Th1 Bias in PBMC Induced by Large Scale Auto-CIK Infusion in Malignant Solid Tumor Patients in China

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OBJECTIVE This article is to verify feasibility and validity of autologous cytokine-induced killer cell (Auto-CIK) treatment in solid malignancy patients.

METHODS Amplification, phenotypic characteristics, cytokine secretion, antitumor cytotoxicity and clinical response to Auto-CIK derived from 65 cases of solid tumor patients with different pathological types and clinical stages were compared with LAKs in a large-scale clinical trial.

RESULTS We found that seriousness of disease and metastatic status had no influence on effective components and antitumor immunological activity of Auto-CIK. Comparing cytotoxicity against various tumor cells with LAKs at various effector to target ratios, CIKs showed more effective cytotoxicity against NK sensitive or non-sensitive solid tumor cell lines at a low E/T ratio (6:1) which suggests indirectly that Auto-CIK had a longer effective time in vivo than LAKs. These results suggest that CIKs are more suitable for immunotherapy for those solid malignancy patients at high risk of relapse or recurrence.

CONCLUSIONS Our experimental data were consistent with the reported conclusion that the potent antitumor activity of Auto-CIK mainly rooted in the $CD4^-$ part of CIKs, including $CD3^+CD56^+$ cells and $CD8^+$ CTLs. The $CD4^+$ part of CIKs seemed to have no direct tumor lytic activity. The results indicate that the special "Th1 bias" and enhanced cytotoxicity against K562 cells occurred in PBMCs after multicycles of Auto-CIK infusions suggesting the induction of a "Th1 shift" and rectification of "Th2 dominance" in PBMC after Auto-CIK treatments.

KEYWORDS: CIKs, antitumor immunity, solid tumor, Th1 bias.

ABBREVIATIONS

Auto-CIK: autologous cytokine induced killer cell; PBMC: peripheral blood mononuclear cells; LAK: lymphokine activated killer cells; TIL: tumor infiltrating lymphocytes; Th: T helper cells; McAb: monoclonal antibody; FCS: fetal calf serum; AML: acute myeloid leukemia; CML: chronic myeloid leukemia; MM: multiple myeloma; IL: interleukin.

It is accepted that for most patients with primary or metastatic carcinoma, standard therapeutic methods can not produce an absolute cure. But immunotherapy, as an accessory treatment to standard therapies, is a promising strategy to achieve those results. As a currently emerging adoptive immunotherapy, treatment with

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cytokine-induced killer cells (CIKs) has shown significant antitumor activity both in preclinical experiments and animal tumor models.

CIKs are a heterogeneous cells subset derived from human peripheral mononuclear cells (PBMCs) which have been cultured in the presence of multiple cytokines, including rhIL-1α, rhIL-2, rhIFN-γ and mouse anti-hCD3 monoclonal antibodies (McAb).[1-3] Compared with traditional LAKs and TILs, CIKs have unique advantages such as enhanced cell proliferation and cytotoxic activity, an enlarged antitumor spectrum, more cytotoxicity toward drug-resistant tumor cell lines, resistance to Fas-mediated apoptosis and lower hemopoietic toxicity.[4-6]

Up to now, most of the autologous CIKs(Auto-CIK) treatments have been targeted against hematological malignancies, such as AML, CML, MM etc.[7-9] But few studies with patients bearing a solid tumor, especially large-scale clinical trails, have been carried out. In this article, we compared amplification, phenotypic characteristics, cytokine secretion, antitumor cytotoxicity and the clinical response to Auto-CIK derived from 65 cases of solid-tumor bearing patients with different types and clinical stages to identify feasibility and validity of employing Auto-CIK in solid malignancy treatment.

MATERIALS AND METHODS

Patients

Sixty-five patients with solid tumor were involved in this clinical trial including the following carcinomas: gastric (15), pulmonary (11), colorectal (11), hepatocystic (6), renal (6), lymphoma (4), breast (3), pancreatic (2), esophageal (2), and others (such as ovarian, thyroid, sarcoma, etc.). The ages ranged from 17 to 72 years (median 62). Exclusion criteria were a history of asthma, venous thrombosis, congestive heart failure, autoimmune disease, active infection, late-phase hepatorenal dysfunction and severe CNS diseases. Twenty-nine of the 65 patients had metastasis in various organs, including local lymph nodes, liver, lung, intestine, colon, bone, ovary, retroperitoneum, thorax, soft tissue, etc. The others had a primary tumor resection before and no metastasis identified at the time of entry into the trail. All biotherapies and chemotherapies ended 1 month prior to starting the clinical trial. Patients were treated as inpatients. Nineteen of the 65 patients (including 4 cases of renal cancers, 4 pulmonary carcinomas, 2 breast cancers, 2 colorectal carcinomas, 3 lymphomas and 1 of each of the following: ovarian carcinoma, thyroid carcinoma, sarcoma and hepatoma) underwent at least 3 cycles of Auto-CIK treatments at 1-month interval. No standard antitumor therapies were given during the whole Auto-CIK treatment period (Table 1).

Cell lines

To monitor antitumor cytotoxicity of the Auto-CIK against different types of malignancy, the following human tumor cell lines were used: non-small cell lung cancer (NSCLC) CALU-6, breast cancer MCF-7, malignant melanoma Mel-526, colorectal carcinoma 823, leukemia K562 and lymphoma Raji. The cells were cultured in RPMI-1640 medium supplemented with 10% FCS (Gibco-BRL, USA), 100 U/ml penicillin and 100 U/ml streptomycin. All cell lines listed above were grown in our laboratory except Mel-526 which was a gift from Dr. Kato (Takara Biotechnology Co. LTD, Japan).

Auto-CIK amplification

Auto-CIK was generated as described. Enriched PBMCs from the patients were prepared using a Ficoll separation and then cultured in RPMI-1640 medium (Gibco-BRL), consisting of 10% human AB serum, 25 mM HEPES, 100 U/ml penicillin and 100 U/ml streptomycin. On day zero the following were added: 100 ng/ml of mouse anti-human CD3 monoclonal antibody (Endogen, USA), 100 U/ml recombinant human IL-1 α (Endogen, USA) and 1000 U/ml recombinant human IFN-γ (Lizhu, Guangzhou). After 24 h of incubation, 300 U/ml recombinant human IL-2 (Shuanglu, Beijing) was added. The cells were incubated for 10-12 days at 37℃ in a humidified atmosphere of 5% CO_2 and subcultured at 3×10^6 cells/ml every 3 days in fresh complete medium with the same final concentration of rhIL-2 and rhIFN-γ.

Table 1. Clinical data of eligible patients

Cancer type	Number of patients	Female/male	Median age	Metastasis		Previous therapies
				Organs	Patient No.	
Gastric carcinoma1	15	5/10	58	Liver	2	Surgery
				Ovary	1	Chemotherapy
				Local LN	4	LAKs
Pulmonary carcinoma	10	3/7	55	Liver	2	Surgery
				Bone	1	Chemotherapy
				Thraox	1	rhIL-2/LAKs
Colorectal carcinoma	11	4/7	60	Liver	2	
				Lung	2	Surgery
				Bone	1	Chemotherapy
				Local LN	2	LAKs
				Soft tissue	1	
Renal carcinoma	6	0/6	51	Lung	1	Surgery
						rhIL-2/IFN-γ LAKs
Hepatocholangio carcinoma	6	1/5	67	Local	2	Surgery
				Intestine	1	Chemotherapy
Lymphoma	4	1/3	48	-	-	Surgery
						Chemotherapy
Breast cancer	3	3/0	42	Liver	1	Surgery
				Bone	1	Chemotherapy
				Local LN	1	rhIL-2
Pancreatic carcinoma	2	0/2	64	Liver	1	Surgery
						Chemotherapy
Esophagus carcinoma	2	1/1	60	-	-	Surgery
						Chemotherapy
Ovary carcinoma	1	1/0	46	-	-	Surgery
						Chemotherapy
Thyroid gland carcinoma	1	0/1	29	Lung	1	Surgery
						LAKs
Sarcoma	4	2/2	49	Lung	1	Surgery
						Chemotherapy
						Radiotherapy
						LAKs

LAKs were cultured for 7 days in same complete medium supplemented only with 1000 U/ml rhIL-2. Viability of both CIKs and LAKs was determined by trypan blue staining.

Phenotypic analysis

The phenotypic expression of paired samples including untreated PBMCs and Auto-CIKs from 22 patients selected randomly from the 65 patients on the trail was examined by 2-color fluorescence. Cells (5×10^5) were resuspended in 20 μl of buffer (PBS, 2% newborn calf serum, and 1% sodium azide) and incubated with 5 μl of appropriate PE- or FITC-labeled various monoclonal antibodies (McAbs) against human CD3, CD4, CD8, CD16, CD20, CD56, CD25, and CD14 (DAKO, DENMARK) cells for 30 min at 4℃. After incubation, the cells were washed twice and resuspended in 1.0 ml of assay buffer. The fluorescence was analyzed by a Coulter X-100 flow cytometer (Beckman-Coulter). Five thousand to 10,000 events were recorded for each

sample using the Expo II Software that simultaneously detects forward scatter, side scatter, FL1 (FITC label), and FL2 (PE label) data. The settings for all of these parameters were optimized at the initiation of the study and maintained constantly during all subsequent analyses. Isotype-matched IgG1 (DAKO) was used as controls. Background staining using irrelevant antibodies was less than 2%.

Cytotoxicity assays

A Cyto Tox 96 non-radioactive cytotoxicity assay (Promega) was performed as described in the operation manual. The assay was used to compare the cytotoxic activity of Auto-CIK with LAKs, and PBMCs before and after Auto-CIK infusion against different tumor cell lines from 9 patients who received at least 3 cycles of the Auto-CIK treatments at different effector to target ratios. As a measurement of cytotoxicity, lactate dehydrogenase (LDH) in the medium was determined, which is released upon cell lysis in much the same way as ^{51}Cr is released in cell toxicity assays. Released LDH activity in the culture supernatant was measured by a colorimetric assay, in which the amount of color formed is proportional to the number of lysed cells. Briefly, 1×10^5 cells/ml target cells were incubated for 4 h in triplicate sets in a U-bottom 96-well tissue culture plate with various ratios of effector to target cells. After incubation, 50 μl aliquots from the wells were transferred to a new flat 96-well plate. A 50 μl substrate mixture was added to each well of the plate followed by incubation at room temperature for 30 min in the dark, after which 50 μl of stop solution was added to each well, and the absorbance was measured at 490 nm using a 96-well plate reader (Labsystem Multiscan MS). Specific cytotoxicity was calculated by {specific cytotoxicity= [(experimental counts-effector control counts −target spontaneous counts) / (target maximal counts-target spontaneous counts)] × 100% }. Basal release of the tumor targets was < 25%. Cytotoxicity against different target cells including K562, CALU-6, MCF-7, Mel-526, 823 and Raji cells was examined. All experiments were performed in triplicate and the mean value calculated.

Cytokine secretion of PBMC

Cytokine secretion in PHA-activated PBMC culture supernatants before and after Auto-CIK treatments was detected by an ELISA assay. The PBMCs from 9 patients who were to receive at least 3 cycles of the Auto-CIK treatments were collected at beginning of the first Auto-CIK culture. One month after the third Auto-CIK infusion, PBMCs were collected again for the cytokine assay. Briefly, microtitre plates were coated with specific anti IFN-γ, IL-2, TNF-α, IL-4, IL-10 and IL-12 McAbs in order to bind the cytokines produced in the cell culture supernatants. After several washes to remove unbound proteins, specific enzyme-linked polyclonal antibodies to the cytokines listed above were added to the multiple cytokines adhering to the plates. The substrate solution was added after washing and the developed color measured at 450 nm using a multiwell plate reader. Concentrations of the multiple cytokines in the samples were determined by comparison to a calibration curve of standards provided by the supplier.

Clinical outcome

The following information regarding the patients was recorded: alterations in symptoms, psychical status, tumor size, serum biochemistry and CT scanning examination, with special concern given to 19 of the 65 patients who received 3 cycles of Auto-CIK treatments. A partial response was defined as a decrease of more than 50% of all measurable tumor manifestations for at least 4 weeks without new manifestations of disease. Stable disease was defined as no decrease of measurable tumor manifestations (WHO 1979).

Statistical analysis

Data were presented as the mean ± standard deviation. Statistical analyses were performed using the student's or matched-pair *t*-test for quantitative data. A P value < 0.05 was considered statistically significant.

RESULTS

Large scale amplification of Auto-CIK

The total sum of Auto-CIKs per cycle used was

16-160× 10^8 (median 51× 10^8). Based on trypan blue staining cellular vitality reached 95% or even higher after 10-12 days of culture.

Phenotypic analysis of Auto-CIK

Auto-CIK and PBMCs were stained with various monoclonal antibodies as outlined above. Compared with the PBMCs of 22 patients, the Auto-CIK showed a significant increase of total T ($CD3^+$) cells, $CD4^+$ Th ($CD3^+CD4^+$)cells, $CD8^+$ CTL ($CD3^+CD8^+$) cells, $CD3^+CD56^+$ cells, and active T ($CD25^+$) cell subsets in relative numbers of Auto-CIK. However a statistical decrease in the proportion of NK cells ($CD16^+CD56^+$), monocytes ($CD14^+$)and B cells ($CD20^+$) was observed (Fig.1).

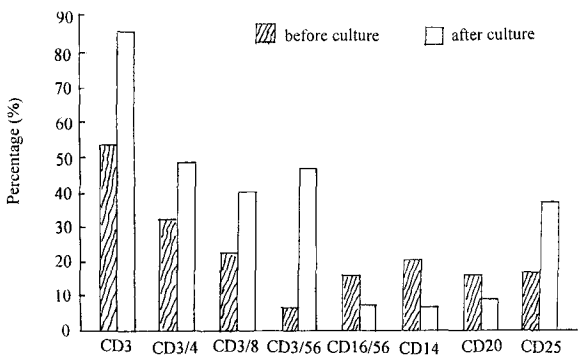

Fig.1. Phenotypic analysis of Auto-CIK before and after 10-12 days of culture. Percentages of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+CD56^+$ and $CD25^+$ cell subsets increased from 50.61 ± 9.22%, 29.37 ± 4.60%, 20.12 ± 6.22%, 3.9 ± 1.87% and 14.55 ± 5.08% to 83.16 ± 10.62%, 45.74 ± 12.45%, 37.52 ± 15.74%, 43.55 ± 14.25% and 34.73 ± 15.22 respectively, with P <0.01. But percentages of $CD16^+CD56^+$, $CD14^+$, $CD20^+$ cell subsets decreased from 13.52± 7.23%, 18.23 ± 11.72% and 13.74 ± 6.39% to 5.41 ± 3.79%, 4.65 ± 1.80% and 7.49 ± 2.72% respectively, with P<0.05.

Phenotypic charactistics of Auto-CIK derived from patients in a different clinical stage were also studied. The proportions of $CD4^+$ Th ($CD3^+CD4^+$) cells, $CD8^+$ CTLs ($CD3^+CD8^+$), $CD3^+CD56^+$ cells, and active T ($CD25^+$) cell subsets in Auto-CIKs derived from metastatic patients declined compared with those post-operation but showed no statistical significance (Table 2).

Table 2. Comparison of phenotypic characteristics of Auto-CIKs derived from patients with a solid tumor at different clinical stage

Membrane protein exprssion	Auto-CIKs metastatic	Auto-CIKs post-operation	*P*
$CD3^+$	91.08 ± 3.1	78.22 ± 11.8	0.05
$CD3^+CD4^+$	46.58 ± 25.5	63.08 ± 26.5	0.35
$CD3^+CD8^+$	49.80 ± 27.6	53.78 ± 30.1	0.83
$CD3^+CD56^+$	42.22 ± 24.1	45.62 ± 22.8	0.82
CD25	33.70 ± 13.8	34.02 ± 18.7	0.98

Cytotoxicity of Auto-CIK in vitro

Auto-CIK or LAKs from different patients were mixed with various types of tumor cell lines at decreasing effector to target ratios from 100:1 to 6:1. Auto-CIK showed more effective lysis of NK-sensitive or non-sensitive tumor cell lines than LAKs at a E/T ratio of 50:1. Mean values of cytotoxicity of CIKs against K562, CALU-6, 823, MCF-7 were 47.37 ± 6.86%, 30.78 ± 10.97%, 35.66 ± 12.87%, 26.26± 6.9%; that of LAKs were 38.58 ± 4.93%, 15.88 ± 3.41%, 13.13 ± 7.98%, 12.41 ± 6.07%, with P values of 0.031, 0.018, 0.037, 0.034, respectively (Fig.2A).

K562 is a NK-sensitive tumor cell line. Cytotoxicity of LAKs against these cells was less than that of Auto-CIK but only at the higher ratios of E/T (100:1 or 50:1) and lowest (6:1) with the differences statistically significant. Mean values of cytotoxicity rates of CIKs against K562 at different E/T ratios from 100:1 to 6:1 were 84.68 ± 14.08%, 47.37 ± 6.86%, 39.26 ± 12.48%, 33.86± 8.13%, 24.84± 6.02%, and that of LAKs were 46.7± 2.26%, 38.58± 4.93%, 37.63± 2.71%, 30.48± 16.2%, 12.39 ± 5.02%, with P values of 0.008, 0.031, 0.932, 0.669 and 0.014, respectively (Fig.2B).

CALU-6 is a NK non-sensitive tumor cell line, and cytotoxicity of LAKs against it was significantly lower than Auto-CIK at each E/T ratio ranging from 100:1 to 6:1. Mean values of cytotoxic rates of CIKs against CALU-6 at different E/T ratios from 100:1 to 6:1 were 45.82 ± 12.7%, 30.78 ± 10.97%, 25.77 ± 15.50%, 15.42± 6.24%, and 6.17± 1.66%; and that of LAKs were 33.2 ± 5.05%, 15.88± 3.41%, 13.79± 3.84%, 5.08 ± 1.52%, and 1.20 ± 1.11%, with P values of

0.044, 0.018, 0.037, 0.029 and 0.013, respectively (Fig. 2C).

Cytotoxic activity of Auto-CIK derived from patients in different clinical stages was also studied. Compared with those post-operation, the cytotoxicity of Auto-CIK derived from metastatic patients against K562, CALU-6 and MCF-7 tumor cells down regulated but showed no statistical significance (Table 3).

Table 3. Comparison of cytotoxicity characteristics of Auto-CIKs derived from patients with a solid tumor at different clinical stages (E/T=50:1)

	Auto-CIK (%)		
Tumor cell lines	Metastatic	Post-operation	*P*
K526	45.85 ± 5.7	46.54 ± 7.2	0.89
CALU-6	25.78 ± 11.6	36.70 ± 8.6	0.21
MCF-7	19.54 ± 10.6	24.47 ± 6.7	0.51

Immunological detection of patients' PBMCs after treatmentment with Auto-CIK

PBMCs were collected from 9 patients who received 3 or more cycles of the Auto-CIK treatments at the beginning of the first CIK culture and a month after the third CIK infusion. The PBMCs were examined for immunological parameters, including phenotypic analysis, antitumor lytic activity, and multiple cytokine secretion.

Phenotypic analysis of the patients' PBMCs after Auto-CIK treatments

PBMCs from the patients before and after 3 cycles of Auto-CIK treatments were stained with various monoclonal antibodies against characteristic surface molecules and analyzed by flow cytometry. No statistically significant difference was observed in the cell subpopulation distribution during treatments (Fig. 3).

Cytotoxicity of patients' PBMCs after Auto-CIK treatments

The cytotoxic effect of PBMCs was monitored using K562 as target cells in a non-radioactive cytotoxicity assay. Cytotoxic activity of the PBMCs significantly

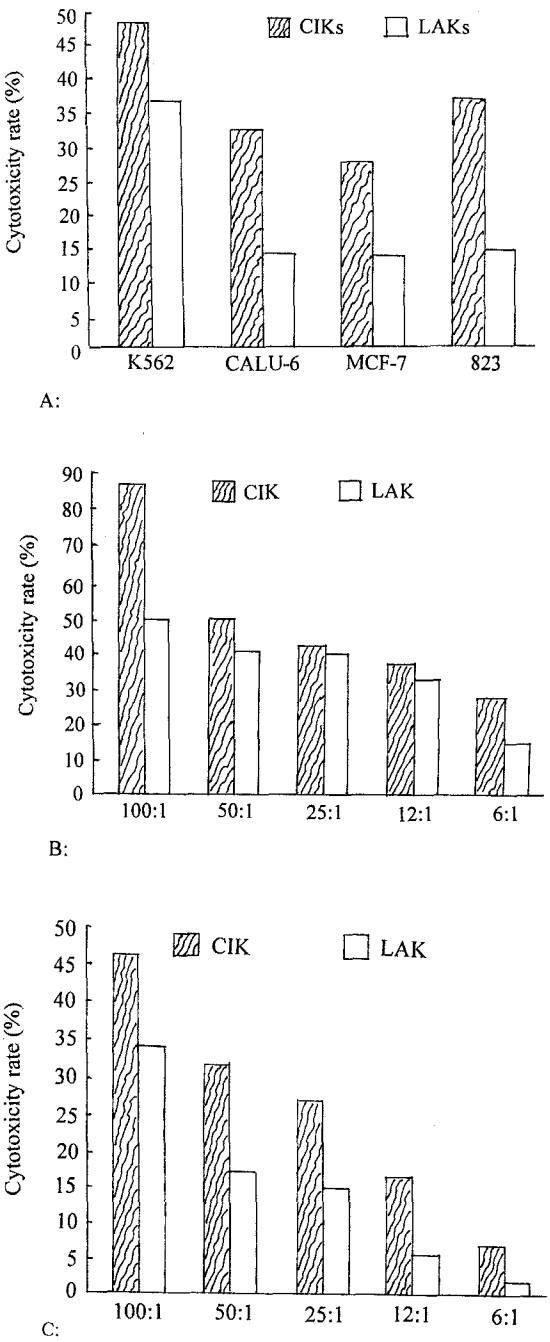

Fig.2. Cytotoxicity of Auto-CIKs and LAKs cells against 4 different tumor cell lines (including K562, CALU-6, MCF-7 and 823) at different effector to target ratios in vitro was examined using a non-radioactive cytotoxic test. All experiments were performed in triplicate.

A: Auto-CIKs showed more effective lysis of NK sensitive or non-sensitive tumor cell lines than LAKs at the 50:1 effector to target cell ratio.

B: Target cells K562, a NK sensitive tumor cell line, cytotoxicity of LAK against which was less than CIK at E/T ratios described above, but only at much higher E/T ratios (100:1 or 50:1) and lowest E/T ratio(6:1) this difference was statistically significant.

C: Target cells CALU-6, a NK non-sensitive tumor cell line, cytotoxicity of LAK against which was significantly lower than CIK at each E/T ratios ranged from 100:1 to 6:1.

increased during treatment at 3 effector to target cell ratios ranging from 40:1 to 10:1 with P <0.05 (Fig.4).

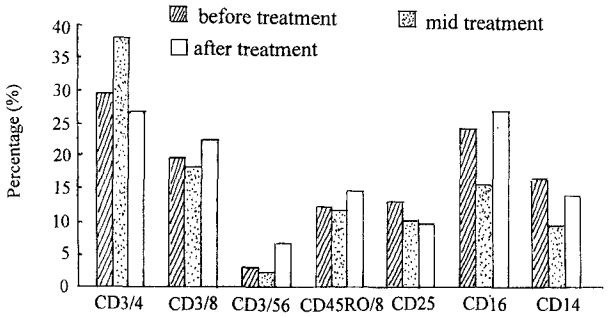

Fig.3. Phenotypic alteration of PBMCs during CIKs treatments. Three groups are listed: distribution of different cell subsets in PBMCs before CIKs treatments, (densely plotted bars); in median of CIKs treatments, (sparsedly plotted bars); after CIKs treatments, (blank bars). No statistically significant differences were observed in the cell subpopulation distribution during the treatments.

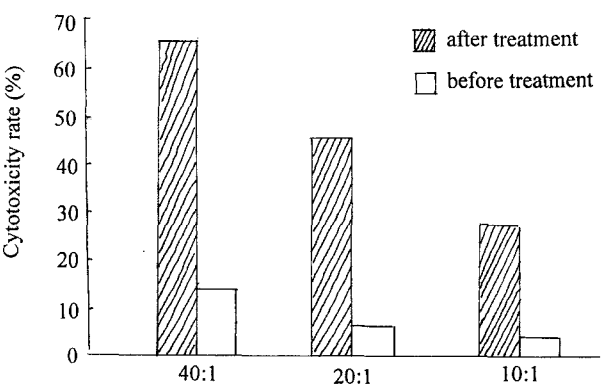

Fig.4. Cytotoxicity of PBMCs against K562 cells before the CIKs treatment (light bar) and after 3 cycles of the CIKs treatment (dark bar) at various effector to target ratios. Cytotoxic activity of PBMCs increased significantly during the treatments at continuative effector to target cell ratio ranging from 40:1 to 10:1. Average cytotoxicity rate of PBMCs after 3 cycles of the Auto-CIK treatments was 62 ± 9.68%, 42.36± 8.84% and 23.91 ± 8.89% at E/T ratio of 40:1,20:1 and 10:1; and that of PBMCs at beginning of the Auto-CIK treatments of the same patients was 10.53± 8.57%, 2.97 ± 1.43% and 0.69 ± 0.59%, with P <0.05.

Cytokine secretion of patients' PBMCs after Auto-CIKs treatments

PBMC samples collected before and after the Auto-CIK treatments were activated by PHA for 48 h before culture supertanants were collected for cytokine analysis by ELISA. Statistically significant elevation in IFN-γ, IL-2 and TNF-α secretion were found, however no statistical differences were identified in IL-4, IL-10 and IL-12 secretion after multicycles of the Auto-CIK treatments (Table 4).

Table 4. TNF-α, IFN-γ, IL-12 and IL-10 secretion in the supernatant of PHA-activated PBMCs after CIKs treatment

Cytokines	Before treatment (pg/ml)	After treatment (pg/ml)	P
IL-2	186.11 ± 126.70	240.40 ± 130.66	0.044
IFN-γ	303.07 ± 176.23	649.18 ± 276.73	0.005
IL-4	16.03 ± 14.09	14.34 ± 10.40	0.722
IL-10	356.87 ± 90.26	438.47 ± 51.61	0.108
TNF-α	892.37 ± 432.18	1323.46 ± 201.93	0.034
IL-12	4.11 ± 5.63	22.22 ± 23.39	0.131

Clinical outcome after the Auto-CIK treatments

Cell administration

Every patient received 2 intravenous infusions of Auto-CIK per cycle with a 1 day interval. Nineteen of the 65 eligible patients had received 3 or more cycles of CIK treatments with an interval of 1 month. Patients received a mean of 50×10^8 Auto-CIK with a range of $3.5\text{-}160 \times 10^8$ (Table 5). Cellular vitality was 95% or higher as determined by trypan blue staining.

Clinical toxicity of treatment

No potential adverse events, such as fever, chill, fatigue were detected during each of the Auto-CIK infusions. Checks of sterility of CIKs and blood cultures were negative at all time. Endogenous pyrogen examinations were negative.

Patient outcome

Nine of the 19 patients who received 3 or more cycles of Auto-CIK treatments were in progressive disease when entering this protocol. With respect to our protocol, clinical outcome was based mainly on comparison of CT scans before and after treatment. Two of those 9 patients died of relapse, one at 8 mouths and the other at 10 months. Neither had any progression of their metastases or primary tumor. Five of the 9 patients had been alive with stable disease since they received the Auto-CIK treatme..ts. Their CT scans showed no

Table.5 Clinical datafrom 19 patients who received at least3 cycles of CIKs treatments

	Cancer type	Clinical phase	Metastasis	Treatment cycles	Infusing cells per cycle	Adverse events	TTP	Outcome	Resp. rate
1	Breast	IV	+	7	3.2×10^9	-	9	alive	SD
2	Renal	IV	+	6	1.7×10^9	-	13	alive	SD
3	Renal	IV	+	4	2.8×10^9	-	8	alive	PR
4	Breast	IV	+	4	5×10^9	-	5	alive	PR
5	Thyroid gland	IV	+	3	4×10^9	-	11	alive	SD
6	Pulmonary	IV	+	3	2×10^9	-	10	dead	-
7	Colorectal	IV	+	3	5.6×10^9	-	5	alive	SD
8	Pulmonary	IV	+	3	2.3×10^9	-	8	dead	-
9	Ovary	IV	+	3	1×10^9	-	13	alive	SD
10	Renal	II	-	10	3.8×10^9	-	14	alive	CCR
11	Colorectal	II	-	10	2.5×10^9	-	16	alive	CCR
12	NHL	III	-	4	1.3×10^9	-	12	alive	CCR
13	NHL	III	-	4	2.6×10^9	-	10	alive	CCR
14	Pulmonary	III	-	4	3.1×10^9	-	8	alive	CCR
15	Hepatoma	III	-	4	1.2×10^9	-	9	alive	CCR
16	NHL	III	-	3	2.3×10^9	-	7	alive	CCR
17	Sarcoma	III	-	3	3.4×10^9	-	7	dead	-
18	Renal	III	-	3	3.5×10^9	-	5	alive	CCR
19	Pulmonary	III	-	3	3.2×10^9	-	8	alive	CCR

Resp.rate: clinical response rate; SD: stable disease; PR: partial response; CCR: continuous complete response.

expansion in tumor size (< 25%) in comparison to scans taken at the start of the CIK treatments. Among another 2 patients out of the 9, one had breast cancer with skin and bone metastasis; the other had kidney cancer with pulmonary metastasis. Based on their clinical and CT diagnostic criteria both had obtained PR after the Auto-CIK treatments (Photo 1, 2). The remaining 10 patients, who had no detectable metastasis at the initiation of this clinical trail, have been showing improvement in symptoms and life quality and maintaining stable status for at least 1 year after starting the Auto-CIK treatments, except for 1 sarcoma patient who relapsed suddenly and died after having 7 months of a stable status.

Among 46 of 65 patients who received less than 2 cycles of the Auto-CIK treatments, 20 patients had distant metastases in the liver, lung, intestine, bone, ovary, etc. Six of those patients died in 3 months after starting the Auto-CIK treatments. Eight patients were alive for at least 9 months and 6 patients lost follow-up. Among another 26 patients without metastases, 3 had died, 6 lost their follow-up, and the remaining 17 patients were alive for at least 4 months after starting the Auto-CIK treatments.

DISCUSSION

Surgery and radiotherapy can eliminate solid primary tumors in stage I and II. But if metastases or relapse occur, chemotherapy often seems to be the suitable treatment. Besides the severe toxicity and inhibition of the host immune system, the ability of malignant cells to survive exposure to cytotoxic agents is still a major obstacle to cure solid tumor patients. Immunological effector cells such as LAKs, TILs, CIKs give a more promising future to those malignant solid tumor patients who have no chance for surgery and are resistant to chemotherapy.[10-12]

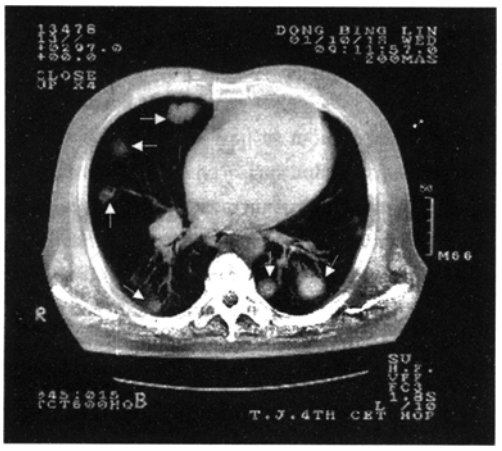

Photo 1. The CT scan of multiple pulmonary metastases (white arrows shown) of a renal cancer patient at the beginning of receiving Auto-CIK treatments.

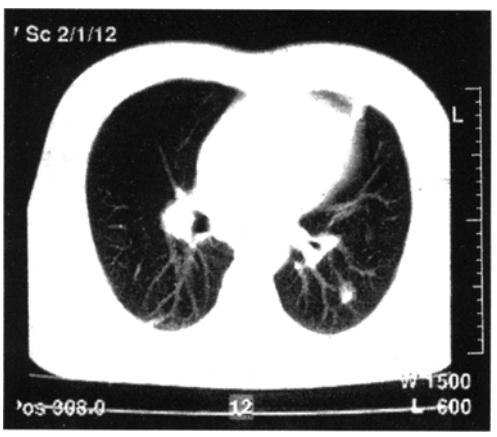

Photo 2. The CT scan showing the shrinkage and disappearance of multiple pulmonary metastases of the same renal cancer patient 1 year after receiving 4 cycles of Auto-CIK treatments.

Large numbers of these effector cells are necessary for effective immunotherapy and for some patients it is difficult to obtain sufficient effector cells since these cells grow poorly in vivo. In this research we examined the possibility for large scale in vitro amplification and the degree of cytotoxicity of Auto-CIKs against various tumor cells in different clinical stages of patients. In all 65 patients entering this trial, either metastatic or post-operative, PBMCs could be proliferated in large scale by stimulation of multiple cytokines. Comparing phenotypic characteristics and cytotoxicity against various tumor cells, no statistically significant differences were observed between the metastatic and postoperative groups. These results suggested that seriousness of disease and metastatic status have no influence on effective cells and antitumor immunological activity of Auto-CIK, therefore CIKs are a more suitable immunotherapy for the late phase of solid malignany patients with metastasis. Comparison of the cytoxicity of LAKs with CIKs against various tumor cells at several effector to target ratios, CIKs showed more effective cytotoxicity against non-NK sensitive solid tumor cell lines at a low effector to target ratio (6:1), which indirectly suggested that Auto-CIK may have a longer effective time in vivo than LAKs. These results indicate that CIKs are more suitable than LAKs for immunotherapy of those solid malignany patients who are at high risk of relapse and recurrence.

Here, we report the results of a considerably large sample clinical trial using Auto-CIK for the treatment of 65 patients with different tumor types and clinical stages of solid malignancy. A previous report indicated that infusion of a large number of immunological effectors would induce some adverse effects to a greater or less extent, such as fever, anemia, and so on.[13] But in our trial, after thoroughly washing off the culture medium and adding a special concentration of HSA to prevent cell congregation, no adverse effects noted before were observed during each CIKs infusion, which suggested that adverse effects could be controlled. This study demonstrates the feasibility and low toxicity of such a new immunotherapeutic approach.

Also in this study, we examined changes in the immunological activity of the PBMC samples from 9 patients before and after the Auto-CIK treatments. In order to eliminate possible interference from the infused Auto-CIK as an explanation of our results, the PBMC samples from the patients who received the Auto-CIKs treatments were collected 1 month after the third Auto-CIK infusion. We found statistically significant increases in IFN-γ, IL-2 and TNF-α, but not in IL-4 and IL-10 levels in PHA activated supernatants of PBMC cultured from the 9 patients who received at least 3 cycles of Auto-CIK treatment. Non-specific cytotoxicity against the K562 tumor cell line was also enhanced. These results suggest that a "Th1 shift" occurred after the multicycle treatments with Auto-CIK. It's known that significant "Th2 dominance"

in PBMC is regarded as one of the important reasons for immunosuppression in cancer patients, and that suppression can be interrupted by immunostimulating cytokines or agents.[14,15] In comparing the distribution of various cell subpopulations in PBMC during CIKs treatment, a 10% elevation in $CD4^+$ Th cells was observed although this change was not statistically significant. This elevation may correlate with large amounts of Auto-CIK continuously infused into patients in short period of time reported in the literature. So the appearance of a "Th1 shift" and rectification of the "Th2 dominance" in PBMCs, as a result of the Auto-CIKs treatment could significantly enhance antitumor immunity against the solid tumor of patients and thereby eradicate tumor cells as well as prevent recurrence; and may also serve as another antitumor immunological mechanism for CIKs in addition to direct cytotoxicity against tumor cells.

The clinical outcomes in all patients, who received at least 1 cycle of Auto-CIK treatment and who were followed up for at least 7 months, showed significant relationship with the cycles administered and the proportion of $CD3^+CD56^+$ cells in the subpopulation of Auto-CIK, but there was no significant relationship with metastasis, total number of cells administered, proportions of $CD4^+$ T and $CD8^+$ CTL cells (data not shown). These results suggested that only a high proportion of $CD3^+CD56^+$ cells in the subpopulation, and continuous CIK treatment are the key guarantee of clinical efficiency, although many factors have effects on immunological activity of CIKs in in vitro experiments.

This is the first report of our large sample clinical trial regarding the effect of the Auto-CIK treatment on multiple types of solid malignancy. The research has provided basic and clinical evidence for more extensive applications using Auto-CIK therapy for solid malignancies in the future.

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REFERENCES

1 Schmidt-Wolf GD, Negrin RS, Schmidt-Wolf IG. Activated T cells and cytokine-induced $CD3^+CD56^+$ killer cells. Ann Hematol. 1997; 74:51-56.

2 Margolin KA, Negrin RS, Wong KK, et al. Cellular immunotherapy and autologous transplantation for hematologic malignancy. Immunol Rev. 1997; 157:231-240.

3 Lu PH, Negrin RS. A novel population of expanded human $CD3^+CD56^+$ cells derived from T cells with potent in vivo antitumor activity in mice with severe combined immunodeficiency. J Immunol. 1994; 153:1687-1696.

4 Schmidt-Wolf IG, Lefterova P, Johnston V, et al. Sensitivity of multidrug -resistant tumor cell lines to immunologic effector cells. Cell Immunol. 1996; 169:85-90.

5 Hoyle C, Bangs CD, Chang P, et al. Expansion of Philadelphia chromosome-negative CD3(+)CD56(+) cytotoxic cells from chronic myeloid leukemia patients: in vitro and in vivo efficacy in severe combined immunodeficiency disease mice. Blood. 1998; 92:3318-3327.

6 Verneris MR, Kornacker M, Mailander V, et al. Resistance of ex vivo expanded $CD3^+CD56^+$ T cells to Fas -mediated apoptosis. Cancer Immunol Immunother. 2000; 49:335-345.

7 Scheffold C, Brandt K, Johnston V, et al. Potential of autologous immunologic effector cells for bone marrow purging in patients with chronic myeloid leukemia.Bone Marrow Transplant. 1995; 15:33-39.

8 Muller M, Scheffold C, Lefterova P, et al. Potential of autologous immunologic effector cells for prediction of progression of disease in patients with chronic myelogenous leukemia. Leuk Lymphoma. 1998; 31:335-341.

9 Linn YC, Lau LC, Hui KM. Generation of cytokine-induced killer cells from leukaemic samples with in vitro cytotoxicity against autologous and allogeneic leukaemic blasts. Br J Haematol. 2002; 116:78-86.

10 Grimm EA, Mazumder A, Zhang HZ, et al. Lymphokine activated killer cell phenomenon: lysis of natural killer resistant fresh solid lymphocytes. J Exp Med. 1982; 155: 1823-1841.

11 Rosenberg SA, Spiess P, Lafreniere R. A new approach to the adoptive immunotherapy of cancer with tumor infiltrating lymphocytes. Science. 1986; 233: 1318-1321.

12 Mehta BA, Schmidt -Wolf IG, Weissman IL, et al. Two pathways of exocytosis of cytoplasmic granule contents and target cell killing by cytokine-induced CD3+ CD56+ killer cells. Blood. 1995; 86:3493-3499.

13 Schmidt -Wolf IG, Finke S, Trojaneck B, et al. Phase I clinical study applying autologous immunological effector

cells transfected with the interleukin-2 gene in patients with metastatic renal cancer, colorectal cancer and lymphoma. Br J Cancer. 1999; 81:1009-1116.

14 Mori T, Takada R, Watanabe R, et al. T-helper(Th)1/Th2 imbalance in patients with previously untreated B -cell diffuse large cell lymphoma. Cancer Immunol Immunother. 2001; 50:566-568.

15 Sato M, Goto S, Kaneko R, et al. Impaired production of Th1 cytokines and increased frequency of Th2 subsets in PBMC from advanced cancer patients. Anticancer Res. 1998; 18:3951-3955.