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Studies of hepatocytes apoptosis induced by TNF-α and its mechanism

Weiqing Song[1, 2], Weihong Lu[2], Huabo Chen[2], Huahui Li[2], Jianmin Wu[1]

[1] Department of Laboratory Medicine, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China
[2] Qingdao Municipal Hospital, Qingdao 266011, China

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Abstract ***Objective:*** To investigate the apoptosis effect of TNF-α on HepG2 cells and its mechanism *in vitro*. ***Methods:*** HepG2 cells were treated with TNF-α (400 U/mL). HepG2 cells treated with TNF-α for 24 h and apoptosis was proved by DNA fragments on gel electrophoresis. Fluorescent quantitative real-time PCR was used to detect Fas and FasL expression. HepG2 cells treated by TNF-α were co-cultivated with normal HepG2 cells. Apoptosis of HepG2 cells was determined by the method of FACS. ***Results:*** After 24 h TNF-α treatment, DNA was collapsed into fragments to form DNA ladder in gel electrophoresis; FasL expression increases and induces HepG2 cells apoptosis. By FACS, 98.4% of the co-cultivated cells were apoptosis, but 16.5% cells in control group were apoptosis. ***Conclusion:*** TNF-α can induce apoptosis of HepG2 cells *in vitro*. FasL expression on TNF-α pre-treated HepG2 cells increased and these cells can lead normal HepG2 cells to apoptosis. This may attribute to the cure of virus hepatitis and hepatoma.

Key words TNF-α; apoptosis; Fas/FasL; hepatocyte

TNF-α is one kind of important cytokines, which, *in vivo* is mainly due to the production by monocytes and macrophages stimulated by pathogenic microorganism and toxin. Several studies have shown that TNF-α is in large scale production by hepatocytes and plays a significant role to cause hepatocyte damage in virus hepatitis and hepatoma through the system of Fas/FasL [1]. In the present study, we cultivated HepG2 as a model to estimate the apoptosis effect on hepatocytes and its mechanism by TNF-α.

Materials and methods

Materials

HepG2 cells; TNF-α was provided by Sigma company, USA; DMEM; Anti-Apo2.7 antibodies from Beckman Coulter, USA.

Cell culture and treatment

HepG2 cells were digested and adjusted to 2.5×10^5/mL. (1) 1 mL/cm^2 cell culture was put to 60 mL culture plate for DNA fragments extraction; (2) 2.5×10^4 cell was added to 24-well plates for Fas/FasL and apoptosis detection. Discarding the supernatant after 24 h cell culture and then co-cultivate with TNF-α (400 U/mL) for 12, 24 and 48 h, respectively. One mL HepG2 cells were treated by TNF-α for 24 h and its culture supernatant was added to the cells in step (2).

DNA fragments detection

The cells were washed with PBS; phenol-chloroform method was used to extract DNA; TE was added to dissolve DNA; 1.5% agarose gel electrophoresis (65 V, 90 min).

Fas/FasL expression by Fluorescent quantitative real-time PCR

GAPDH was used as house keeping gene. Total RNA was extracted with RNA isolation kit and reverse transcripted to cDNA. PCR was performed in a 20 μL reaction volume containing 5 × Buffer 2 μL, dNTPs (10 mmol/L) 0.4 μL, sense and anti-sense primers (10 μmol/L) 0.4 μL, Taq DNA polymerase (10^6 U/L) 1.0 μL, BSA (1 mg/mL) 1.0 μL, SYBR Green I 1.0 μL, cDNA 1.0 μL, $MgCl_2$ (25 mmol/L) 3.2 μL. Initial pre-denaturation was at 95 ℃ 7 min and denaturation was at 95 ℃ 7 s. Amplification conditions were listed in Table 1. A novel formula was used to calculate relative gene expression by the method of GED [2]. PCR efficiency and CT was got by LinRegPCR [3] and Light Cycler Software 3.5 respectively.

Correspondence to: Jianmin Wu. Email: wjml186@yahoo.com.cn

Table 1 Primer sequences and PCR conditions

Gene	Primer sequences	Product (bp)	Anneal	Extension	Fluorescence detection
GAPDH	Upstream GAAGGTGAAGGTCGGATGC Downstream GAAGATGGTGATGGGATTTC	226	60 ℃, 5 s	72 ℃, 20 s	88 ℃, 1 s
Fas	Upstream AGCTTGGTCTAGAGTGAAAA Downstream GAGGCAGAATCATGAGATAT	180	51 ℃, 5 s	72 ℃, 10 s	83 ℃, 1 s
FasL	Upstream CACTTTGGGATTCTTTCCAT Downstream GTGAGTTGAGGAGCTACAGA	160	57 ℃, 5 s	72 ℃, 10 s	79 ℃, 1 s

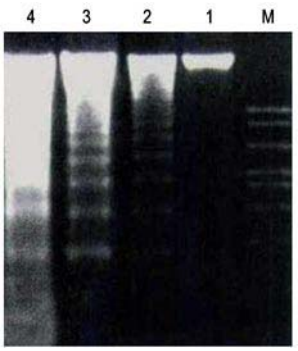

Fig. 1 DNA fragments on agarose gel. M: DNA Marker; 1: HepG2 cells; 2, 3, 4: HepG2 cells treated by TNF-α for 12, 24, 48 h respectively

$$rER=\frac{Rnorm(SOI)}{Rnorm(ref)}=\frac{(1+E(HKG;SOI))^{C_T(HKG;SOI)}}{(1+E(GOI;SOI))^{C_T(GOI;SOI)}}*\frac{(1+E(HKG;ref))^{-C_T(HKG;ref)}}{(1+E(GOI;ref))^{-C_T(GOI;ref)}}$$

HKG: house keeping gene GOI: gene of interest
SOI : sample of interest Ref: reference sample
CT: cycle of threshold

Apo2.7 positive apoptotic cells detection by FACS

Cells were fixed with 4% paraform; 70% methanol to increase cell membrane permeability; labeled with mouse anti-human Apo2.7 McAb and sheep anti-mouse IgG-FITC; FACS detection.

Statistical analysis

All data were expressed as $\bar{\chi} \pm s$. Two-sample t test was performed with SPSS 11.0 for Windows. Statistical significance was defined as $P < 0.05$.

Results

DNA fragments detection

After treatment with TNF-α for 24, 48 h, DNA fragments by agarose gel electrophoresis shew typical DNA ladder (Fig. 1). This proved that HepG2 cells had been apoptosis.

Fas/FasL expression by Fluorescent quantitative real-time PCR

Specificity of PCR products

From Melting curve and PCR results of FasL and Fas, we could see that PCR products were specific (Fig. 2).

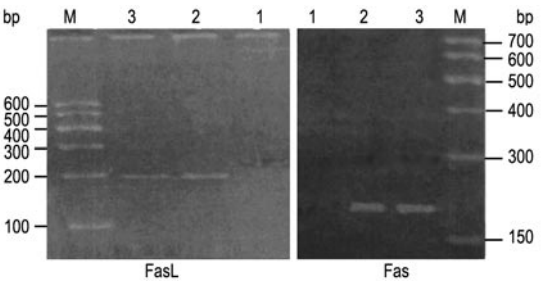

Fig. 2 PCR results of FasL and Fas on agarose gel. 1: blank; 2: HepG2 cells treated by TNF-α for 24 h; 3: HepG2 cells; M: marker

Fas/FasL relative expression

rER of Fas and FasL mRNA was shown in Table 2. There was no difference in Fas expression. However, FasL expression was higher in TNF-α-treated HepG2 cells than normal HepG2 cells, $P < 0.01$.

Apo2.7 positive apoptotic cells detection by FACS

In the co-cultivation with normal HepG2 cells and TNF-α pre-treated HepG2 cells, the amount of Apo2.7 positive apoptotic cells reached about 98.4%, that of control cells was 16.5% and that of TNF-α pre-treated HepG2 cells was 19.6% (Fig. 3). This showed that after co-cultivation, HepG2 cells had been apoptosis.

Discussion

HepG2 cells apoptosis is important in the pathology of virus hepatitis. And apoptosis is associated with severity of hepatitis. TNF-α is one kind of important cytokines. We have thought highly of the function of TNF-α in HBV patients. Hepatitis virus infection activates immune system and many cytokines production increases, such as TNF-α [4]. TNF can induce many pathological changes: activating immune system [5], killing tumor cells and inducing apoptosis in those cells infected by virus [6]. Our study reveals that apoptosis occurs when HepG2 cells were treated with high concentration of TNF-α. DNA fragments prove apoptosis induced by TNF-α. Fluorescent quantitative real-time PCR revealed that FasL expression on TNF-α-pre-treated cells increased and these cells induced other cells apoptosis.

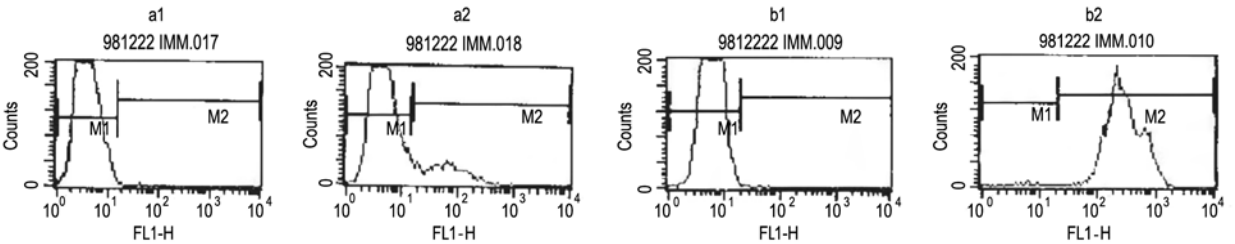

Fig. 3 Apoptosis detection by FACS. (a1) blank; (a2) normal HepG2 cells + culture supernatant (control cells); (b1) TNF-α pre-treated HepG2 cells; (b2) co-cultivated cells

Table 2 Fas/FasL relative expression

Groups	Fas	FasL
HepG2 cells	1.00	1.00
HepG2 cells treated by TNF-α for 24 h	0.96 ± 0.02	1.35 ± 0.02[a]

[a] As to HepG2 cells, $P < 0.01$

So reducing TNF concentration or inhibiting its receptors may contribute to the cure of virus hepatitis. After administration of anti-TNF drug for three months in HCV patients, Marotte reports that HCV RNA and serum transaminase do not increase [7]. However, TNF may induce apoptosis through other pathways and there are many cytokines in virus hepatitis [8]. These cytokines interfere the roles of TNF-α. So, further studies should be done to investigate the role of TNF-α, so as to make advances in the cure of virus hepatitis.

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