Pathologic characteristics of immunologic injury in primary cultured rat hepatocytes and protective effect of glycyrrhizin in vitro

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ABSTRACT

AIM: To explore the pathologic characteristics of immunologic injury in primary cultured rat hepatocytes, and to evaluate the protective effect of glycyrrhizin (Grz) in vitro. METHODS: Sprague-Dawley rats were initiated with BCG vaccine in vivo, and then the primary cultured rat hepatocytes were stimulated with lipopolysaccharides (LPS) 10 mg/L in vitro (BCG+LPS treatment), the hepatoprotection by Grz was evaluated in the hepatocytes treated as above. Supernatant AST and LDH activities were measured, and nitric oxide (NO) was evaluated by Giress reaction. Aminoguanidine was used to confirm the target of Grz action. Expression of intercellular adhesion molecular-1 (ICAM-1) was determined by immunocytochemistry. Percentage of apoptosis was detected with fluorescence microscope and flow cytometer. RESULTS: In LPS-treated group, supernatant AST was not increased compared with that in control, while supernatant AST and LDH in BCG+LPS treatment were increased significantly (P<0.05). The enhancements of AST and LDH were inhibited by co-culture with Grz (P<0.05). Both supernatant NO and ICAM-1 expression, and percentage of apoptosis in hepatocytes were elevated by BCG+LPS treatment (P<0.01), and these elevations could be decreased by Grz co-incubation either (P<0.05). The NO generation could be decreased by aminoguanidine treatment (P<0.05). CONCLUSION: The elevations of supernatant NO, ICAM-1 expression, and apoptosis in hepatocytes could be taken as three important pathologic changes in the immunologic hepatotoxicity induced by BCG+LPS treatment in primary cultured rat hepatocytes. NO synthesis mechanism was involved in the immunologic hepatotoxic process. Grz could downregulate both supernatant NO and hepatocyte apoptosis in the culture system.

INTRODUCTION

The immunologic hepatotoxicity of primary cultured rat hepatocytes could be induced by BCG vaccine combined with lipopolysaccharides (LPS) (BCG+LPS treatment) in vitro. The pathogenesis of this type...
(NO)\(^{3,4}\). The cellular adhesion mediated by intercellular adhesion molecule-1 (ICAM-1) was necessary for the cytotoxicity of T lymphocytes\(^{5}\). In physiological situations, there was little expression of ICAM-1 in hepatocytes, while in acute hepatitis or the active phase of chronic hepatitis, the expression of ICAM-1 on hepatocytes increased obviously\(^{6}\). Recently, apoptosis was found to be closely associated with the pathogenesis of liver diseases\(^{7}\). The aim of present study therefore was to explore whether the supernatant NO, ICAM-1, and apoptosis in hepatocytes were involved in the immunologic hepatotoxicity induced by BCG in vivo combined with LPS in vitro, and to evaluate the hepatoprotection of glycyrrhizin (Grz) against the cytotoxicity described above.

**MATERIALS AND METHODS**

**Animals** Sprague-Dawley rats (male, 200±20 g, Grade II, Certificate No 2001001, Experimental Animal Center, Zhejiang Province) were fed with a standard laboratory diet and housed in an air-conditioned room, 55 %±5 % humidity, and 12-h light/12-h dark, with water provided.

**Reagents and drugs** Collagenase (type IV) and LPS (E. coli 0111:B4) were purchased from Sigma Chemical Co. Modified eagle’s medium (MEM) was obtained from Gibco BRL. Naphthylethylenediamine dihydrochloride and acridine orange (AO) were the products of Fluka Co. Glycyrrhizin (Grz), purchased from Zhengda Tianqing Co and aminoguanidine, the product of Sigma, were dissolved in MEM. AST kit and LDH Reagent Co.

**Preparation of hepatocytes** Hepatocytes were harvested from rats (treated with or without BCG) using a two-step collagenase perfusion technique\(^{8,9}\). Hepatocytes, viability more than 95 % were used routinely.

**Induction of immunologic rat hepatotoxicity** Hepatocytes were incubated in 24-well plate or dish at a density of 2.5x10\(^5\) cells per well or 5x10\(^6\) cells per dish under the condition of 95 % O\(_2\) with 5 % CO\(_2\). After 16 h, the plating medium was replaced by the fresh dexamethasone-free medium and then treated with LPS 10 mg/L to cause the injury of hepatocytes pretreated with BCG in vivo. Simultaneously, Grz 6.25, 12.5, 25 mg/L and aminoguanidine 185, 370, 740 mg/L were co-incubated with hepatocytes, respectively. After 3, 6, 12, and 24 h, supernatant was collected to measure the biochemical index, and cultured hepatocytes were washed twice for ICAM-1 expression and apoptosis analysis.

**Determination of supernatant AST and LDH activities** The activities of AST and LDH in supernatant were measured spectrophotometrically using a Beckman 700 autoanalyzer with rate mode.

**Measurement of supernatant NO production** Supernatant NO was measured by Griess reaction\(^{10,11}\). The nitrite concentration was determined using a curve calibrated on sodium nitrite standard.

**Evaluation of expression of ICAM-1** The expression of ICAM-1 on hepatocytes was evaluated by immunocytochemistry (ICC). Positive cells were stained brown and negative ones were not stained. The degree of immunoreactions was graded as follows: no positive cells (-), the number of positive cells <25 (+), 25 % - 49 % (++), 50 % - 75 % (+++), >75 % (++++).

**Quantitation of apoptosis with AO fluorescence staining** Cultured hepatocytes were washed with PBS twice and gently mixed with 50 µL AO dye solution (0.01 %). After 10 min, hepatocytes were examined with fluorescence microscope. Condensed and fragmented nuclei, typical morphologic changes of apoptosis, were easily distinguished from intact nuclei and percentages were calculated by counting. Three to five randomly chosen fields of view in each well from 3 different wells in each group were observed after exposure to the conditions indicated, with a minimum number of 300 cells scored in each condition\(^{12}\).

**Analysis of apoptosis with flow cytometer** Hepatocytes were fixed in 75 % ice-cold methanol at 4 °C for more than 24 h until analysis. Thirty minutes before analysis, the fixed hepatocytes were resuspended in PBS and filtered with 42-µm mesh. The cell density was regulated to 1x10\(^5\)/L and the hepatocytes were stained with ethidium bromide 50 mg/L and incubated at 4 °C in dark for more than 30 min. The percentages of apoptosis (with sub-diploid DNA staining) were analyzed with the Partec flow cytometer\(^{13}\).

**Statistical analysis** Data were presented as means±SD and statistically assessed by ANOVA followed by LSD-test for multiple comparisons. Single comparisons were evaluated by Dunnett’s t test. Statistical analysis was conducted with SPSS statistical software. P<0.05 was considered as statistically significant.

**RESULTS**

**Activities of supernatant AST and LDH** In...
control group, supernatant AST and LDH activities had no significant change in 24 h. Compared to that in control group, supernatant AST activity of LPS-treated hepatocytes was not elevated in 48 h ($P>0.05$) (Fig 1). In BCG+LPS treatment group, supernatant AST and LDH activities were elevated at 3, 6, 12, and 24 h, respectively, in a time-dependent manner ($P<0.01$), and with the maximum values at 12 h. The enhancements of supernatant AST and LDH activities, induced by BCG+LPS treatment, were all prevented by Grz 6.25, 12.5, and 25 mg/L at 3, 6, 12, and 24 h, respectively ($P<0.05$) (Fig 2).

Production of supernatant NO In control group, supernatant NO production could hardly be detected in 24 h, all the values were less than 1 µmol/L, while in BCG+LPS treatment group, supernatant NO produc-

![Fig 1. Time-course of supernatant AST activity in control group (normal primary cultured rat hepatocytes) and LPS-treated group (LPS-treated primary cultured rat hepatocytes). $n=6$ cells from 6 rats, respectively. Mean±SD.](image)

![Fig 2. Effects of glycyrrhizin (Grz) on supernatant AST and LDH activities in immunologic hepatotoxicity of BCG+LPS on primary cultured rat hepatocytes. $n=6$ cells from 6 rats, respectively. Mean±SD. $bP<0.05$, $cP<0.01$ vs BCG+LPS group. $dP<0.01$ vs control group.](image)

Tab 1. Time-course of BCG+LPS on supernatant NO and percentage of apoptosis. $n=6$ cells from 6 rats, respectively. Mean±SD. Dunnett’s $t$ test, $P<0.01$ vs control group.

<table>
<thead>
<tr>
<th>Time/h</th>
<th>NO/µmol·L⁻¹</th>
<th>Percentage of apoptosis/%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>BCG+LPS</td>
</tr>
<tr>
<td>3</td>
<td>&lt;1</td>
<td>3.2±1.0</td>
</tr>
<tr>
<td>6</td>
<td>&lt;1</td>
<td>3.3±0.7</td>
</tr>
<tr>
<td>12</td>
<td>&lt;1</td>
<td>3.6±0.8</td>
</tr>
<tr>
<td>24</td>
<td>&lt;1</td>
<td>3.9±0.7</td>
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treatment, was prevented by Grz within 24 h ($P<0.05$).

In the presence of aminoguanidine, the NO generation during all the courses was decreased significantly ($P<0.05$) (Fig 3).

Expression of ICAM-1 There was no or a little expression of ICAM-1 in the control hepatocytes without BCG+LPS treatment (degree: -), while the degree of ICAM-1 expression in hepatocytes was reinforced to ++++ by BCG in vivo. In vitro treatment with LPS 10 mg/L enhanced the degree of ICAM-1 expression to ++++ with or without BCG in vivo, and increased the positive rate of ICAM-1 expression to more than 90% (Tab 2), and enhanced obviously the number of stained cells at 3, 6, 12, and 24 h under the microscope (Fig 4).

Percentage of hepatocyte apoptosis Morphologic changes of hepatocyte were investigated with AO staining. DNA in nuclei of fixed hepatocyte appears yellow-green (450-490 nm filter). In control group, only a few apoptotic cells were detected at 3, 6, 12, and 24 h, while the cell numbers with apoptosis were increased in BCG+LPS treatment group at the same time
In all Grz groups, the rate of apoptosis induced by BCG+LPS treatment was lowered at 12 h ($P<0.01$) (Fig 5, 6).

DNA content of hepatocytes at 12 h was analyzed by flow cytometry and expressed in DNA histogram of FL3-channel. In control group, only a few apoptotic
cells could be detected. In BCG+LPS group, the apoptotic cells were increased \((P<0.01)\). This increase could be prevented by Grz 6.25, 12.5, and 25 mg/L \((P<0.01)\) (Fig 5, 7).

**DISCUSSION**

Little NO was generated in control hepatocytes, while a large amount of NO was released from hepatocytes treated by BCG in vivo combined with LPS in vitro. Grz, a clinically used hepatoprotective agent with membrane-stabilizing activity, could inhibit the NO generation induced by such a treatment. At the presence of aminoguanidine, an effective and specific inhibitor of iNOS\(^{[14]}\), the NO production was decreased remarkably. The results showed that the release of NO from hepatocytes was mainly due to high expression of iNOS. NO synthesis mechanism was involved in the immunologic hepatotoxic process, and the amount of NO production could be one of the pathologic changes of BCG+LPS-induced immunologic cytotoxicity-like model using primary rat hepatocytes. The hepatoprevention of Grz may owe to inhibiting the pathway of NO generation, such as the down-regulation of iNOS expression.

LPS itself could not increase the supernatant AST activity but could induce the expression of ICAM-1 in hepatocytes, which could be enhanced after BCG treatment in vivo. This result confirmed that LPS could not induce the cytotoxicity of hepatocytes, and BCG treat-
ment in vivo followed by LPS exposure in vitro could induce immunologic cytotoxicity that related to the BCG-mediated immune reaction. The high expression of ICAM-1 mainly mediated by LPS could be taken as one of the pathologic changes of (BCG+LPS)-induced immunologic cytotoxicity-like model using primary rat hepatocytes. Because over-expression of ICAM-1 will increase the cytotoxicity of cytotoxic T-lymphocyte and induce the liver injury[15], the down-regulation of ICAM-1 on hepatocytes by kinds of ways, such as interrupting the pathway of LPS-mediated high expression of ICAM-1, was expected to be one of the therapeutic strategies for hepatitis.

In this study the percentage of apoptotic cell increased significantly under the treatment of BCG combined with LPS, while this increase could be prevented by Grz at 12 h. A large quantity of hepatocyte apoptosis could be taken as another important pathologic change of (BCG+LPS)-induced immunologic cytotoxicity-like model using primary cultured rat hepatocytes. There might be new drug targets of liver-protective drugs in the regulation of apoptosis.

Grz could not inhibit the TNF-α-dependent hepatocyte apoptosis in mice[16], but in this research, Grz indeed decreased the hepatocyte apoptosis induced by BCG combined with LPS. It suggested that Grz had selective inhibition of apoptosis induced by immunologic cytotoxicity.
REFERENCES