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• BRIEF REPORTS •

Role of activation-induced cell death in pathogenesis of patients with chronic hepatitis B

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Abstract

AIM: To study and compare the difference of activation-induced cell death (AICD) in peripheral blood T-lymphocytes (PBL-Ts) from patients with chronic hepatitis B (CHB) and the normal people *in vitro*, and to explore the role of AICD in chronic hepatitis B virus (HBV) infection and the pathogenesis of CHB.

METHODS: Twenty-five patients and fourteen healthy people were selected for isolation of PBL-Ts. During cultivation, anti-CD3 mAb, PMA and ionomycin were used for AICD of PBL-Ts. AICD ratio of PBL-Ts was detected with TdT-mediated dUTP nick end labeling and assessed by flow cytometry.

RESULTS: When induced with anti-CD3, PMA and ionomycin *in vitro*, AICD ratio of PBL-Ts from CHB patients was significantly higher than that from healthy control (17.24±1.21 *vs*. 6.63±1.00, $P<0.01$) and that from CHB patients without induction (17.24±1.21 *vs*. 9.88±1.36, $P<0.01$). There was a similar AICD ratio of PBL-Ts between induction group and without induction group, but no difference was found before and after induction in healthy control. The density of INF-γ in culture media of induction groups of CHB was lower than that of other groups ($P<0.01$). There was no difference between these groups in density of IL-10 ($P>0.05$).

CONCLUSION: When induced during cultivation *in vitro*, PBL-Ts from CHB have AICD very commonly. This phenomenon has a potentially important relation with pathogenesis of CHB and chronicity of HBV infection.

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INTRODUCTION

Currently, the exact pathogenesis of chronic hepatitis B (CHB) and the reason of chronic hepatitis B virus (HBV) infection are still not completely understood. Activation-induced cell death (AICD) is related with lymphocytes decrease and functional defect. This phenomenon can cause decrease of immune clearance. Alloreactive T cells can effectively be depleted from allogeneic T cells by induction of AICD to prevent graft-versus-host disease[1]. AICD is essential for the function, growth and differentiation of T-lymphocytes[2]. This may be an important reason of persistent infection of HBV. AICD in peripheral blood T-lymphocytes (PBL-Ts) of CHB *in vivo* has been approved by some reports, but does AICD occur more easily in PBL-Ts of CHB than in those of healthy control? In order to explore the role of AICD in chronic HBV infection, we studied and contrasted the difference of AICD in PBL-Ts from patients with CHB and from normal people *in vitro*.

MATERIALS AND METHODS

Patients

Twenty-five patients (17 men, 8 women, aged 19-49, mean age 35.6 years) with CHB between March 2000 and April 2001 were selected from the Second Affiliated Hospital of Harbin Medical University. The diagnoses of all the patients were in accord with the Fifth National Conference on the Diagnostic Criteria of Virus Hepatitis (Beijing, 1995). And 14 healthy persons were selected as control.

PBL-Ts isolation

10 mL peripheral blood was taken and heparin was added for anticoagulation. After equivalent Ficoll-Paque (from Amersham-Pharmacia, USA) was gently added, peripheral blood monocytes (PBMCs) were isolated by density gradient centrifugation (600 g, 20 min). Then PBL-Ts were purified with negative selection technique using immune-magnetic beads as follow. After PBMC was washed, mouse-anti-human anti-CD_{14}, anti-CD_{16}, anti-CD_{19} (2 μg· mL^{-1}, DAKO Company, Denmark) were added and incubated for 30 min at 0 ℃, centrifuged for removing the uncombined antibody. Then goat-anti-mouse CD3 mAb coating with magnetic beads (1 cell *vs.* 30 beads, Promega Company, USA) was added and incubated for 30 min at 0 ℃. B cells, NK cells and monocytes were all linked with immune-magnetic beads and absorbed by magnetic stock (DAKO Company, Denmark). After the liquor was gently extracted by centrifugation, PBL-Ts were purified. The viability (95 %) of the cells was confirmed by trypan blue staining. When detected by flow cytometry, the purity of PBL-Ts was over 97 %.

Cultivation and AICD induction of PBL-Ts in vitro

After washed 3 times with PBS, 2×10^6· mL^{-1} PBL-Ts were added to a 24 well plate (NENC Company, USA) for cultivation. The samples were divided into treatment group and control group. The culture medium was RPMI1640 (GIBCO Company, USA) containing 10 % calf blood serum (BANDIN TECH Company, China), penicillin (100 U· mL^{-1}, BANDIN TECH Company, China) and streptomycin (100 U· mL^{-1}, BANDIN TECH Company, China). The wells of treatment group were pre-coated with anti-CD_3 mAb (5 μg· mL^{-1}, DAKO

Company, Denmark). Phorbol 12-myrisate 13-acetate (PMA) (50 ng· mL^{-1}, Sigma Company, USA) and ionomycin (50 ng· mL^{-1}, Sigma Company, USA) were added to the culture medium[3]. The culture medium of control group did not contain CD_3, PMA and ionomycin. The liquid of culture medium was adjusted to 1 mL. After cultured for 14 h (37 ℃, 5 % CO_2), PBL-Ts were harvested for AICD detection.

Observation by fluorescence microscope

Some PBL-Ts were put on the carry sheet glass, dried naturally and fixed by 4 % formaldehydum polymerisatum. Then, all the cells were stained with TdT-mediated dUTP nick end labeling (TUNEL, procedure according to clarification of the kit) (Promega Company, USA). The positive cells of TUNEL staining were detected by fluorescence microscope (BG-12, Olympass, Japan).

Flow cytometry detection

1×10^6 PBL-Ts were washed, fixed by1 % formaldehydum polymerisatum, stayed overnight in 70 % ethanol (-20 ℃) and stained with TUNEL for apoptosis detection (procedure according to clarification of the kit). The apoptotic ratio of PBL-Ts was detected by a flow cytometer (Fort, B-D Company, USA).

Cytokine detection

100 μl supernatant of medium was collected respectively from each group after cultured for 14 h and frozen in -20 ℃ refrigerator for detection. The contents of IFN-γ and IL-10 were detected by using an ELISA kit. The parallel sample was set up for each sample. The OD450 value of each sample was measured with an enzyme label meter (550 model, Bio-RAD Program, USA), and then the content of each sample was converted according to the standard curve.

Statistical analysis

The data were presented as $\bar{x}\pm s$. ANOVA was used to compare the means.

RESULTS

Observation by fluorescence microscope

The apoptotic PBL-Ts presented DNA breakage. The breakage DNA could be linked by fluorescence labeling dUTP when TUNEL staining was adopted. The apoptotic cells took on kelly fluorescence under fluorescence microscope (Figure 1). This was named positive TUNEL staining. The plasm of PBL-Ts with AICD took on red fluorescence and the nuclei took on kelly fluorescence when TUNEL and PI double staining were adopted. But the cells without AICD only took on red fluorescence (Figure 2). The positive cells of TUNEL staining in PBL-Ts of CHB (with and without anti-CD3mAb, PMA and ionomycin) were more excessive than that of healthy control.

Results of flow cytometry detection

After cultivated for 14 h with induction, the PBL-Ts of CHB patients displayed distinct apoptosis. Apoptosis was also found in groups without anti-CD3 and other inductions, but their apoptotic ratio was lower. There was a similar AICD ratio of PBL-Ts between induction group and healthy control without induction. AICD ratio of PBL-Ts from CHB patients (with or without induction) was significantly higher than that from healthy control (Table 1).

Results of cytokine detection

Activated T lymphocytes may produce plentiful endogenous cytokine. Th_1 mainly produces IFN-γ, IL-2 and TNF-α. But Th_2 mainly produces IL-4, IL-5, IL-6, IL-10, etc. Cytokine IFN-γ, IL-10 were detected in this test. In all groups, the density of INF-γ in culture media of healthy control with induction group was the highest, and the patient in groups with induction was the lowest. But there was no difference among these groups in density of IL-10 (Table 2).

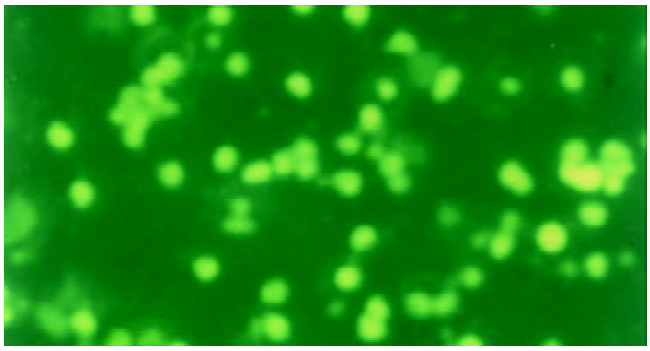

Figure 1 PBL-T with AICD took on kelly fluorescence and the cell without AICD did not take on any fluorescence (TUNEL staining, 200×, fluorescence microscope).

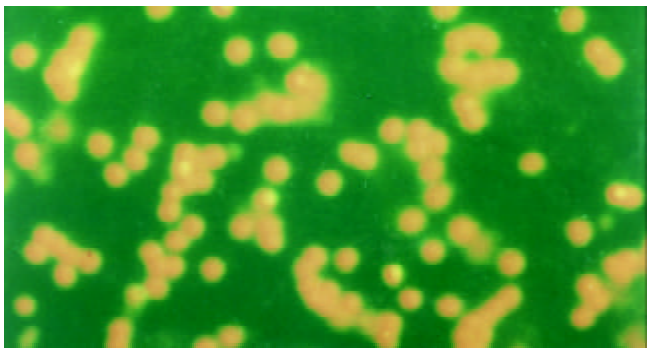

Figure 2 The plasm of PBL-T with AICD took on red fluorescence and the nucleus took on kelly fluorescence. But the cell without AICD only took on red fluorescence (TUNEL and PI double staining, fluorescence microscope, 200×).

Table 1 AICD ratio of each group ($\bar{x}\pm s$)

Group	*n*	AICD Ratio (%)
Patient with induction	26	$17.24\pm1.21^{a,b,c}$
Patient without induction	26	9.88 ± 1.36^{b}
Healthy control with induction	15	6.63 ± 1.00^{c}
Healthy control without induction	15	6.44±1.01

$^aP<0.01$ (F=164.34) *vs*. other groups; $^bP<0.01$ (F=660.45) *vs*. healthy control groups; $^cP<0.01$ (F=326.37) *vs*. without induction groups.

Table 2 The density of IFN-γ and IL-10 from medium of each group ($x\pm s$, pg·mL^{-1})

Group	*n*	IFN-γ	IL-10
Patient with induction	26	$728.32\pm149.59^{a,b,c}$	175.75 ± 34.65^{d}
Patient without induction	26	$1\,313.35\pm403.98^{b}$	74.48±37.21
Healthy control with induction	15	$2\,255.18\pm465.56^{c}$	188.86±66.26
Healthy control without induction	15	2 379.22±465.33	190.58±46.65

$^aP<0.01$ (F=7.37) *vs*. other three groups; $^bP<0.01$ (F=232.94) *vs*. healthy control groups; $^cP<0.01$ (F=25.90) *vs*. groups without induction; $^dP>0.05$ (F=0.02) *vs*. other three groups.

DISCUSSION

Chronic HBV infection is mainly related to the immune function of patients. In a large degree, immune tolerance, especially neonatal immune tolerance, results in persistence of chronic HBV infection. Because naive T cells are sensitive to Fas-mediated AICD and more easily deleted by Ag restimulation than primed T cells[4]. AICD of PBL-Ts plays a key role in central and peripheral immune tolerance[5,6]. AICD is one kind of apoptosis of reactivated lymphocytes when these lymphocytes are induced by activation signals (especially by complex of TCR/CD_3). Ashwell and his colleagues first detected the AICD phenomenon in 1987 when they studied T lymphocyte hybrid tumors. AICD plays an important role in the negative selection of T lymphocytes in thymus, peripheral elimination and clearance of T lymphocytes that have already cleaned the foreign antigens. Therefore, AICD is an important mechanism in maintaining immunoregulation and achieving immune system homeostasis[6,7]. If one' s AICD mechanism is disordered (up-regulation or down-regulation), immune tolerance or autoimmune disease would occur.

In this experiment, AICD of PBL-Ts was successfully induced using anti-CD3 mAb, PMA and ionomycin. The responses of PBL-Ts from CHB and healthy control were different. The results indicated that when induced with anti-CD3, PMA and ionomycin *in vitro*, AICD ratio of PBL-Ts from CHB patients was significantly higher than that from healthy control and that from CHB patients without induction. But there was a similar AICD ratio of PBL-Ts between induction group and healthy control without induction. The results imply that AICD exists in PBL-Ts of CHB and causes decrease of T lymphocytes especially Th_1 cells and functional defect. Specific immune response aiming directly at HBV should not occur. Finally, immunology tolerance to HBV would occur. Ji *et al* using staphylococcus aureus enterotoxin B and rHBcAg proved that AICD of PBMCs in patients would lead to persistent infection of HBV[8].

Reduction of deferent cytokines in culture medium implies apoptosis of deferent subtype T lymphocytes, because the types of cytokine secreted by Th1 and Th2 are different. The detection results revealed that the density of INF-γ in culture media of induction groups from CHB was lower than that of other groups ($P<0.01$). There was no difference between these groups in density of IL-10 ($P>0.05$). These results imply AICD cells are mainly Th1 cells.

After infection of HBV, the virus elimination depends on specific cell immunity of the body. Mostly, specific cell immunity responses are induced by Th1 lymphocytes, but humoral immunity responses are induced by Th2 type lymphocytes. The sensitivity of the two types of T lymphocytes is not equal. The occurrence of AICD is easily induced by Th1 but not Th2 when induced by Anti-CD3 and corresponding antigen[9-11]. Fan *et al* have proved that enhanced Th2 responses are present in chronic HCV infection, and this should be responsible for the persistent HCV infection[12-14]. So, if specific PBL-Ts of CHB are reactivated by HBV antigens, AICD would occur mostly in Th1 type lymphocytes. Thus, specific cell immunity response aiming directly at HBV would be defective, and HBV permanent infection would occur. However, it would be a possible method to surmount immune tolerance and to clean HBV of CHB patients that we have managed to block the apoptosis of activated T lymphocytes[6,15] and raise the amount of specific T lymphocytes.

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