Int J Clin Exp Med 2015;8(4):5601-5610
www.ijcem.com /ISSN:1940-5901/IJCEM0005543

Original Article

Cytokine-induced killer cells combined with dendritic cells inhibited liver cancer cells

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Received January 5, 2015; Accepted April 1, 2015; Epub April 15, 2015; Published April 30, 2015

Abstract: Objectives: To investigate the prognosis of advanced liver cancer patients treated with CIK-DCs and the mechanism of apoptosis of HEPG 2 cells. Methods: 67 patients were enrolled in the study. Peripheral blood mononuclear cells (PBMCs) were separated, of which adherent PBMCs used granulocyte 2 macrophage colony2 stimulating factor (GM2CSF), tumor necrosis factor 2α (TNF2α), and interleukin 24 (IL24) to induce DCs, which were sensitized with antigen of autologous or exogenous cancer cells to obtain Ag-DCs; suspended PBMCs used interferon 2γ (IFN2γ), IL-2, and CD 3 monoclonal antibody (CD3mAb) respectively, to induce CIK cells. DCs and CIK cells were cultured together. Flow cytometry was used to detect the phenotypes of DCs and CIK cells, and the blood retransfused into patients. Western blot and flow cytometer were used to analyze the growth cycle of HepG 2 cells and the expression of BAX and PCNA. Results: No patients underwent complete remission, 5 obtained partial remission and 29 had stable disease. Of the 31 patients whose lesions could not be evaluated, 17 received effective treatment, showing that the immune response was enhanced. *In vitro* laboratory experiments revealed that DC-CIK cells markedly affected the growth cycle of HepG 2 cells. Analysis showed that DC-CIK cells enhanced the gene expression of BAX and inhibited the activity of PCNA. Conclusions: Co-cultured DCs and CIK cells inhibit the proliferation and migration of liver cancer cells by down-regulating PCNA and up-regulating BAX. This approach may be an effective method to treat advanced liver cancer.

Keywords: CDs-CIK, HEPG2 cells, liver cancer patients, Bel associated protein (Bax), proliferating cell nuclear antigen (PCNA)

Introduction

Primary liver cancer is the sixth most common cancer worldwide with up to 750,000 new cases diagnosed every year and is the third most common cause of death from cancer resulting in > 600,000 deaths annually [1]. Liver cancer is the sixth most common cancer in the world. The age-standardized rate of incidence of this cancer is more than six times higher in Eastern Asia (20.9 per 100,000) compared with that in Northern Europe (3.1 per 100,000). In China, liver cancer is the second major cause of cancer deaths, with a mortality rate of 26.26 per 100,000 (males: 37.55 and females: 14.45 per 100,000), accounting for 19.33% of all cancers. Accordingly, the estimated annual incident cases and deaths of liver cancer are 360,000 and 350,000, respectively and can be considered to be an epidemic [2]. The liver tumor-related death rate ranks in second place among all malignant tumors. Because of the insidious onset, the diagnosis of the majority of primary liver cancers is usually delayed. The survival time is unacceptably short, with a median survival time for untreated patients of only 6 months. In recent years, although various treatment regimens have been developed, the therapeutic effects are not ideal, mainly because the overall survival time has not been obviously prolonged. The main treatment options available for liver cancer are

surgery and chemotherapy and other techniques are sometimes used depending on the patient's circumstances. As there are limited ways to prevent or treat liver cancer, research into effective treatments has a high priority in China and indeed worldwide. Thus, new approaches are being developed that use different mechanisms from standard chemotherapy agents and target specific parts of cancer cells or their surrounding environments. For example, in the field of tumor therapy, autologous cell immunotherapy has become a supplementary means for the treatment of many types of tumor [3, 4]. Among the clinically used immunological effector cells in China, cytokine induced killer (CIK) and dendritic cells (DCs) have become the first choice [5]. CIK cells are a heterogeneous population of cells mainly consisting of $CD3^+$, $CD56^+$ and T lymphocytes, with the characteristics of high antitumor activity due to the T lymphocytes and non-major histocompatibility complex (MHC) restriction of natural killer cells [6]. To date, immunotherapy with CIK, the preferred alternative for tumor biotherapy, has achieved a better prognosis in the treatment of solid and hematopoietic tumors [7]. In addition, DCs are the most potent professional antigen presenting cells, which can uniquely stimulate primary T lymphocytes [8]. DC-based vaccines have been successfully utilized in the treatment of malignant melanoma and other tumors [9]. DCs are initiators of the immune response, which can induce a lasting and potent specific antitumor response, while CIK cells possess powerful oncolytic activity, with the characteristic of non-MHC restriction.

Recent studies have reported that Tregs and Tregs-related cytokines can inhibit the antitumor activity of cytokine-induced killer (CIK) cells, but dendritic cells co-cultured with CIK (DC-CIK) cells can be used to induce a specific immune response by blocking Tregs and TGF-β, IL-10 [10, 11]. DC clearly down-regulates the expression of Tregs and Tregs-related IL-35 and simultaneously up-regulates the proliferation ability as well as cytotoxic activity of CIK cells against leukemia cell lines [12]. The higher effectiveness of DC-CIK therapy was proved in a comparative study [13]. Therefore, DC-CIK therapy is used for the treatment of advanced liver cancer patients who are either unwilling or unable to undergo an operation, radiotherapy and/or chemotherapy [14].

The mechanism by which DC-CIK cells can specifically kill the liver cancer cells remains unclear. We therefore used DCs to induce CIK cells specifically targeted to kill liver cancer cells. The prognosis of 67 advanced liver cancer patients treated with DC-CIK cells was evaluated, and the mechanism of apoptosis of HEPG2 cells investigated in laboratory experiments conducted *in vitro*.

Patients and methods

Effect of DC-CIK cells on the apoptosis of human liver cancer cells

HEPG2 cells, in the logarithmic phase, were inoculated at a density of 1×10^6 cells/mL into a 6-pore plate. DC-CIK cells in a same density or half were implanted into Transwell chambers matched with the 6-pore plate. DC-CIK cells in the two different density were placed into the 6-pore plate, respectively. Then, DCs, CIK cells and HEPG2 cells were cultured together under the circumstance of 5% CO_2, 37°C and saturated humidity for 24 h and 48 h, respectively. HEPG2 cells cultured alone were served as control. The cells were collected and processed with Annexin V-PI staining (C1063, Byotime, Beijing, China). Flow cytometry (BD FACS Calibur, Franklin Lakes, New Jersey, USA) was used to detect the apoptosis of HEPG2 cells. The specifics were as follows: A volume of 5 μl Annexin V-FITC was added to 100 μl cell suspension (50 to 100 thousand cells), and gently mixed. They were incubated from light at a room temperature of 20-25°C for 10 min. And then 10 μl 20 ng/mL propidium iodide (PI) was added, gently mixed, and placed from light at a room temperature in an ice bath. The apoptosis of HEPG 2 cells were analyzed by the flow cytometry.

Effect of DC-CIK cells on the growth cycle of human liver cancer cells

1) The cell grouping was the same as above.

2) Detection of the HEPG2 cell cycle: The cells were collected and the sample of each tube contained $1 \times 10^4 \sim 2 \times 10^6$ cells. Routine PI (Sigma-Aldrich, St. Louis, MO, USA) staining was performed. The change of cell cycle was detected by flow cytometry which was set at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The results were

analyzed by Cell Quest acquisition software (BD Biosciences, Franklin Lakes, New Jersey, USA).

Patients

The tissues or cancer cell specimens of 67 advanced liver cancer cell patients from October 2010 to December 2012 were collected by the Pathology Department of Tumor Research Institute of Chongqing City. The inclusion criteria were: advanced primary liver cancer at stage III or IV confirmed histologically or cytologically; patients who were unwilling or inappropriate for other treatments, such as target therapy, radiotherapy or chemotherapy; patients who had not received autologous cellular immunotherapy before; those whose blood routine examination results and heart, liver and renal function were normal with a Karnofsky Performance Status (KPS) score > 60 and an expected life time greater than 3 months. Among those patients, 49 cases were male and 18 female with a median age at 57 years (range 37 to 74 years). According to the method of TNM staging, 27 cases were at stage III and 40 were at stage IV.

The study was approved by the Ethical Committee of our institute. Informed consent was obtained from each patient and their legal representative. All the patients received one follow-up visit every three months after the efficacy evaluation when an imaging and/or hematology examination was carried out.

Preparation of tumor antigens

The human liver cancer cell line SMMC27721 and tissues harvested from previous operations or biopsy specimens that were preserved in liquid nitrogen, underwent phacofragmentation, an ultra-sonicator (Ningbo Instrument, Ningbo, China) and were then centrifuged at 600 × g for 30 min. The tissue or cell splitting supernatants, i.e. tumor antigen, were filtered through a thick 0.45 membrane and were collected for use in sensitizing DCs.

Separation and cultivation of DC-CIK cells

The preparation of tumor antigen and the separation and cultivation of cells was carried out according to reference 13 and the prepared tumor antigen was used to sensitize DCs.

Quality control of DC and CIK

The proliferation of DC and CIK cells and they were contaminated was determined by inspection under an inverted microscope during the process of cell cultivation. Bacterial and fungal cultivation and endotoxin detection were performed before cell collection. It was shown unequivocally that the exogenous factors of cell products including bacteria, fungi and endotoxins showed a negative result. The cell survival rate was > 90%, while the resurgent cell survival rate after being cryopreserved was > 70%.

Detection of cell phenotype

The DCs cultured on the first day and the seventh day and CIK cells on the first day and the twelfth day, were collected. After being washed in PBS and centrifuged, anti-human CD14 (eBioScience, San Diego, CA, USA), CD83 (eBioScience, San Diego, CA, USA) and CD86 (eBioScience, San Diego, CA, USA) monoclonal antibody, marked by FITC, and anti-human HLA-DR monoclonal antibody (eBioScience, San Diego, CA, USA), marked by PE, were added to the DC; while anti-human CD3 and CD4 monoclonal antibody (eBioScience, San Diego, CA, USA), marked by FITC, and anti-human CD8 and CD56 monoclonal antibody, marked by PE, were added to the CIK. After incubation for 20 min at room temperature, cells were washed twice in PBS and then placed in a flow cytometer to analyze the cell phenotype.

Cell transfusion

Bacteria, fungi and endotoxins were detected as described above, 48 h before transfusion or on the day of transfusion. After the exogenous factors were confirmed to be negative, the adherent matured DCs cultured on the 10th to 12th day were collected, washed twice in PBS, resuspended in 1 mL of 0.9% sodium chloride solution at cell numbers > 1×10^6. The solution was injected into the bilateral axilla and bilateral inguinal lymph nodes by multiple subcutaneous injections. The suspended matured CIK cells cultured on the 12th to 14th day, with a cell number > 1×10^{10}, were centrifuged at 500 × g for 15 min, washed twice in PBS, resuspended in 100 mL 0.9% sodium chloride solution containing 10% human albumin, and administered to each patient by intravenous drip within 0.5 h. Patients were given a muscu-

lar injection of 20 mg diphenhydramine (Liqun Pharmaceutical Co. Ltd., Datong, China) or an intravenous injection of 2.5 mg dexamethasone (Liqun Pharmaceutical Co. Ltd., Datong, China) to prevent anaphylactogenesis before transfusion, depending on the circumstances. Generally, the treatment was designed according to the patient's clinical condition. Two courses were strengthened in the previous 6 months, one course was consolidated later than 6 months, and one course was maintained every year until the disease took its course. All the patients only received cellular therapy because they were unwilling or inappropriate for other types of treatment.

Therapeutic evaluation and clinical benefit rate

The lesion size was measured mainly during imaging examinations such as B ultrasound (iu22G4, Philips, Holland), CT (64 sclice Brilliance, Philips, Holland) or MRI (ACHIEVA 1.5T, Philips, Holland). The short-term efficacy was evaluated according to the WHO Response Evaluation Criteria in Solid Tumors (RECIST), that is, the therapeutic effect was estimated according to the imaging examination results including complete remission (CR), partial remission (PR), stable disease (SD), and progressive disease (PD) before and after treatment. If the preliminary evaluation result was CR or PR, it was re-evaluated 4 weeks later. The CR, PR and SD statistics confirmed 4 weeks later were used in the disease control rate (DCR) calculation. On the basis of body mass increasing by > 7% or the KPS score exceeding > 20, and pain relief accounting for more than 50% for more than 4 weeks, the clinical benefit rate (CBR) was determined. The CBR included two assessments of effectiveness (at least one of the above criteria and others having no change) and stabilization (all having no change), respectively.

Detection of tumor markers and immune function

The serum AFP and other tumor-related antigens were detected before and after DCs-CIK cellular therapy in order to evaluate the effect of DCs-CIK cellular therapy on the tumor markers. FCM was used to detect the positive rate of serum $CD3^+$, $CD8^+$ and $CD56^+$ expression before and after DCs-CIK cellular therapy, so that the immunological function improvement due to immune cellular therapy could be evaluated.

Determination of adverse reactions

Every time that CIK or DCs-CIK cells were transfused, whether symptoms of chill, fever and/or allergy occurred was noted. Adverse reactions were divided into 0-4 degrees of severity according to the WHO uniform standard [15].

Detection of the effect of DC-CIK cells on the proliferation and migratison of human liver cancer cells

HEPG2 cells during the logarithmic phase were inoculated at a density of 1×10^6 cells/mL into a 6-pore plate. The cells were cultured at 37°C and saturated humidity under 5% CO_2, until they were all developing on the plate. The scratch test was performed, that is, a 1 mL sterile spearhead was used to draw a scratch. Then DC-CIK cells at the same density were implanted into Transwell chambers matched to the 6-pore plate. Then, the Transwell chambers were placed in the 6-pore plate. DC-CIK cells and HepG2 cells were cultured together. HepG2 cells cultured alone served as the control and a pure nutrient solution acted as the blank control. An Image-Pro-Plus image analysis system (Media Cybernsetics Inc, Bethesda, MD, USA) was used to measure the average residual area of the scratch and the healing rate (HR = [1-AI/AO × 100%]) of the scratch was calculated.

Effect of DC-CIK cells on the growth of the human liver cancer-proliferation curve

Liver cancer HepG2 cells at a density of 4×10^4 cells/2 mL were inoculated into four 6-pore plates. DC-CIK cells at the same density were implanted into Transwell chambers matched with the 6-pore plate. Then the Transwell chambers were placed in the 6-pore plate. DC-CIK cells and HepG2 cells were cultured together. HepG2 cells cultured alone served as the control and the pure nutrient solution as the blank control. One 6-pore plate was drawn on day 1, 3, 5 and 7, respectively. New culture medium and CCK-8 was added to each pore. The cells were continuously cultured for 4 h under the same conditions, vibrated for 30 s and then mixed. A UV performance analyzer (VICTOR X2

Table 1. Comparison of phenotype changes in dendritic cells and CIK cells (x ± s%, *n* = 110)

	DC				CIK			
Day	$CD14^+$	$CD83^+$	$CD86^+$	HLA-DR^+	$CD3^+$	$CD4^+$	$CD3^+$ $CD8^+$	$CD3^+$ $CD56^+$
D0	25.14 ± 1.22	5.38 ± 0.86	34.42 ± 4.31	40.15 ± 3.84				
D10	8.45 ± 0.67	56.39 ± 3.45	75.64 ± 6.67	81.83 ± 5.62				
D0					54.32 ± 5.36	49.25 ± 4.24	16.54 ± 1.15	4.24 ± 0.86
D12					97.15 ± 8.48	26.43 ± 1.62	45.68 ± 3.84	17.65 ± 1.52

Table 2. Changes in concentration of AFP in the blood of patients pre- and post-treatment

Time	$AFP_{ρB}$/(ng.mL^{-1})
Before treatment	236.55 ± 48.38
After treatment	141.37 ± 27.63*

AFP: Alpha-fetal protein; *P < 0.05.

Multilabel Plate Reader, PerkinElmer, USA) was used to detect the optical density at a wavelength of 450 nm. The same experiment was repeated in triplicate. The average optical density value was used as the Y-axis and time as the X-axis to construct the proliferation curve.

Effect of DC-CIK cells on the PCNA gene of human liver cancer

A specific amplification primer was designed according to the full-length cDNA sequence of PCNA and glyceraldehyde-3-phosphate dehydrogenase, respectively. PCNA upstream: 5'-ATGGCCGGAGCTGGCGCCCTGG-3', downstream: 5'-CGCT ACAGGCAGGCGGGAAG-3'; GAPDH upstream: 5'-CGCGGGGCTCTCCAFAAC AT-3', downstream: 5'-GTGGGGGACTGAGTGTGGCAGG-3'. The RNA of DC-CIK and HepG2 cells cultured together were used as a template to amplify the cDNA of PCNA using the reverse transcription-polymerase chain reaction (RT-PCR) (Takara Biotechnology (Dalian), Co., Ltd., China). The cDNA of PCNA was amplified by taking the RNA of unprocessed cells as a template to act as one control as well as the cDNA of amplified GAPDH. GoldView™ (G8140, SBS Genetech Co., Ltd., Beijing, China) staining was used to confirm the PCR products.

Quantitative analysis of the effect of CIK cells on the PCNA expression of human liver cancer

The Image-Pro Plus was used for gray level (GL) quantitative analysis of the expression of PCNA in HEPG2 cells, which were cultured together with DC-CIK cells at the same mRNA level and for optical density (OD) quantitative analysis in the protein level, so that the effect of CIK cells on the PCNA expression of human liver cancer could be quantified.

Detection of effect of CIK cells on the PCNA protein of human liver cancer

By adopting the Western blot method, the total proteins of HEPG2 cells in the observation group cultured together with DC-CIK cells were transferred to a nitrocellulose membrane, soaked in the confining liquid containing PCNA a mouse monoclonal antibody (sc-25280, Santa Cruze Biotechnology Inc., Dallas, Texas, USA) (1:300), and incubated at 4°C overnight. After adding horseradish peroxidase conjugated goat anti-mouse antibody (A0216, Byotime, Beijing, China), the proteins were incubated at 25°C for 1 h and combined with the chemiluminescent substrates (34080, Pierce Biotechnology Inc., Rockford, IL, USA) for 5-10 min, and an image taken. The corresponding detection of the same unprocessed cells served as the control and the β-actin (relative molecular mass: 4.3×10^4) as the internal control.

Statistical analysis

Statistical analysis was carried out using SPSS (version 12.0, IBM Corp., Armonk, New York, USA). The data are expressed as the mean ± SD. The comparison between groups adopted the *Chi*-square test at a significance level of a = 0.05. Values of $P < 0.05$ were considered to be statistically significant.

Results

Detection of the immunophenotype of DC-CIK cells

The FCM method was used to detect the immunophenotype of DCs and the results demonstrated that when DCs were mature on day 8, the proportion of $CD83^+$, $CD86^+$ and $HLA2DR^+$

Table 3. Comparison of the expression of immunity markers in 110 patients pre- and post-treatment

Time	$CD3^+$	$CD8^+$	$CD56^+$
Before treatment	48.65 ± 3.75	31.57 ± 2.63	4.81 ± 0.34
After treatment	64.15 ± 5.28*	53.24 ± 3.32*	7.49 ± 1.20*

*$P < 0.05$.

was obviously increased, while the proportion of $CD14^+$ was reduced compared to non-cultured cells; the difference was statistically significant ($P < 0.05$, **Table 1**).

The immunophenotype of CIK cells was analyzed by the FCM method and the results showed that the proportion of $CD3^+$, $CD3^+$, $CD8^+$ and the $CD3^+$ $CD56^+$ double positive cell mass was obviously increased, while the proportion of $CD4^+$ was obviously reduced compared with non-cultured cells; the difference was statistically significant ($P < 0.05$, **Table 2**).

Therapeutic evaluation and DCR of DCs-CIK

After all of the 67 advanced primary liver cancer patients had received cellular therapy, image examinations (B ultrasound, CT or MRI) were carried out and the results showed that 0 case achieved CR, 5 PR and 21 had SD, the DCR being 38.8%.

Changes in concentration of the tumor marker AFP

The results of measurements of AFP tumor related antigen in the serum of 67 advanced primary liver cancer patients suggested that the expression quantity of AFP antigen was reduced after treatment, and the average level was statistically different from that before treatment ($P < 0.05$, **Table 2**).

Changes in patients' immune functions after DCs-CIK treatment

The FCM method was used to detect the positive rate of $CD3^+$, $CD8^+$ and $CD56^+$ in peripheral blood T lymphocytes before and after treatment in order to evaluate the effects of the therapy on the patients' immune functions. The results showed that the average positive rate of $CD3^+$, $CD8^+$ and $CD56^+$ in the patients' serum T lymphocytes after DCs-CIK cellular therapy was enhanced compared to before treatment, an action that was statistically significant ($P < 0.05$, **Table 3**).

Adverse reactions

After DCs-CIK cellular therapy, the 67 advanced primary liver cancer patients' heart, liver and renal functions were not obviously affected. The most common adverse reaction was fever. Their temperatures were all < 38.5°C (4.8%, 3/67) and generally no special processing was needed. The temperature reduced to normal within 2 to 4 hours. No chills or other adverse reactions were detected.

Effects of DCs-CIK cells on the proliferation, migration and growth of HepG2 human liver cancer cells

Why the DCR was so high under the DC-CIK treatment on liver and lung cancer remains a mystery. Therefore, we investigated the effect of DCs-CIK cells on the proliferation, migration and growth of HepG2 human liver cancer cells. First, we observed and calculated the 5-day average residual area of the scratch (cm^2). We found that the area for HepG2 cells was 5.49 ± 0.42 cm^2, the area for HepG2 cells cultured together with DC-CIK cells at the same density was 9.14 ± 0.86 cm^2 and the area for HepG2 cells cultured together with DC-CIK cells at half density was 6.64 ± 0.83 cm^2. When compared with uncultured HepG2 cells together with DC-CIK cells, the average residual area of the scratch for the HepG2 cells cultured together with DC-CIK cells at the same or half density was significantly different ($P < 0.05$, **Figure 1**). This finding suggests that DC-CIK cells, at a relatively low density, can inhibit the proliferation and migration of HepG2s. Meanwhile, the detection results using the CCK-8 method also demonstrated that the inhibitory effect of DC-CIK cells on the growth of HepG2 cells increased with the increasing cultivation time. There was a statistically significant difference when compared with cells in the control group. The detection of the cell cycle was employed to evaluate the effect of DC-CIK cells on the growth of HepG2 cells. It was found that 24 h after DC-CIK cells at half density were added to the HepG2 cells, the percentage of HepG2 cells blocked in the S phase increased by 291.0% ($P < 0.01$) and 10.7% in the G2/M phase. However, the percentage blocked in the G1 phase was reduced by 14.8% demonstrating that HepG2

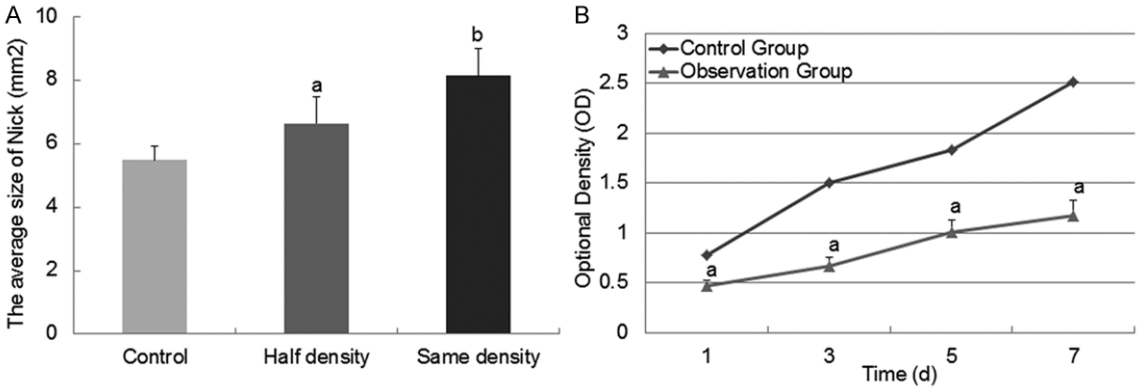
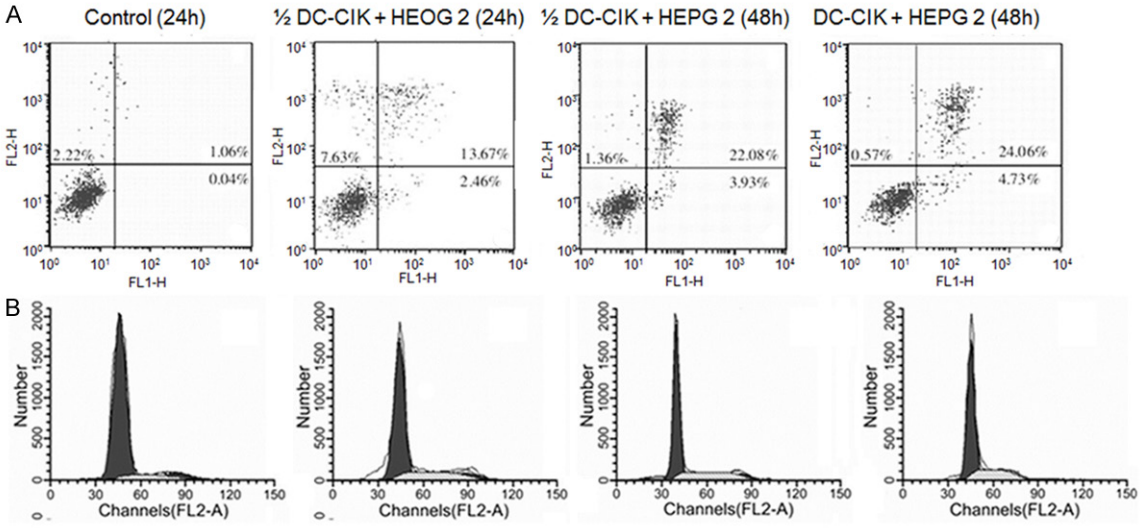


Figure 1. Effects of DCs-CIK cells on the proliferation, migration and growth of HepG2 cells. A: Average residual area on the fifth day; B: The growth and proliferation curve of HepG2 cells.



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Group	Time	G_1	S	G_2M
Control	24h	87.77 ± 8.31	6.58 ± 1.06	5.66 ± 0.81
1/2 DC-CIK-HEPG2	24h	74.79 ± 8.15*	19.15 ± 1.38*	6.05 ± 1.21
Control	48h	87.43 ± 8.35	7.09 ± 1.03	5.48 ± 0.95
1/2 DC-CIK-HEPG 2	48h	73.55 ± 6.15*	21.68 ± 1.52*	4.77 ± 0.51
1/2 DC-CIK-HEPG 2	48h	74.17 ± 6.35*	23.58 ± 1.74*#	2.25 ± 0.13

*p < 0.01 vs. Control; #p < 0.05 vs. 1/2 DC-CIK + HepG2.

Figure 2. Effect of DC-CIK cells in a different concentration and processed in a different time on the cell cycle of HepG2 cells.

cells were blocked in the S phase. When the number of DC-CIK cells was doubled, it was found that the percentage of HepG2 cells blocked in the S phase increased by 332.5%, which was significantly increased compared to conditions when the number of DC-CIK cells was half ($P < 0.05$). DC-CIK cells obviously inhibited the growth of liver cancer cells in S phase, and the blocking ability was increased as the DC-CIK cell numbers increased. Thus, the proliferation and migration of HepG2 cells can be regulated and controlled (**Figure 2**).

Effect of DC-CIK cells on the mRNA and protein expression of PCNA and BAX in human liver cancer cells

The expression ability of PCNA in HepG2 cells cultured together with DC-CIK cells at the same density was obviously reduced (**Table 4**).

Table 4. Quantitative analysis of the effect of CIK cells on the expression of PCNA in human liver cancer cell (mean ± SD)

Groups	GL (mRNA) level
HEGP2 cells uncultured together with DC-CIK cells	220.20 ± 10.86
HEPG2 cells uncultured together with DC-CIK cells in a same density	72.38 ± 3.24[a]
GAPDA	219.68 ± 10.29

[a]When compared with the control and the internal control, $P < 0.01$.

Figure 3. Effects of DC-CIK cells cultured together with HepG2 cells on the expression of PCNA and BAX protein in human liver cancer.

The expression ability of PCNA in HepG2 cells cultured together with DC-CIK cells was relatively weak but it is noteworthy that there was a relatively strong BAX antigen dye band. However, the expression ability of PCNA in uncultured HepG2 cells together with DC-CIK cells was very strong but the BAX protein expression level was relatively low (**Figure 3**). This finding suggests that when DC-CIK cells are cultured together with HepG2 cells, the DC-CIK cells inhibit the expression of PCNA and enhance BAX protein expression leading to suppression of the growth of HepG2 cells. Therefore, it can be deduced that the mechanism of action of DC-CIK cells in the treatment of advanced liver cancer is likely that DC-CIK cells enter the liver tissue and reduces PCNA expression and promotes the expression of BAX.

Discussion

Although advanced liver cancer patients are often treated with radiotherapy and chemotherapy or are operated on, the curative effect is very limited and the survival time far too short. In the present study, DC-CIK cellular therapy was proven to be effective in the treatment of primary advanced liver cancer with a DCR of 38.8%. The reason for the improvement of the curative effect is most likely that after DCs are co-cultured with CIK cells, the proportion of $CD83^+$, $CD86^+$ and $HLA2DR^+$ was increased, while the proportion of $CD14^+$ was reduced and $CD83^+$ and $CD86^+$ have the role of activating T cells. DC cells can stimulate the proliferation of primary T cells and thus establish the primary immune response, and also possess the ability to mi-grate to local T lymphocytes. CD3, the T cell surface marker and CD 56, the natural killer cell surface marker, coat the CIK cell surface, so that CIK cells have the characteristics of antitumor activity of T lymphocytes and the non-MHC limit of natural killer cells. After being cultured together with DCs, the proliferation capacity and the killing ability of CIK cells on targeted cells was greatly enhanced. This killing ability mainly relies on the T lymphocytes which can simultaneously express $CD3^+$ and $CD56^+$ [16].

For the method of DC-CIK cells cultured together, Wang et al. [13] deemed that compared with autologous DC-CIK cells co-cultured, semi-allogeneic DC-CIK cells significantly enhanced antitumor activity, increased the proliferation capacity of CIK cells, improved the ratios of $CD3^+$ $CD56^+$ cells, and $CD3^+$ $CD8^+$ cells, promoted the secretion of IFN-a and maintained the activity of CIK cells. The establishment of semi-allogeneic DC-CIK cells provides a basis for a new, safe and effective immunotherapy. Up to now in China, several studies have implied that the relative levels of $CD3^+$, $CD4^+$, $CD8^+$ and $CD56^+$ may be important in reducing the tumor burden. However, only some of these studies suggest that these changes will impact on clinical outcomes and prognosis [17]. Angela et al. [18] used idiotype-pulsed DCs from patients with melanoma to co-culture with autologous CIK cells. CIK cells were able to lyse cancer cells at the lower target ratio of 5:1. CIK cells showed high lytic activity against cancer cells, which could be enhanced by co-culturing with antigen-specific pulsed DC.

Finally, results from *in vitro* co-cultivation of DC-CIK cells and HEPG2 cells showed that when HEPG2 cells were cultured together with DC-CIK cells at half the density for 24 h and 48 h, respectively, the cell apoptosis ratio was statistically different ($P < 0.01$). When HEPG2 cells were cultured together with DC-CIK cells at the same or half density for 48 h, the cell apoptosis ratio was also statistically different ($P < 0.05$),

suggesting that DC-CIK cells derived substances could kill HEPG2 cells and induce apoptosis. Moreover, apoptosis showed a time-dependent and cell number-dependent trend (**Figure 1**). The mechanism of apoptosis is probably related to the change of PCNA and BAX expression. PCNA is an accessory protein of DNA polymerase and is also an essential substance during the DNA synthesis phase, which increases in the late G1 phase, reaching a maximum in the S phase, declining in the G2 phase, and could not be detected in the M phase. Therefore, PCNA is closely related with cell proliferation activity and is usually taken as the marker of the cell proliferation status [19, 20]. When HepG2 cells were co-cultured with DC-CIK cells, the content of PCNA was reduced showing that the descended ability of DNA synthesis by HepG2 cells is the likely mechanism that DC-CIK cells use to block the growth of HepG2 cells. The results of FCM analysis showed that the number of HepG2 cells was increased during the G1 phase (**Figure 2**), so that the HepG2 cells were mainly blocked in the G1 phase. Bax is a pro-apoptotic protein studied widely. In most tumor cells, BAX expression was reduced showing that the factors inhibiting tumor growth were weakened. In the present study, when HepG2 cells and DC-CIK cells were co-cultured, the BAX content was significantly increased showing that the ability to promote apoptosis of HepG2 cells was enhanced [21]. Our results have important significance. The way that the DC-CIK cells and HepG2 cells were co-cultured can effectively inhibit HepG2 cells in the G1 phase and simultaneously increase the expression of proteins, which in turn can promote apoptosis of HepG2 cells.

In our study, DC-CIK cellular autoimmunological therapy was proven to be effective clinically to treat advanced liver cancer. The *in vitro* experiments using semi -allogeneic DC-CIK cells co-cultured with HepG2 cells confirms the reason for the efficacy i.e. liver cancer cells were blocked at the G1 phase, PCNA was down -regulated and BAX protein expression was up-regulated. Thus, the proliferation and migration of liver cancer cells was effectively inhibited.

Acknowledgements

This research is supported by the foundation of Chongqing Application and Development Projects (cstc2014yykfA11003), Chongqing Municipal Health Bureau of Medical Science and Technology Research Project (2010-2-301) and Chongqing Health Bureau of Medical Research Major Project (2012-1-127).

Disclouse of conflict of interest

None.

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