**Original Article**

# The Antitumor activities and Effects of CIK Cells Against Gastric Cancer

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**ABSTRACT** **Aim:** To characterize the anticancer function and anti-proliferation effect of cytokine-induced killer cells (CIK cells) on MGC-803 cells. **Methods:** Peripheral blood mononuclear cells (PBMC) from healthy donors and patients with Gastric Cancer were incubated in vitro and induced into CIK cells in the presence of various cytokines and monoclonal antibody (mAb) against CD3. The phenotype and characterization of CIK cells were identified by flow cytometric analysis. The mechanisms were studied with a tetrazolium dye-based (MTT) assay. Morphological changes were observed with inverted microscope, and electron microscope. The TdT-mediated dUTP nick and labeling (TUNEL) method was used to detect the apoptosis-induced by CIK cells. **Results:**The CIK cells were shown to be a heterogeneous population with different cellular phenotypes. The percentage of CD3+/CD56+ positive cells, the dominant effector cells, in total CIK cells from healthy donors and gastric cancer patients, significantly increased from 0.1～0.13% at day 0 to 19.0～20.5% at day 21 incubation. After 28 day in vitro incubation, the CIK cells from patients with gastric cancer and healthy donors increased by more than 300-fold and 500-fold in proliferation cell number, respectively. CIK cells originated from gastric cancer patients possessed a higher in vitro antitumor cytotoxic activity on autologous gastric cancer cells than the autologous lymphokine-activated killer (LAK) cells and PBMC cells. Inverted microscope observation showed that CIK cells were closer to the target cells and formed a typical “rose” shape. The scanning electron microscope showed that most target cells had undergone apoptosis and many “apoptotic bodies,” and that transmission electron microscopy showed condensed chromatin, disintegration of the nucleolus, vacuoles in the cytoplasm, and apoptotic bodies appearing in most target cells. TUNEL analysis showed that apoptotic cells contract and turn navy blue in nuclei or perinuclei.

**Conclusion:** CIK cells induce apoptosis and have an antiproliferative effect on human MGC-803 gastric cancer cells and might serve as an alternative adoptive therapeutic strategy for Gastric Cancer patients.

**KeyWords:** CIK cells; gastric cancer cell; MGC-803; apoptosis; in vitro

## Introduction

With the development of tumor immunology, molecular biology, and techniques of gene engineering, cytokine therapy is one of the promising antitumor means in molecular therapeutics. In recent years, many studies have demonstrated that some factors, such as chemical drugs, oncogenes, cytokines, traditional medicines, and others can regulate cell apoptosis. Cellular immune therapy has become widely studied since the discovery that lymphocytes were shown to control the regression and metastasis of tumors if recombinant interleukin-2 (IL-2) was given to animals with nonimmune systematical tumors. In 1991[1], the marrow transplantation program at the Stanford University School of Medicine (Stanford, CA) first reported that cytokine-induced killer (CIK) cells have a strong antiproliferative capacity and cytotoxicity. Gastric cancer is the most common malignant tumor of alimentary tract in China, with an average annual mortality rate

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of 25.16/100,000 nationwide, and produce great harm to people's health. Studies on inducing apoptosis of gastric cancer cells were started recently, which mainly focus on the research of in vitro cells, and the induction methods include: radiation, biological response modifiers, chemotherapy drugs, and so forth. With the rapid advance of molecular biology technology, the application of cytokines in the control of cancer is thought to be a promising strategy for cancer treatment. However, further studies have demonstrated that CIK cells produced by lymphocytes with the stimulation of multiple cytokines has an absolute advantage over cytokines or lymphokine-activated killer (LAK) cells activated by IL-2 alone, as far as their activity of killing tumor cells is concerned; thus it has great prospects for the application in tumor treatment and is receiving increasing attention. CIK cells, a group of heterogenous cells derived from the incubation of human peripheral blood mononuclear cells with various cytokines (such as CD3McAb, IL-2, interferon gamma (IFN-γ), and so forth) in vitro, are non-major histocompatibility complex (non-MHC) and non-T cell receptor (non-TCR) restricted immunologically competent cells, which mainly composed of CD3+ and CD56+ T-cells, so it can be assumed that CD56 is an important antigen for CIK cells to enhance cytotoxic activity. For the CIK cells cultured in this study, the effect of CD3 was to promote proliferation, while the effect of IFN-γ was to enhance cytotoxic activity, which was the most powerful NK cells activator. IFN-γ can enhance the cytotoxic activity of CIK cells only if added 24 hours prior to the addition of IL-2. CIK cells themselves can also secrete many cytokines, such as IL-2, interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF), so as to enhance cytotoxic activity or modulate the sensitivity of tumor cells to CIK cells.

Apoptosis, which is also called programmed cell death, is referred to as a process in which the death mechanism in a cell is initiated with the stimulation of an apoptosis signal and results in programmed cell degeneration and death through a series of signaling pathways[2].

Cytokine-induced killer (CIK) cells are the major histocompatibility complex-unrestricted cytotoxic lymphocytes and generated by incubation of peripheral blood monocytes (PBMC) in the presence of various types of cytokines such as CD3 monoclonal anti-body, interleukin-2 (IL-2), interleukin-1 (IL-l) and interferon-gamma[3]. CIK cells are the population of heterogenous effector cells possessing enhanced cytotoxicity and a higher proliferation rate as compared with lymphokine-activated killer(LAK) and tumor infiltrating lymphocytes (TIL) cells. The high anti-tumor activity of CIK cells is mainly due to the high proliferation of double CD3+ and CD56+ positive cells. Some reports indicated that CIK cells, other than LAK and TIL cells, can be efficiently employed as an adjuvant in anticancer immunotherapeutic strategy for the eradication of residual cancer cells and prevention or delay of tumor relapse.

This study investigated CIK cells inducing human gastric cancer MGC-803 morphological changes by light microscope, inverted microscope, electron microscope, and in vitro cell apoptosis detection (TUNEL) and explored some possible mechanisms involving gastric cancer cell apoptosis by immunohistochemistry. This study will provide an experimental basis for future clinical tumor therapy.

## MATERIALS AND METHODS

### *Medicine and Reagents*

Recombiant human interleukin-2, mouse antihuman CD3 McAb, and lymphocytes separating medium were purchased from Beijing BangDing Biology Medicine Co. Ltd. (Beijing, China). Recombinant human IFN-γ was purchased from Shanghai Clone Biology High-Tech Co. Ltd.(Shanghai, China). RPMI medium and IMDM medium were purchased from Life Technologies, Inc. (Grand Island, NY). Fetal calf serum (FCS) was purchased from Lanzhou Minhai Biologyl Engineering Co. Ltd. (Lanzhou, China) MTT was purchased from Sigma (St. Louis, MO). An in vitro cell apoptosis detection kit was purchased from Bohringer Mannheim Inc. (Indianapolis, IN). Lymphocytes separating medium was purchased from the Blood Institute, Chinese Academy Institutes (Beijing, China).

### *Cell culture*

MGC-803 gastric cancer cell line was supplied by Prof. Yinchang Zhang of the Tumor Institutes of the Chinese Medical University (Shenyang, China). Leukocytes extraction component was supplied by volunteer donors in the YanBian state (Yanji, China). Preparation of effector cells: peripheral blood mononuclear cells (PBMC) were separated from a component of white blood cell (WBC) of a healthy person through density gradient centrifugation with the lymphocyte-separating medium (1.077 ± 0.001 in density), dissolved, and kept in IMDM medium (containing 10% FCS). PBMC was adjusted to 1 × 106/ml in concentration, supplemented with 2000 U/mL IFN-γ at day 0, cultured for 24 hours in an incubator with 5% CO2 in air at 37°C. At day 1, 5U/mL CD3 McAb and 1000 U/mL IL-2 were added to the medium for future culture. A half-dose of the medium was changed every 3 days, 1000 U/mL IL-2 was supplemented and cell concentration was adjusted to 1 × 106/ml. The cells were harvested after 15 days.

*Cell separation*

The patients with gastric cancer were submitted to cytopheresis after their writing contents were signed. An enriched peripheral blood mononuclear cells (PBMC) product was collected with specific program of the Cobe Spectra blood separator. Cells were re-suspended in phosphate buffered saline (PBS) without calcium and magnesium. The separation of PBMC was performed as previously described. The concentrated PBMC cells were used immediately for CIK cell culture.

*Generation of cytokine–induced killer (CIK) cells*

CIK cells were generated as described previously. Briefly, non-adherent Ficoll-separated human peripheral blood mononuclear cells were prepared and incubated in RPMI1640 medium containing 100ml•L-1 FCS and various types of cytokines added according to the reported protocol with minor modifications. The final concentrations of the cytokines and antibody added were as follows:IL-2,1000×103U•L-1; IL-1, 100×103U•L-1; IFN-γ, 100×103U•L-1; mAb CD3, 50μg•L-1. Cells were incubated at 37 in a humidified atmosphere of 50ml•L-1 CO2 and fed every 3 days in fresh complete medium with 100ml•L-1 FCS and various types of cytokines at 0.5×109cells•L-1.

*Phenotype analysis*

Cells were obtained from CIK cultures for phenotype analysis with the appropriated monoclonal antibodies, including CD3-FITC, CD4-FITC, CD8-PE, CD56-APC, and CD56-PE. One million CIK cells were washed once in PBS containing 1% bovine serum albumin (BSA) and resuspended in 100μL of PBS/BSA buffer. The cells were incubated with various conjugated monoclonal antibodies for 20 minutes at 4℃, washed twice in PBS, and resuspended in 400μL of PBS. A flow cytometric analysis was performed on a FACSCalibur flow cytometry (BD Biosciences), and the data were analyzed using the WinMDI statistical software (Scripps, La Jolla, CA, U.S.A.). Forward and side scatter parameters were used to gate on live cells (Han et al., 2005b).

*Invert microscope*

CIK cells and MGC-803 gastric cancer cells were seeded into a 12-well plate. Both of The samples were observed by invert microscope.

*MTT Assay*

Gastric cancer cells in a logarithmic growth period were stained with trypan blue (MTT) and counted. A gastric cancer cell suspension with a concentration of 10×104 cells/mL and CIK cultured for 15 days at a concentration of 1×106/mL effector cells were mixed in a 96-well plate at ratios of 10:1, 20:1, and 40:1. Eight wells of target cells and 8 wells of effector cells were cultured at 37°C, with 5% CO2 for 24 hours. Twenty milliliter (5 mg/mL) of MTT was added at 48 hours. After another 4 hours of culture, the supernatant was removed and 160 uL of dimethyl sulfoxide (DMSO) was added following 10 minutes of oscillation. The optical density (OD) value was determined with an enzymelinked immunosorbent assay (ELISA) detection machine at a wavelength of 490 nm. The kill rate was calculated according to the formula: inhibition rate (100 %)=[1-(A value in experimental well-A value in effector cell wells)/A value in target cell wells]×100%.

*Scanning invert microscope by immunofluorescence staining*

CIK cells and target cells were seeded into a 24-well plate at a ratio of 40:1 for 5, 14, and 24 hours, cultured at 37℃, with 5% CO2, and observed in invert microscope after immuneofluorescence staining at declined times. Simply cultured gastric cancer cells were stained with bioluminescence at proliferative phase.

*TUNEL Staining*

After the interaction of CIK cells and target cells at a ratio of 40:1 for 5, 14, and 24 hours, the cells were concentrifugated, smeared, dried at room temperature, fixed with 4% formalin for 30 minutes, and then underwent TUNEL staining. All the procedures were performed according to the manufacturer's instruction. Positive substance for cell apoptosis presented as blue, located in the nucleus. Ten fields were selected for each smear in a 40×10 high-fold microscope to count apoptotic cells and total cell number, so as to calculate the apoptosis index (AI) and its mean value (AI%=the number of apoptotic cells / the total number of cells× 100%). Gastric cancer tissue section containing positive substance was used as a positive control, and TdT buffer not containing TdT in replacement of TUNEL reaction mixture was used as a negative control.

*Statistical Analysis*

SPSS 11.0 software was used. The comparison of inhibitory rate detected by MTT assay was performed with a t test; the comparison of AI was carried out with analysis of variance

(ANOVA) and a paired q test; an intergroup comparison was disposed with an x2 test. The Wilcoxon matched-pairs test was used to analyze for statistical significance. The P value less than 0.05 was considered as significant difference.

## RESULTS

*Proliferation and phenotype of CIK cells*

Figure 1A and B showed the proliferation of CIK cells from healthy donors and patients with gastric cancer at different incubation days. During cell generation, there was a steady increase in both the absolute number and the percentage of CD3+/CD56+ cells, e.g. the percentage of CD3+/CD56+ cells was 7.5% on 14d and 51.3% on 56d of in vitro incubation, respectively. After 14d in vitro incubation, the number of total incubated CIK cells increased significantly (500 fold from 8.07×105 to 1.02×108). The majority (as high as 82%±6.4%) of these cells were positive for TCR-α/β. Cells expressing TCR-α/βwere relatively rare (4.5%±2.6%). The proliferation capability of PBMC obtained from normal donors was slightly higher than that of PBMC obtained from the HCC patients. The percentage of double CD3+/CD56+ positive cells varied during CIK cell generation. The percentage of CD3+/CD56+ positive cells in total CIK cells from healthy donors and HCC patients significantly increased from 0.1%～0.13% at day 0 to 18.95%～20.5% at day 21, which suggested that the CD3+ CD56+ positive cells proliferated faster than other populations of CIK cells in the protocol used in this study. In peripheral blood, 24.2% of CD56+ cells coexpressed CD3+ as compared to 36.2% in LAK cell cultures and 76% in CIK cell cultures (Figure 2). Conversely, only 17.8% and 42.1% of CD3+ cells in peripheral blood and LAK cells co-expressed CD56+, whereas 76.6% of CIK CD3+ cells co-expressed CD56+. At day 28 of CIK cell generation, the percentage of CD3+ cells co-expressing CD56+ increased to 82.4%.

Table 1 Phenotype of CIK cells and cytotoxic activity of CIK subsets

| Subset | Percentage positively of CIK | LU/106 cell stained cells | Cell number×$10^6$ | Total LU per culture |
|---|---|---|---|---|
| CIK | | 43.6±4.8 | 712±24.3 | 29074 |
| TCR-α/β | 82.0±6.4 | 20.6±3.8 | 583±41.6 | 12088 |
| CD56 | 30.4±5.6 | 78.4±6.9 | 214±70.3 | 13948 |
| CD16 | 15.8±5.4 | 60.4±6.0 | 89±40.9 | 4106 |

Note: Total LU per culture was calculated by multiplying the number of LU per million cells by the total number of cells

*Phenotype analysis of CIK Cells*

CD3+CD56+ CIK cells are rare in fresh human PBMC, but they will markedly expand and be generated from T cell precursors. After two weeks of culturing human PBMC, the absolute number of total cells increased by 235-fold (Data not shown). The viability of expanded cell populations on day 14 was 85～95%. First, we examined the phenotypes of cultured cell population with fluorescence-activated cell sorting analyses (Fig. 1A). Cell population showed 92% CD3+CD56-, 7% CD3-CD56+, and 38% CD3+CD56+. In addition, most cells were CD8+, but not CD4+. Compared with fresh PBMC, cultured cell population showed dramatic increase in CD3+, CD3+CD56+, and CD8+, whereas decrease in CD4+ (Fig. 1B). It could be theoretically calculated that the absolute cell number of CD3+CD56+ cells increased by greater than 1,500-fold (235 fold of total cell number×6.6 fold of percent of CD3+CD56+). Morphologically, the expanded CIK cells (day 14) were large and granular, and growing in aggregate form, as opposed to single cells (Data not shown).

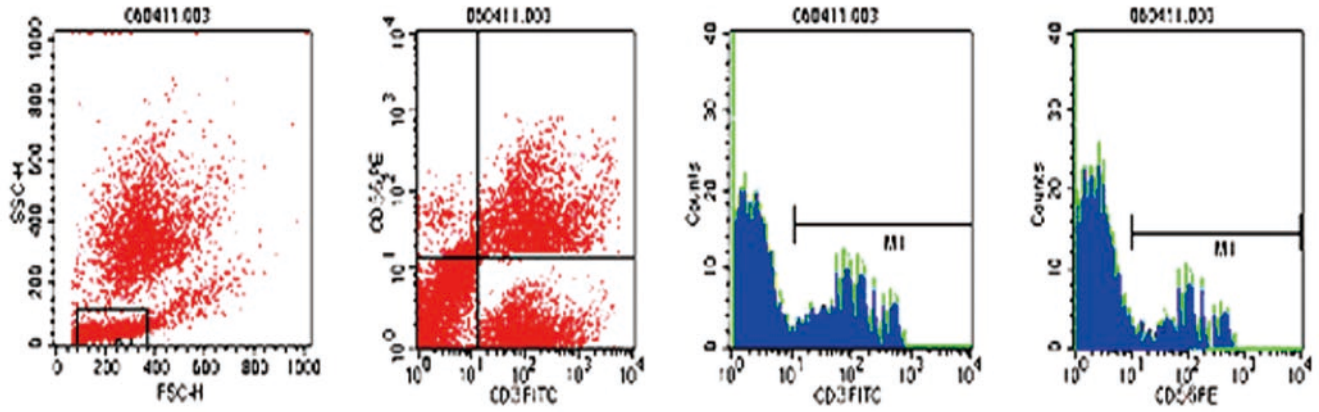


Fig. 1. The phenotypic characterization of CIK cells. CIK cells cultured for 14 days were stained with human monoclonal antibodies, such as anti-CD3-FITC plus anti-CD56-PE, anti-CD4-FITC plus anti-CD8-PE plus anti-CD56-APC. A representative histogram depicting one of eight experiments was shown (A). Phenotypes of fresh PBMC and CIK cells were compared (B). Data were expressed as the mean ± SD of eight separate experiments. The viability of the expanded cell population at each time interval was 85%～95%. Statistical significance was determined using the Student's t-test versus fresh PBMC (* p<0.01).

*Observed by Invert microscope*

It was found that CIK cells congregated increment spread all over the visual fields, the cell body near to round and small (Fig2. a,b), but the tumor cells were bigger than CIK cells, the cell body look like polygon and nucleus deep stained(Fig2. c,d).

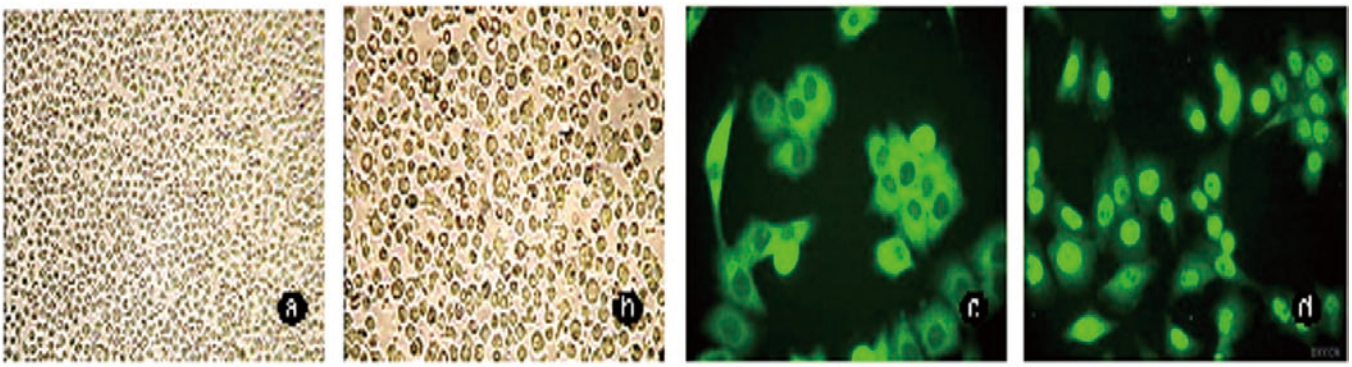


Fig. 2. CIK cells congregated increment spread all over the visual fields( a,inverted microscope×100 b, inverted microscope×200), The tumor cells were bigger and look like polygon, the nucleus deep stained( c,d Inverted fluorescence microscope ×400)

*Inverted Microscope Observations of the Effect of CIK Cells on MGC-803 Gastric Cancer Cells*

As observed in invert microscope, CIK cells displayed the ability to move and congregate around gastric cancer cells, presenting with typical rosette-like change around the target cells. Granular substances appeared in gastric cancer cells, then the gastric cancer cells blurred and disappeared, making the adhering cells float. Some CIK cells entered the cytoplasm or nucleus of gastric cancer cells to kill the tumor cells. The number of tumor

cells was significantly reduced after 24 hours of co-culture of effector cells and target cells, and gastric cancer cells no longer adhered to the walls after 48 hours and were accompanied by the appearance of fragments in the culture medium. In the control group, gastric cancer cells still grew, adhering to the surface, and remained in good condition. Movement was not seen from CIK cells cultured alone and they were distributed uniformly.

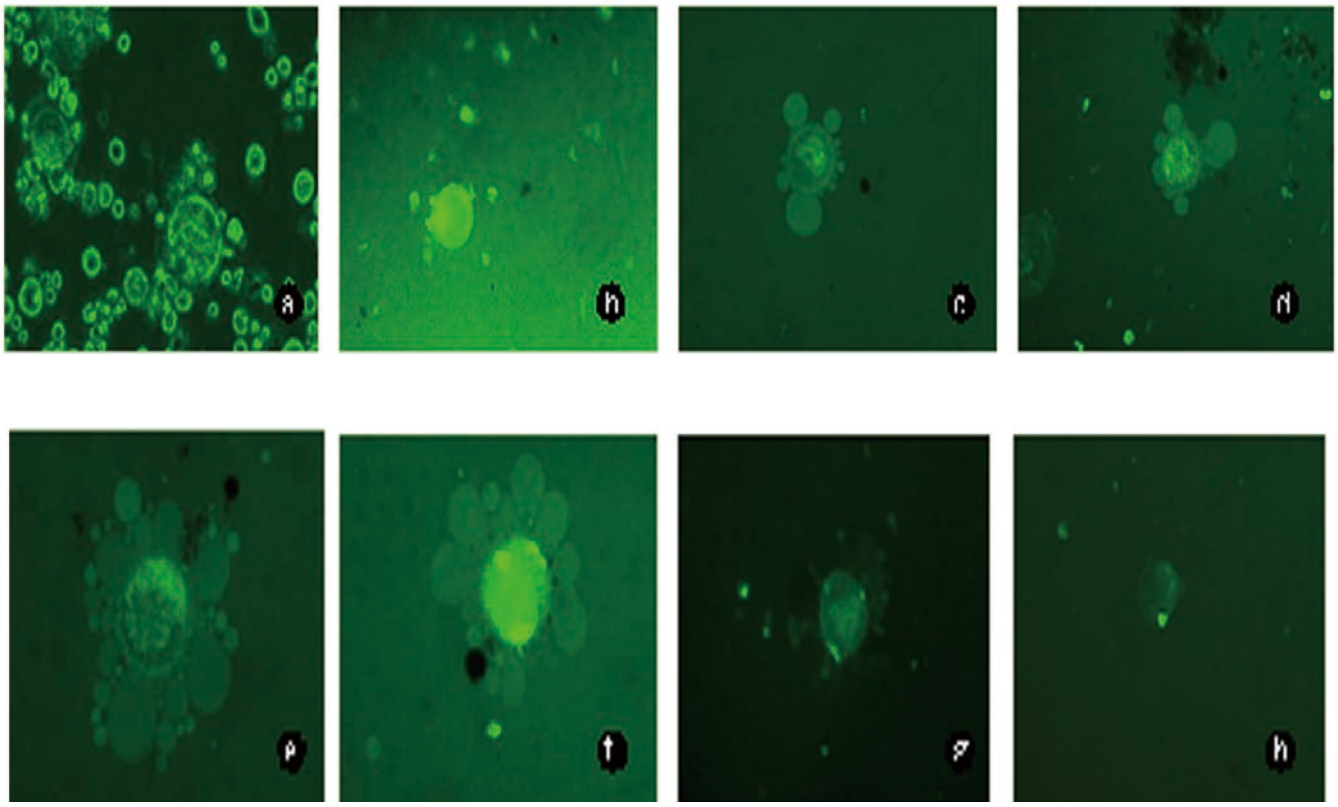

Fig. 3. Effect of CIK cells were observed in immunofluorescence staining(×400).

a . Gastric cancer cells in control group grew in good condition

b. Co–culture of CIK cells and gastric cancer cells.

c,d,e,f CIK cells congregated around tumor cells, presenting as rosettes–shaped

g,h Gastric cancer cells no longer adhered to the walls and broke into fragments in the culture medium

*Antiproliferation and Cytotoxic Effect of CIK Cells on MGC–803 Gastric Cancer Cells Detected by MTT Assay*

The cytotoxic effect was obviously increased with the increase in effector cells/target cells ratio (E/T) and the elongation of action time. There was a significant difference between those with the same action time but different E/T ratio ($p<0.01$) and also between those with the same ratio of E/T but different action time ($p<0.01$). When the E/T ratio was 40:1, the inhibitory rate was 46.23%, approximately half-inhibition concentration with 24 hours of effective action time, as seen in Table 2.

*Determination of the apoptosis–inducing effect of CIK cells on MGC–803 gastric cancer cells by TUNEL staining*

Two kinds of changes occurred in target cells. Cell apoptosis, characterized by shrunken target cells, decreased cytoplasm, condensed nucleus, and blue granules of different size, dispersed throughout the nucleus or concentrated at the border of the nucleus membrane. Swollen, necrotic target cells with ruptured membranes and an outflow of cell contents were seen. Negative cells (including necrotic cells) were not stained. In the 5-hour experimental group fixed with 2.5% glutaral, apoptotic tumor cells, negative tumor cells, and lymphocytes could be clearly recognized. In the early period of action (5～14 hours), the apoptosis rate was increased ($p<0.01$), while with the elongation of action time, apoptosis rate

was reduced in the late period (14～24 hours). The gastric cancer cell in the control group showed uniform staining (Table 3).

Table 2. Inhibitive Rate of CIK on MGC-803 Tested by MTT Assays

| | Inhibitive rate (%) | |
|---|---|---|
| Effect/Target (E/T) | 24 hours | 48 hours |
| 10:1 | 18.41 | 61.64[b] |
| 20:1 | 35.94[a] | 72.35[a,b] |
| 40:1 | 46.23[a] | 86.34[a,b] |

a . compared to the former at the same time $p<0.01$.

b . compared to the former at the same E/T $p<0.01(n=8)$.

CIK cells, cytokine–induced killer cells.

Table 3. AI of MGC-803 Treated with CIK Cells at Different Times (x±s, %)

| Time | AI (%) |
|---|---|
| 0 hours | 2.6 ±1.5 |
| 5 hours | 49.9±2.5[a] |
| 14 hours | 58.2 ±3.5[a,b] |
| 24 hours | 27.2 ±6.46[a,b] |

a. compared to control $p<0.01$. b. compared to the former $p<0.01$ ($n=5$).

CIK cells, cytokine–induced killer cells; AI, apoptosis index.

## DISCUSSION

The aim of this study was to investigate the effect of CIK cells on the apoptosis of MGC-803 gastric cancer cells by morphology and biochemical index observation, as well as its role in gene regulation by the immunocytochemical staining method in order to explore the mechanism of action. we found that autologous CIK cells had a higher proliferation rate and enhanced cytotoxic activity compared with lymphokine-activated killer cells.

It was proven by inverted microscope, light microscope, scanning electron microscope, and transmission electron microscopy that CIK cells had a strong ability to attack and kill MGC-803 gastric carcinoma cells. Target cells died in two different ways; one way is by cell apoptosis, as characterized by the formation of apoptotic body, and the other was lytic necrosis characterized by lyzed nucleus and disappeared structure. This suggested that CIK cells first approached target cells by deformation and chemotaxis, then released a number of toxins and chemicals into the medium to induce the lysis, necrosis and apoptosis of target cells[4]. There was a particular bubble-like structure of microtubules with a smooth surface in some CIK cells combined with target cells, which was believed to be associated with the killing effect of CIK cells, and similar results were also reported by Von Lilienfeld et al[5]. Lysosomes also can be seen in CIK cells, which was related to the killing activity. Those CIK cells to plenty of lysosomes were strong in their killing effect[6,7]. In this study, TUNEL assay was used to detect the apoptosis of MGC-803 gastric cancer cells induced by CIK cells. Thus, it could be inferred that in the early-period action of CIK cells (i.e., 5～14 hours), the apoptosis rate of MGC-803 gastric cancer cells was increased, while it dropped in the late period with time (i.e., 14～24 hours). This may be because the target cell died mainly by apoptosis in the early period and necrosis in the late period because necrotic cells increased with time the action reported by Kornacker M et al. [8] , that a majority of target cells underwent apoptotic death after 9～14 hours of co-culture

of CD16+ LAK cells and lung adenocarcinoma cells, which was consistent with our results.

The detailed mechanism need further research. Further studies should be performed to investigate how CIK cells modulate body immunity, the mechanism of other effects of CIK cells on MGC-803 gastric cancer cells, and the possible effect of CIK cells on other tumor cells. The results of this study indicated that CIK cells possess great potential in antitumor treatment and provide experimental evidence for the application of CIK cells in other tumor animal models and clinical management.

## CONCLUSION

CIK cells have an antiproliferative effect on human gastric cancer MGC-803 cells through the killing of cancer cells by inducing apoptosis in the early stage and necrosis in the late stage. The CIK cell is a kind of new typical, effective, immunologically competent cells which have a stronger capacity for the killing of gastric cancer cells in vitro, which would become adoptive immune therapy of late gastric cancer in clinics.

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