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Preliminary Study of Local Immunotherapy with Autologous Cytokine-Induced Killer Cells for Glioma Patients

Li Lin[1]
Yonggao Mu[2]
Zhongping Chen[2]

[1] Department of Lung Cancer Chemotherapy, Tianjin Medical University Cancer Institute & Hospital, Tianjin 300060, China.
[2] Department of Neurosurgery/Neuro-Oncology, Cancer Center, Sun Yat-sen University, Guangzhou 510060, Guangdong Province, China.

Correspondence to: Li Lin
E-mail: linli76@163.com

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CJCO http://www.cjco.cn
E-mail: 2008cocr@gmail.com
Tel (Fax): 86-22-2352 2919

OBJECTIVE Cytokine-induced killer (CIK) cells are T-cells that display effective anti-tumor activity. In this study, we investigated the anti-tumor activity of CIK cells in vitro, and conducted a preliminary investigation using autologous CIK cells to treat glioma patients through local administration.

METHODS The CIK cells were derived from peripheral blood monocytes (PBMCs) of the glioma patients. The anti-tumor activity of the CIK cells against human T98-G glioma cell was tested in vitro. In addition, the autologous CIK cells were locally administrated into the tumor cavity in the malignant glioma patients through an Ommaya reservoir which was pre-inserted during tumor resection. The 4×10^8 CIK cells in a 5 ml suspension were injected once a week 2 times per cycle. Five hundreds KU of IL-2 was injected every other day.

RESULTS (*i*) With incubation, the CIK cells showed dual staining of $CD3^+CD56^+$ with a positive rate of 3.45% on day 10 and 55.2% on day 30. In vitro anti-tumor activity (against T98-G cells) of the CIK cells reached the highest level after 18 days of incubation with different effector/target (E:T) ratios. (*ii*) Six patients received autologous CIK cell treatment (10 cycles). Two patients showed no recurrence and are still alive (24 and 10 months), while 4 cases had a recurrence 3 of which have died. The mean survival time from the first CIK cell treatment to the end of follow-up was 12.5 months. The main side-effects of the local CIK cell treatment was brain edema, which was controlled by mannitol in most of the cases. However for one patient injection of CIK cells and IL-2 had to be discontinued.

CONCLUSION In vitro CIK cells are effective anti-glioma T-cells. Local therapy with CIK cells has potential anti-glioma efficacy and tolerable side-effects.

KEY WORDS: cytokine-induced killer (CIK) cells, glioma, local immunotherapy.

Introduction

Gliomas are the most common primary tumors of the central nervous system (CNS). Despite advances in surgery, radiation and chemotherapy, malignant neuroectodermal tumors carry a poor prognosis with a median survival in adults of only 51 weeks for high-grade gliomas. Thus, innovative treatments for this disease are desperately needed. Immunotherapy is one of the most encouraging alternatives in cancer therapy. In 1991, a novel protocol was reported by Schmidt-Wolf et al.[1] for the generation of highly efficient cytotoxic effector cells, i.e. cytokine-induced killer (CIK) cells. The CIK cells are

major-histocompatibility complex-unrestricted cytotoxic lymphocytes which are generated by incubation of peripheral blood monocytes (PBMCs) in the presence of various types of cytokines. Some reports have indicated that CIK cells, other than lymphokine-activated killer (LAK) cells and Tumor-infiltrating lymphocytes (TIL), could be efficiently employed as adjuvants in an anticancer immunotherapeutic strategy for the eradication of residual cancer cells and prevention or delay of a tumor relapse[2,3].

In the present study we attempted to characterize the anticancer efficiency of CIK cells and to develop an alternative immunotherapy for patients with glioma. We tested the anticancer capability of CIK cells derived from glioma patients, and conducted a preliminary study using local immunotherapy for malignant glioma patients.

Materials and Methods

In vitro study

Subjects

Eighteen patients who had been pathologically confirmed with WHO grade I~II glioma were enrolled in this study. They included 12 males and 5 females, ages from 8 to 58 years (average 33 years). The patients received no chemotherapy or radiotherapy or for at least 3 months prior to the study. Ten members of the hospital staff were selected as normal controls.

Generation of CIK cells

CIK cells were prepared as described according to Schmidt-Wolf et al.[2] Briefly, peripheral blood mononuclear cells (PBMCs) were collected and separated from glioma patients (glioma group) or from volunteers as a control group (normal group) using a CS-3000 Plus blood cell separator (Baxter, USA) or by ficoll gradient separation. The cells were grown in RPMI-1640 (Hyclone Co. USA) culture medium (CM) and 1,000 U/ml rhIFN (Shanghai Clonbiotech Co, LTD, China) was added on d 0, and 24 hr later, 50 ng/ml of mouse monoclonal antibody (mAb) against CD3 (R&D Co., USA), 100 U/ml rhIL-1 and 300 U/ml rhIL-2 (Bejing Sihuan Biological Engineer Materials Co LTD, China) were added. The initial cell density was about 1×10^6/ml. Fresh CM with IL-2 was replaced every 3 days[4,5].

Generation of lymphokine-activated killer (LAK) cells

The PBMCs were collected from glioma patients and separated on a ficoll gradient. LAK cells were generated by incubation of the cells in fresh CM with IL-2. The CM was exchanged every 3 days.

Flow cytometry analysis

On days 0, 10, 14, 18, 22 and 30, 50 ul of a CIK cell suspension were stained with 10 μl Phycoerythrin (PE) anti-human CD3 (R&D Co, USA) antibody and cychrome anti-human CD56 (Bejing Sihuan Biological Engineer Materials Co, LTD, China) antibody to determine the immunophenotype using a FACScalibur flow cytometer[6].

Cytotoxicity test

The CIK cells obtained from the glioma patients and healthy donors were incubated for 10, 18 and 26 days with T98-G glioma cells at various ratios of effector cells to target cells (E:T, 25:1; 50:1; 100:1). The LAK cells were incubated at an E:T ratio of 50:1. Target cells were seeded in a microplate at a density of 1×10^5/ml and effector cells were mixed into each well at a density of 5×10^6/ml. After 18 hr of incubation, the CIK cells and non-adhesive T98-G glioma cells were removed, and then 10 μl of MTT was added. The cytotoxicity rate (CR) was calculated by the following formula: CR = (1-A_{570} experiment/A_{570} T control) × 100%[7].

Primary clinical study

Patients

The following standards were used to select the patients to receive local treatments of autologous CIK cells; those with glioma WHO grade III or grade IV; after tumor resection, the tumor cavity did not communicate with the ventricle; the patient KPS was higher than 60; there was a period of 6 weeks following radiotherapy or chemotherapy.

Autologous CIK cell treatment for glioma patients

The autologous CIK cells were locally administrated into the tumor cavity of those patients with malignant glioma through an Ommaya reservoir which was preinserted during tumor resection. The CIK cells (4×10^8) in a 5 ml suspension were injected once a week 2 times per cycle, adding 500 KU of IL-2 every other day.

Statistical analysis

The paired t test was used with SPSS for Windows, version 10.0 (SPSS, Chicago) to analyze the statistical significance. $P < 0.05$ was considered statistically significant.

Results

Phenotype of the CIK cells

There was a steady increase in the percentage of $CD3^+CD56^+$ cells during culture (Figs.1&2 and Table 1). The percentage of $CD3^+CD56^+$ cells was 3.45% on day 0 and 55.2% after 30 days of incubation; $CD3^+$ cells were 70.2% on day 0 and 94.8% after 30 days; $CD56^+$ cells were 11.1% on day 0 and 57.0% after 30 days. These results showed that $CD3^+CD56^+$ cells were the major population of the CIK cells.

Table 1. Percentage of $CD3^+$, $CD3^+CD56^+$ and $CD56^+$ during cell culture (%).

Group	0 d	10 d	14 d	18 d	22 d	30 d
$CD3^+$	70.2 ± 10.2	80.0 ± 9.58	92.5 ± 4.46	91.5 ± 8.17	90.0 ± 7.91	94.83 ± 4.91
$CD56^+$	11.1 ± 4.85	13.3 ± 5.76	13.7 ± 5.79	23.5 ± 10.3	41.0 ± 10.48	57.02 ± 7.72
$CD3^+CD56^+$	3.45 ± 0.98	9.03 ± 2.94	11.1 ± 3.56	17.42 ± 5.5	40.8 ± 4.68	55.2 ± 9.74

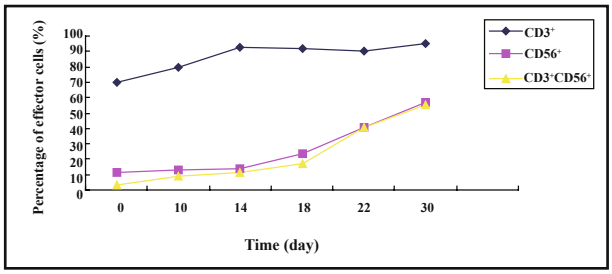

Fig.1. Phenotypic analysis of $CD3^+CD56^+$ cells determined by flow cytometry at different culture times.

Cytotoxic activity of CIK cells against T98-G cells

Figs.3&4 and Table 2 show the cytotoxic activity of CIK cells against glioma cells. With an increase in the E:T ratio, the cytotoxic effect of CIK cells correspondingly became stronger. At ratios of 25:1; 50:1 and 100:1, the patient CIK-cell kill rate was 37.1%, 60.7% and 71.1%, respectively. At a ratio of 50:1, the patient CIK-cell kill rate on the 10, 18, and 26 day was 52.4%, 67.3% and 58.9%, respectively. The high cytotoxic effect was maintained about 2 weeks, from the second week to the third week. At about 18 days the cytotoxic effect reached the highest level. There was a significant difference between the kill rate of CIK cells (67.3%) and LAK cells (55.8%) of the patients at the ratio of 50:1 ($t = 2.526$, $P = 0.019$). For the healthy donors at ratios of 25:1; 50:1 and 100:1, the CIK-cell kill rate was 52.7%, 73.3% and 84.2%, respectively. There was a significant difference of CIK activity between the healthy donors and patients ($P < 0.05$).

Clinical observations

Six patients received 10 cycles of local CIK cell immunotherapy (Table 3). All of the patients were followed up for 7~24 months. Mean survival time from the first CIK-cell treatment to the end of follow-up was 12.5 months. There was no recurrence in 2 cases who are still alive (24 and 10 months). Four cases recurred of which 3 died. Side effects included the following: fever, 2 patients, 4 incidences, ~ 38℃ 1 case; > 38℃ 1 case; headaches, 3 patients, 4 incidences; cerebrum edema, 6 cases; neurological dysfunction, aphasia 1 case; hemianesthesia 1 case. MRI follow-up for 4 cases at 4~12 weeks showed complete tumor remission in one case, no change of tumor size in 1 case, and mass enlargement in 1 case who had received a second surgery. Another case using MRI showed stable disease at the 4th week but finally recurred after 20 months.

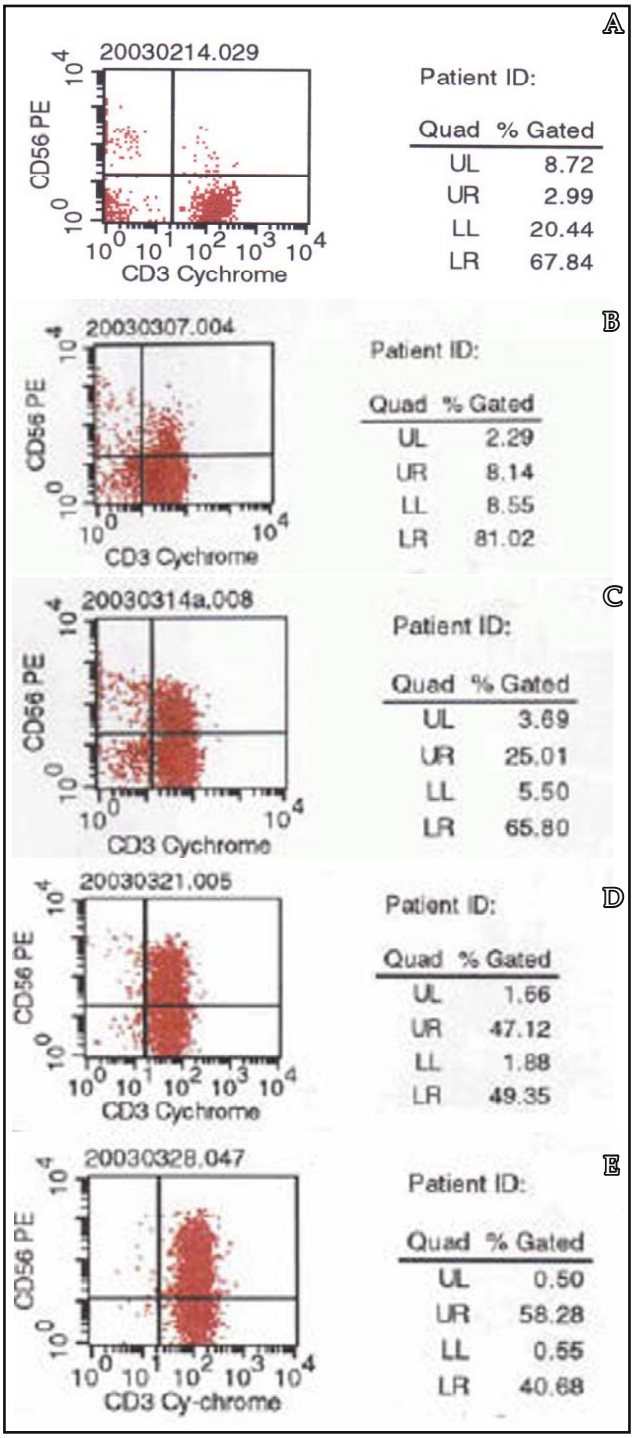

Fig.2. Phenotypic analysis of $CD3^+CD56^+$ cells from one patient. The cultured cells were analyzed by flow cytometry at various culture times. A, 0 d; B, 9 d; C, 16 d; D, 23 d; E, 30 d.

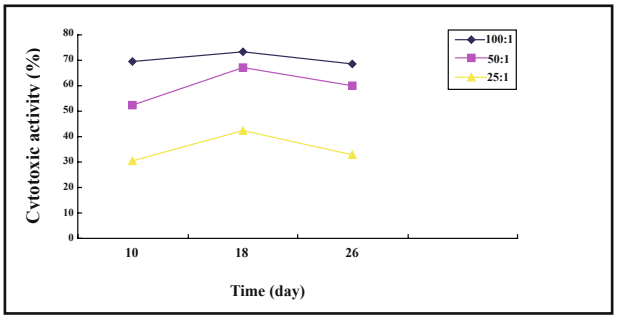

Fig.3. Cytotoxic activity of the patient and healthy donor CIK cells at 10, 18 and 26 days.

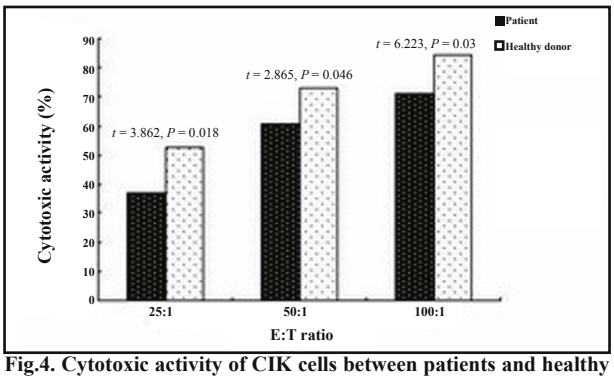

Fig.4. Cytotoxic activity of CIK cells between patients and healthy donors at the same ratio.

Table 2. Comparison of the CIK-cell anti-tumor activity between healthy donors and patients.

Group	E:T	10 d (%)	18 d (%)	26 d (%)
Patient	100:1	69.43 ± 10.45	73.32 ± 8.48	68.8 ± 16.99
	50:1	52.39 ± 13.73	67.31 ± 15.51	59.89 ± 21.03
	25:1	30.60 ± 14.49	42.45 ± 21.79	32.74 ± 15.77
Healthy donors	100:1	82.41 ± 10.93	82.71 ± 8.52	87.64 ± 5.78
	50:1	71.73 ± 4.6	76.67 ± 10.43	69.64 ± 15.24
	25:1	51.57 ± 16.88	56.21 ± 15.41	48.16 ± 14.60

Table 3. Clinical data from patients receiving local CIK-cell treatment.

Patient: Age/Sex	Diagnosis	Surgery	CIK cell treatment (cycles)	Outcome
25/M	R-Parietal-occipital lobe GBM	Partial resection	1	Died
39/M	R-Frontal-parietal lobe GBM	Subtotal resection	1	Died
37/F	L-Frontal-parietal lobe glioma (WHO III)	Total resection	4	CR
57/M	R-Temporal lobe glioma (WHO III)	Subtotal resection	1	Died
58/M	L-Temporal lobe glioma (WHO III)	Subtotal resection	1	PR
37/M	L-Temporal-parietal lobe glioma (WHO III)	Total resection	1	CR

Discussion

Some articles have reported that CIK cells have a higher proliferation rate and enhanced cytotoxic activity compared to lymphokine-activated killer cells[8,9]. Our results suggest that the CIK effector cells are a subpopulation that coexpresses $CD3^+/CD56^+$, based on the finding that CIK cells possess a higher proliferation rate and higher antitumor cytotoxic activity in vitro compared to LAK cells. Though LAK cells recognize target cells by a non-MHC restricted mechanism similar to CIK cells, the CIK cells were found to have the greatest lytic activity.

In our study, there was a significant difference in the kill rate between CIK cells (67.3%) and LAK cells (55.8%) of the patients at the ratio of 50:1. We found that autologous CIK cells had enhanced cytotoxic activity. We also found at the cell ratio of 50:1,the patient CIK-cell kill rate on the 10, 18 and 26 days was 52.4%, 67.3% and 58.9%, respectively. At about 18 days, the cytotoxic effect reached its highest level. The high cytotoxic effect was maintained about 2 weeks, from the 14 d to 21 d, this showing that the high cytotoxic effect of CIK cells in vitro can be maintained a long time. This provides a basis for the antitumor effect in vivo. Furthermore, the cytotoxic activity of CIK cells from healthy donors was showed to be stronger than those from the patients with glioma.

Huang et al.[10] reported that the proliferative level and killer activity of CIK cells from the patients with chronic hepatitis B was lower compared to healthy people, which may be the reason for persistent development of HBV infection. Our results might reflect the function of CIK cells in glioma patients is probably subject to inhibition. Zhang et al.[11] reported that CIK cells significantly increase the cytotoxic activity after being co-cultured with autologous dendritic cells. Whether the dysfunctional dendritic cells led to lower proliferation and function of the CIK cells is unknown. This finding needs further experimental study.

In addition to vitro experimental results, studies have

showed that CIK cells have antitumor effects in a nude mice model [12,13]. Based on these results, many patients have been treated with CIK cells for different kinds of tumors. Jiang et al.[14] reported 57 patients were divided into 2 groups: chemotherapy plus CIK biotherapy and chemotherapy alone. Results showed that the serum levels of the tumor markers were significantly decreased, the host immune function was increased, the short-term curative effect as well as the quality of life (QOL) was improved and the 2-year life-span was prolonged in the group treated by chemotherapy plus CIK cells compared to the group treated with chemotherapy alone. Weng et al.[15] reported on a study in which autologous cytokine-induced killer (CIK) cells were transfused via the hepatic artery into 85 HCC patients. The 1-year and 18-month recurrence rates of the immunotherapy group were 8.9% and 15.6%, compared with 30.0% and 40.0% of the group not receiving adjuvant therapy (both $P < 0.05$). The data suggest that CIK cell transfusion was an effective treatment as it boosted the immunologic function in the HCC patients and played an important role in reducing the recurrence rate of HCC.

Reports of CIK cell immunotherapy for treating glioma are rare. Bu et al.[16] reported that microsurgical resection combined with interstitial chemotherapy and sensitive radiotherapy plus autologus immunotherapy was a safe and effective method for individual comprehensive therapy in malignant human gliomas. No similar studies with glioma patients using local immunotherapy with autologous cytokine-induced killer cells have been published. Our study, the first of its kind, was developed to examine the safety and efficacy of local immunotherapy with cytokine-induced killer cells. Six glioma patients accepted the treatment. The main side-effects were fever and brain edema, which possibly could have been related to usage of IL-2. Fortunately, brain edema was controlled through administration of mannitol in most of the cases, except for one patient for whom CIK cell treatment was discontinued. The fever is also mild and controllable. Our primary clinical results indicated that local CIK cell therapy has potential anti-glioma efficacy, and that the side-effects are acceptable. The clinical significance of local CIK cell treatment for glioma needs to be studied further with an expanded sample size.

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