Studies on Hepatocyte Apoptosis, Proliferation and Oncogene c-fos Expression in Carbon Tetrachloride-induced Cirrhotic Rat Liver

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Summary: To investigate the significance of hepatocyte apoptosis, proliferation and oncogene c-fos expression in carbon tetrachloride (CCl₄)-induced cirrhotic rat liver. Rat cirrhosis was induced by subcutaneous injection of 50 % (v/v 1 : 1) CCl₄. Hepatocyte apoptosis, proliferation and oncogene c-fos expression were examined with TUNEL, PCNA and c-fos immunohistochemical methods in control group and treatment group 72 h, 5, 7, 11 and 15 weeks after CCl₄ induction. Hepatocyte apoptosis was rarely seen in control rat liver. The hepatocyte apoptosis was obviously increased 72 h after treatment. Fifteen weeks after treatment, the apoptosis was still more obvious in treatment group than that in controls. PCNA was constantly expressed in CCl₄ group, with highest level at middle phase. C-fos was positive 7 and 11 weeks after CCl₄ treatment. The results suggest that: 1) apoptosis is involved in rat liver damage at the early phase in CCl₄-induced injury, and the process can alleviate nodule reconstruction or eradicate potentially mutational hepatocytes at the later phase; 2) hepatocytes constantly proliferate in CCl₄-induced rat liver cirrhosis, especially at the middle phase; 3) c-fos might modulate hepatocyte proliferation in CCl₄-induced rat liver cirrhosis.

Key words: liver cirrhosis;  apoptosis; cell proliferation; TUNEL; oncogene

Apoptosis has been intensively studied in hepatobiliary diseases. However, data about the relationship between liver cirrhosis and apoptosis is skimpy. This experiment was to investigate the roles of hepatocyte apoptosis, proliferation in liver cirrhosis and their relationship with oncogene c-fos by studying the changes of hepatocyte apoptosis, proliferation in different rat cirrhotic phases.

1 MATERIALS AND METHODS

The methods reported by Wu[4] was used to induce rat liver cirrhosis. Briefly, 2 weeks of oral feeding of 0.05 % phenobarbital, 50 rats weighing about 200 g each were subcutaneously injected with 50 % CCl₄ (CCl₄ : cotton oil = 1 : 1) 2 ml/kg every 4 days, with food and water ad libitum. Fifty rats without receiving CCl₄ served as controls. Seventy-two h, 5 weeks, 7 weeks, 11 weeks and 15 weeks after first CCl₄ injection, 10 experimental (CCl₄-treated) and control rats were intraperitoneally anesthetized with 30 mg/kg barbitol and sacrificed. Livers of the animals were harvested and fixed with 10 % polymethyldehydro for 24—48 h. Livers were cut into 5 μm slices and subjected to HE, TUNEL staining, PCNA and c-fos immunohistochemical procedures. TUNEL kit were procured from B.M. Co. and anti-PCNA, anti-c-fos monoclonal antibodies were obtained from sigma Co.

1.1 HE Staining
Conventional technique was used for HE staining.

1.2 TUNEL Procedure
After digestion with proteinase K (1:100) 50 μl for 15 min at 37 °C, each specimen was incubated with TdT-Digoxin-dUTP-buffer 10 μl for 1 h (37 °C). They were allowed to react with anti-Digoxin-AP 50 μl at 37 °C for 30 min. Each specimen was labeled with fresh BCIP/NBT in darkness for 30 min.

1.3 PCNA and c-fos Immunohistochemical Staining
After incubation with 3 % H₂O₂ for 5 min and heating at 96 to 100 °C with microwave for 15 min, each specimen was co-incubated with 20 μl monoclonal anti-PCNA or anti-c-fos antibody overnight at 4 °C and
subsequently exposed to ABC 20 μl for 20 min at 37 ℃. Then, each specimen was stained with 0.5 mg/ml fresh 3, 3-diaminobenzidine tetrachloride (DAB) in darkness for 5 min.

1.4 Data Analysis

Data were analyzed with Chi square analysis and a P less than 0.05 was considered statistically significant.

2 RESULTS

2.1 HE Staining

Nodules of rat livers in controls were morphologically intact and clear, without necrosis or degeneration. 72 h after CCl₄ injection, piecemeal or bridge necrosis was observed in the livers of the experimental rats. Fibrogenesis and incomplete pseudonodules were seen in rat livers from the 5th week to 11th week in experimental group. At the 15 week in CCl₄ group, full-fledged pseudonodules were found in rat livers.

2.2 Hepatocyte Apoptosis

Findings of hepatocyte apoptosis in control and experimental groups were shown in table 1.

| Table 1 TUNEL positive hepatocytes in control and different phases in CCl₄ group (TUNEL+/HP) |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Control group                  | Experimental group              |
| 72 h                           | 72 h                            | 7 weeks                         | 11 weeks                        | 15 weeks                        |
| 0.84±0.82                      | 7.24±3.17                       | 3.24±1.19                       | 2.76±1.10                       | 1.17±0.87                       |

| Table 2 PCNA positive hepatocytes in control and different phases in experimental group (PCNA+/HP) |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Control group                  | Experimental group              |
| 72 h                           | 5 weeks                         | 7 weeks                         | 11 weeks                        | 15 weeks                        |
| 2.14±1.12                      | 5.64±2.19                       | 13.33±2.28                     | 12.6±73.02                      | 12.28±3.69                     | 6.68±2.84                      |

2.3 Hepatocyte Proliferation

Findings of hepatocyte proliferation in control and experimental groups were shown in table 2.

PCNA positive hepatocytes were rare in the livers of control rats. In the experimental group, PCNA positive hepatocytes were increased significantly, reaching the highest at the 5th week after first CCl₄ injection. Difference between control and experimental groups was significant (P<0.05). Difference among different phases in experimental group was significant (P<0.05).

2.4 C-fos

C-fos was negative in control and it was negative 72 h, 5 weeks and 15 weeks after treatment in the livers of the experimental rats. At the 7th week and 11th week in experimental group, c-fos was positive in nuclei and cytoplasm of liver pseudonodules.

3 DISCUSSION

Apoptosis is one means of cell death induced by activation of endonucleases due to changes in transcription/translation in cells under injurious stimulation. Apoptosis is morphologically defined by chromosome congregation, deep staining, cell fragmentation and breaking up of cell into membrane-bound bodies containing structurally intact, viable organelles, referred to as apoptotic bodies. Apoptosis has been found in normal livers and diseased livers with hepatitis, hepatoma, liver transplantation, liver injury, etc. Hypoxia and many toxicants such as bile salt, alcohol, cyclohexemide, 2-AAF cause hepatocyte apoptosis, with apoptosis level correlating with the dosage and duration of intoxication. Data about hepatocyte apoptosis induced by CCl₄ has

TUNEL positive hepatocytes were scarcely in control rat livers. At 72nd h after CCl₄ injection in experimental group, TUNEL positive hepatocytes were increased maximally, mainly near necrotic areas. At 7th week, 11th week and 15th week in experimental group, TUNEL positive hepatocytes were decreased, but higher than control. Difference between controls and experimental groups was significant (P<0.05), difference among separate phases in experimental group was significant.