Pathologic characteristics of immunologic injury in primary cultured rat hepatocytes and protective effect of glycyrrhizin *in vitro*[1]

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KEY WORDS rats; hepatocytes; lipopolysaccharides; nitric oxide; apoptosis; glycyrrhizin

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 aminoguarnidine 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*[1]. The pathogenesis of this type of hepatocyte injury may be related to the recruit and sensitizing of monocytes or macrophages into the rat liver after priming with immune enhancing factor such as BCG *in vivo*. The monocytes or macrophages could be sensitized when they were challenged with LPS released soluble factors including free radicals, lerkotrienes, TNF, interleukin-1, interleukin-6, protease, *etc*, but the accurate mechanism of this kind of injury was not elucidated so far[2]. In acute hepatitis, the expression of inducible nitric-oxide synthase (iNOS) on hepatocytes was increased drastically, which catalyze the *L*-arginine to generate a large quantity of nitric oxide

[1] Project supported by the National Natural Science Foundation of China, No 30070904.
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Received 2002-08-12 Accepted 2003-03-11

(NO)[3,4]. The cellular adhesion mediated by intercellular adhesion molecular-1 (ICAM-1) was necessary for the cytotoxicity of T lymphocyte[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 kit were the products of Ningbo Cicheng Biochemical 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 2.5×10^5 cells per well or 5×10^6 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 1×10^9/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 mean±SD and statistically assessed by ANOVA followed by LSD-test for multiple comparisons. Single comparisons were evaluated by Dunnett's *t* test. Statistic 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 production was elevated to more than 3 μmol/L at 3, 6, 12, and 24 h , respectively, in a time-dependent manner, and the value was maximum at 12 h (Tab 1). The supernatant NO generation, induced by BCG+LPS 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).

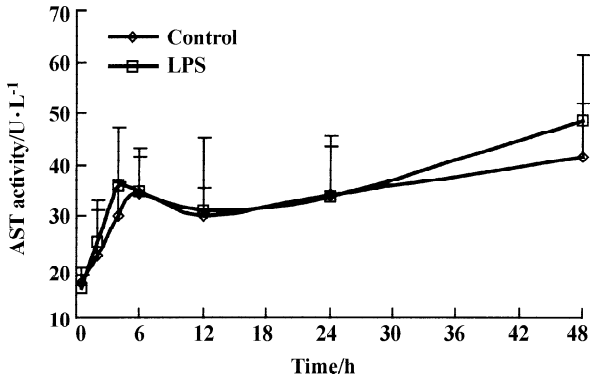

Fig 1. Time-course of supernatant AST activity in control group (normal primary cultured rat hepatocytes) and LPS-treated group (LPS-treated primary cultured rat hepatocytes). n=6 cells from 6 rats, respectively. Mean±SD.

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, ^cP<0.01 *vs* control group.

Time/h	NO/μmol·L^{-1}		Percentage of apoptosis/%	
	Control	BCG+LPS	Control	BCG+LPS
3	<1	3.2±1.0	9.2±1.3	27.1±0.4^c
6	<1	3.3±0.7	8.4±0.8	27.4±0.9^c
12	<1	3.6±0.8	9.4±0.6	30.9±0.4^c
24	<1	3.9±0.7	9.3±0.5	30.6±1.0^c

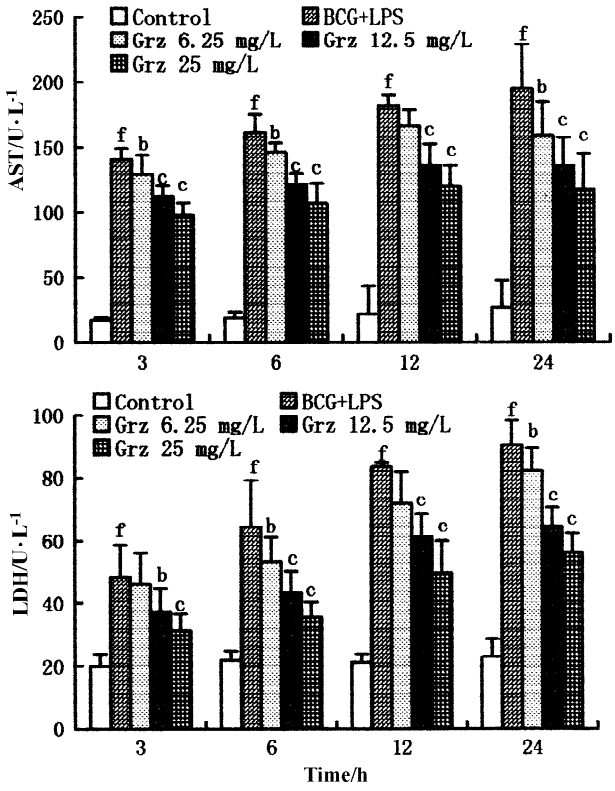

Fig 2. Effects of glycyrrhizin (Grz) on supernatant AST and LDH activities in immunologic hepatotoxicity of BCG+LPS on primary cultured rat hepatocytes. n=6 cells from 6 rats, respectively. Mean± SD. ^bP<0.05, ^cP<0.01 *vs* BCG+LPS group. ^fP<0.01 *vs* control group.

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

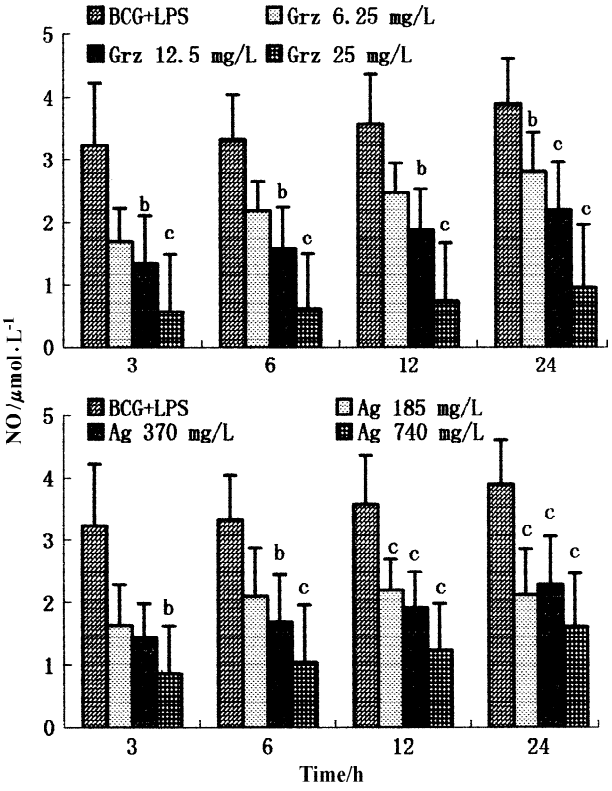

Fig 3. Effects of Grz and aminoguanidine on supernatant NO production in immunologic hepatotoxicity of BCG+LPS on primary cultured rat hepatocytes. *n*=6 cells from 6 rats, respectively. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* BCG+LPS group.

($P<0.01$) (Tab 1). In all Grz groups, the rate of apoptosis induced by BCG+LPS treatment was lowered at 12 h ($P<0.01$) (Fig 5, 6).

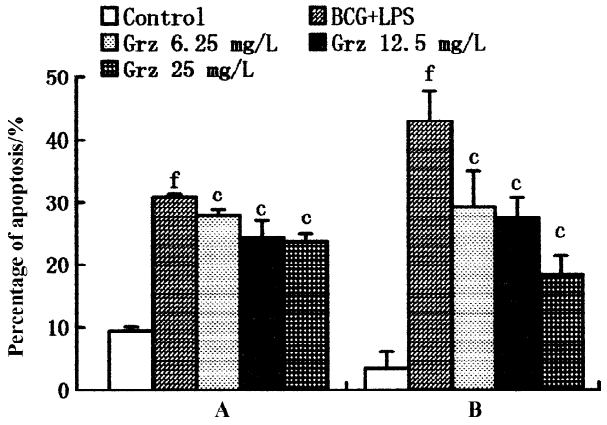

Fig 5. Effects of Grz on hepatocyte apoptosis in immunologic hepatotoxicity of BCG+LPS on primary cultured rat hepatocytes. Apoptosis was measured by (A) AO staining (*n*=6 cells from 6 rats, respectively) and (B) flow cytometry assay (*n*=4-6 cells from 4-6 rats, respectively). Mean± SD. $^{c}P<0.01$ *vs* BCG+LPS group. $^{f}P<0.01$ *vs* control group.

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

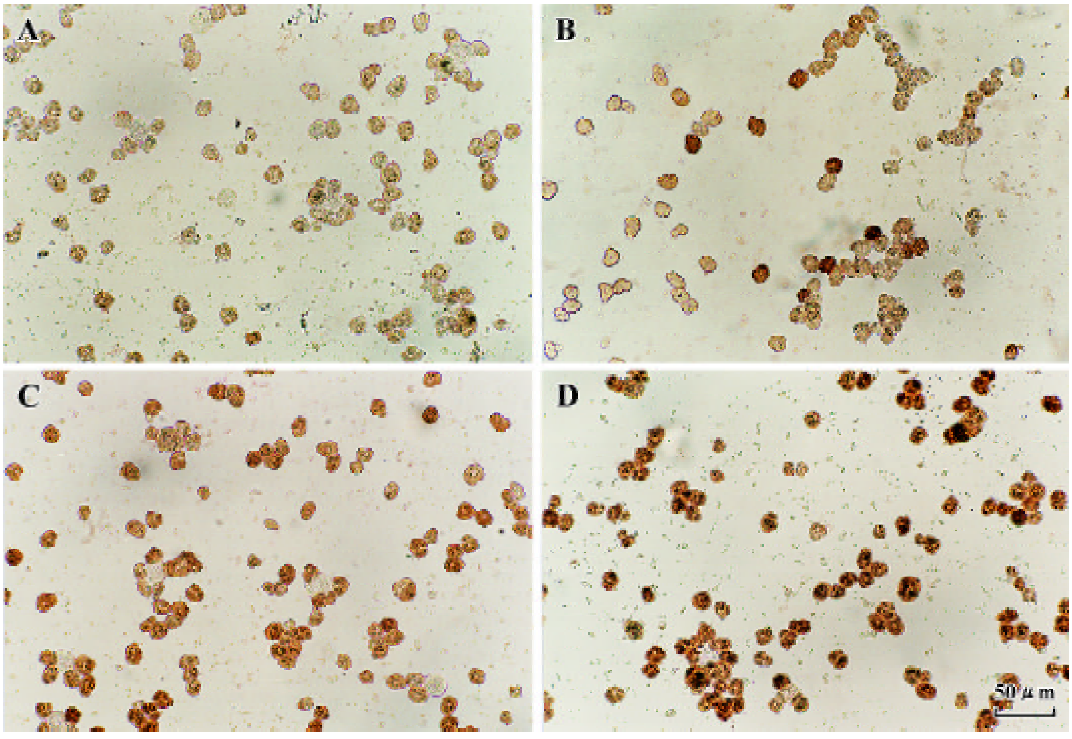

Fig 4. Expression of ICAM-1 in primary cultured rat hepatocytes at 12 h by immunocytochemistry assay. A: Control group; B: BCG group; C: LPS group; D: BCG+LPS group. SABC stain, ×200.

Tab 2. Effects of BCG and/or LPS on expression of ICAM-1 in primary rat hepatocytes (Stain degree: "-" No stain, "+"stain < 25 %, "++" stain 25 %-49 %, "+++" stain 50 %-75 %, "++++" stain > 75 %). *n*=6 cells from 6 rats, respectively. Dunnett's test, $^cP < 0.01$ *vs* control group.

Time/h	Degree of ICAM-1 expression				Positive rate of ICAM-1 expression/%			
	Control	BCG	LPS	BCG+LPS	Control	BCG	LPS	BCG+LPS
3	-	+	++++	++++	0.28±0.28	22.45±1.90	79.56±7.01	85.30±9.86
6	-	+	++++	++++	0.48±0.37	22.82±3.08^c	87.10±3.22^c	88.91±9.21^c
12	-	+	++++	++++	0.71±0.32	23.41±3.76^c	91.95±3.84^c	93.65±4.28^c
24	-	++	++++	++++	0.63±0.40	25.35±4.71^c	92.05±3.43^c	95.05±3.66^c

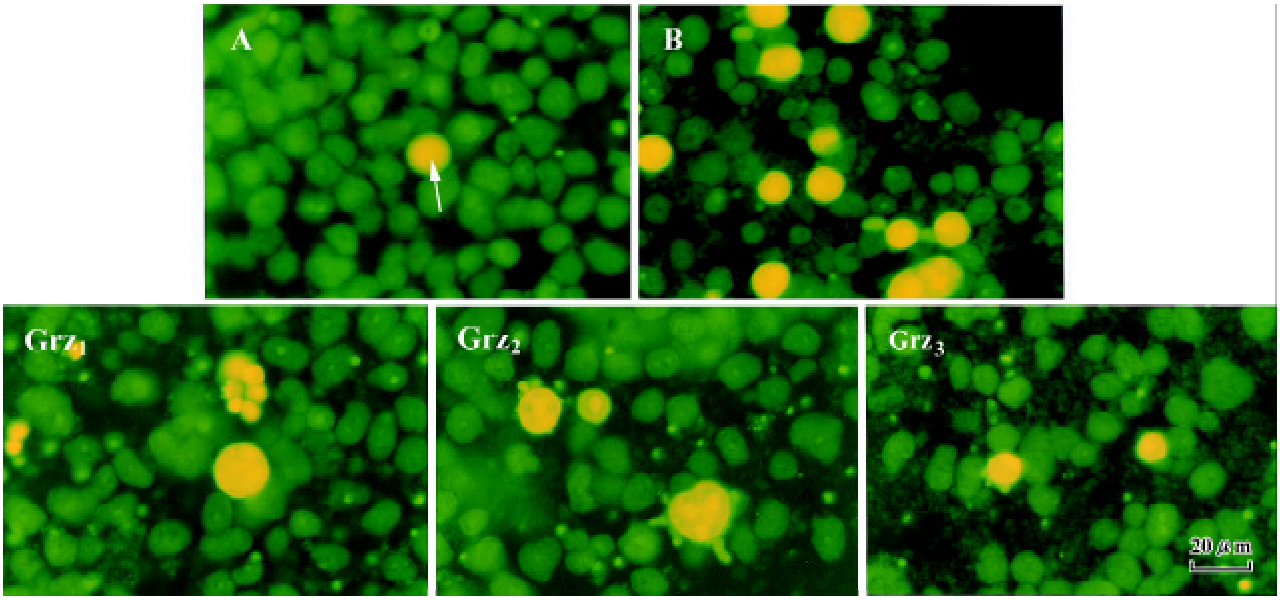

Fig 6. Fluorescence micrographs of hepatocytes with acridine orange staining. A: Control group; B: BCG+LPS group; Grz_1, Grz_2, and Grz_3: Grz 6.25, 12.5, and 25 mg/L groups. Nuclei of control hepatocytes were dyed with light, even, and yellow-green fluorescence; Nuclei of apoptotic cells (-) were dyed with thick, strong, and yellow-green fluoresscence. ×500.

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-

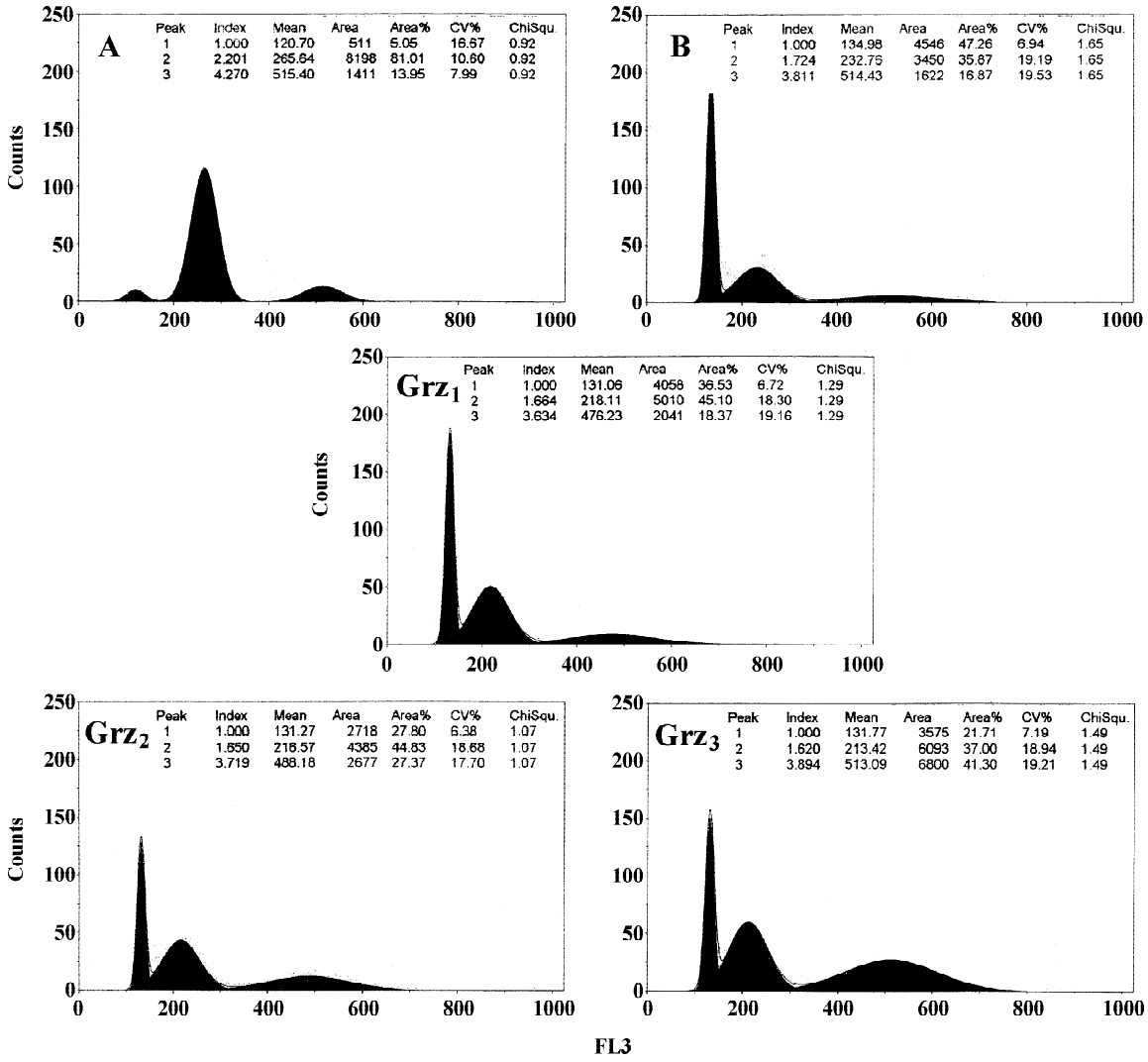

Fig 7. Analysis of hepatocyte at 12 h by flow cytometry. A: Control group; B: BCG+LPS group; Grz_1, Grz_2, and Grz_3: 6.25, 12.5, and 25 mg/L Grz groups. Hepatocytes were stained with ethidium bromide and analyzed by Partec flow cytometry analytical software. In the DNA histogram of FL3-channel, a typical sub-G_1 peak before the G_1/G_0 peak represents the apoptosis peak.

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.

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