

Research Article

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The possible molecular regulation mechanism of CIK cells inhibiting the proliferation of Human Lung Adenocarcinoma NCL-H157 Cells

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Abstract: Cytokine-induced killer (CIK) cells were isolated and proliferation from human peripheral blood and cultured in appropriate growth medium. The biological characteristics of CIK cells were further determined by the characterization of surface markers by flow cytometry. CIK cells inhibited the proliferation of human lung adenocarcinoma NCL-H157 cells. Vascular endothelial growth factor (VEGF) expression was down-regulated in CIK cells co-cultured with NCL-H157 cells by western blotting analysis. Furthermore, in comparison with cells untreated by CIK, the NCL-H157 had a lower proliferation capacity. We proposed that the pharmacological mechanisms of NCL-H157 promoted by CIK can be estimated possibly with different biological significance that can be ascribed to down-regulated VEGF expression *in vitro*. The results suggest that the VEGF pathway guides developmental inhibiting of NCL-H157, and we speculate that the function of VEGF pathways is to guide NCL-H157 to inhibition by abundant CIK.

Keywords: NCL-H157, inhibition, CIK, VEGF

1 Introduction

Human lung cancer is one of the most common malignant diseases and a leading cause of death annually in much of the developed world especially in China [1]. Adenocarcinoma, the most common form of lung cancer, is one of main human malignant tumors, and its occurrence and development are highly correlated with inactivation of tumor suppressor genes. Many genes play a role in negatively regulating lung tumor growth and apoptosis [2-3]. Beyond that, there is little that can be definitively said. The standard test to confirm multipotency is differentiation of the cells into osteoblasts, adipocytes, and chondrocytes, as well as myocytes and neurons. A type of immunological cell, cytokine-induced killer (CIK) cells are a heterogeneous cell population that was first discovered in the 1990s and can be generated from lymphocytes co-cultured with an anti-CD3 antibody and many other cytokines *in vitro* [4-5]. Numerous studies have demonstrated that CIK cells exhibit active and potent antitumor cytotoxicity against multifarious tumor cells *in vitro* and *in vivo* [6]. CIK cells alter the cytokine secretion profiles of dendritic cells, T cells, and natural killer cells to induce a more anti-inflammatory or tolerant phenotype. This phenomenon has been documented in specific cells and tissues in living animals and their counterparts growing in cell culture [7].

It is well known that CIK cells used in biological systems are required to be anti-tumor. More and more scientists are devoting themselves to the field of growth and differentiation of NCL-H157. *In vitro*, CIK cells have greater expansion capability and a faster growth rate. However, the conquest of NCL-H157 by CIK is still a great challenge to medical science. In this study, we evaluated the inhibition of NCL-H157, which was significant with prolonged cellular doubling time and the possible molecular regulation mechanism of the proliferation of NCL-H157 by CIK.

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2 Materials and methods

2.1 CIK cells isolated and expanded?

CIK cells were isolated and cultured according to standard protocol. Peripheral blood (50 mL) was drawn from the patients with heparin as an anticoagulant. Mononuclear cells were isolated by Ficoll-Conray (GE Healthcare, Fairfield, CT, USA) density gradient centrifugation, and then the remaining cells were washed twice with phosphate buffer saline (PBS). The viability and concentration of mononuclear cells were determined with trypan blue and a hemacytometer. Approximately 2.0×10^6 /mL suspension and mononuclear cells were cultured in six-well culture dishes with medium containing RPMI 1640 plus 1.0×10^6 U/L human interferon gamma (IFN- γ), 10% heat inactivated fetal bovine serum, 25 mM HEPES and 2 mM L-glutamine and incubated overnight at 37 °C in 5% carbon dioxide. After 24 hours, 50 μ g/L monoclonal antibody (MAb) against CD3 and rhIL-2 (1×10^6 U/L) were added. Fresh complete medium with rhIL-2 (1×10^6 U/L) was changed every 2 to 3 days, and the cells were harvested on day 7, 14 and 21. All CIK cell cultures were tested for contamination (bacteria, fungi, and mycoplasma) throughout the study to assure culture quality and transfusion safety. The morphological changes were observed under an inverted phase contrast microscope at 200 \times magnification.

2.2 CIK cell surface maker detected by flow cytometer measurement

In order to assay the percentages of cell surface antigen, FITC- and APC-stained cells were analyzed. After washing with ice-cold PBS two times, CIK cells were re-suspended in 200 μ L binding buffer (10 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂) and co-incubated with 10 μ L FITC-annexin V (25 g/mL) and 5 L propidium iodide (PI, 50 g/mL) in the absence of light for 15 minutes at room temperature. To confirm the cellular identity of cultured cells, CIK cells were subjected to fluorescence-activated cell sorting using CD3FITC, CD56APC positive markers. Finally, fluorescent intensities of stained cells were analyzed with a FACScalibur Flow cytometer (Becton Dickinson Corp., USA).

2.3 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay

A 200 μ L suspension of NCL-H157 cells per well was seeded in a 96-well plate at a density of 5×10^4 cell/mL. NCL-H157 cells were grown for 12 hours after seeding, and then treated with CIK. After a 24-hour incubation, the medium was removed, and the cells were treated with CIK cells for 72 hours. Effector-to-target cell ratios (E/T ratio) ranged from 10:1, 20:1 and 30:1. NCL-H157 cells were re-suspended in a fresh culture medium after being centrifuged at 1000 rpm for 10 minutes; 20 μ L MTS (5 mg/mL) was added to each well, and incubation was allowed to continue for an additional 4 hours. Finally, all media were removed by the centrifugation; 150 μ L DMSO was added to each well and shaken for 10 minutes. The absorbance was read at a wavelength of 550 nm with a Benchmark Microplate Reader (Bio-Rad Corp. USA).

2.4 Western blotting analysis to detect vascular endothelial growth factor (VEGF) in NCL-H157 treated by CIK cells

Western blotting analysis was performed to evaluate the influence of DMOG on the expression of VEGF in NCL-H157 cells. The cells were cultured in regular medium with the addition of different concentrations of CIK (concentration of effector-to-target cell ratios (E/T ratio) ranged from 10:1, 20:1 and 30:1). The total protein was harvested from the cultured cells according to standard protocols. Then the protein concentration was measured with a bicinchoninic acid (BCA) *protein* assay kit (Thermo Scientific, Waltham, MA, USA). The cell lysates were separated on SDS-PAGE with 12% gels and transferred to nitrocellulose membranes. The membranes were then incubated with primary antibodies of β -actin (1:1000, Santa Cruz, USA), VEGF (1:500, Santa Cruz, USA). Then the membranes were incubated with gentle agitation for 2 hours at 37°C with horseradish peroxidase-conjugated secondary antibody diluted in 5% skim milk powder at 1:7500. After washing three times in Tris-Buffered Saline-Tween-20 (Beyotime, China) (10 minutes each wash), the membranes were developed by an enhanced chemiluminescence (ECL) western blotting detection system. Immunoreactive proteins were then detected by ChemiDoc MP System #170-8280 (Bio-Rad, USA). Images were captured and analyzed by Quantity One software (Bio-Rad, USA).

2.5 Statistical Analysis

All of the *in vitro* experiments were performed in triplicate, and data were presented as the mean \pm standard deviation (SD) of three independent experiments. Wherever appropriate, the data were subjected to statistical analysis by a one-way analysis of variance (ANOVA) test followed by the Student-Newman-Keuls test for comparison of all pairs of means. A value of $P < 0.05$ was considered to be statistically significant. SPSS 11.5 for Windows software was used for the statistical analysis. P -values < 0.05 were considered significant.

Ethics Statement: The study was approved by the institutional review board (CWO) of Hebei Chest Hospital Ethics Committee, Shijiazhuang, The People's Republic of China. All people provided written informed consent.

3 Results

3.1 Isolation and expansion of CIK cells with cytokine induced methods

Cultured CIK cells began to grow within 3 days and entered the proliferation stage within 5 days. Following 7 days of culture, the proliferation rate of CIK cells significantly grew faster and faster. After 21 days, cultured cells reached a density of $(149.42 \pm 4.59) \times 10^7$. Cells were harvested and examined under an inverted microscope, which revealed that the cells grew as colonies in suspension (Figure 1). Cell volume was markedly increased over time, and the survival rate of cultured cells was $> 95\%$.

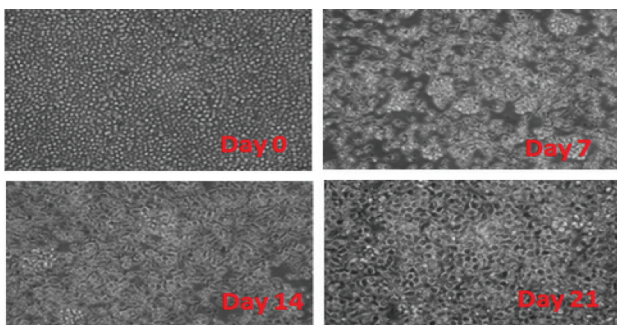


Figure 1: Representative image of CIK cell morphology at days 0, 7, 14 and 21 of culture (magnification, $\times 100$).

3.2 Immune phenotype of CIK cells

Intensive and strict studies on the immune phenotype of CIK cells have been conducted. The CIK cells were analyzed by flow cytometric analysis and gated for granularity, size and surface markers. The gated cells were analyzed for the expression of cell membrane protein markers and found to be positive for the expression of a heterogeneous cell population, comprising $CD3+CD56+$, which are generally considered markers of CIK cells (Figure 2). $CD3+CD56+$ T cells expression of CIK cells was $39.2 \pm 1.1\%$.

3.3 CIK inhibited the proliferation of NCL-H157 cells

The effects of CIK on the viability of NCL-H157 cells were evaluated by MTS assay (Figure 3). The results showed that exposure to CIK for 48 hours resulted in a dose-dependent increase in CIK, with a statistically significant difference between different groups. Compared to the untreated control in DMEM/F12 cultured medium, the MTS assay showed CIK was more active against or with NCL-H157 cells in an ascending order. Figure 2 shows the effect of CIK on the inhibition of NCL-H157. As shown in Figure 2, when NCL-H157 was incubated with CIK, dose-dependent decreases of NCL-H157 viability from 21.4%, 54.0% and 71.3% were observed after increasing the CIK concentration of effector-to-target cell ratios (E/T ratio) ranging from 10:1, 20:1 and 30:1, respectively, compared with NCL-H157 in the control group. The concentration-dependent decreases of cell viability were observed as shown in Figure 2.

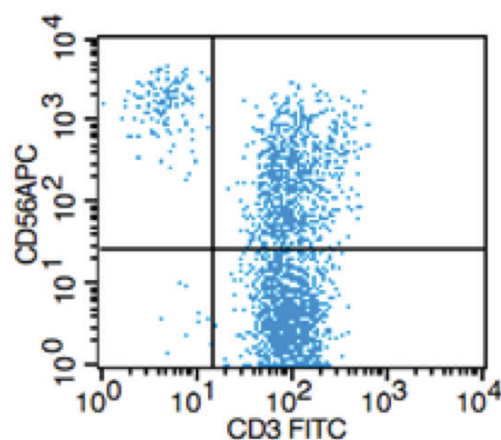


Figure 2: Flow cytometry was used to detect the surface antigen of CIK. CIK can be characterized by a panel of surface markers, which is positive for markers CD3 and CD56.

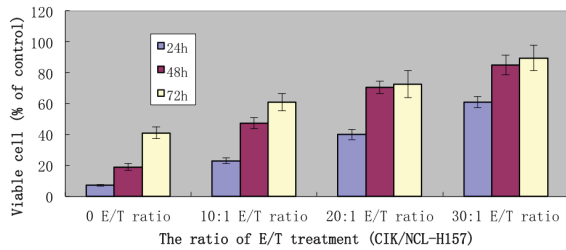
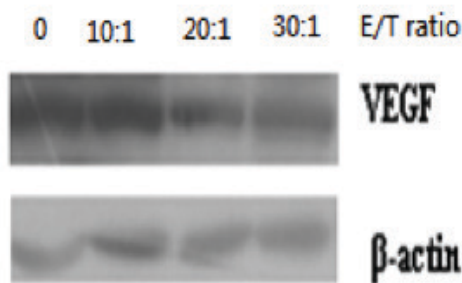


Figure 3: Effects of CIK on NCL-H157 viability ($n = 5$). Cell viability of NCL-H157 treated with different CIK concentrations of effector-to-target cell ratios (E/T ratio) ranged from 10:1, 20:1 and 30:1, for 24h, 48h, and 72h. (*, significant difference between the two groups, $p < 0.05$)

Also, the time-dependent decreases of cell viability were observed as in Figure 2. The cell viability was decreased from 66.8% to 79.8% to 86.9% after the incubation time 24 hours, 48 hours and 72 hours, respectively.

3.4 Molecular expression changes in CIK co-cultured with hUCNCL-H157

To evaluate the influence of CIK on the expression of VEGF in NCL-H157, the protein levels of VEGF in NCL-H157 treated with different concentrations of CIK were detected by western blotting. The results showed the level of VEGF protein was enhanced in response to CIK treatment in a dose-dependent manner. VEGF is a molecule associated with the proliferation of NCL-H157 and the important proteins associated with it. In order to discover the molecular mechanism underlying the proliferation effect of CIK on NCL-H157 *in vitro*, we investigated the expressions of the proteins in NCL-H157 by western blotting (Figure 4 A). The results showed that CIK co-cultured with NCL-H157 by transwell chamber for 48 hours resulted in the



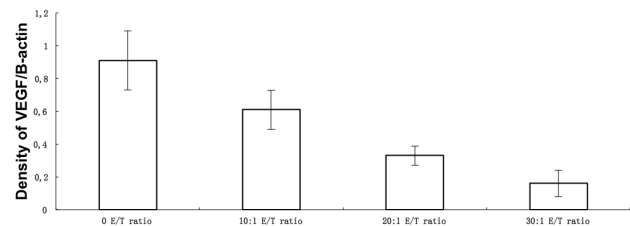
A

significant down-regulation of VEGF as compared to controls. We found increased VEGF levels in culture media of NCL-H157 cells. Irradiated NCL-H157 showed an inverse pattern, that is, decrease of VEGF.

4 Conclusions

To our knowledge, this is the first study of inhibition of NCL-H157 treated by CIK. In the event our study is viewed as merely phenomenological, we stress that our experiments were carefully designed to facilitate the inhibition of NCL-H157 cells and to record these effects, as well. Instead of focusing on NCL-H157 activity, we were more interested in following the inhibition of NCL-H157 that involves cell signal pathways and the breakout function of biological material-CIK cell. We found that the CD3+CD56+ subset of CIK cells was variably naturally active. The CD3+CD56+ subset could be polarized toward either Th1 or Th2 phenotype that, in turn, shapes its anti-tumor activity [8-9].

In this study, we assessed the NCL-H157 proliferation inhibited by CIK using the inverted phase contrast microscope and flow cytometer. NCL-H157 cells were detected after incubation with CIK at designed concentrations and times. CIK plays a critical role in providing the essential micro-environment for NCL-H157 at different CIK concentrations of effector-to-target cell ratios (E/T ratio) ranging from 10:1, 20:1 and 30:1 for 24 hours, 48 hours, and 72 hours, respectively. These images are shown in Figure 1. These results were further confirmed by MTS analysis as shown in Figure 2. The percentage of NCL-H157 in the control group was less than the treated group. When the incubation time was prolonged to 72 hours, a marked



B

Figure 4: A shows VEGF over-expression in NCL-H157 treated by CIK with western blotting analysis. In order to elucidate the pathway leading to proliferation, we examined the activation of VEGF, which was reported to initiate NCL-H157 generation by various stimuli. NCL-H157 treated with CIK were analyzed for the enzymatic activity by western blot. The expression of VEGF was not activated after CIK treatment in NCL-H157 was down-regulated. The activity of VEGF had significantly changed. B shows densitometric analysis of the levels of VEGF by an image analysis system. ($n=6$. Mean \pm SD. * $P < 0.01$ vs control group) (ANOVA with subsequent multiple comparisons test)

decrease in the percentage of NCL-H157 cells was observed in CIK-treated groups. The results obtained from MTS analysis suggest that CIK can induce lack of proliferation of NCL-H157, which is consistent with the observation with the inverted phase contrast microscope. In the present study, however, the percentage of inhibition viability cells is not much more than that of the control group.

CIK cells establish an appropriate scaffold and a complex network of cytokines, adhesion molecules, and extracellular matrix proteins. We showed that a VEGF pathway controls the proliferation of NCL-H157 treated by CIK [10-11]. NCL-H157 express some genes, suggesting the genicity and the superiority for clinical use. CIK cells appear to have cytotoxic effects, indicating their superior role in the management of clinic treatment, and CIK cells were examined for safety and efficacy for carcinoma treatment [12-15]. We observed markedly enhanced anti-tumor cytotoxic activity of CIK cells after co-culture with NCL-H157. Furthermore, CIK regulates NCL-H157 activity in a time- and concentration-dependent manner. Effective adoptive cell transfer faces numerous challenges, such as systemic immune tolerance and tumor local immune escape. The homing of immune cells to the tumor site is reduced, and the antitumor immune functions are inhibited by tumor microenvironment and immunomodulatory properties of suppressive cell populations [16-18]. Collectively, these results demonstrate that VEGF signals coordinate and regulate the dynamics of CIK during VEGF-induced migration of NCL-H157 in varying states of differentiation. Genetic experiments indicate that the VEGF pathway functions independently of pathways governing NCL-H157 inhibition. Future studies using other gene expression should address the extent of the relative stiffness of NCL-H157 mobility as seen in our experiments and elucidate the understanding of the mechanism of NCL-H157 inhibition.

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Competing financial interests: The authors declare that they have no competing financial interests.

References

- [1] Jemal A, Bray F, Center MM, Ferlay J, Ward E, et al. Global cancer statistics. *CA Cancer J Clin* 2011; 61(2): 69-90
- [2] Zohre S, Kazem NK, Abolfazl A, Mohammad RY, Aliakbar M, Effat A, Zahra D, Hassan D, Nosratollah Z. Trichostatin A-induced Apoptosis is Mediated by Kruppel-like Factor 4 in Ovarian and Lung Cancer. *Asian Pac J Cancer Prev* 2014;15(16):6581-6586
- [3] Zongjuan M, Meihua J, Wei L, et al. Bioinformatics analysis and expression study of fumarate hydratase in lung cancer. *Thoracic Cancer* 2014;5(6):543-549
- [4] Mesiano G, Todorovic M, Gammaitoni L, Leuci V, Giraudo DL, et al. Cytokine induced killer (CIK) cells as feasible and effective adoptive immunotherapy for the treatment of solid tumors. *Expert Opin Biol Ther* 2012; 12: 673-684
- [5] Ardiani A, Gameiro SR, Kwilas AR, Donahue RN, Hodge JW. Androgen deprivation therapy sensitizes prostate cancer cells to T-cell killing through androgen receptor dependent modulation of the apoptotic pathway. *Oncotarget* 2014;5(19):9335-9348
- [6] Zoll B, Lefterova P, Csipai M, Finke S, Trojaneck B, Ebert O, Micka B, Roigk K, Fehlinger M, Schmidt-Wolf GD, Huhn D, Schmidt-Wolf IG. Generation of cytokine-induced killer cells using exogenous interleukin-2, -7, or -12. *Cancer Immunol Immunother* 1998; 47: 221-226
- [7] Dengrui L, Sumin G, Hui L, et al. Effect of inhibition proliferation in human lung adenocarcinoma A549 cells by cytokine-induced killer cells. *Thoracic Cancer* 2014;6(4):458-463
- [8] Wang Z, Liu JQ, Liu Z, Shen R, Zhang G, et al. Tumor-derived IL-35 promotes tumor growth by enhancing myeloid cell accumulation and angiogenesis. *Immunol* 2013;190: 2415-2423
- [9] Li H, Yu JP, Cao S, Wei F, Zhang P, et al. CD4+CD25+ regulatory T cells decreased the antitumor activity of cytokine-induced killer (CIK) cells of lung cancer patients. *J Clin Immunol* 2007;27(3): 317-326
- [10] Escoubas P. Molecular diversification in CIKs: a web of combinatorial peptide libraries. *Molecular Diversity* 2006;10 (4): 545-554
- [11] Zhao P, Bu X, Wei X, Sun W, Xie X, et al. Dendritic cell immunotherapy combined with cytokine-induced killer cells promotes skewing toward Th2 cytokine profile in patients with metastatic non-small cell lung cancer. *Int Immunopharmacol*. 2015;25(2):450-456
- [12] Ai YQ, Cai K, Hu JH, Jiang LW, et al. The clinical effects of dendritic cell vaccines combined with cytokine-induced killer cells intraperitoneal injected on patients with malignant ascites. *Int J Clin Exp Med*. 2014;7(11):4272-81
- [13] Tomita Y, Yuno A, Tsukamoto H, et al. Identification of promiscuous KIF20A long peptides bearing both CD4+ and CD8+ T-cell epitopes: KIF20A-specific CD4+ T-cell immunity in patients with malignant tumor. *Clin Cancer Res*. 2013; 19 (16): 4508-4520
- [14] Lu G, Xing J, Liu GQ, et al. Clinical Efficacy of DC and CIK Immunotherapy Combined with Chemotherapy and Its Impact on Treg Cells in Newly Diagnosed Multiple Myeloma. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 2015 ;23(3):737-741
- [15] Suehiro Y, Hasegawa A, Iino T, et al. Clinical outcomes of a novel therapeutic vaccine with Tax peptide-pulsed dendritic cells for adult T-cell leukaemia /lymphoma in a pilot study. *Br J Haematol* 2015;169(3):356-367
- [16] Chen Q, Wang L, Ma Y, Wu X, Jin L, Yu F. Increased hepcidin expression in non-small cell lung cancer tissue and serum is associated with clinical stage. *Thoracic Cancer* 2014;5(1):14-24

- [17] Li W, Wang Y, Zhao L, et al. Efficacy of RetroNectin-Activated Cytokine- Induced Killer Cell Therapy in Metastatic Brain Tumor Patients. *Oncol Res Treat* 2015;38(4):160-165
- [18] Wang S, Zhang H, Liu C, Jiao X, Liu D, et al. Human leukocyte antigen- haploidentical donor-derived cytokine-induced killer cells are safe and prolong the survival of patients with advanced non-small cell lung cancer. *Oncol Lett.* 2014;8(6):2727-2733