Treatment of Advanced Gastric Cancer by Chemotherapy Combined with Autologous Cytokine-induced Killer Cells

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Abstract. *The effects of autologous cytokine-induced killer (CIK) ($CD3^+CD56^+$) cells together with chemotherapy were investigated in patients who suffered from advanced gastric cancers (stage IV). Fifty-seven patients were divided into two groups: chemotherapy plus CIK biotherapy and chemotherapy alone. CIK cells were induced from autologous peripheral blood mononuclear cells in vitro and separated by flow cytometry and then transfused into the patients. The T-lymphocyte subgroups ($CD3^+$, $CD4^+$ or $CD8^+$), CIK cells and NK cells ($CD3^-CD56^+$) were separated and determined by flow cytometry and the serum levels of MG7-Ag, CA72-4, CA19-9 and CEA were determined by ELISA or ECLIA. It was demonstrated that the cytotoxic activity of CIK cells reached a maximum between days 14 to 21 (68.7±10.9% and 65.3±10.4%, respectively). The amounts of CIK cells were gradually increased from day 0 to day 21 and slightly decreased in the further incubations. Thereafter, the CIK cells on days 14 to 21 (with the highest population of CIK cells) transfused back to the patients. The serum levels of the tumor markers were significantly decreased, the host immune function was increased and the short-term curative effect as well as the quality of life (QOL) were improved in the patients treated by chemotherapy plus CIK cells compared to the patients treated by chemotherapy alone. Moreover, the 2-year life-span was prolonged in the group treated by chemotherapy plus CIK cells compared to the group treated with chemotherapy alone. It is concluded that chemotherapy plus CIK cells has obvious benefits for patients who suffer from advanced gastric cancers.*

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Gastric cancer is one of the most common causes of cancer death in China (1). In spite of the standardization of surgical operations and multimodal therapies, the postoperative survival of patients with advanced gastric cancer remains very poor (2). The main cause of recurrence and metastasis of gastric cancer is the failure of cancer therapies (3), making the development of a new treatment modality necessary. Biotherapy is becoming an important and effective combination method for cancer therapy, especially for patients at later stages (4, 5). A variety of studies involving lymphokine-activated killer (LAK) cells (6), tumor-infiltrating lymphocytes (TIL) (7) and antiCD3 monoclonal antibody-induced killer (CD3-AK) cells (8) have been reported. However, the therapeutic effectiveness of adoptive immunotherapy is limited, depending on its inherently low cellular antitumor activity *in vivo* (9). It has been demonstrated that cytokine-induced killer (CIK) cells can proliferate abundantly *in vitro* and kill tumor cells directly (10). Moreover, CIK cells can regulate and increase the host cellular immune function *in vivo* (11). We previously reported that CIK cells possess more potent antitumor activity than TILs *in vitro* (12).

In the present study, the potential benefits of the combination of autologous CIK cells together with chemotherapy were investigated in patients suffering from advanced gastric cancer (stage IV) and were compared to patients treated with chemotherapy alone.

Several clinical parameters, including serum levels of tumor markers, host cellular immune function, short-term curative effect and survival rate, were examined in these two patient groups.

Materials and Methods

Patients and treatments. Fifty-seven gastric cancer patients were divided into two groups: *i.e.*, group I treated with chemotherapy alone (18 men and 7 women, aged from 28-76, median age 52) and group II treated with chemotherapy plus autologous CIK biotherapy (21 men and 11 women, aged from 29-78, median age 54). The characteristics of the patients are listed in Table I. All the patients had been

Table I. *Patient characteristics.*

	Chemotherapy (n=25)	Chemotherapy+CIK (n=32)
Tumor size (cm, mean±SD)	6.4±2.3	6.2±2.2
Histological types		
Glandular carcinoma	23	30
Mucoid adenocarcinoma	2	2
Depth of tumor invasion		
T1	0	0
T2	0	0
T3	17	22
T4	8	10
Lymph node metastasis		
N0	0	0
N1	6	5
N2	19	27
Distant metastasis		
Without	23	30
With	2	2

diagnosed as stage IV (13) and treated by palliative gastrectomy. After surgery, all the patients obtained three cycles of chemotherapy with the protocol of FOLFOX4 (folinic acid, 200 mg, 5-Fluorouracil, 400 mg and oxaliplatin, 85 mg/m^2 body surface) as described previously (14). The group II patients received autologous CIK biotherapy five times after each chemotherapy. CIK cells (1.0x10^9) were transfused into patients within 1 h every second day. The curative effects were evaluated after each treatment.

Separation of peripheral blood mononuclear cells (PBMCs) and induction of CIK cells. Blood (50-100 ml) was drawn from patients using heparin as an anticoagulant. The PBMCs were isolated by Ficoll-Conray density gradient centrifugation, as described previously (15). The viability of the PBMCs was assessed by trypan blue exclusion. PBMCs (2.0x10^6 /mL) were plated onto 6-well dishes (Nunc, Denmark) and cultured with the Medium I containing RPMI 1640 in the presence of human interferon-gamma (IFN-γ, 1.0x10^6 U/L, Shanghai Fosun Pharma Co., China), recombinant human interleukin 2 (IL-2, 5.0x10^5 U/L, Shangdong Quangang Pharmaceutical Co., China), 10% inactivated human serum, 25 mM HEPES, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were incubated in a humidified atmosphere with 5% CO_2 at 37˚C. After 24 h, monoclonal antibody (MAb) against CD3 (100 μg /L, Antibody Diagnostic Inc., USA) and IL-1α (1.0x10^5 U/L, Promega, USA) were added. After another 48 h of culture, the supernatant was removed by aspiration and the cells were cultured in Medium II (Medium I in the absence of IFN-γ). The medium was then changed every 3 days.

CIK cell identification and cytotoxic examination. The cells were identified and sorted by flow cytometry (FACS, Beckman-Coulter) on days 1, 7, 14, 21, 28 and 35. In addition, the patient's whole blood cells were also separated and sorted by FACS before and after treatments. The cytotoxic activity of the CIK cells was determined by co-incubation with the NK-sensitive K562 cell line (ATCC, USA), as previously described (15). Before transfusion of the CIK cells, they were washed twice with 0.9% NaCl and, they were re-suspended in 100 ml 0.9% NaCl containing 1% human albumin. The CIK cells (1.0x10^9 cells) were transfused into the patient within 1 h for each biotherapy.

Evaluation of curative effects. Curative effects were short-term and determined by tumor markers in the blood, cellular immune responses and cumulative survival rate. The short-term curative effect was estimated by a standard procedure (16). The serum levels of tumor markers, including MG7-Ag, CA72-4, CA19-9 and CEA, were determined by either the ELISA method (Biocheck, USA) or the ECLIA method (Roche, Switzerland). The cellular distribution of T-cell subgroups, CIK cells ($CD3^+CD56^+$) (15) and natural killer cells (NK, $CD3^-CD56^+$) (17) were determined by FACS. The cumulative survival curve was estimated by the Kaplan-Meier method (18).

Statistical analysis. The data are expressed as means±SD and were analyzed by the Student's *t*-test. The cumulative survival curve was estimated by the Kaplan-Meier method and the differences in the distributions were compared by the log-rank test. A *p*-value less than 0.05 was considered as significant.

Results

Induction and identification of CIK cells. Autologous CIK cells identified as $CD3^+CD56^+$ (15) were induced from PBMCs and separated by FACS (Figure 1). The number of CIK cells was gradually increased during cell cultures, reaching the highest on day 14 and then slightly decreasing on days 28 and 35 (Figure 2). Similar patterns were found for the NK cells ($CD3^-CD56^+$) and the T-cell subgroups, including $CD3^+$, $CD4^+$ and $CD8^+$ (Figure 2). The cytotoxic activity of the CIK cells was highest on day 14 (68.7±10.9%) (Figure 3), on which day they were transfused into the patients.

Changes in serum levels of tumor markers and cellular immune responses. As shown in the Table II, the serum levels of tumor markers, including MG-7Ag, CA72-4, CA19-9 and CEA, were significantly decreased after treatment but were more pronounced in the patients treated by chemotherapy plus the CIK cell transfusion. The distribution of lymphocytes in the patients treated by chemotherapy plus CIK cells (Table III) was also determined. The proportions of CIK cells, NK cells, T-cell subgroups $CD3^+$ and $CD4^+$, and the ratio of $CD4^+/CD8^+$ were significantly increased, whereas the proportion of $CD8^+$ cells was decreased.

Short-term curative effects, evaluation of clinical QOL and survival rate. The short-term curative effects were evaluated after treatments. As shown in Table IV, the total remission rate (CR+PR+MR) was statistically significantly increased

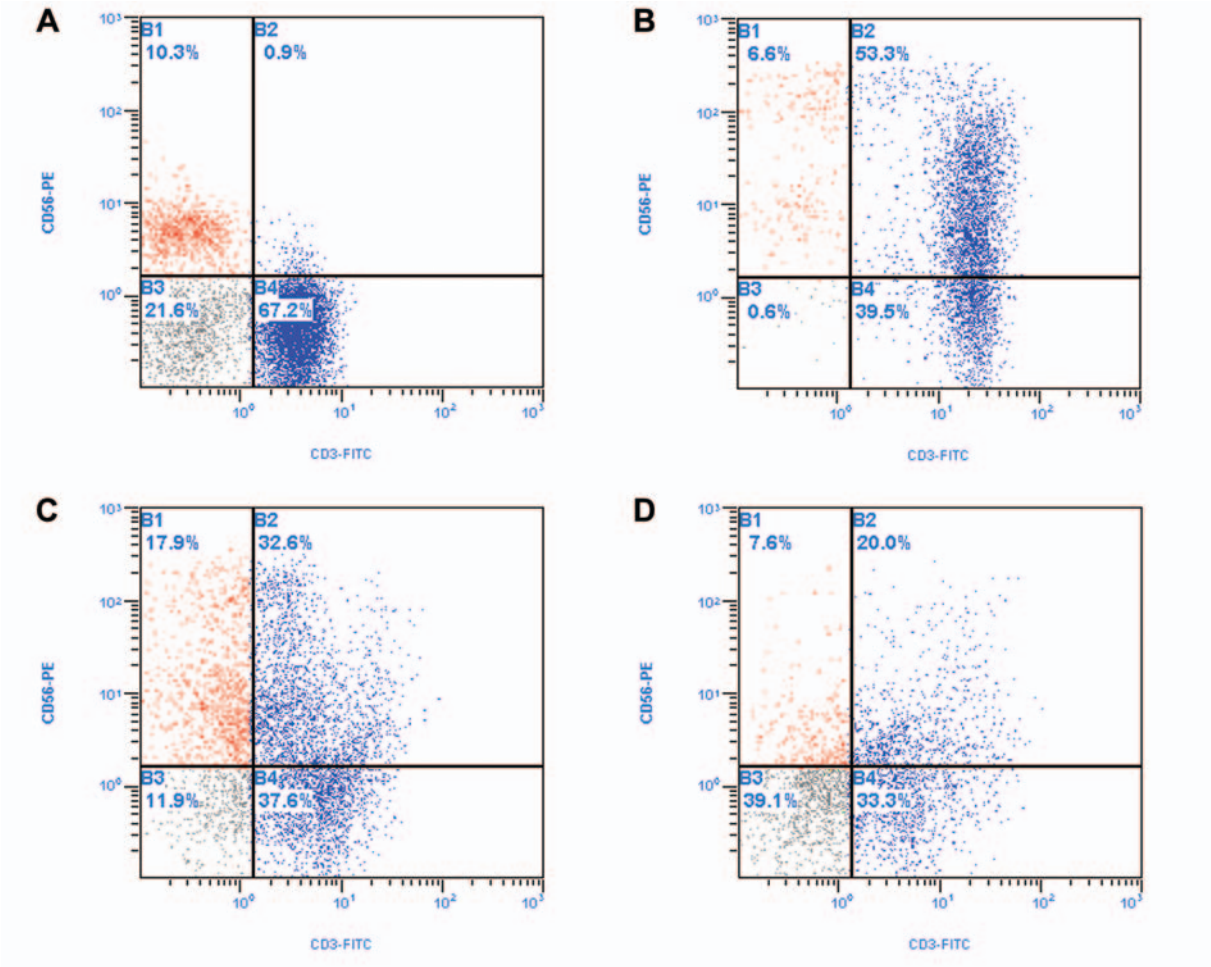

Figure 1. *Separation and identification of CIK cells. The CIK cells were separated and identified by FACS after incubation at different time-intervals: (A) day 1; (B) day 14; (C) day 21; and (D) day 28. The population of CIK cells ($CD3^+CD56^+$) was largest on day 14 of incubation.*

in the group treated by chemotherapy plus CIK cells (56.3%) compared to the group treated by chemotherapy alone (48.0%). The patients' quality of life (QOL) was also evaluated according to a standard protocol (19), demonstrating that the QOL increased in patients treated by chemotherapy plus CIK cells. However, the QOL decreased in the group treated with chemotherapy alone (Figure 4). Patient survival time was recorded and the survival rate was analyzed. The survival rate within 2 years increased in the group treated with chemotherapy plus CIK cells compared to that of the group treated with chemotherapy alone. However, there was no difference between the groups after 2 years (Figure 5).

Side-effects of CIK cells transfusion. During and after the CIK cell transfusion, side-effects, including chill, fever, headache, nausea and vomiting, were recorded for all patients. Three patients had chill, 14 patients had fever

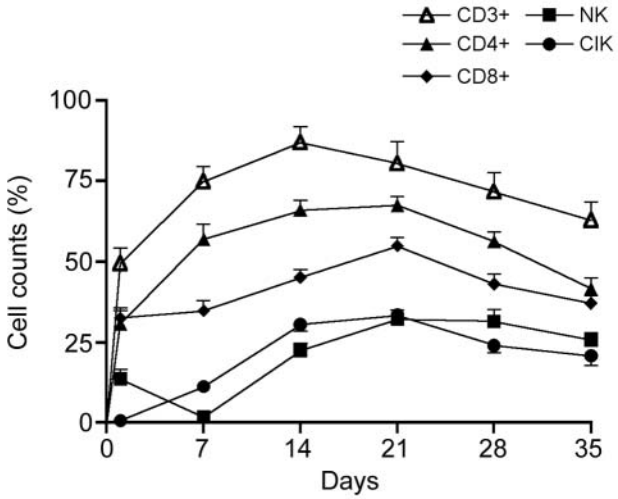

Figure 2. *Changes in T-cell subgroups, CIK cells and NK cells during the incubation. The CIK cells were separated and identified by the FACS after incubation at different time-intervals. The data are presented as mean±SD.*

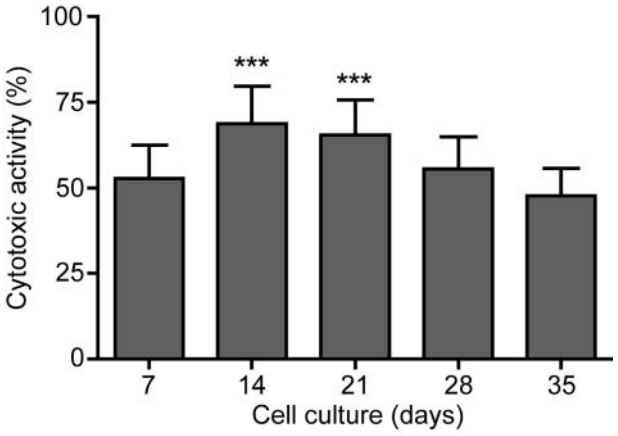

Figure 3. *Cytotoxic activity of CIK cells. The data is presented as mean±SD. ***p<0.001 vs. 7-day incubation.*

(37.8˚C to 39.5˚C) and two patients suffered from nausea and vomiting. These side-effects could be treated by simple allopathy. There were no severe or unusual side-effects recorded after CIK cell infusion.

Discussion

The present study demonstrated that the transfusion of autologous CIK cells in chemotherapeutic patients had potential benefits compared to chemotherapy alone. Both cell numbers and the cytotoxic activity of the CIK cells reached a peak from days 14 to 21 after being induced by cytokines. Thus, 14-day CIK cells were considered best for the therapy. The serum levels of tumor markers were significantly decreased and the host immune responses were significantly increased in patients treated by chemotherapy plus CIK. The short-term curative effect and the QOL were also improved. Moreover, the 2-year life-span, though not the 5-year life-span, was significantly prolonged compared to the group treated by chemotherapy alone.

In China, gastric cancer is often diagnosed at a relatively advanced stage after metastasis has occured to other organs (20). A series of treatments such as surgical resection combined with chemotherapy (21, 22), radiotherapy (23, 24), thermotherapy (25, 26) and/or traditional Chinese medicine (27, 28) have been introduced, but the 5-year survival rate of advanced stage patients remains very poor. It had been suggested that cellular immunotherapy, that directly or indirectly regulates the biological interaction between the host and the tumor (29), is another choice to improve patient survival. Autologous CIK cells represent one of the promising cellular immunotherapies (30). In the present study, the potential effects of autologous CIK cells were investigated in patients suffering from stage IV gastric cancer. The results demonstrated that the combination of chemotherapy together with autologous CIK cells had beneficial effects compared to chemotherapy alone. Although the patients had not been divided randomly, most treated with chemotherapy plus CIK cells had a better QOL and longer 2-year life-span than the patients treated with chemotherapy alone. However, the 5-year life-span did not differ between the groups. More cases are needed to explore these beneficial effects.

Table II. *Serum levels of tumor markers before and after treatments. Mean±SD (positive cases).*

	Chemotherapy (n=25)		Chemotherapy+CIK (n=32)	
	Before	After	Before	After
MG-7Ag	8.4±1.3(12)	6.0±1.4(5)[a]	8.3±1.2(15)	5.3±1.3(5)[b]
CA72-4	23.8±16.9(15)	18.1±11.1(6)[a]	24.5±17.1(19)	16.4±9.6(6)[b]
CA19-9	47.0±15.8(17)	28.1±13.0(10)[a]	46.5±15.2(22)	21.5±10.2(11)[b]
CEA	27.8±6.9(9)	24.5±4.7(7)	28.3±7.1(12)	10.8±3.1(4)[c]

[a]$p<0.05$, [b]$p<0.001$ *vs.* before treatment, [c]$p<0.05$.

Table III. *Cellular immune responses before and after CIK treatment. (Mean±SD, n=32).*

	Pre-therapy	Post-treatment
$CD3^+$	49.6±11.7	58.2±11.7[a]
$CD4^+$	31.5±8.4	38.5±9.7[a]
$CD8^+$	32.7±9.5	28.6±5.1[a]
$CD4^+/CD8^+$	0.9±0.1	1.4±0.2[a]
$CD3^-CD56^+$	15.6±3.3	26.4±7.0[a]
$CD3^+CD56^+$	0.7±0.2	3.2±1.7[a]

[a]$p<0.001$ *vs.* pre-therapy.

Table IV. *Short-term curative effects.*

Group	Cases	CR	PR	MR	NC	PD	Remission rate (%)
Chemotherapy	25	0	4	8	6	7	48.0
Chemotherapy+CIK	32	0	7	11	8	6	56.3[a]

[a]$p<0.05$ *vs.* chemotherapy. CR (complete remission) was defined as the disappearance of all measurable tumors for at least 1 month. PR (partial remission) was defined as a 50% decrease of all measurable tumors for at least 1 month. MR (minor remission) was defined as a reduction of 25-49%. NC (no change) was defined as a reduction of <25%. PD (progressive disease) was defined as an increase in tumor burden. Total remission rate=CR+PR+MR.

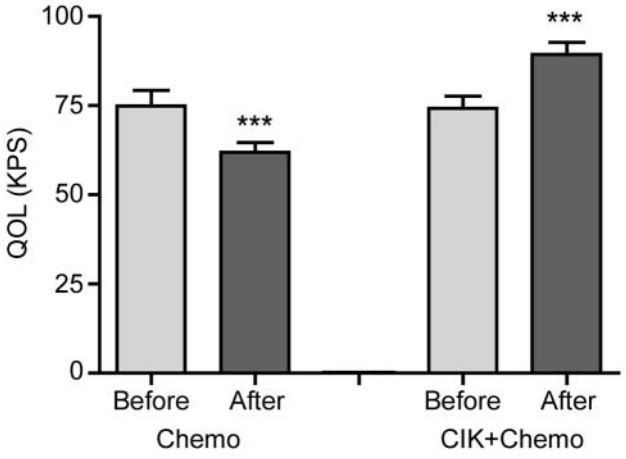

Figure 4. *Evaluation of QOL in patients administered different treatments. The patients' QOL before and after different treatments was evaluated by a standard method (KPS). ***p<0.001 vs. before treatment.*

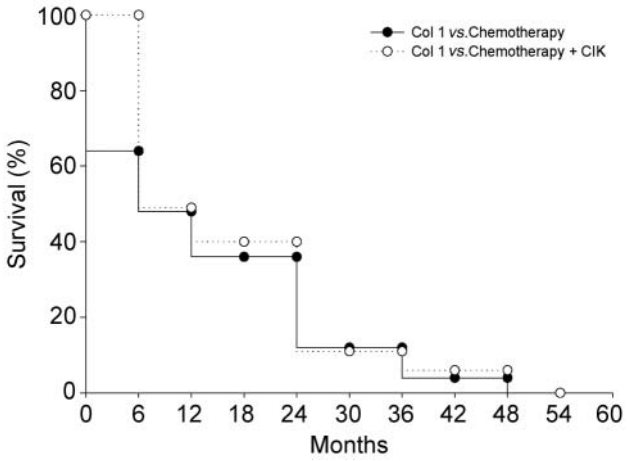

Figure 5. *Cumulative survival rate of the patients. The cumulative survival curve was analyzed by the Kaplan-Meier method. No patient was alive after 5 years in either group.*

In the present study, the CIK cells were obtained from PBMCs induced by the combination of IFN-γ, IL-2, anti-CD3MAb and IL-1α. Both the cell number and cytotoxic activity of the CIK cells were highest from days 14 to 21, indicating that these CIK cells were suitable for transfusion into the patients. The serum level of the tumor marker MG-7, an antigen of gastric cancer, is considered to be an index of severity (31). CA72-4, CA19-9 and CEA are also related to gastric cancer (32). In the present study, MG-7, CA72-4, CA19-9 and CEA were combined as an index to evaluate disease severity. These markers significantly decreased in the patients treated with chemotherapy plus CIK compared to those treated with chemotherapy alone. As shown in Table III, the percentage distribution of T-cell subgroups, CIK cells and NK cells in the blood was also changed after the treatments, indicating that the transfusion of autologous CIK cells could improve the patient cellular response. *In vitro* experiments will be performed in order to examine the biological changes in CIK cells under cytokine induction.

It can be concluded that chemotherapy combined with CIK cells has obvious benefits for patients suffering from stage IV gastric cancer, compared to chemotherapy alone. The detailed mechanisms of action require further investigation.

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