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Kupffer cell and apoptosis in experimental HCC

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INTRODUCTION

Our previous study has proved that Kupffer cells may have an inhibitory effect on the process of hepatocarcinogenesis[1], however, their inhibitory mechanism needs exploring deeply. We performed a comparative study on the expression of PCNA, Bax, P53 and apoptosis of liver cancer cells using immunohistochemical technology and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-dig oxigenin nick end labeling (TUNEL) in the diethylnitrosamine-induced hepatocell ular carcinoma (HCC) in rats with or without pretreatment with gadolinium chlori de or zymosan which might effectively block or enhance the activity of Kupffer cells in order to know the role of Kupffer cells in apoptosis in the experimental HCC and explore further the inhibitory mechanism of Kupffer cells on the process of hepatocarcinogenesis.

MATERIALS AND METHODS

Establishment of animal models and pathological examination

One hundred and forty male Sprague-Dawley rats were divided into six groups. ① DENA group, 40 rats received diethylnitrosamine (DENA) at a dose of 70 mg/kg in distilled water once/wk till wk15. ② GC+DENA group, gadolinium chloride was injected iv at a dose of 10 mg/kg once 2 wk to suppress Kupffer cells in 40 rats till wk15 and these rats simultaneous ly rec eived DENA just as DENA group. ③ ZM+DENA group, zymosan was injected iv at a do se of 20 mg/kg once 2 wk to activate Kupffer cells till wk15 and DENA was received just as DENA group. ④ GC group, gadolinium chloride was in jected iv at a dose of 10 mg/kg in 0.85% NaCl once 2 wk till wk 15. ⑤ ZM group, zymosan was injected iv at a dose of 20 mg/kg in 0.85 % NaCl once 2 wk till wk15. ⑥ Control group, these rats were maintai ned on a standard laboratory diet and tap water. All rats were killed at the wk21 of hepatocarcinogenesis. The liver samples were taken, fixed in 40 mL/L paraformaldehyde and embedded in paraffin. Each specimen was cut into 5 μm serial slices, stained with hemotoxylin-eosin and subjected to histopathological examination.

Immunohistochemical staining

The SP method was used, the 1st antibody was mouse-anti-human PCNA monoclona l antibody (Calbiochem Co., dilution 1 : 50), rabbit-anti-human Bax (Santa Cruz, dilution 1 : 50) and mouse-anti-human P53 monoclonal antibody (Novocastra Laboratories, dilution 1 : 50). The SP kit was purchased from Boehringer Mannheim , Germany. DAB staining was used. The dark brown staining of nuclei was taken as PCNA and P53-positive reaction. The dark brown granules in cytoplasm were taken as Bax-positive reaction. We used PBS to replace the 1st antibody as negati ve control.

Terminal deoxynucleotydial transferase-mediated biotinylated-dutp nick end labeling method (TUNEL method)

In situ Cell Apoptosis Detection kit was purchased from Boehringer Mannheim, Germany. After deparaffinization and rehydration in ethanol, the sections were incubated with 0.2 mol/L HCl for 20 min at room temperature (RT). They were then deproteinized by incubation with 30mg/L proteinase (in 50mM Tris-HCl, pH 8.0; 5mM EDTA) for 30 min at RT. Terminal deoxynucleot idyl transferase (TdT) and biotinylated dUTP in TdT buffer (30mM Trizma base, pH 7.2; 140mM sodium cacodylate; 1mM cobalt chloride) were added to cover the sections, which were then incubated for 60 min in a humid atmosphere at 37℃. The sections then reacted with alkaline phasphatase (ALP) for 30 min at RT. Visulization was carried out with NBT/BCIP. Nuclei with clear blue staining w ere regarded as positive. TUNEL incubation solution without terminal deoxynucleo tidyl transferase was used as negative control.

Proliferating index (PI) and apoptosis index (AI)

PI and AI were determined by Leitz ASM 68K image analyzing system purchased from Germany. Five visual fields (orginal magnificantion, 10 × 40)

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from every positive slide would be chosen and the TUNEL and PCNA-positive parenchymal cells as well as the total number of parenchymal cells in the same field would be counted . PI or AI was expressed as the percentage of the number of PCNA or TUNEL-pos itive parenchymal cells in the total parenchymal cells of the same field.

$$\text{Proliferating index (PI)} = \frac{\text{number of PCNA-positive cells}}{\text{number of total cells}} \times 100\%$$

$$\text{Apoptosis index(AI)} = \frac{\text{number of TUNEL-positive cells}}{\text{number of total cells}} \times 100\%$$

Statistical analysis

Fisher's exact test, Student's test and Spearman rank correlation were employe d.

RESULTS

Macroscopic observation and histopathological examination (at wk21 of hepatocarcinogenesis)

Meither the control group, nor the GC group and ZM group showed any changes macro and microscopically; on the contrary, the liver surface in DENA group and GC +DENA group were covered with a lot of white nodules. The diameter of the larges t nodules was 0.5 cm in DENA group and 2.0 cm in GC+DENA group. Thes e nodules were diagnosed as HCC histologically. Some white grey focal nodules sc attered over the liver surface in ZM+DENA group, were also diagnosed as HCC with many apoptotic cells and apoptotic bodies.

Bax staining

Cytoplasm of cancer cells with clear brown staining was regarded as positive (Figure 1). The positive rates of Bax in ZM+DENA group, DENA group and GC+DENA group were 84.6% (11/13), 28.6% (2/7) and 27.3% (3/11) respectively. It was significantly higher in ZM+DENA group than that in DENA group (Fisher's exact test, $P<0.05$).

P53 staining

A clear brown staining of the nuclei in cancer cells was regarded as positive (Figure 2). The positive rates of P53 in ZM+DENA group, DENA group and GC+DENA group were 76.9% (10/13), 14.3% (1/7) and 36.4% (4/11) respectively. It was markedly higher in ZM+DENA group than that in DENA group (Fisher's exact test , $P<0.05$).

PCNA staining

Nuclei with clear brown staining were regarded as positive (Figure 3).

No expressions of Bax, P53 and PCNA in the control group, GC group and ZM group were found.

Apoptosis of cancer cells using TUNEL method

Apoptotic cells were observed in DENA group, GC+DENA group and ZM+DENA group by means of TUNEL method. The cells with clear nuclear labeling were defined as TUNEL-positive cells. Apoptotic cells were scattered in the cancer tissue (Figure 4).

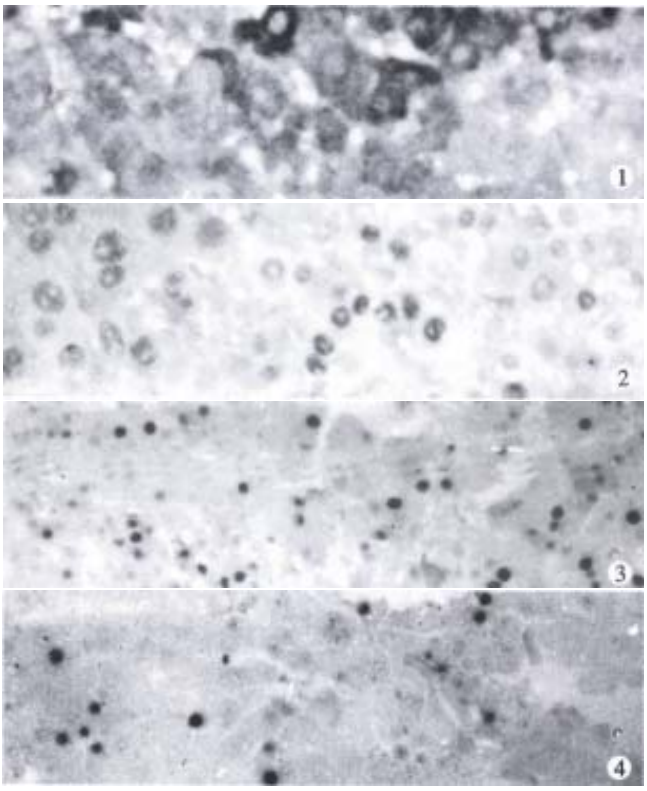

Figure 1 Expression of Bax in rat HCC in ZM+DENA group. SP × 400

Figure 2 Expression of P53 in rat HCC in DENA group. SP × 400

Figure 3 Expression of PCNA in rat HCC in DENA group. SP × 200

Figure 4 Apoptotic cells in rat HCC in ZM+DENA group. TUNEL × 200

Proliferating index (PI) and apoptosis index (AI) (Table 1)

Table 1 PI and AI in ZM+DENA group, DENA group and GC+DENA group

Groups	Number	PI(%, $x \pm s$)	AI(%, $x \pm s$)	PI/AI
ZM+DENA group	13	15.22 ± 2.17[b]	7.53 ± 1.61[b]	2.20
DENA group	7	24.97 ± 2.53	4.36 ± 1.18	5.73
GC+DENA group	11	38.69 ± 3.17[b]	2.52 ± 0.81[b]	15.40

[b]$P<0.01$, *vs* DENA group, Fisher's exact test.

Correlation between apoptosis index, Bax and P53 expression

There was a positive correlation between apoptosis index and Bax or P53 protein reactivity in ZM+DENA group, DENA group and GC+DENA group (Table 2).

Table 2 Correlation between apoptosis index and Bax or P53 expression

Groups	AI *vs* Bax			AI *vs* P53		
	Bax-positive cases / total	r_s	P	P53-positive cases / total	r_s	P
ZM+DENA group	11 / 13	0.63	<0.05	10 / 13	0.73	<0.05
DENA group	2 / 7	0.79	<0.05	1 / 7	0.61	
GC+DENA group	3 / 11	0.74	<0.01	4 / 11	0.82	<0.05

Spearman rank correlation.

DISCUSSION

Gadolinium chloride is believed to be a specific suppressor of Kupffer cells[1,2]. Zymosan is an immunopotentiator and may be used to activate Kupffer cells[3]. We performed a comparative study on apoptosis in DENA-induced hepatocellular carcinoma in rats with or without pretreatment with gadolinium ch loride or zymosan which might effectively block or enhance the activity of Kupffer cells in order to clarify whether the Kupffer cells play arole in apoptosis of the experimental hepatocarcinogenesis or not.

Proliferating cell nuclear antigen (PCNA) is an auxiliary protein of DNA polymerize-delta. It accumulates little in the resting stage cell, but prominently in the nuclei of proliferating cells during G_1-late phase and S-phase and decreases in G_2-phase and M-phase. It is associated with cell proliferation an d regarded as a biological marker for cell proliferation[4]. Bax is a M_r21 000 protein with extensive amino acid homology with Bcl-2. This protein has been shown to form heterodimers with apoptosis inhibiting protein, B cl_2, so it can induce cell apoptosis[5]. When DNA is injured by toxica nt, wild-type p53 drives cell to G_1 and arrests or inhibits cell prolifer ation until DNA is repaired. When nuclear DNA is badly damaged or cannot be rep aired, wild-type p53 induces transcription of apoptotic genes and drives the cell to be in apoptosis[6]. Gottlieb *et al*[7] thought that overexpression of P53 was related to cell apoptosis. Zhao *et al*[8] observed that there w as a positive correlation between apoptosis index and P53 protein, and supported the role of P53 in regulating apoptosis in human HCC. The positive correlation between apoptosis index and P53 protein immunoreactivity observed in our study also supports these findings. A new method to detect apoptosis *in situ*, terminal deoxynucleotidyl transferase-mediated (TdT) dUTP-digoxigenin nick end labeling (TUNEL) was recently developed by Gavrieli *et al*[9], and was applied to detect cell apoptosis of human HCC and other tumors[10].

Our results showed that the positive rates of Bax and P53 protein were markedly higher in ZM + DENA group than in DENA group with significant differences. Proliferating index and apoptosis index respectively increased or decreased in ZM+DENA group, DENA group and GC+DENA group successively. These results demonstrated that the blockage of Kupffer cells with gadolinium chloride might suppress cell apoptosis and the activation of Kupffer cells with zymosan might promote cell apoptosis in the experimental hepatocarcinogenesis.

One of the characteristic features of cancer is the continuous growth and the ratio of cell proliferation and cell apoptosis determine the fate of tumor growth[11-13]. In normal tissues, apoptosis is an efficient way of eliminatin g transformed cells. However, inability of cells to undergo apoptosis may advanc e their development, both by allowing the accumulation of dividing cells and by impairing the elimination of genetic mutants that may harbor malignant potential[11,12]. Our results proved that Kupffer cells could promote apoptosis in the experimental hepatocarcinogenesis and revealed further that they played an inhibitory role in hepatocarcinogenesis through inducing apoptosis of tumor cells.

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