Oncology and Translational Medicine
DOI 0.1007/s10330-015-0078-8

December 2015, Vol. 1, No. 6, P275–P279

ORIGINAL ARTICLE

In vitro incubation of cytokine-induced killer cells from patients with and without hepatitis B virus and a cell subset analysis

Xuebin Ma[1] (✉), Cong Ma[1], Wei Qiu[2], Hongxia Yuan[2], Ping Yang[2], Jinbo Kang[2]

[1] Department of Clinical Laboratory, Navy General Hospital, Beijing 100048, China
[2] Tumor Diagnosis and Treatment Center, Navy General Hospital, Beijing 100048, China

Abstract

Objective The aim of the study was to explore the difference between immune cell subsets during the incubation of cytokine-induced kill cells (CIKs) from patients with and without hepatitis B virus (HBV).
Methods Peripheral blood samples were extracted from 50 tumor patients, and were divided into two groups according to the presence or absence of HBV. The proliferation rate and activity of CIK cells were examined based on counts on days 1, 5, 7, 9, 11, 13, and 15 of culture. Additionally, the $CD3^+$, $CD4^+$, $CD8^+$, $CD3^+CD8^+$, $CD3^+CD4^+$, and $CD3^+CD56^+$ T cell populations were analyzed by flow cytometry on days 5, 7, 10, 13, and 15 of culture.
Results Proliferation over a 15-day period was higher in the HBV-positive group than in the negative group (280-fold vs. 180-fold increase, respectively), but there was no significant difference between the two groups at each time point. The frequencies of $CD3^+$, $CD8^+$ T, $CD3^+CD8^+$, and $CD3^+CD56^+$T cells increased over time, while those of $CD4^+$ and $CD3^+CD4^+$ T cells decreased over time, and these changes were greater in the positive group than in the negative group. The differences in $CD8^+$ T cells and $CD3^+CD4^+$ T cells between the two groups were significant ($P < 0.05$).
Conclusion The proliferative capacity of CIK cells was higher for patients in the HBV-positive group than those in the HBV-negative group, and immune cell subsets were more favorable in the HBV-positive group than the negative group.

Received: 16 March 2015
Revised: 14 June 2015
Accepted: 25 August 2015

Key words: hepatitis B virus (HBV); cytokine-induced killer cells (CIKs); immune cell subset

The prevalence of hepatitis B virus (HBV) is high in our country; it has a positive rate of 8.57% among 15–59 year olds and 8.98% among tumor patients [1], and there is no statistical difference in its prevalence between these groups. HBV infection is not only an important factor causing liver cancer, but also has a close relationship with other tumors. In recent years, immunotherapy has emerged as a treatment modality with encouraging efficacy and slight adverse effects in cancer therapy. Among various kinds of immunotherapy, cytokine-induced killer (CIK) cell therapy has moved from the "bench to bedside" and is a promising method. Compared with traditional immune cells, such as lymphokine-activated killer cells and tumor-infiltrating lymphocyte cells, CIK cells can proliferate rapidly *in vitro* and have enhanced antitumor activity and a broader spectrum of targeted tumors. CIK cells are a subset of T lymphocytes with a natural killer T cell phenotype expressing both the CD3 and CD56 markers. They exhibit potent non-major histocompatibility complex-restricted cytotoxicity against a variety of tumor target cells, similar to NK cells. There are reports that CIK cells can inhibit HBV replication with non-toxic side effects [2]. However, there are no reports regarding the influence of hepatitis B surface antigen (HBsAg) in the peripheral blood of cancer patients on cultured CIK cell proliferation and cell surface molecule expression. Accordingly, the primary objective of this study was to investigate the effect of HBsAg on CIK properties.

✉ Correspondence to: Xuebin Ma. Email: 1965394316@qq.com

Materials and methods

Patients

Fifty advanced-stage cancer patients were recruited from PLA Navy General Hospital between October 2012 and June 2013, including 15 cases of lung cancer, 10 cases of liver cancer, 7 cases of nasopharyngeal carcinoma, 6 cases of breast carcinoma, 3 cases of gastric cancer, 2 cases of renal carcinoma, 2 cases of cervical cancer, and 5 cases of other malignant tumors. Written informed consent was obtained from all patients, and the study was approved by the Ethics Committee of Navy General Hospital.

The patients enrolled in this study were aged 53.42 ± 13.32 years, including 28 males and 22 females, and had malignant tumors confirmed by radiography and pathologic examinations. HBsAg, anti-HCV and -HIV antibodies, and syphilis antibodies were examined in all patients, and all were negative except for HBsAg.

The 50 patients with malignant tumors were divided into two groups according to HBsAg status: a positive group (14 cases) and a negative group (36 cases).

Instruments and reagents

Instruments

The following instruments were used: a CO_2 incubator (3111 Series, ThermoFisher, USA), a low-speed centrifuge (4000 Type; Kubota, Japan), BCM Biological Clean Bench (Suzhou Antai Air Technology Co. Ltd, China), an Epics XL MCL Flow Cytometer (Beckman Coulter, USA), an XE-2100 Automatic Blood Cell Analyzer (Sysmex, Japan), and an LX-20 Automatic Biochemical Analyzer (Beckman Coulter, USA).

Reagents

The following reagents were used in the study: fresh serum-free GT-T551 (Takara, Japan), human lymphocyte separation liquid (Tianjin Virtue, ASM Pacific Technology, Ltd., Honk Kong), buffer D-PBD (Takara, Japan), rhIL-2 (Beijing Double Dew Pharmaceutical Companies, China), IFN-gamma (Shanghai Kaimao Bio Pharmacy, China), CD3 monoclonal antibody (Center for Molecular Immunology, Cuba), CD3, CD4 CD56, and CD8 fluorescence-labeled antibodies (Beckman Coulter, USA), blood cell analysis reagent (Sysmex, USA), and biochemical detection reagent (North Kowloon Strong Biological Technology Co. Ltd., USA).

Methods

Comparison of lymphocytes and biochemical properties before treatment

Blood samples from all patients were obtained for lymphocyte and biochemical detection at the same time that CIK cultures were initiated.

Ex vivo generation of CIK cells

Informed consent was obtained from all the patients. Heparinized peripheral blood (54 mL) was obtained from each patient. Peripheral blood mononuclear cells were separated by Ficoll-Hypaque density centrifugation and resuspended at 1×10^6 cells/mL in fresh serum-free GT-T551 medium containing 800 U/mL recombinant human IFN-γ. Autologous plasma (10%) was added to a flask that was pre-coated with CD3 (5 g/mL) monoclonal antibody, and samples were incubated at 37 ℃ in a humidified atmosphere containing 5% CO_2 for 24 h. Then, 1000 U/mL recombinant human IL-2 was added to the media. Cells were cultured for up to five days according to cell growth and proliferation with adjustments for cell density, and transferred to the GT-T610 (A) Permeability Culture Bag for expansion. Fresh IL-2 and fresh medium were added every 2–3 days, and the cell density was maintained at 2×10^6 cells/mL. At days 7, 10, 13, and 15, a fraction of cells was obtained for phenotype analysis and cell viability staining with trypan blue. CIK cells were harvested on day 15. A fraction of harvested CIK cells was collected for phenotype analysis, and most fresh CIK cells were infused to patients immediately after harvesting.

CIK cell phenotype analysis

For each patient, the phenotypes of autologous CIK cells after culturing for 5, 7, 10, 13, and 15 days were characterized by flow cytometry (Beckman Coulter Epics XL MCL, USA) using four-color fluorescence. The following monoclonal antibodies were used: anti-CD3-PC5, anti-CD4-FITC, anti-CD8-PE, and anti-CD56-PE (all obtained from Beckman Coulter, USA). The cultured cells and antibodies were mixed gently and incubated in the dark at room temperature for 20 min. Phosphate-buffered saline was added followed by centrifugation at 1500 × g/min for 8 min. The supernatant was discarded, cells were resuspended in 500 μL of phosphate-buffered saline, and the proportion of cells in each subgroup ($CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, and $CD3^+CD56^+$) was calculated based on cell density.

Statistical analysis

All statistic analyses were performed using SPSS16.0 Statistical Software. Statistical comparisons between groups were performed using Student's *t*-tests, one-way ANOVA (analysis of variance), or multiple comparison tests. A *P*-value of less than 0.05 was considered statistically significant.

Results

General data

All enrolled tumor patients were divided into two groups according to the HBsAg results: a positive group ($n = 14$ cases) and a negative group ($n = 36$ cases), and the properties of each group were summarized in Table 1.

Table 1 Comparison of the HBsAg-positive and -negative groups

	Negative	Positive	*F*	*P*
Age (years)	51.36 ± 13.56	47.71 ± 9.54	3.521	0.243
lymphocyte (× 10^9/L)	1.28 ± 0.74	0.81 ± 0.44	4.415	0.041*
Album (g/L)	38.89 ± 4.33	42.62 ± 5.07	6.378	0.015*
Alanine Transaminase (g/L)	68.87 ± 7.14	70.63 ± 5.30	0.653	0.423
Alanine Transaminase (U/L)	17.86 ± 8.94	28.92 ± 15.87	9.300	0.004*
Total bilirubin (umol/L)	10.09 ± 3.46	13.61 ± 5.82	6.623	0.013*
Conjugated bilirubin (umol/L)	5.26 ± 1.65	6.99 ± 3.48	5.502	0.023*

* Compared with the negative group ($P < 0.05$)

Table 2 Comparison the proliferation of CIK cells (× 10^7/L)

Day	Negative	Positive	*F*	*P*
1	6.00 ± 2.53	4.32 ± 1.75	2.223	0.142
5	12.49 ± 4.23	11.75 ± 3.90	0.399	0.53
7	42.88 ± 18.20	38.51 ± 10.75	4.020	0.051
9	148.53 ± 100.66	129.65 ± 53.01	1.741	0.193
11	357.81 ± 206.73	314.07 ± 175.11	0.511	0.478
13	636.47 ± 361.34	676.29 ± 484.37	0.989	0.325
15	1074.6 ± 711.72	1198.6 ± 926.06	1.332	0.254

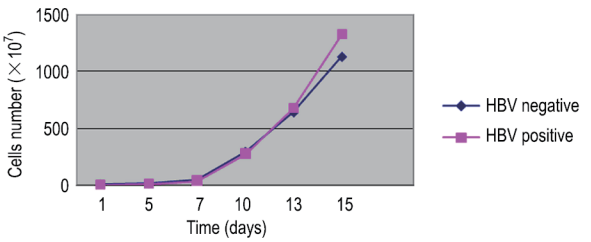

Fig. 1 CIK cell growth curves for the two groups

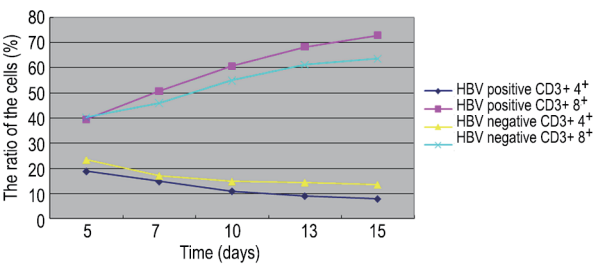

Fig 2 Trend in subtype frequencies over time in the two groups

Comparison of the proliferation of CIK cells

Cell viability and cell density were estimated on days 1, 5, 9, 11, 13, and 15. Cell viability was more than 98% (Table 2), and the cell growth curve was shown in Fig. 1. There was no significant difference in the rate of CIK cell proliferation between the two groups. CIK cells entered a period of rapid growth at approximately days 7–15 in the two groups. These cells were still in the logarithmic growth phase at day 15, but approximately 280 × amplification was observed in the positive group, while it was only approximately 180 × in the negative group, and this difference between groups was significant.

Phenotypic characteristics of ex vivo-generated CIK cells

The phenotypic characteristics of *ex vivo*-generated CIK cells were analyzed at 5, 7, 10, 13, and 15 days. The proportion of $CD3^+$, $CD8^+$, $CD3^+CD8^+$, and $CD3^+CD56^+$ T cells in the positive group increased more than that in the negative group. The $CD4^+$ and $CD3^+CD4^+$ T cells decreased more in the positive group than in the negative group. Based on the ANOVA, there were statistically significant differences in the proportion of $CD8^+$ and $CD3^+CD4^+$ T cells between the two groups ($P < 0.05$). These results were presented in Table 3 and Fig. 2.

Discussion

Immune therapy is helpful for HBsAg seroconversion, and to restore or improve the antiviral ability of defective T cells. CIK cell infusion can inhibit HBV DNA replication, decrease HBV levels in chronic HBV patients,

Table 3 Sub-cellular groups of T cells in the peripheral blood samples of patients with or without HBsAg (%)

Subgroup	Negative					Positive				
	D5	D7	D10	D13	D15	D5	D7	D10	D13	D15
$CD3^+$	58.9 ± 13.7	69.3 ± 16.9	80.6 ± 11.9	90.3 ± 5.3	91.6 ± 3.6	60.52 ± 6.7	72.6 ± 11.4	82.8 ± 7.0	90.1 ± 3.1	93.1 ± 3.4
$CD4^+$	35.9 ± 12.7	30.0 ± 14.0	24.6 ± 12.5	19.3 ± 14.0	18.3 ± 12.7	31.5 ± 11.1	26.0 ± 9.1	18.5 ± 7.5	15.1 ± 7.8	12.9 ± 8.1
$CD8^+$	51.6 ± 13.1	61.5 ± 14.8	68.3 ± 11.3	70.8 ± 11.3	72.2 ± 14.6	51.6 ± 8.2	64.4 ± 8.4	73.6 ± 6.9	79.4 ± 5.8	81.8 ± 4.3*
$CD3^+4^+$	23.4 ± 10.1	17.1 ± 8.9	14.8 ± 8.4	14.4 ± 12.5	13.6 ± 11.1	18.8 ± 5.9	14.7 ± 8.6	10.9 ±6.8	8.9 ± 5.9	7.8 ± 5.9*
$CD3^+8^+$	40.3 ± 13.1	46.0 ± 15.0	54.9 ± 12.3	61.3 ± 13.1	63.6 ± 17.1	39.6 ± 11.7	50.8 ± 7.5	60.7 ± 6.8	68.4 ± 5.8	72.9 ± 6.6
$CD3^+56^+$	1.23 ± 0.56	2.07 ± 0.92	3.47 ± 1.57	5.17 ± 1.76	7.72 ± 4.16	1.9 ± 1.3	2.7 ± 1.6	4.3 ± 2.4	5.7 ± 2.9	7.7 ± 4.9

* Compared with the negative group ($P < 0.05$)

and decrease viral loading in patients with a higher proportion of $CD3^+CD56^+$ T cells. The number of immune cells and immune function recovery are closely related to antiviral ability [3]. Many factors affect tumor development and prognosis during tumor progression [4–5]; however, the relationship between HBsAg levels and CIK cell proliferation in patients with different tumor types is unknown. In this study, the use of CIK cells in the treatment of different malignant tumors was examined, and patients who were HBsAg-positive exhibited better cell properties than those who were HBsAg-negative. In order to study the effect of HBsAg levels on the proliferation of CIK cells, we analyzed the effect of CIK cell treatment for a sample of 50 patients in our hospital.

With respect to the initial lymphocyte count of patients, that of patients in the HBsAg-positive group was significantly lower than that of patients in the negative group. Average levels of albumin, serum transaminase, total bilirubin, and direct bilirubin were significantly higher in the positive group than the the negative group, showing that HBsAg-positive expression is a negative indicator of the general condition of patients, consistent with an effect of HBsAg on human health [6–7]. CIK cell proliferation was similar in the HBsAg-positive and –negative groups. At about the seventh day, CIK cells entered a period of rapid growth, at that point, the number of cells in the two groups was not significantly different, but on the fifteenth day, the positive group exhibited much higher proliferation than that of the negative group (280-fold vs. 180-fold increase, respectively). This may reflect the contact between lymphocytes and tumor cells, influencing HBsAg activation, as well as with HBV, resulting in double stimulation and activation. This suggests that CIK cells from HBsAg-positive tumor patients have stronger recognition capability and tumor- and virus-killing activity [8]. CIK cells with double recognition and activation are more likely to grow and proliferate *in vitro*, so the multiplication observed after 15 days in the positive group was significantly higher than that observed in the negative group.

In an analysis of the phenotypic characteristics of CIK cells, $CD3^+$, $CD8^+$, $CD3^+CD8^+$, and $CD3^+CD56^+$ T cells increased as the culture time increased. These increases were more extensive in the positive group than the negative group; $CD4^+$ T cells and $CD3^+CD4^+$ T cells decreased over time, and this reduction was greater in the positive group than the negative group. There was a significant difference in the frequency of $CD8^+$ and $CD3^+CD4^+$ T cells between the two groups ($P < 0.05$), while the other differences were not statistically significant. In the heterogeneous CIK cell populations, the main effector cells are $CD3^+CD8^+$ T cells and $CD3^+CD56^+$ T cells in the positive group, while $CD3^+CD4^+$T cells have an inhibitory effect on CIK-mediated cell killing [9]. During the culture process, the $CD3^+CD4^+$ population decreased to a minimum, and this is an important factor in improving the effect of CIK cell-killing activity. We discovered that the percentage of $CD3^+CD4^+$ T cells in the positive group was reduced more significantly than that in the negative group. This provides a theoretical basis for improved CIK cell transfusion effects in HBsAg-positive patients. With respect to CIK cell culture techniques, the addition of IL-6 can reduce the proportion of cells associated with inhibition and increase effector cells and toxicity, resulting in increased efficacy [10].

Immune therapy is a promising therapeutic strategy for malignant tumors and HBV [11–14]. An important issue in this field is the development of therapies with improved antitumor and antiviral effects. Accordingly, additional, detailed studies are needed to expand on our results.

Conflicts of interest

The authors indicated no potential conflicts of interest.

References

1. Wang MJ, Li XX, Han BB, *et al.* The analysis of HBsAg results in 10724 cancer patients. China Cancer (Chinese), 2010,19: 623–625.
2. Shi M, Fu J, Shi F, *et al.* Transfusion of autologous cytokine-induced killer cells inhibits viral replication in patients with chronic hepatitis B virus infection. Clin Immunol, 2009, 132: 43–54.
3. Tang Z, Shi M, Zhang B, *et al.* Effect of treatment of autologous cytokine induced killer cells (CIKs) on the suppression of hepatitis B virus and its mechanism. Medical J Chin PLA (Chinese), 2011, 36: 901–903.
4. Ruan Jian, Luo Rongcheng. Micro environment of tumor metastasis and its clinical significance. Transl Med J (Chinese), 2012, 1: 111–114.
5. Zheng YW, Li RM, Zhang XW, *et al*. Current adoptive immunotherapy in non-small cell lung cancer and potential influence of therapy outcome. Cancer Invest, 2013, 31: 197–205.
6. Wang H, Xue L, Yan R, *et al.* Comparison of FIB-4 and APRI in Chinese HBV-infected patients with persistently normal ALT and mildly elevated ALT. J Viral Hepat, 2013, 20: e3–e10.
7. Sarin SK, Kumar M. Should chronic HBV infected patients with normal ALT treated: debate. Hepatol Int, 2008, 2: 179–184.
8. Pan CC, Huang ZL, Li W, *et al.* Serum alpha-fetoprotein measurement in predicting clinical outcome related to autologous cytokine-induced killer cells in patients with hepatocellular carcinoma undergone minimally invasive therapy. Chin J Cancer, 2010, 29: 596–602.
9. Ma Y, Zhang Z, Tang L, *et al*. Cytokine-induced killer cells in the treatment of patients with solid carcinomas: a systematic review and pooled analysis. Cytotherapy, 2012, 14: 483–493.
10. Lin G, Wang J, Lao X, *et al.* Interleukin-6 inhibits regulatory T cells and improves the proliferation and cytotoxic activity of cytokine-induced killer cells. J Immunother, 2012, 35: 337–343.
11. Jiang J, Wu C, Lu B. Cytokine-induced killer cells promote antitumor immunity. J Transl Med, 2013, 11: 83.
12. Yang L, Ren B, Li H, *et al*. Enhanced antitumor effects of DC-activated CIKs to chemotherapy treatment in a single cohort of advanced non-small-cell lung cancer patients. Cancer Immunol Immunother, 2013, 62: 65–73.

13. Dong M, Liang D, Li Y, *et al.* Autologous dendritic cells combined with cytokine-induced killer cells synergize low-dose chemotherapy in elderly patients with acute myeloid leukaemia. J Int Med Res, 2012, 40: 1265–1274.
14. Zhang J, Zhu L, Wei J, *et al.* The effects of cytokine-induced killer cells for the treatment of patients with solid tumors: a clinical retrospective study. J Cancer Res Clin Oncol. 2012, 138: 1057–1062.

DOI 10.1007/s10330-015-0078-8
Cite this article as: Ma XB, Ma C, Qiu W, *et al.* In vitro incubation of cytokine-induced killer cells from patients with and without hepatitis B virus and a cell subset analysis. Oncol Transl Med, 2015, 1: 275–279.