

Original Article

Effects of lymphocyte co-culture on proliferation and apoptosis of hepatitis B virus (HBV)-expressing normal human mesangial cells (NHMCs)

Aihua Qu^{1*}, Xiaochun Han^{2*}, Yiguo Wang³, Changhong Liu^{3,4}, Sen Hong⁵, Qian Liu³

¹Department of Nephrology, Zuoping Hospital Affiliated to Taishan Medical College, Zuoping 256200, Shandong, China; ²Department of Preventive Medicine, College of Traditional Chinese Medicine (College of Basic Medical Sciences), Shandong University of Traditional Chinese Medicine, Jinan 250355, Shandong, China; ³Department of Gastroenterology, Qianfoshan Hospital Affiliated to Shandong University, Jinan 250014, Shandong, China; ⁴Department of Biochemistry and Molecular Biology, School of Medicine, Shandong University, Jinan 250012, Shandong, China; ⁵Department of Gastroenterology, The Fifth People's Hospital of Jinan City, Jinan 250012, Shandong, China. *Equal contributors.

Received September 30, 2015; Accepted December 19, 2015; Epub February 15, 2016; Published February 29, 2016

Abstract: Objective: This study is to investigate the effects of the lymphocyte co-culture on the proliferation and apoptosis of the hepatitis B virus (HBV)-expressing normal human mesangial cells (NHMCs). Methods: NHMCs were transfected with the PHY106-CHBV plasmid containing C genotype HBV with liposome. These cells were co-cultured with human peripheral blood lymphocytes. The HBsAg and HBeAg contents in the supernatant were detected at 24 h, 48 h, and 72 h after transfection. Cell proliferation was assessed with the MTT assay, and apoptosis was detected with flow cytometry. Results: In the normal control and negative control groups, negative results for the HBsAg and HBeAg detection were observed, at all the time points. However, in the transfected NHMCs, positive results were observed for the HBsAg and HBeAg detection at 48 h and 72 h after transfection. These HBV-expressing NHMCs were co-cultured with human peripheral blood lymphocytes for 48 h. Results from the MTT assay showed that, the proliferation of NHMCs was not significantly affected by the transfection of the empty plasmid. However, compared with the control group, the proliferation rate was dramatically declined in HBV-expressing NHMCs. Moreover, the lymphocyte co-culture further significantly decreased the proliferation of HBV-expressing NHMCs. In addition, our results from the flow cytometry showed that, the early apoptosis rate in the co-culture group was significantly higher than the HBV-expressing group. Conclusion: Co-culture of peripheral blood lymphocytes could inhibit proliferation and enhance apoptosis of HBV-expressing NHMCs. These findings might contribute to the understanding of the pathogenic mechanisms of the HBV-associated glomerulonephritis.

Keywords: Hepatitis B virus-associated glomerulonephritis (HBV-GN), hepatitis B virus (HBV), normal human mesangial cells (NHMCs), peripheral blood lymphocytes, co-culture

Introduction

Hepatitis B virus-associated glomerulonephritis (HBV-GN) is one of the common extrahepatic damages caused by HBV infection, which accounts for about 3% of the HBV infection in the population [1]. The pathogenesis of HBV-GN is still unclear. There has already been the evidence of the infection and *in situ* replication of HBV in the kidney inherent cells [2]. Some studies have demonstrated the deposition of the HBV markers and viral particles in the renal tissues [3], indicative of the HBV infection. Our previous studies indicate that, HBV could

directly induce apoptosis of the renal cells, and lead to the transdifferentiation of the tubular cells [4, 5].

Activated cytotoxic T lymphocytes (CTLs) could clear the virus in the following two ways: (1) the lysis pathway, in which the infected target cells are directly killed by the CTL-secreted cytotoxic molecules (such as perforin and granzyme), or through the Fas/FasL pathway-induced apoptosis; and (2) the non-lysis pathway, in which the viral replication is inhibited by the secreted cytokines [6, 7]. Studies have shown that the CTLs are the important immune cells that would

cause pathological damages in the pathogenesis of hepatitis B. When the activated CTLs clear the virus through the lysis pathway, the infected target cells (such as HBV-infected liver cells and kidney cells) would undergo cellular apoptosis. Severe injuries of the target cells might result in the pathophysiological injuries and functional impairment in the target organs, even after reconstruction, leading to corresponding clinical symptoms.

It has been shown that the hepatitis B virus core antigen (HBcAg)-positive renal interstitial tissues are associated with the T lymphocyte infiltration, accompanied by more severe renal tubular lesions, interstitial inflammation, and fibrosis [8]. All these findings suggest that HBV infection in the renal tissues might not only cause immune complex glomerulonephritis (through the formation of the antigen-antibody immune complexes), but also induce the infiltration of CTLs, aggravating the kidney damages. However, the experimental evidence supportive of the hypothesis is still needed to be established.

In this study, the role of the peripheral blood lymphocyte in the pathogenesis of the HBV-GN was investigated. The NHMCs expressing HBV markers were co-cultured with human peripheral blood lymphocytes, and the effects of lymphocyte co-culture on the proliferation and apoptosis of these NHMCs were observed.

Methods and materials

Cell line and cell culture

Normal human mesangial cells (NHMCs) were preserved by our own laboratory. These cells were cultured with DMEM low-glucose medium (Gibco, Gaithersburg, MD, USA), containing 10% fetal bovine serum (FBS; Sijiqing, Hangzhou, Zhejiang, China), 100 U/mL penicillin, and 100 µg/L streptomycin, in a 37°C, 5% CO₂ incubator.

PHY106-CHBV extraction, identification, and transfection

The competent bacteria DH5α was transformed with 2 mL PHY106-CHBV, and the incubated in the Amp^r plate at 37°C overnight. The monoclonal was picked up and cultured with 20 mL Amp^r LB medium at 37°C overnight. Then the

plasmid was extracted with the plasmid extraction kit (Invitrogen, Carlsbad, CA, USA). The target plasmid was identified using the double digestion with HindIII and NsiI, followed by agarose gel electrophoresis. Moreover, sequencing was performed by the LiuheHuada Genomics Technology Co., Ltd., Beijing, China.

For the cell transfection, the NHMC cells in the logarithmic growth phase were seeded onto the 6-well plates, at the density of 1×10⁴ cells/mL. The cell culture was ready for transfection when 80%-90% confluence was reached. 4 µg PHY106 empty vector or PHY106-CHBV plasmid (C genotype HBV, a kind gift from Prof. Jun Cheng, Beijing Ditan Hospital, Beijing, China) was added into and mixed with 250 µL opti-MEM. Moreover, 10 µL liposome (Invitrogen) was diluted with another 250 µL opti-MEM, and placed at room temperature for 5 min. The above mixture were gently mixed together, and placed at room temperature for 20 min. For transfection, the culture medium was discarded, and 1500 µL DMEM low-glucose medium was added into each well. Then 500 µL liposome-PHY106 (negative control) or liposome-PHY106-CHBV plasmid was added into the cell culture. 500 µL DMEM low-glucose medium was added instead in the control group. After 6 h, the medium was replaced by DMEM low-glucose medium containing 10% FBS.

Human peripheral blood lymphocyte isolation and cell co-culture

Totally 10 mL heparin-anticoagulated blood was obtained and diluted with PBS. The diluted blood sample was added into the tube containing lymphocyte separating solution (1:1), followed by centrifugation at 1500 rpm for 15 min. Then the lymphocyte layer was slowly obtained and washed with PBS twice. After centrifugation at 2000 rpm for 10 min, the precipitation was cultured with DMEM low-glucose medium, containing 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin, 60 µg/mL PHA, and 100 U/mL rIL-2, in a 37°C, 5% CO₂ incubator. Half of the culture medium was changed twice a week.

For the cell co-culture, human peripheral blood lymphocytes were added in to the culture of HBV-expressing NHMCs, at the density of 1×10⁶ cells/mL, for 48 h.

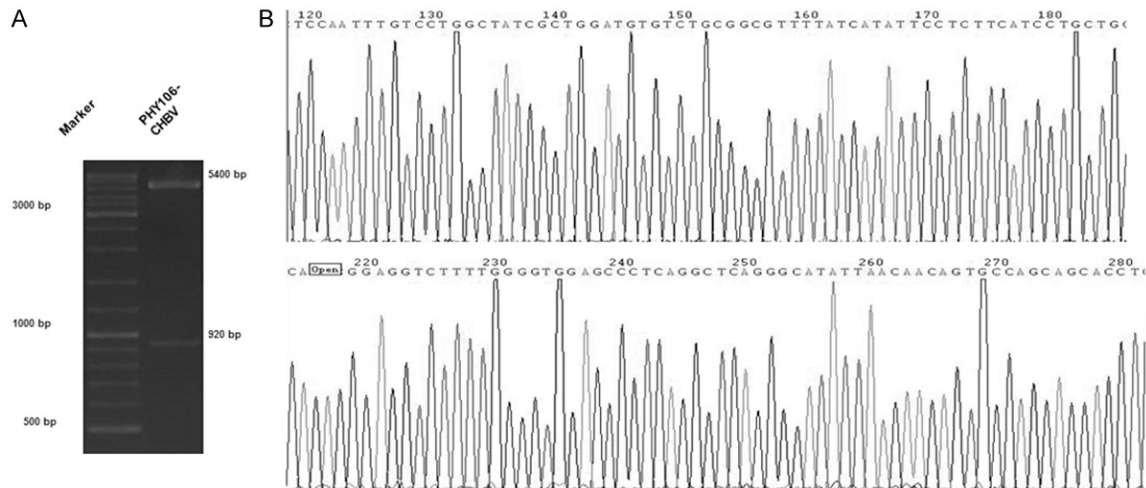


Figure 1. Identification and characterization of PHY106-CHBV plasmid. A. The PHY106-CHBV plasmid was digested with HindIII and NsiI restriction endonucleases, and then subjected to the agarose gel electrophoresis. B. Results of the sequencing of the PHY106-CHBV plasmid.

HBsAg and HBeAg content detection

After transfection, the culture supernatant was collected, and the HBsAg and HBeAg contents in the supernatant were determined in the laboratory of our hospital.

MTT assay

The cell proliferation was assessed with the MTT assay. The cells were planted onto 96-well plates. 10 μ L MTT was added to incubate the cells for 4 h. Then 100 μ L dimethyl sulfoxide (DMSO) was added, and the plate was kept shaking for 10 min. The optical density (OD) was detected with a Model 680 microplate reader (Bio-Rad, Hercules, CA, USA), with the excitation wavelength of 570 nm and the emission wavelength of 630 nm.

Flow cytometry

Cellular apoptosis was detected with flow cytometry. The cells were harvested and washed with PBS twice. The cells were then re-suspended with 500 μ L 1 \times binding buffer, and stained with 5 μ L Annexin V-FITC and 10 μ L PI in dark at 2-8°C for 5 min. The fluorescence was detected with a flow cytometer (BD Biosciences, San Jose, CA, USA) within 1 h.

Statistical analysis

Data were expressed as mean \pm SD. The SPSS 17.0 software was used for the statistical anal-

ysis. The *t*-test was performed for the group comparison. *P*<0.05 was considered statistically significant.

Results

Identification and characterization of PHY106-CHBV plasmid

When the obtained PHY106-CHBV plasmid was digested with HindIII and NsiI restriction endonucleases, target fragments of 5400 bp and 920 bp were observed (**Figure 1A**). Moreover, the gene sequencing further confirmed that the obtained plasmid harbors the full-length C genotype HBV (**Figure 1B**), which was suitable for the following experiments.

Detection of HBsAg and HBeAg contents in culture supernatant

To confirm the expression of hepatitis B markers in the transfected NHMCs, the determination of HBsAg and HBeAg contents in the cell culture supernatant was performed, at 24 h, 48 h, and 72 h after transfection. Our results showed that, in the normal control and negative control groups, negative results for the HBsAg and HBeAg detection were observed, at all the time points (i.e., 24 h, 48 h, and 72 h) (**Table 1**). However, in the transfection group, positive results were observed for the HBsAg and HBeAg detection at 48 h and 72 h after transfection (**Table 1**), with statistical signifi-

Lymphocytes affect HBV-expressing NHMCs

Table 1. Levels of HBsAg and HBeAg in the culture supernatant of NHMCs

Group	N	HBsAg level (ng/mL)			HBeAg level (PEIU/mL)		
		24 h	48 h	72 h	24 h	48 h	72 h
Normal control	3	0.10 ± 0.06	0.11 ± 0.02	0.13 ± 0.02	0.14 ± 0.02	0.18 ± 0.03	0.14 ± 0.02
Negative control	3	0.11 ± 0.02	0.12 ± 0.01	0.11 ± 0.02	0.15 ± 0.03	0.13 ± 0.03	0.13 ± 0.03
HBV-NHMCs	3	0.16 ± 0.04	0.59 ± 0.08*	1.77 ± 0.91*	0.19 ± 0.02	1.08 ± 0.15*	1.64 ± 0.30*

Note: Quantitative reference intervals: 0-0.2 ng/mL for HBsAg and 0-0.5 PEIU/mL for HBeAg. Compared with the normal control group, * $P < 0.05$.

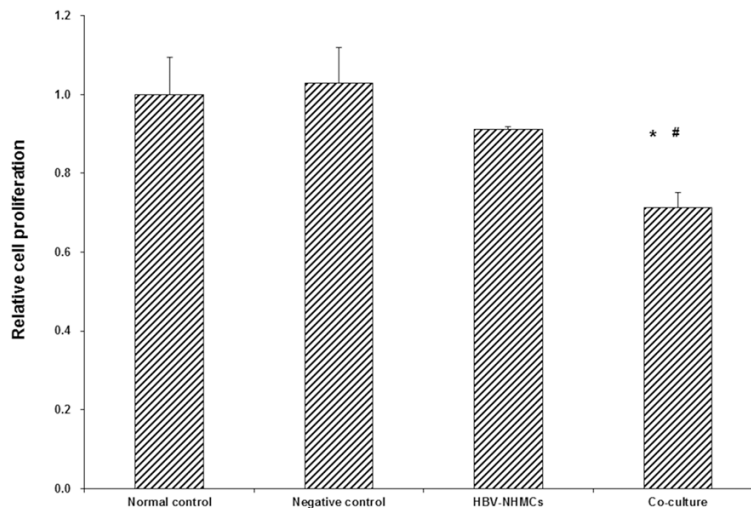


Figure 2. Effects of lymphocyte co-culture on proliferation of HBV-expressing NHMCs. HBV-expressing NHMCs (HBV-NHMCs) were co-cultured with human peripheral blood lymphocytes for 48 h, and then the proliferation rates of the HBV-NHMC and the co-culture groups were detected with the MTT assay. The control group was treated with the medium alone, and the negative control group was transfected with the plasmid vector. Compared with the control group, * $P < 0.05$; compared with the HBV-NHMC group, # $P < 0.05$.

cance compared with the control group (both $P < 0.05$). These results suggest that the transfected NHMCs express hepatitis B markers as expected.

Effects of lymphocyte co-culture on proliferation of HBV-expressing NHMCs

The HBV-expressing NHMCs were co-cultured with human peripheral blood lymphocytes for 48 h, and then the proliferation of these NHMCs was detected with the MTT assay. Our results showed that, compared with the normal control group, the proliferation of NHMCs was not significantly affected by the transfection of the empty plasmid ($P > 0.05$) (Figure 2). However, compared with the normal control group, the proliferation rate was dramatically declined in the HBV-expressing NHMCs. Moreover, the lymphocyte co-culture further significantly de-

creased the proliferation rate of HBV-expressing NHMCs ($P < 0.05$) (Figure 2). These results suggest that, co-culture with human peripheral blood lymphocytes could significantly decline the proliferation of HBV-expressing NHMCs.

Effects of lymphocyte co-culture on apoptosis of HBV-expressing NHMCs

The apoptosis of the HBV-expressing NHMCs co-cultured with human peripheral blood lymphocytes was detected with flow cytometry after the AnnexinV-FITC/PI double staining. As shown in Figure 3, the cells in the lower right quadrant were early apoptotic cells. No significant difference was observed in

the early apoptosis rate between the normal control and negative control groups ($P > 0.05$). However, compared with the control group, the early apoptosis rate was significantly higher in the HBV-expressing NHMCs ($P < 0.05$). Moreover, the apoptosis rate was further elevated by the lymphocyte co-culture in the HBV-expressing NHMCs ($P < 0.05$) (Figure 3). These results suggest that, co-culture with human peripheral blood lymphocytes could significantly elevate the early apoptosis of HBV-expressing NHMCs.

Discussion

Ever since the first report on HBV-GN [9], more evidence has been accumulated concerning the association between the HBV infection and kidney diseases. A previous study has shown the deposition of HBV markers and viral parti-

Lymphocytes affect HBV-expressing NHMCs

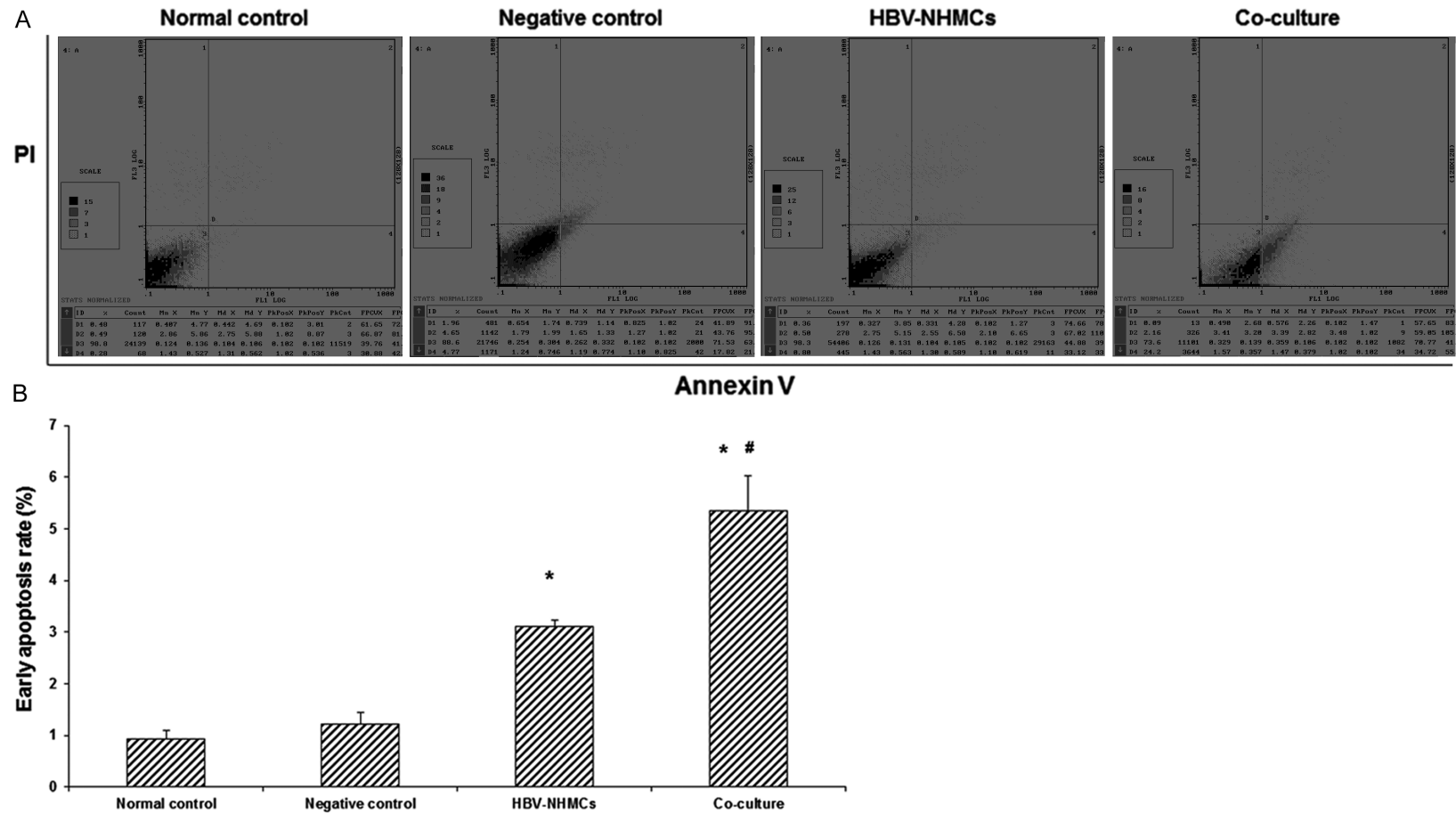


Figure 3. Effects of lymphocyte co-culture on apoptosis of HBV-expressing NHMCs. A. HBV-expressing NHMCs (HBV-NHMCs) were co-cultured with human peripheral blood lymphocytes, and then cellular apoptosis rates of the HBV-NHMC and the co-culture groups were detected with flow cytometry. The control group was treated with the medium alone, and the negative control group was transfected with the plasmid vector. B. Statistical analysis of the apoptosis rates. Compared with the control group, * $P < 0.05$; compared with the HBV-NHMC group, # $P < 0.05$.

cles in the renal tissues [3], indicating that HBV could infect kidney cells. Moreover, the infiltration of local CD4⁺ and CD8⁺ cells has also been observed in the renal interstitial tissues, in the children with HBV-GN [10]. Furthermore, the expression of perforin would also be detected in the renal interstitial tissues, glomerular, and tubular, in patients with HBV-GN [11]. The perforin-mediated CD8⁺ CTL specific cytotoxic effects could induce the cellular injuries and apoptosis, leading to kidney damages. In order to demonstrate the effects of sensitized lymphocytes on the renal cell injuries, the HBV-expressing NHMCs were co-cultured with peripheral blood lymphocytes from healthy subjects inoculated with the hepatitis B vaccine, and the cell proliferation and apoptosis were investigated. Our results showed that, compared with the HBV-expressing NHMC group, the proliferation was significantly declined and the apoptosis rate was significantly elevated by the lymphocyte co-culture.

It has long been accepted that the specific CTLs mainly work through the lysis pathway. HBV induces high expression of Fas/FasL in liver cells and peripheral blood lymphocytes. Thus, the clearing of HBV might cause serious damages to liver cells. On the other hand, some studies showed that the non-lysis pathway also plays an important role. Activated CTLs secrete certain cytokines, inhibiting the viral replication, and recruit a large number of non-specific T lymphocytes to the liver, increasing the liver cell injuries. Therefore, the pathological damages in the liver tissue might not only be attributed to the Fas system-mediated apoptosis, but also to other involved factors, including virus products and immune cytokines [12]. The role of cytokines in the pathogenesis of HBV-GN cannot be ignored. Studies have found that, in the HBV-transgenic mice, activated CTLs could rapidly accumulate in the liver, which induce liver cell apoptosis via direct contact and secrete large amounts of IFN- γ at the same time. IFN- γ could inhibit the expression and replication of HBV, and clear the viral nucleocapsid in the cytoplasm. Moreover, IFN- γ could change the viral RNAs integrated into the nucleus through an SSB/La dependent way, further reducing the viral gene products in liver and body fluid [13].

A previous study has been shown that, the incubation with the serum from chronic hep-

atitis B patients could increase the apoptosis of the renal tubular epithelial cells, which is associated with the serum TGF- β 1 level and the viral replication [14]. Moreover, TGF- β 1 has been found to directly induce the apoptosis of the renal tubular epithelial cells in mice, increasing the renal tubular damages [15]. TGF- β 1 binds to the receptors on tubular epithelial cells, and activates the MAPK signaling pathway through Smad and p38, leading to the cellular apoptosis [15]. Based on these findings, we suppose that, in the pathogenesis of HBV-GN, peripheral blood lymphocytes could not only directly attack the target cells, but also produce certain cytokines via the immune system to activate the cellular apoptosis pathway in kidney cells, increasing kidney damage. However, further in-depth studies are still needed to investigate the signaling pathways involved in the disease pathogenesis.

In conclusion, our results showed that the co-culture of human peripheral blood lymphocytes could significantly inhibit proliferation and enhance apoptosis of HBV-expressing NHMCs. These findings might contribute to the understanding of the pathogenic mechanisms of HBV-GN.

Acknowledgements

This work was supported by the Shandong Province Science and Technology Development Program (2007GGWZ02054).

Disclosure of conflict of interest

None.

Address correspondence to: Yiguo Wang and Changhong Liu, Department of Gastroenterology, Qianfoshan Hospital Affiliated to Shandong University, No. 16766, Jingshi Road, Jinan 250014, Shandong, China. Tel: 86-13791126821; E-mail: 1856008-2133@163.com (YGW); Tel: 86-15165055679; E-mail: liuchanghong5675@126.com (CHL)

References

- [1] Xu G and Huang T. Hepatitis B virus-associated glomerular nephritis in East Asia: progress and challenges. *Eur J Intern Med* 2011; 22: 161-166.
- [2] Wang NS, Wu ZL, Zhang YE and Liao LT. Existence and significance of hepatitis B virus DNA in kidneys of IgA nephropathy. *World J Gastroenterol* 2005; 11: 712-716.

- [3] Feng J, Zhou X and Ren Q. The pathogenic role of HBV in renal tissues detected with in situ hybridization. *Chin J Nephrol* 1995; 2: 107.
- [4] Wang Y, Liu C, Hong S, Zhang P and Liu Q. Effect of recombinant Hepatitis B virus on human glomerular mesangial cell apoptosis. *Biotechnol Biotechnol Equip* 2014; 28: 689-696.
- [5] Liu C, Chen F, Han X, Xu H and Wang Y. Role of TGF- β 1/p38 MAPK pathway in hepatitis B virus-induced tubular epithelial-myofibroblast transdifferentiation. *Int J Clin Expe Pathol* 2014; 7: 7923.
- [6] Guidotti LG, Borrow P, Brown A, McClary H, Koch R and Chisari FV. Noncytopathic clearance of lymphocytic choriomeningitis virus from the hepatocyte. *J Exp Med* 1999; 189: 1555-1564.
- [7] Guidotti LG, Rochford R, Chung J, Shapiro M, Purcell R and Chisari FV. Viral clearance without destruction of infected cells during acute HBV infection. *Science* 1999; 284: 825-829.
- [8] Wang NS, Wu ZL, Zhang YE, Guo MY and Liao LT. Role of hepatitis B virus infection in pathogenesis of IgA nephropathy. *World J Gastroenterol* 2003; 9: 2004-2008.
- [9] Furuse A, Hattori S, Terashima T, Karashima S and Matsuda I. Circulating immune complex in glomerulonephropathy associated with hepatitis B virus infection. *Nephron* 1982; 31: 212-218.
- [10] Ma L, Pan T, Yang Q, Yan Y and Zhou Z. Tubulointerstitial lesion in pediatric patients with hepatitis B related membranous nephropathy. *Chinese Journal of Integrated Traditional and Western Nephrology* 2003; 4: 159-160.
- [11] Wang X, Zhao Y and Xu C. Expression and significance of perforin in hepatitis B virus-associated nephritis. *Journal of The Fourth Military Medical University* 2007; 28: 18-20.
- [12] Xing WB, Liu LN and Lu S. Expression of Fas and FasL in the hepatocytes and peripheral blood lymphocytes from patients with chronic hepatitis B. *Chin J Clin Hepatol* 2007; 1: 003.
- [13] Moriyama T, Guilhot S, Klopchin K, Moss B, Pinkert CA, Palmiter RD, Brinster RL, Kanagawa O and Chisari FV. Immunobiology and pathogenesis of hepatocellular injury in hepatitis B virus transgenic mice. *Science* 1990; 248: 361-364.
- [14] Deng CL, Song XW, Liang HJ, Feng C, Sheng YJ and Wang MY. Chronic hepatitis B serum promotes apoptotic damage in human renal tubular cells. *World J Gastroenterol* 2006; 12: 1752-1756.
- [15] Dai C, Yang J and Liu Y. Transforming growth factor- β 1 potentiates renal tubular epithelial cell death by a mechanism independent of Smad signaling. *J Biol Chem* 2003; 278: 12537-12545.