In vivo anti-tumor effect of DC-CIK cells on human lymphoma cell line Raji

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Abstract: To research on the effect of DC-CIK cells on human lymphoma cell line Raji the immunophenotype of DC-CIK cells was analyzed using flow cytometry, and its proliferation inhibition effect was detected using MTT assay. 24 nude mice (4-6 weeks old) were employed and inoculated Raji cells on right axillaries for constructing human Burkitt lymphoma model. MTT results showed that DC-CIK cells had a significant inhibitory effect on Raji cells with obvious dose- and time- dependent effect. Western Blot results confirmed that DC-CIK cells could significantly down regulate the expression of BCL-2 (P<0.05). DC-CIK cells possesses significant anti-tumor effect on human Burkitt lymphoma bearing nude mice, and down regulation of Raji induced BCL-2 cell apoptosis may be one of the inhibitory mechanisms of DC-CIK cells.

Keywords: Immunotherapy, DC-CIK cells, lymphoma, nude mice.

INTRODUCTION

Malignant Lymphoma (ML) is a malignant tumor of hematopoietic system, which is primary in lymph nodes and extranodal tissues or organs with invasion and high heterogeneity, and there is an upward trend of incidence in recent years. It can be divided into Hodgkin's lymphoma (HL) and non Hodgkin's lymphoma (NHL) according to clinical pathological characteristics. Burkitt lymphoma is one common type of NHL. The response rate of existing treatment methods has been reported to be only 40%, where higher dosage of chemotherapy could not eliminate lymphoma cells completely. Consequently, minimal residual disease (MRD) might cause a significant proportion of relapse in patients after several months or years, and 60% still dead of this disease (Siemińska et al., 2015; Yin., 2014).

Mobilization of the body's immune system via cellular immunotherapy could lead to the improvement of tumor microenvironment and anti-tumor immunity, and ultimately to control and kill tumor cells. There are four development phases in cellular immunotherapy, including lymphokine-activated killer cell (LAK), tumor infiltrating lymphocytes (TIL), cytokine-induced killer cell (CIK) and dendritic cells (DC) cytokines induced killer cell, and its clinical effect enhanced from phase to phase. CIK cells possess high proliferation capacity and high cytotoxicity. Compared with LAK and TIL cells, the kill activity of CIK cells was more potent (Blusková et al., 2014; Wang et al., 2015; Kowalczyk et al., 2017). DC is the most powerful antigen presenting cell so far. It can induce the proliferation of T lymphocytes in resting stage and produce specific cellular immunity as an initiator and a participant in immune response (Baxi et al., 2015). It has been found that the amplification ability CIK could be enlarged and its cytotoxicity was significantly enhanced after combining its high efficiency with the strong tumor antigen presenting ability of DC (Neilands et al., 2014).

The treatment of colon cancer, hepatic carcinoma and chronic hepatitis b using DC-CIK cells (Bittar, et al., 2014; KM Sands et al., 2015) have been reported, but few of Burkitt lymphoma. This study therefore seeks to evaluate the antitumor effect and safety of DC-CIK cells on Burkitt lymphoma bearing nude mice and explore its mechanism, while throwing more light on the clinical application of adoptive cellular immunotherapy. Burkitt lymphoma model was established in the axilla of Balb/c- nu/nu nude mice in this study. The model was given DC-CIK cells, cyclophosphamide and physiologic saline intervention respectively after the tumor was formed in nude mice. Tumor volume changes and adverse reaction of tumor bearing mice were observed.

MATERIALS AND METHODS

Materials
Tumor cell line: Burkitt lymphoma cell line Raji (purchased from Beijing union medical college cell resource center) was cultured in RPMI-1640 medium with 10% fetal bovine serum.

Experimental animal
24 male Balb/c- nu/nu nude mice (4-6 weeks old, 17-21 g, purchased from Chinese Academy of Medical Sciences Laboratory Animal Center) were fed at specific pathogen free animal (SPF) environment.

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Main reagents
RPMI-1 (Blusková et al., 2014; Wang et al., 2015; Kowalczyk et al., 2017).
640 medium (GIBCO, America); fetal bovine serum (HyClone, America); recombinant human interferon-γ (IFN-γ) (Strthmann Biotech GmbH, Germany); Mouse Monoclonal Antibody against Human CD3 antigen (Wuhan Institute of Biological Products); recombinant human IL-2 (Beijing Sihuan Biological Engineering Products Company); recombinant human IL-4 (Shanghai Science Peptide Biological Technology Co., LTD); recombinant human TNF-α (Suzhou Biological Products Co., LTD); lymphocyte separation medium (Shanghai Hengxin Chemical Reagent Co., LTD); monoclonal antibody against BCL-2 and rabbit-anti-mouse conjugated antibody (Cell Signaling Technology, America); healthy full-term birth umbilical cord blood was supplied by our hospital maternity.

The establishment of Burkitt lymphoma cell line Raji bearing mice model
Raji cells were resuspended in PRMI-1640 culture medium after centrifugation and counting, and then the cell concentration was adjusted for the injection in each Balb/c-nu/nu nude mice right anterior axillary with 1×10^7 cells. After the tumor formation, 25 nude mice were randomly divided into 3 groups: 9 mice were treated with DC-CIK cells (experimental group), 8 in cyclophosphamide chemotherapy group (positive control) and 8 in normal saline group (blank control).

Culture of DC-CIK cells from umbilical cord blood
Latezza et al. 2016
15mL healthy full-term birth umbilical cord blood (applying heparin as anticoagulant) was collected after maternal delivery with given consent from their family. It was diluted and mixed with the same volume of normal saline solution, and mononuclear cells were separated by centrifugation using a lymphocyte separating medium. Conventional washing were performed twice using normal saline and then the cell density was adjusted to 2 × 10^9/L with RPMI 1640 complete medium, followed by incubation in a humidified incubator at 37°C in 5% CO₂ for 3h. The non-adherent cells were collected and transferred to new culture flasks. DC were induced by adherent cells after adding recombinant GM-CSF and recombinant IL-4 with final concentration of 1.0×10^5 U/L and 50µg/L, respectively. Then, the medium was added with complete culture medium containing 5×10^5 U/L IL-2 every 3 days. DC activated CIK cells (DC-CIK) were obtained after the mixture of DC and CIK with the ratio of 1:20 at the ninth day, and its amplification was performed by adding recombinant IL-2 with the concentration of 2.5 × 10^5 U/L.

Detection of DC-CIK cells surface antigen
DC-CIK cells were collected and counted after co-cultured for 6 days. Flow cytometry was applied to analysis the expression of CD3 and CD56 in DC-CIK cells.

Detection the cytotoxic effect of DC-CIK cells
The cell suspension was collected after DC and CIK cells were co-cultured for 4 days, and MTT assay was employed to measure the lethal effect of DC-CIK cells with different concentrations and different action time (12 h and 24h) on target cell (Raji cells). The effector and target ratios were 10:1, 20:1 and 50:1, respectively. An enzyme mark instrument was further applied to measure the absorbance of the liquid culture medium at 570 nm for cytotoxicity rate analysis.

Grouping and intervention of tumor bearing mice
Mice in DC-CIK cells treatment group were given subcutaneous injection of DC-CIK cells at right axillary beside the tumor at the ninth day after bearing, and the effector cells were given every two days with 6×10^7 per mouse. Cyclophosphamide chemotherapy group was administrated intraperitoneal injection of cyclophosphamide at a dose of 75mg/kg every 3 days while the Normal control group received intraperitoneal injection of normal saline (0.2ml per mouse). All groups were treated 3 times.

Effect of DC-CIK cells on the expression of apoptosis protein BCL-2 in Raji cells
Raji cells were collected after the action of DC-CIK cells, and were washed twice by precooling PBS, followed by lysis for 30min. Then the protein concentration of supernatant was quantitated by conventional BCA method after the centrifugation at 4°C, 11600 rpm for 20 min. The protein was separated by 12% SDS-PAGE gel electrophoresis and transferred to nitrocellulose membrane by half dry transfer printing method. Subsequently, it was blocked overnight with buffer containing 5% skim milk, followed by hatch overnight with BCL-2 monoclonal antibody and β-actin antibody at 4°C, respectively. Then, the membrane was washed 3 times for 10 min. The conjugated antibody marked with biotin was washed 3 times for 10min per time after hatching at 37°C for 1h. Chemiluminescence method was employed to color the exposure, and Gel-proanalyzer software was used for gray
analysis, while statistical analysis was carried out with reference to β-actin.

Observation index
Mobility, eating and defecation situation of experimental animals were observed alongside tumor formation at the site of injection every 3 days. The tumor volume was measured every day, with Vernier caliper, until the volume was approximately equal to major axis × minor axis\(^2/2\). The average tumor volume of nude mice at different time points were measured and the tumor growth curve was drawn accordingly.

STATISTICAL ANALYSIS
SPSS 18.0 software was used for statistical data processing. All the data were shown with mean and standard deviation, and the comparison among groups was performed using single factor variance analysis of randomized block design. \(P<0.05\) was considered statistically significant.

RESULTS

Observation of tumor formation and experimental animals
The tumor was formed at 16-19 days after the inoculation of Raji cells, and the average time was 17.4±0.9 day (table 1). All the mice of blank control and chemotherapy groups were observed with tumor formation, while one mouse without tumor formation was observed in DC-CIK cells treatment group. The total tumor formation rate was 96.0%. The nude mice had no significant reduction in activity. Diet and pellet morphology were normal after inoculation, and there was no significant change in body weight compared with initial.

Cytotoxic effect of DC-CIK cells
In this study, DC-CIK cells acted as the effector cells and cytotoxicity test was carried out with effector-target ratio of 10:1, 20:1 and 50:1, respectively. The results (fig. 1) showed that DC-CIK cells had cytotoxic activity to Raji cells, and its cytotoxic effect depended on effector-target ratio. The cytotoxicity of DC-CIK cells was the strongest with effector-target ratio of 50:1, and the \(P\) value was less than 0.05 compared with the other two groups. It was obvious that the cytotoxicity decreased with effector-target ratio. Additionally, the longer the action time, the stronger the cytotoxicity of DC-CIK cells on Raji cells. These results showed that there was an obvious dose- and time-dependent cytotoxic effect of DC-CIK cells.

DC-CIK cells phenotype
DC-CIK cells from umbilical cord blood were analyzed by flow cytometry after co-culture for 6 days. The results showed that double positive rate of CD3\(^+\)CD56\(^+\) was 32.6% and single positive rate of CD3\(^+\) was 98.2%.

Therapeutic action of DC-CIK cells on burkitt lymphoma bearing nude mice
It was found that the tumor volume growth rate of normal saline group was the fastest after calculating the tumor volumes and drawing its growth curve. On the other hand, the tumor volume growth rate of cyclophosphamide group and DC-CIK cells group were significantly slower compared with the control group, with DC-CIK cells treatment group exhibiting the slowest growth rate (fig. 2). Furthermore, it was shown that the tumor weight of DC-
**In vivo anti-tumor effect of DC-CIK cells on human lymphoma cell line Raji**

CIK cells and cyclophosphamide groups was decreased significantly compared with normal saline group after dissection and weighing. There was no obvious difference between DC-CIK cells group and cyclophosphamide group. The tumor weight of DC-CIK cells treatment group was statistically significant (P<0.05) compared with normal saline group (fig. 3).

**Adverse reaction**
There was no significant adverse reaction in tumor bearing nude mice during the treatment period, and the skin of the injection area remained same without ulceration, congestion, edema or nodule formation.

**Influence of DC-CIK cells on the expression of apoptotic protein BCL-2**
In order to further explore the mechanism of antitumor effect of DC-CIK cells on Burkitt lymphoma, western blot was employed to measure the level of apoptotic protein BCL-2 in Raji cells after being treated with DC-CIK cells (fig. 4). Gray value of stripe was analyzed with Gelproanalyzer software, and its results showed that the area percentage value of protein expression of BCL-2/β-actin was 1.36±0.07, and that of DC-CIK cells treatment group was 0.59±0.07, which was statistically significant (P<0.05) compared with control group.

**DISCUSSION**
Burkitt lymphoma is a highly aggressive B cells lymphoma and the treatment effect of high-dose chemotherapy with stem cells support in refractory-relapsed patients has been so limited with no great improvement in the prognosis of Burkitt lymphoma. Series of studies have shown that adoptive cellular immunotherapy, as a new powerful weapon in the treatment of malignant tumor after traditional treatment methods (such as surgery, chemotherapy and radiotherapy), could effectively clear MRD and improve the organism immune status (Laterza *et al.*, 2016). Due to its high speed proliferation and antitumor activity, DC-CIK cells are getting more and more attention as immune effector cells in antitumor therapy (Peng *et al.*, 2015).

Previous reports have established that the secretion level of IL-12 in CIK cells was significantly increased after co-cultured with DC cells, and the killing activity of CIK cells on target cells was also significantly enhanced (L Tan *et al.*, 2014). Another clinical study showed that the complete remission rate (CRR) after 3 years was up to 75% in acute leukemia patient who received treatment of DC-CIK cells in paracmasis, and their median survival time was 26 months (Takamura *et al.*, 2013). However, there has been no *in vivo* study on the treatment of Burkitt lymphoma with DC-CIK cells. This study was aimed at verifying the antitumor effect of DC-CIK cells *in vivo*, and primarily exploring the possible antitumor effect mechanism of DC-CIK cells by detecting the changes of proliferation and apoptosis of Raji cells, as well as the novel therapeutic strategy of Burkitt lymphoma.

Nude mice are the most commonly used tumor bearing animals since its transplanted tumor growth could not be influenced by autologous cellular immune system due to the dysfunction of T cells. The Burkitt lymphoma bearing...
nude mice model was successfully established in this study, and the time of tumor formation was generally at the range of 16-19 days. The tumor was given different interventions (normal saline, cyclophosphamide and DC-CIK cells, respectively) after formation, and the measurement of tumor volume was followed by the drawing of tumor growth curves. It was found that the tumor growth rate of DC-CIK cells treatment group was slower and its tumor weight was significantly reduced with statistical difference. In addition, the significant inhibitory effect of DC-CIK cells on Raji cells was further confirmed by in vitro experiment, and exhibited obvious dose- and time-dependent effects.

BCL-2 is a gene firstly found in follicular B cells lymphoma. Overexpression of BCL-2 may not only inhibit the apoptosis of various cells, but also have relation with multidrug resistance of tumor cells (Montanari et al., 2010; Elboga et al., 2015) In order to further explore the mechanism of tumor inhibition effect of DC-CIK cells, this study examined the expression of BCL-2 protein in Raji cells after treatment with DC-CIK cells. Results showed that the expression of BCL-2 was significantly downregulated by DC-CIK cells, suggesting that one of the antitumor mechanisms of DC-CIK cells might be apoptosis of Raji cells induced by the down-regulation of BCL-2.

In this study, the in vivo and in vitro experiments demonstrated that the DC-CIK cells could promote apoptosis by the activation of endogenous apoptotic pathway, and inhibit the proliferation of Burkitt lymphoma line Raji cells thereby, further lowering the tumor growth rate of tumor bearing nude mice. The findings in this study supply a new idea for the clinical and therapeutic schedule of Burkitt lymphoma. However, its complete mechanism of inducing apoptosis still needs to be studied further.

REFERENCES


