCC cells by regulating the expression of caspase-8.

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KEY WORDS: Semen coicis; traditional Chinese medicine; Bcl-2; caspase-8; HepG2 cells; apoptosis

Introduction

Epidemiologists have suspected for a long time that the low cancer rates in some areas of China might be related to Coix lacryma-jobi, a grass-like relative of maize that is a dietary staple in those regions and a key ingredient of many traditional Chinese herbal medicines. Injectable Semen coicis (SC) is extracted from Coix seeds using advanced pharmaceutical technology, and its injection inhibits some types of tumor cells. A phase I trial of this extract was approved by the United States Food and Drug Administration (FDA) and conducted at the Huntsman Cancer Institute (Salt Lake City, Utah, USA) in 2001. The FDA also approved a phase II trial to test its efficacy in treating non-small cell lung cancer.

For hepatocellular carcinoma (HCC) patients who have lost the opportunity of surgery, SC injection combined with transcatheter arterial chemoembolization is an effective method. Combined with chemotherapy, SC injection has a good effect in the treatment of advanced HCC, and life span and quality of life could improve.

Our previous study showed that injectable SC stops cells in the G2+M phase of the cell cycle and then reduces the number of cells entering the G0 and G1 phase. As a result, the percentage of cells in the S phase and karyokinesis is reduced, preventing hyperplasia and causing apoptosis. However, the mechanism of this apoptotic effect is still unknown.

The Bcl-2 gene (B-cell lymphoma/leukemia-2) is a proto-oncogene which inhibits apoptosis. Apoptosis may be affected by the intracellular anti-oxidative function of Bcl-2, and the inhibitory effect of the transmembrane movement of calcium ions. More importantly, Bcl-2 inhibits the activation of caspases by suppressing the mitochondrial release of cytochrome c, thus inhibiting apoptosis. Caspase-8 is a protease, also called FLICE, MACH or Mch5, which plays an important role in the apoptotic cycle of human cells. It is usually in the form...
of a proenzyme, and is activated in the apoptotic signal transduction process. Caspase-8 is considered to be an upstream caspase in apoptotic signal transduction. In Fas-receptor and TNFR-1-mediated apoptotic processes, caspase-8 is activated and forms a dimer, composed of p18 and p10.[9,10]

It is known that Bcl-2, IAP, c-myc, p53 and p35 genes affect apoptosis through regulating the activation of caspase-8.[11] The mechanism by which injectable SC inhibits tumors ex vivo, particularly by inducing apoptosis in HCC and influencing Bcl-2 and caspase-8 expression were investigated in this study, in order to facilitate the evaluation of this treatment for HCC.

Methods
Materials
The HCC cell line HepG2 was from KeyGen Biotechnology Ltd. Co. (Nanjing, China); RPMI-1640 medium and digestive pancreatin were from Gibco (Billings, MT, USA); Annexin V-FITC apoptosis ICT was from Beyotime Biotechnology Institute (Nanjing, China); Bcl-2 mouse anti-human monoclonal antibody and caspase-8 mouse anti-human monoclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); caspase-8 Alexa fluor 488 F(ab')2 fragment of goat anti-mouse IgG (H+L)*2 mouse IgG1 isotype control/PE radio-labeled antibody was from Molecular Probes (Billings, MT, USA); and mouse IgG1 isotype control/PE homotype radio-labeled antibody, stationary liquid, and rupturing liquid were from Jingmei GU Technology Ltd. (Beijing, China). Injectable Semen Coicis was from Zhejiang Kanglaite Pharmaceutical Co., Ltd. (Hangzhou, China). RPMI-1640 containing 10% fetal bovine serum was used with the HCC cell line HepG2 at 37 °C in a culture chamber containing 5% CO₂. The cells were cultured until the exponential phase of growth. When the cells were 80% confluent (within two or three days) the rate of trypsinization was 0.25%.

Effects of different concentrations of SC on HepG2 cells
We adjusted the cellular concentration to 1×10⁵/mL and the HepG2 cells were cultivated in a 6-well culture dish for 24 hours, then discarded the culture medium. The concentrations of injectable SC were 5, 10, 15 and 20 μL/mL. The cells were collected at 12, 24 and 48 hours, as for the control group with 5-fluorouracil (5-FU; 75 ng/mL).

Detecting apoptosis with fluorescence-activated cell sorter
The cells were washed twice in phosphate buffered saline (PBS), cooled to 4 °C and re-suspended in 250 μL binding buffer. The cell concentration was adjusted to 1×10⁶/mL. Aliquots (100 μL) of this cell suspension were placed in 5 mL tubes with 5 μL Annexin V/FITC and 10 μL 20 μg/mL propidium iodide. They were mixed thoroughly and incubated at room temperature away from sunlight for 15 minutes. PBS (400 μL) was then added. The suspension was analyzed with a fluorescence-activated cell sorter (FACS).

Determining apoptosis factor caspase-8 and Bcl-2 with FACS
Both caspase-8 and Bcl-2 which are located in the cell membrane were isolated by fixing and rupturing the membrane. Collected cells were centrifuged for 5 minutes at 100 rpm and the supernatant was removed. The cells were then re-suspended in 100 μL PBS. Immobilization buffer (1 mL per 10⁶ cells) was added and mixed thoroughly. After incubation at room temperature for 5 minutes, the mixture was washed twice with PBS, and then centrifuged. Bcl-2 and caspase-8 monoclonal antibodies were added after re-suspension. Ten microliters of IgG1 antibody of the same type was added to the control group. The Bcl-2 mixture was placed in an ice bath away from light, and then washed with PBS. We then assessed the mixture using FACS.

The caspase-8 mixture was incubated at room temperature for 30 minutes, washed with PBS, and centrifuged for 5 minutes at 2000 rpm. The mixture was then incubated with 10 μL type-2 antibody at room temperature for 30 minutes. The mixture was washed with PBS and centrifuged again. FACS was used immediately.

Statistical analysis
The data were analyzed by SPSS 10.0 statistical software (SPSS Inc., Chicago, IL, USA). Group means were compared with single factor variance analysis; the LSD-t test was used for pairwise comparison. The results were expressed as mean±SD. A P value less than 0.05 was considered statistically significant.

Results
HepG2 cell apoptosis induced by different concentrations of injectable SC
Apoptosis in HepG2 cells with different concentrations of SC was assessed by FACS after 24 hours (Fig.). Apoptosis was higher in the experiment group than in
HepG2 cell apoptosis induced by injectable Chinese herb

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Fig. Apoptosis in HepG2 cells induced by 24 hours treatment with injectable SC detected by FACS. In each scatter, upper left quadrant shows naked nucleus cell mass, upper right quadrant shows necrotic cell mass, lower left quadrant shows survival cell mass, lower right quadrant shows apoptotic cell mass. The numbers in the upper left quadrant indicate the rate of apoptosis. A-O: scatter plots of control group treated for 24 hours with: 75 ng/mL 5-fluorouracil (A-C); 5 μL/mL SC (D-F); 10 μL/mL SC (G-I); 15 μL/mL SC (J-L); 20 μL/mL SC (M-O).

The control group (P<0.01). In addition, at the same concentration, apoptosis increased over time (P<0.01) (Table 1).

Effect of injectable SC on Bcl-2 expression

Bcl-2 expression declined with increasing SC concentration, but the differences in expression rates between the experimental and control groups were not significant (Table 2).

Influence of injectable SC on caspase-8 expression

Higher concentrations of SC were associated with higher expression of caspase-8 at the same time points; and caspase-8 expression was higher in each of the experimental groups than in the control group (P<0.01) (Table 3). Moreover, at the same SC concentrations, the caspase-8 expression increased with time (P<0.01).

Table 1. Apoptosis rate induced by injectable SC in HepG2 cells (mean±SD, %)

<table>
<thead>
<tr>
<th>Groups</th>
<th>12-h</th>
<th>24-h</th>
<th>48-h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.37±0.43</td>
<td>17.05±3.09</td>
<td>18.09±2.06</td>
</tr>
<tr>
<td>5 μL/mL</td>
<td>15.85±1.35*</td>
<td>24.20±2.84*</td>
<td>30.89±1.55*</td>
</tr>
<tr>
<td>10 μL/mL</td>
<td>28.31±1.68*</td>
<td>37.12±2.22*</td>
<td>49.91±2.03*</td>
</tr>
<tr>
<td>15 μL/mL</td>
<td>37.35±3.11*</td>
<td>45.72±1.70*</td>
<td>60.78±3.31*</td>
</tr>
<tr>
<td>20 μL/mL</td>
<td>51.12±3.30*</td>
<td>58.57±4.56*</td>
<td>73.73±5.71*</td>
</tr>
</tbody>
</table>

*: P<0.01, compared to the control group.

Table 2. Effect of injectable SC on Bcl-2 expression (mean±SD, %)

<table>
<thead>
<tr>
<th>Groups</th>
<th>12-h</th>
<th>24-h</th>
<th>48-h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.11±1.86</td>
<td>17.27±2.42</td>
<td>10.33±2.20</td>
</tr>
<tr>
<td>5 μL/mL</td>
<td>25.55±1.73*</td>
<td>17.63±2.24*</td>
<td>9.89±2.55*</td>
</tr>
<tr>
<td>10 μL/mL</td>
<td>26.16±1.48*</td>
<td>17.12±2.36*</td>
<td>9.71±2.06*</td>
</tr>
<tr>
<td>15 μL/mL</td>
<td>24.05±2.81*</td>
<td>16.32±1.51*</td>
<td>9.72±2.45*</td>
</tr>
<tr>
<td>20 μL/mL</td>
<td>24.28±2.32*</td>
<td>16.87±2.54*</td>
<td>9.78±2.71*</td>
</tr>
</tbody>
</table>

*: P>0.05, compared to the control group.

Table 3. Influence of injectable SC on caspase-8 expression (mean±SD, %)

<table>
<thead>
<tr>
<th>Groups</th>
<th>12-h</th>
<th>24-h</th>
<th>48-h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.23±0.87</td>
<td>1.77±0.64</td>
<td>3.17±1.46</td>
</tr>
<tr>
<td>5 μL/mL</td>
<td>9.23±1.42*</td>
<td>13.56±0.98*</td>
<td>15.83±1.42*</td>
</tr>
<tr>
<td>10 μL/mL</td>
<td>19.90±2.01*</td>
<td>26.56±0.68*</td>
<td>30.26±1.41*</td>
</tr>
<tr>
<td>15 μL/mL</td>
<td>26.23±1.88*</td>
<td>29.83±2.22*</td>
<td>32.55±1.27*</td>
</tr>
<tr>
<td>20 μL/mL</td>
<td>29.76±1.57*</td>
<td>33.52±1.87*</td>
<td>35.54±1.62*</td>
</tr>
</tbody>
</table>

*: P<0.01, compared to the control group.

Discussion

Many Chinese herbs, especially injectable herbal preparations, have been confirmed to have anti-tumor effects. However, most reports focus on the clinical results rather than the mechanisms of the effect. It is not well known how Chinese herbs induce apoptosis in tumor cells. This may have affected the credibility of the clinical reports.

Apoptosis has been studied widely. However, the underlying mechanisms have not been completely elucidated. The process of apoptosis has been divided into three phases: a derivative phase, an effector phase and a degradative phase. During the derivative phase, a wide range of processes are induced by a variety of...
signals; in the effector phase, cells die by a sequential and irreversible process. The molecular regulation that determines the destiny of cells to live or die includes the production of a series of protooncogenes and anti-oncogenes.\cite{17}

The anti-apoptotic properties of the protooncogene Bcl-2 are supported by convincing evidence. Many studies showed that elevated expression of Bcl-2 inhibits apoptosis in many cell types, thus inducing cancer. The anti-apoptotic effect of Bcl-2 was first observed in blood lymphocytes and then in many other cell types. Its excessive expression can contribute to the resistance of cells against most cellular toxins. These findings imply that there is a mutual pathway or crosstalk in the signal transduction pathway regulated by Bcl-2. However, in recent years, studies have found that, in addition to this pathway, there are other pathways which are apparently insensitive to Bcl-2. For example, in T-cells, Bcl-2 does not inhibit apoptosis when the pathway is mediated by certain members of the TNF receptor family. In these cases, the apoptosis inducer may have its effect downstream of the Bcl-2 pathway or by apoptosis pathways other than the Bcl-2 pathway.\cite{18,19}

The caspase-3 protein is the key promoter in the apoptosis pathway mediated by the TNF-receptor, and plays an important role in both the pathways mediated by the receptor and by mitochondria, and in the crosstalk between the two pathways. Caspase-8 can switch on the caspase cascade reaction when activated. When caspases 3, 6 and 7 are activated, the functional and structural proteins degrade, and then the cell finally goes through apoptosis.\cite{20,21}

Our study demonstrated a significant apoptotic effect of injectable SC on HepG2 cells.\cite{7} The expression of caspase-8 was enhanced with rising SC concentration (P<0.01). Therefore, caspase-8 was one of the apoptosis-inducing effects of SC in HepG2 cells. Caspase-8 is a downstream molecule in the cellular pathway of FAS for transmitting death signals.\cite{21} Our previous studies showed that treatment of HepG2 cells with SC caused the upregulation of Fas and FasL mRNA.\cite{23} So the enhanced expression of caspase-8 may be the result of FAS expression.

On the other hand, no statistically significant difference was found in Bcl-2 expression at different SC concentrations. This may be due to the complexity of the apoptosis pathways in HCC cells. Wu et al\cite{7} showed that the expression of Bcl-2 protein in pancreatic cancer cells decreases after 72 hours with application of 20 μL/mL SC, indicating that SC induces apoptosis by down-regulating the expression of Bcl-2 genes. However, in HL-60 cells, there was no significant change in the gene expression of Bcl-2 after 24 hours of treatment with 10 μL/mL SC.

The data raise some questions as to whether the apoptosis pathway of HepG2 cells is free of Bcl-2 mediation and whether the potential target of SC lies downstream of the Bcl-2 pathway. In addition, other studies have reported that the expression rate of Bcl-2 is 22.73% in HCC tissues—no different from surrounding normal tissues. Perhaps that implies that Bcl-2 has no influence on the occurrence or development of HCC. These problems need further study.

In conclusion, injectable SC induced apoptosis of HepG2 cells in a concentration- and time-dependent manner, and the expression of caspase-8 was elevated and prolonged. However, SC did not influence the expression of Bcl-2. Thus injectable SC may induce apoptosis of HCC cells by regulating the expression of caspase-8.

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Ethical approval: Not needed.

Contributors: JZX proposed the study. ZBY wrote the first draft. JZX analyzed the data. All authors contributed to the design and interpretation of the study and to further drafts. LY is the guarantor.

Competing interest: No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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