Original article

Effects of Norcantharidin on Proliferation and Anti-tumor Activity of Cytokine-induced Killer Cells in Vitro

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ABSTRACT **objective :**To explore the effects of Norcantharidin (NCTD) on proliferation and killing activity of cytokine-induced killer cells (CIK). **Methods** :Mononuclear cells of Cord blood were extracted by Ficoll density gradient method and then induced into CIK cells. The experiment was divided into NCTD+CIK group with a final concentration of 30 μmol/L NCTD and control group CIK without NCTD. The changes of cell proliferation, immune phenotype and cytotoxic activity of CIKs were observed using cell counting, flow cytometry and MTT assay, respectively. **Results:**The rate of cell proliferation and anti-tumor activity in NCTD+CIK group were significantly higher than those in the control CIK group ($P<0.05$). These results suggested that NCTD promoted the proliferation of CIK cells and enhanced their anti-tumor activity, which will provide a new approach for adoptive immunotherapy of CIK.

Key Words: Norcantharidin; Cytokine-induced killer cells; Cell proliferation; Anti-tumor activity

One of the commonly used traditional Chinese medicine cantharidin (CA), has a crucial function of lossing sore, expelling blood Sanjie as well as anti-tumor activity. However, it limited its clinical application due to CA's high toxicity and severe irritation to the urinary system[1]. Norcantharidin (NCTD) derived from the hydrogenated adduct of furan and maleic anhydride, was short of two methyls at the site of 1 and 2 compared with CA, but showed the same three-dimensional configuration. Extensive attention was paid for its reduced toxicity, strong inhibition of tumors and a good whitening effect [2-3]. To date, no report was found about the effects of NCTD on cytokine-induced killer cells (CIK). Therefore, this paper will explore the effects of NCTD on proliferation and cytotoxic activity of CIK.

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MATERIALS AND METHODS

Sampling

Cord blood with heparin (20u/mL) anticoagulant were collected under sterile conditions from healthy pregnant woman after birth of normal full-term fetus in Liwan Hospital of Guangzhou Medical College. Human chronic myelogenous leukemia cell line (K562) was conventionally preserved in our laboratory.

Main reagents and apparatus

rhIFN-γ, rhIL-β, rhIL-2 and CD3mAb were provided by Peprotech Company. NCTD (Shandong Lunan Pharmaceutical Company) were dissolved in RPMI-1640 medium (Gibco) and dubbed into 3.2 mmol/L stock solution after sterile filtration. FBS and human lymphocyte separation medium were purchased from Tianjin Hao Yang Biological Products Co., Ltd. MTT, dimethyl sulfoxide (DMSO) were obtained from Sigma Company. Fluorescent monoclonal antibody (CD3+, CD56+, CD4+ and CD8+), Flow cytometry (FACSCalibur) and the corresponding software were products of BD Company.

Preparation and identification of CIK cells

Mononuclear cells were extracted by Ficoll density gradient method from 50 mL of fresh cord blood and were adjusted to 5×106 cells /mL with RPMI-1640 medium. After IFN-γ(1000 U/mL) was added, mononuclear cells were placed in 37℃, 5% CO2 incubator overnight. The next day, the suspension of the cells were transferred to 6 well plates and were added with rhIL-β (100 u/mL), rhIL-2 (300 u/mL) and CD3mAb (50 ng/mL) respectively. Medium was half-changed every 3 days and the equal rhIL-2 were added as describe above. 7 days latter, the suspended cells were collected and observed under the microscope for identification of CIKs.

CIK cell proliferation and phenotype testing

CIK cells were adjusted to 5×106/mL after 7 day's culture and 100 μL of CIKs were seeded in 48 well plates. 24h later, 100 μL of RPMI-1640 medium and NCTD (with the final concentration of 30 μmol/L) were added to the control CIK group and NCTD+CIK group, respectively. Each group has 24 parallel wells and was incubated in 37℃, 5% CO2 incubator. Half medium was changed every two days and equal amount of medium or NCTD was added correspondingly. On the 10th, 12th, 14th and 21th day, CIKs were counted with trypan blue staining. Meanwhile, CIKs in the control group and NCTD+CIK group were washed with PBS solution respectively, incubated with FITC or PE labeled monoclonal antibody CD3 +, CD56+, CD4 + and CD8 +, at 4℃ for 30 min, and then tested with flow cytometry assay.

Detection of cytotoxic activity of CIK cells

After 10 days of culture, CIKs were divided in A group (without NCTD) and B group (with a final concentration of 30 μmol/L of NCTD) and continued to incubate in 5% CO2 incubator at 37℃. 24 h, 36 h, 48 h, 60 h and 72 h later, CIKs were collected in A and B group respectively and adjusted to 2×106 cells/mL. In addition, K562 cells at exponential growth phase were also adjusted to 1×105 cells/mL. CIK cells in group A and B were mixed with K562 cells at a ratio of 20:1 respectively and seeded in a 96 well plate. After incubation for 24 h at 37℃ and 5% CO2, each well added with 150 μL MTT (5 mg/mL) continued to incubate for 4 h and then added with 150 μL DMSO. A value at a wavelength of 570 nm for each group was measured on the microplate reader. The kiling rate of CIK cells (%) = [A value of target cells(K562)+ A value of effector cells(CIK)－A value of the experimental group(CIK+ K562)] / A value of target cells(K562)×100%.

Statistical analysis

SPSS12.0 statistical package were used for data analysis. $\bar{x}$ ±s and t test were used and P <0.05 shows statistically significant.

RESULTS

Effect of NCTD on proliferation Of CIK cells

It was found that NCTD+CIK and control CIK cells both grew slowly and their expansion was not obvious at initial stage of culture. 4～5 days later, NCTD+CIK proliferate quickly, grew larger and became a great number of cell colonies. However, CIKs in the control group had a slower proliferation and smaller volume (Fig.1 and Fig.2). When cultured for 14 ~ 21 days, growth rate of CIK reached its peak, the average amplification times of CIKs and NCTD+CIKs were 36.8±2.5 and 101.3 ± 7.3 days respectively, which indicated that the proliferation rate of NCTD+CIKs was significantly higher than that of the control group CIKs, and the difference was statistical significance (P <0.05).

Immunophenotype analyses of CIK cells

As showed in Table 1, NCTD+CIK and CIK cells after 10 days of culture, the amount of CD3+CD4+ cells were higher than that of CD3+CD8+ cells. As the culture time increased, the proportion of CD3+CD8 and CD3+CD56+ cells increased gradually, while that of CD3+CD4+ cells decreased. On the 14th day, CD3+ CD8+ cells in NCTD+CIK group had a percentage of (58.34 ± 4.12) %, in which CD3+CD56+ cells (the main effector of CIK cells) were up to (21.29 ± 1.25) %, which was significantly higher than that in the control group CIK (P < 0.05).

Effects of NCTD on anti−tumor activity of CIK cells in vitro

MTT assay showed that the killing activity of CIKs in the experimental group NCTD+CIK and the control group without NCTD was both associated with the culture time (Fig.3). At initial stage of culture, the cytotoxicity of NCTD+CIK cells on target cells K562 was weak and it enhanced gradually over time. After 48 h, the cytotoxicity reached its peak, and then began to decline. The killing activity of NCTD+CIK were significantly higher than that of the control CIK (P<0.05), which indicated that NCTD can enhance the anti-tumor activity of CIK cells.

DISCUSSION

Cytokine-induced killer cells (CIK) is a group of heterogeneous

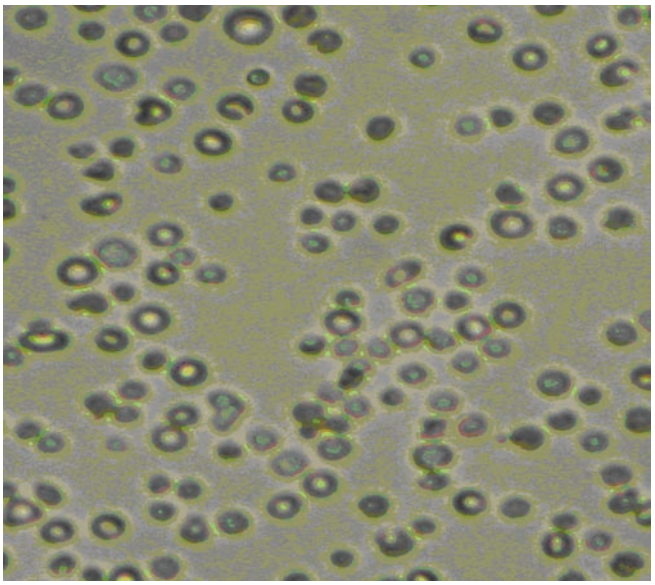

Fig.1 The morphology of control group CIK cells (X400)

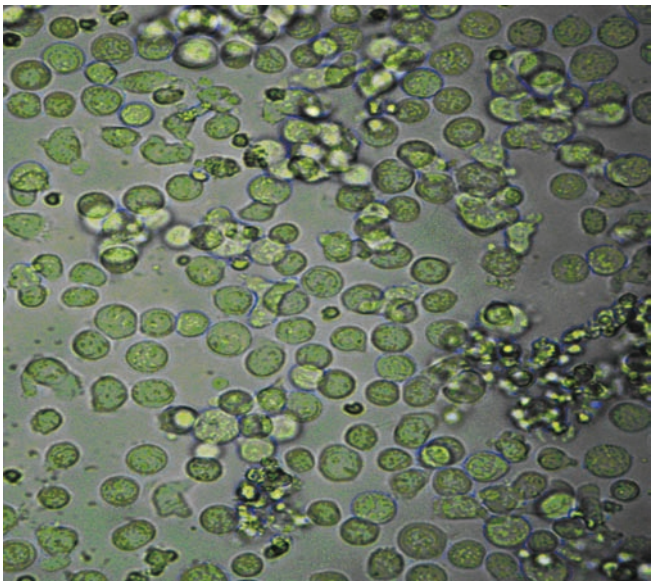

Fig.2 The morphology of experimental group CIK cells treated with NCTD (X400)

Table 1 Effects of NCTD on immunophenotype of CIK cells (%, $\bar{x}$ ±s)

culture time	CIK			NCTD+CIK		
(day)	CD3+CD4+	CD3+CD8+	CD3+CD56+	CD3+CD4+	CD3+CD8+	CD3+CD56+
10	26.54±2.32	21.15±1.03	6.15±0.09	24.18±1.62	22.36±1.98	7.38±0.52
12	21.14±1.83	34.18±2.35	10.18±1.12	20.15±1.72	48.16±2.23	12.32±1.06
14	16.38±1.82	45.09±4.23	12.63±1.04	15.36±1.22	59.34±4.12**	21.29±1.25**
21	14.22±1.13	50.92±3.84	14.37±1.18	13.50±1.03	68.83±5.08**	26.67±1.84**

** means P<0.05 compared to CIK group

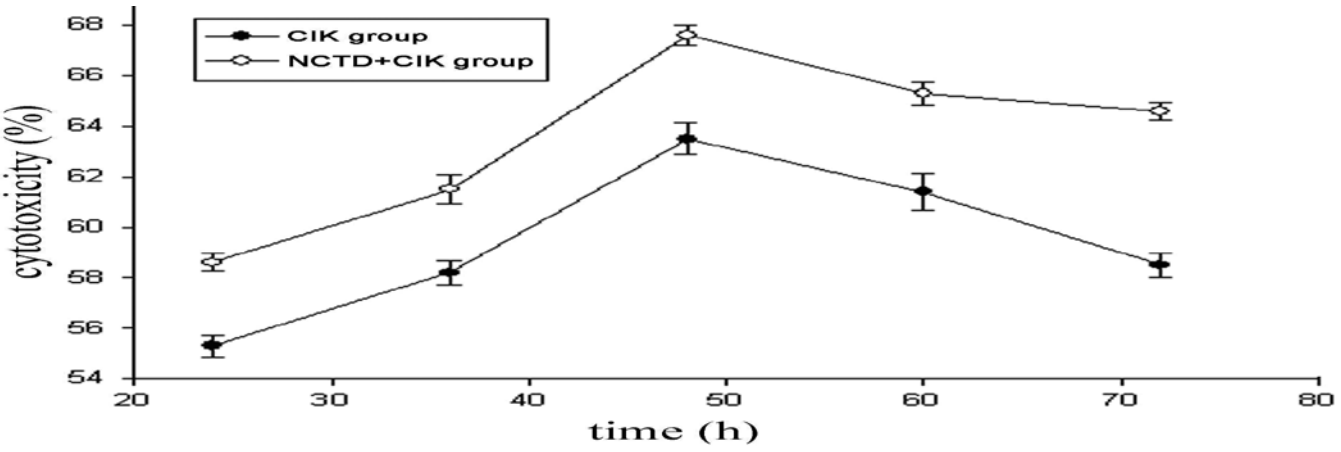

Fig. 3 Effects of NCTD on cytotoxic activity of CIK cells

cells induced by a variety of cytokines and antibodies (IFN-γ, IL-2, IL-1α and CD3McAb)[4]. It expresses two membrane protein molecules (CD3 and CD56), and is also known as NK-like T lymphocytes, which not only has strong killing activity of T lymphocyte, but also has a non-MHC restricted characteristic of NK cells [5]. CIK cell has a rapid proliferation, a strong anti-tumor activity, and a broad spectrum of killing tumors, which is regarded as another more efficient immune cells in comparision with LAK, TIL, and CD3AK cells [6-7]. Meanwhile, CIK has been used as the preferred option for tumor adoptive immunotherapy in present. However, although some cancer patients received CIK adoptive immunotherapy, the vast majority of patients suffered little significant improvement clinically, which may be related to the input amount of CIK cells, their killing activity or other factors. Thus, how to improve the amplification and cytotoxic activity of CIK cells has become one of the hot issues of tumor immunotherapy.

NCTD is a new anti-tumor drug synthesized through demethylation at the site of 1 and 2 of the traditional Chinese medicine cantharidin. It reserves the anti-cancer function with reducing adverse reactions and elevates the amount of white blood cells as well as immune function[8-9]. Previous studies showed that NCTD could kill K562 and Raji cells which were sensitive to NK and LAK cells respectively in a dose dependent manner, which suggested that NCTD has a potential cytotoxic stimulation to lymphocytes[10]. Liu et al.[11] found that combination therapy of NCTD, the immune modulator (TCDO) and chemotherapy drugs (ELP) markedly prolonged the survival time of DBA/2 mice infected by Friend Leukaemia Virus (FLV), which demonstrated that NCTD and ELP can inhibit retrovirus infection and enhance immune function. In addition, some studies have reported that Chinese herbs such as astragalus and ginseng saponins possess immuno-modulatory effects, and they can promote T cells proliferation, improve the immune activity of T and TIL cells, increase LAK cell expansion, activate the production of tumor necrosis factors, and lead to dramatically enhanced anti-tumor effects [12-13].

Our study showed that NCTD+CIK had much higher proliferation and killing activity than that of CIK cells without NCTD treatment, and the difference was statistically significant. Moreover, on the 14th day of culture, the expression level of surface-related antigens such as CD3+CD8+ and CD3+CD56+ on NCTD+CIK cells was significantly higher than that on the control CIKs ($P < 0.05$), and the tendency of their changes of immunophenotype was similar to that of anti-tumor activity in vitro. Therefore, NCTD can improve CIK cells proliferation, increase their with the previous report on NCTD promoting cytotoxicity of peripheral blood lymphocytes[9], but further studies are required urgently to illustrate the molecular mechanism of NCTD enhancing anti-tumor effect of CIK cells, anti-tumor activity, and thereby enhance immune function, which is consistent.

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