Abstract. – OBJECTIVE: Primary liver cancer is a sort of the most common solid tumors occurred in the digestive system. The incidence and mortality rate maintain at a high level, thus leading to heavy economic and psychological burdens for patients. Next-generation biological therapy, such as cellular immune treatment, improves the medicine efficacy. Cytokine-induced killer (CIK) cells can effectively clear residual tumor lesion and inhibit metastasis or recurrence. Dendritic cells (DCs) can specifically eliminate tumor cells via modulating cellular immune function of the host. This study aimed to investigate the function of DC+CIK combined treatment on rat liver model and its effect on immune functions.

MATERIALS AND METHODS: RH-35 tumor cell was used to prepare live cancer model on Wistar rats, which were further divided into control, CIK and DC+CIK groups, in which rats received autograft CIK and DCs. Tumor size was later measured along with liver function index. The secretions of IFN-γ, IL-4, and TNF-α were measured by Real-time PCR and Western blotting.

RESULTS: Both CIK and DC+CIK treatment significantly reduced tumor size and improved liver function, increased secretion of IFN-γ, IL-4, and TNF-α, decreased Bcl-2 expression and enhanced Bax expression (p < 0.05 compared to control group). DC+CIK combined therapy presented significantly better efficacy than CIK did.

CONCLUSIONS: DC+CIK combined therapy can protect the host against tumors invasion via modulating body immune or liver function, regulating apoptosis/anti-apoptosis balance, which shows better efficacy than CIK alone, and can work as a novel biological therapeutic strategy for liver cancer.

Key Words: Liver cancer, Dendritic cells, Cytokine induced killer cells, Immune function, Apoptosis.

Introduction

Liver cancer represents the most common solid tumor in digestive tract, being a leading malignant tumor worldwide. Liver cancer can be further divided into primary liver cancer, which derived from epithelial or mesenchymal tissues with higher incidence in China. The overall mortality of liver cancer is still high, making it the second deadly tumor only next to pulmonary carcinoma. The progression of liver cancer involves a complicated pathological process, which is associated with factors such as genetics, physics, chemistry, environment and life styles. Moreover, hepatitis B, hepatitis C viral infection, alcohol, aflatoxin, sex hormone, liver cirrhosis, water pollution, nitrosamine compound and trace elements are all related with liver cancer pathogenesis. Among them, HBV infection is closely related to the occurrence of liver cancer. The pathogenesis and precise molecular mechanism of primary liver cancer are still unknown. Therefore, it is of critical importance to reveal the pathogenesis and the progression of liver cancer for further development and diagnosis/treatment.

With the progression of biological therapy, the targeted treatment on tumor has been shown with high efficiency, low toxicity and...
DC+CIK treatment of cancer

Side effects, which is promising for tumor treatment\(^{30}\). Cytokine induced killer (CIK) cells\(^{11,12}\) are endowed with a MHC-unrestricted antitumor activity, and belong to CD3+CD56+ subset of cytotoxicity lymphocytes\(^{13}\). The application of CIK cells in tumor treatment facilitates the removal of residual minimal lesion or metastatic tumor, thus inhibiting tumor metastasis or recurrence, improving immune potency of patients, and presenting the primary choice for immune therapy of tumors\(^{14}\). Dendritic cells (DCs) are a type of specific tumor killer cells, which contributes to the modulation of body cell immune functions. In addition to the induction of proliferation of antigen specific cytotoxic T lymphocytes, DC can also promote B cell proliferation and activation by direct or indirect ways, thus playing important roles in body defense against tumors via the modulation on both humoral and cell immune response\(^{15}\). A previous study\(^{16}\) showed co-culture of CIK cells and DCs favors cell proliferation and enhance immune functions. Therefore, we investigated the role of DC+CIK combined therapy on rat liver cancer model and its regulation on immune functions.

Materials and Methods

Animals

Healthy male Wistar rats (2 months age, SPF grade, body weight at 250±20 g) were purchased from Laboratory Animal Center of our University and were kept in a SPF grade facility. The room temperature was maintained at 21 ± 1°C while the relative humidity was maintained at 50-70% with a 12 h/12 h light/dark cycle.

Equipment and Materials

RH-35 tumor cells were purchased from ATCC Cell Bank (Manassas, VA, USA). Ficon lymphocyte separation buffer was purchased from Haoyang Bio (Guangzhou, Guangdong, China). Pentobarbital sodium and lidocaine were purchased from Zhaohui Pharm (Shanghai, China). Polyvinylidene difluoride (PVDF) membrane was a product of Pall Life Sciences (Port Washington, NY, USA). Western blotting reagents were purchased from Beijing (Beijing, China). ECL reagent was produced by Amersham Biosciences (Little Chalfont, UK). Rabbit anti-human Bel-2 monoclonal antibody, rabbit anti-human monoclonal antibody, goat anti-rabbit horseradish peroxidase (HRP)-labeled IgG secondary antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). IFN-\(\gamma\), recombinant human GM-CSF, IL-4, IL-2, IL-1 and anti-CD3 monoclonal antibody were purchased from Life Technology (Waltham, MA, USA). ELISA kit for IFN-\(\gamma\), IL-4 and TNF-\(\alpha\) were purchased from B&D (San Jose, CA, USA). RNA extraction kit and reverse transcription kit were all purchased from Axygen (Waltham, MA, USA). Microscopic surgical instruments were purchased from Suzhou Medical Inst. (Suzhou, Jiangsu, China). Microplate reader was purchased from BD (San Jose, CA, USA). ABI7500 fluorescent quantitative PCR was purchased from ABI (Waltham, MA, USA). Other common reagents were purchased from Sangon (Shanghai, China).

Animal Grouping and Treatment

Rat tumor cell RH-35 were prepared in \(1\times10^8\) ml suspensions, and were inoculated underneath the hepatic capsula for 0.04 ml \textit{in situ} as previously described\(^{17}\). After 2 weeks, a total of 45 liver cancer rats were randomly divided into 3 groups (\(N = 15\) each), namely, control group, CIK group (which received back-transfusion of autograft CIK cells), and DC+CIK group, which was back-transfused with CIK plus DCs.

Preparation of Autologous CIK Cells

15 ml blood samples were collected from rat-tail vein under sterilized conditions, and were kept in heparin-treated tubes. Ficon lymphocyte separation buffer was used to separate PBMCs. After washing in RMPI-1640, cells were re-assorted by autologous serum, and were seeded into 6-well plate at \(2 \times 10^6\) per ml. Under 2 h incubation in a humidified chamber with 5% \(\text{CO}_2\) at 37°C, the supernatant was discarded to obtain attached cells. Fresh medium containing 1000 U/ml recombi-
nant human GM-CSF and 500 U/ml IL-4 was added. The medium was changed every 2-3 days. Suspended cells were collected at day 7 as DCs.

**Back-transfusion of Autologous CIK Cells and DCs**

In CIK group, CIK cells were prepared into 25 ml suspensions by adding sterilized saline, and were back transfused to rats via tail vein. In DC+CIK combined group, autologous CIK cells and DCs were mixed at ratio of 5:1, and were prepared into 25 ml cell suspensions, which were then back-transfused to the rat via the tail vein.

**Liver Function Assay**

A fully automatic biochemical analyzer was used to record the change of liver function indexes from serum samples, which were collected 2 weeks before and after treatment.

**Observation of Tumor Growth**

After the occurrence of tumor, the growth pattern was observed every 3 days for 2 consecutive days. The long and short diameters (in mm) were measured in rat tumor and area was calculated by the long diameter × short diameter.

**Sample Collection**

Abdominal aorta blood samples were collected by vacuum tubes. After incubation at room temperature for 30 min until coagulation, the sample was centrifuged at 4°C for 10 min at 3600 rpm. The supernatant was collected and frozen at -20°C for further use. Liver tumor tissues were also collected and stored at -80°C for further use.

**ELISA Assay for Secretion Level of IFN-γ, IL-4 and TNF-α**

Rat serum samples were assayed for the measurement of IFN-γ, IL-4 and TNF-α levels using ELISA kit following manual instruction of test kit. Linear regression function was calculated based on standard concentrations and respective OD values. Sample concentrations were then calculated based on OD values on the regression function.

**Real-time PCR for Bcl-2 and Bax mRNA Expression**

TRIzol reagent was used to extract total RNA, which was used to synthesize DNA by reverse transcription based on manual instruction of the test kit. Primers were designed based on target gene sequence using Primer 6.0 software and were synthesized by Sangon (Shanghai, China) as shown in Table I. Real-time PCR was performed on target genes under the following conditions: 55°C for 1 min, followed by 35 cycles each containing 92°C 30 s, 58°C 45 s and 72°C 35 s. Data were collected by ABI 7500 fluorescent-quantitative PCR cycler and build-in software. Using GAPDH as the reference, fluorescent quantification was performed to calculate CT values of all samples and standards. The standard curve was firstly plotted followed by semi-quantitative analysis using 2^(-ΔΔCt) method.

**Western Blotting for Bcl-2 and Bax Protein Expression**

The protein from tumor tissue was extracted from all groups. In brief, lysis buffer was added to rupture cells on ice for 15-30 min, along with ultrasound treatment (5 s, 4 times). The mixture was centrifuged at 10000 g for 15 min at 4°C. The supernatant was transferred to a new tube for protein quantification and was kept at -20°C for storage. In Western blotting, proteins were firstly separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and were transferred to polyvinylidene difluoride (PVDF) membrane by semi-dry method. Non-specific binding sites were removed by 5% defatted milk powder for 2 h. Rabbit anti-human Bcl-2 (1:1000) or anti-human Bax monoclonal antibody (1:1500) was added for 4°C overnight incubation. After Phosphate-buffered saline and Tween 20 (PBST) washing, goat anti-rabbit secondary antibody (1:2000) was added for 30 min incubation at room temperature. ECL reagent was added for developing the membrane, followed by X-ray exposure. The result was obtained by protein imaging system and

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
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<tr>
<td>GAPDH</td>
<td>ACCAGGTATCTGCTGGTTG</td>
<td>TAACCATGATGTCGGTGGT</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>GACCTAATCTGACTGCTTG</td>
<td>TCCGGTTCACACTCTCTTTA</td>
</tr>
<tr>
<td>Bax</td>
<td>TTACATGAGTACTCTCTTA</td>
<td>TGTGACTGCTGCTTTCA</td>
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Table I. Primer sequence.
Quantity One software (Hercules, CA, USA) for measuring band density. Each experiment was replicated for four times (N = 4) for statistical analysis.

**Statistical Analysis**

SPSS19.0 software (IBM Corp. IBM SPSS Statistics for Windows, Armonk, NY, USA) was used for data analysis. Measurement data were presented as mean±standard deviation (SD). One-way analysis of variance (ANOVA) with LSD as post-hoc test was used for comparing means of multiple groups. A statistical significance was defined when $p < 0.05$.

**Results**

**The effect of DC+CIK Combined Therapy on Tumor Growth of Rat Liver Cancer**

We compared the effect of CIK treatment or DC+CIK combined therapy on tumor volume. Results showed that both treatments significantly retarded the increase of volume of tumors in rat liver cancer model ($p < 0.05$, compared to control group). DC+CIK combined therapy presented significantly better efficacy than CIK group ($p < 0.05$, Figure 1).

**Effect of DC+CIK Combined Therapy on Liver Function**

We analyzed the effect of autologous CIK and DC+CIK combined therapy on liver functions of rats. Results indicated that the combined therapy significantly depressed levels of ALT, AST and TBL, and increased ALB ($p < 0.05$ compared to control group). DC+CIK combined therapy showed more potent efficacy than CIK treatment ($p < 0.05$, Table II). This result showed that autologous DC+CIK combined therapy facilitated the recovery of liver functions in cancer model.

**Serum Secretion of IFN-γ, IL-4 and TNF-α in Liver Cancer Rats**

ELISA was used to detect change of serum levels of IFN-γ, IL-4 and TNF-α. We found that after CIK or DC+CIK therapy, serum levels of IFN-γ, IL-4 and TNF-α were significantly increased ($p < 0.05$ compared to control group), the levels of which were statistically elevated in the group with DC+CIK combined therapy compared to that with CIK treatment ($p < 0.05$, Figure 2A to Figure 2C). These results collectively suggested that autologous CIK-DC combined therapy could facilitate both cellular and humoral immune response and exert anti-tumor effects.

**Effect of DC+CIK Combined Therapy on Bcl-2 mRNA Expression in Liver Cancer Rats**

Real-time PCR was used to detect the expressional change of Bcl-2 mRNA in rat model of liver cancer. Results revealed that both CIK and DC+CIK treatments significantly suppressed Bcl-2 mRNA level in tumor tissues ($p < 0.05$ compared to control group). DC+CIK combined therapy contributed to better inhibition of Bcl-2 level than CIK group ($p < 0.05$, Figure 3).

### Table II. Effect of DC-CIK combined therapy or CIK treatment on liver functions.

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>TBL (µmol)</th>
<th>ALB (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>97.5 ± 11.8</td>
<td>101.6 ± 15.6</td>
<td>34.1 ± 9.7</td>
<td>32.5 ± 2.3</td>
</tr>
<tr>
<td>CIK</td>
<td>72.1 ± 9.1*</td>
<td>83.5 ± 11.3*</td>
<td>23.2 ± 3.2*</td>
<td>35.5 ± 6.6*</td>
</tr>
<tr>
<td>DC-CIK</td>
<td>48.6 ± 10.3**</td>
<td>62.3 ± 9.5**</td>
<td>12.6 ± 5.1**</td>
<td>42.7 ± 3.5**</td>
</tr>
</tbody>
</table>

*Note:* *,$p < 0.05$ compared to control group; #,$p < 0.05$ compared to CIK group.
Bax mRNA Expression of DC+CIK Combined Treatment in Liver Cancer Rat Model

Real-time PCR was also performed to quantify the expression level of Bax mRNA in liver cancer rat model. Results indicated that both CIK and DC+CIK treatments significantly enhanced Bax mRNA level in tumor tissues ($p < 0.05$ compared to control group). DC+CIK combined therapy presented stronger induction of Bax level than CIK group did ($p < 0.05$, Figure 4).
Protein Expressions of Bcl-2 and Bax in Rat Liver Cancer Tissues

Western blotting was also performed to measure the expression profile of Bcl-2 and Bax proteins in rat liver cancer tissues. Results showed that similarly to the change in Bcl-2 and Bax mRNA, both CIK and DC+CIK treatment significantly elevated Bax protein expression in tumor tissues, and depressed Bcl-2 protein expression ($p < 0.05$ compared to control group). Significantly favorable proapoptotic effect was shown in the treatment of DC+CIK compared with that in CIK group ($p < 0.05$, Figure 5 and Figure 6).

Discussion

Current treatment for liver cancer includes surgery, radio-, chemo- or intervention therapy; all of which, however, had unsatisfactory efficacy such as high recurrent and metastatic frequency, and worse prognosis, leading to shorter survival span, worse life quality of patients, as well as heavy economic burdens. So far, there have been few effective drugs targeting liver cancer. The establishment of anti-liver cancer reagent was thus of critical importance for improving treatment efficacy. Immune therapy has been recognized as a type of promising treatment measure for tumors as it can elongate patient survival time and improve life quality. CIK cells were induced by IL-2, IFN-γ and CD3 monoclonal antibody. In addition to its MHC unrestricted tumor lytic effect, it also presents specific anti-tumor effects concentrated on tumor site. Current study believed efficient proliferation of CIK cells in vivo compared to other immune therapy cells such as LAK or CD3AK, along with the abundant secretion of cytokines with the properties of tumor killing effects, which were independent of exogenous IL-2 cytokine stimulus and were absence of toxicity effects. CIK cell therapy has been shown to have satisfactory efficacy in breast cancer, pulmonary carcinoma or colon cancer when being used singly or in combination with chemotherapy. DCs are the most potent antigen presenting cells (APCs) and are the only APC sub-type to activate junior T cells. It can induce the proliferation and differentiation of cytotoxic T cells and B cells via direct or indirect pathways. Researchers found that DC could significantly potentiate CIK proliferation and accelerate its maturation. Therefore, the combination of DC with CIK cells conducts high effective cytotoxicity and exerts synergistic effects against tumor growth. The function and immune modulation of DC+CIK in rat liver cancer model, however, has not been illustrated. Thus, we firstly generated a rat liver cancer model through in vivo transplantation of tumor cells, followed by the back-transfusion of autologous DC+CIK cells. Our data revealed that DC+CIK combined therapy significantly reduced tumor size and improved liver function indexes, suggesting that the combined therapy of autologous CIK and DC could exert anti-liver cancer effects, in addition to the function on liver recovery. Further study found that DC+CIK combined therapy could elevate serum levels of IFN-γ, IL-4 and TNF-α in serum of liver cancer rats. As an important regulatory factor for cell immune response, IFN-γ could facilitate Th0 cells to differentiate into Th1 cells, while IL-4 could induce the Th2 cell differentiation from Th0 cells. Both Th1 and Th2 cells are significantly involved in cellular and humoral immune response, respectively. Therefore, these results showed that DC+CIK combined therapy could up-regulate both cellular...
and humoral immune response during the inhibition of tumors. The over-expression of Bcl-2 and inhibition of Bax are closely related with anti-apoptosis/apoptosis imbalance of liver cancer cells, as the disruption of such balance causes abnormal cell activity\(^2\). This work at mRNA and protein levels demonstrated that DC+CIK combined therapy decreased Bcl-2 while elevated Bax in liver cancer tissues, suggesting an anti-tumor effect via proapoptotic function as consistent with previous study\(^2\).

**Conclusions**

DC+CIK combined therapy could exert anti-tumor effects via modulating apoptosis and anti-apoptosis balance, as well as improving body immune functions, which may serve as a novel biological therapy for liver cancer in the future.

**Acknowledgements**

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**Conflict of Interest**

The Authors declare that they have no conflict of interests.

**References**


