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Original Article

Cytokine-induced killer cells (CIK) from healthy donors for treatment of advanced breast cancer

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Abstract: Objectives: The aim of this study was to investigate clinical efficacy and safety of CIK cells isolated from healthy donors for treatment of advanced breast cancer. Methods: Peripheral blood monocytes were collected from 10 patients with advanced breast cancer and 12 healthy donors. CIK cells were prepared from each sample. Proliferation rates, CD3/CD56 antigen amounts, and killing activity of each CIK sample were compared. Patients were then treated with CIK cells from healthy donors, administered by intravenous drip, once a day, for 6 consecutive days. Results: Proliferation rates, CD3/CD56 antigen amounts, and killing activity of CIK cells isolated from healthy donors were unsurprisingly better than that of CIK cells isolated from cancer patients ($P < 0.01$). Efficacy summary of 10 advanced breast cancer cases: CR (complete remission)-9 cases (90%); PR (partial remission)-1 case; (10%); total effectiveness rate was 100%. Follow up of 10 patients showed 1 survival for more than 1 year, 1 survival for more than 3 years, 4 survivals for more than 5 years (with 'tumor-free' diagnoses), and 4 survivals of 2-4 years (with 'tumor-free' diagnoses). Conclusion: Proliferation rates, CD3/CD56 mark rates, and killing activity of CIK cells isolated from healthy donors are better than CIK cells isolated from advanced breast cancer patients. CIK cells isolated from healthy donors are able to treat advanced breast cancer with good efficacy and safety and should be promoted in clinical application.

Keywords: Healthy donors, CIK cells, advanced breast cancer

Introduction

Breast cancer is a female malignant cancer, with its highest incidence in China [1, 2]. Incidence of this disease has rapidly increased in recent years, tripling between 1990 and 2013. Although various therapies, such as chemotherapy, radiation therapy, and targeted therapy, have been adopted to treat advanced breast cancer and have achieved considerable success [3], 5-30% of the cancer relapses and metastasizes following treatment with traditional therapies [4]. The median survival period of relapsed and metastatic patients is no longer than 2 years [5].

Cytokine-induced killer (CIK) cells are a subset of the T lymphocytes with a natural killer T-cell (NKT)-phenotype, expressing both CD3 and CD56 markers. CIK cells are mostly $CD8^+$, express a heterogeneous TCR repertoire, are CD1d independent, and can be reproducibly expanded *in vitro* from bone marrow or PBMC over a 3-week time-period [6-8]. CIK cells are capable of broad MHC-unrestricted anti-tumor activity against both syngeneic and allogeneic hematological malignancies, as documented both *in vitro* and *in vivo* using murine models [9]. This study attempted to apply CIK cells from healthy donors to treat relapsed and metastatic advanced breast cancer in patients unsuccessfully treated by traditional therapies.

Materials and methods

Overview of patients

The Medical Center received 10 patients with advanced breast cancers since 2007. Of these, 3 were under 60 years old and 7 were above 60. Of these patients, 6 had infiltration ductal carcinomas, 2 experienced infiltration of lobular carcinoma, and 2 had unclear tumors. In 9 patients, cancer was found to have relapsed

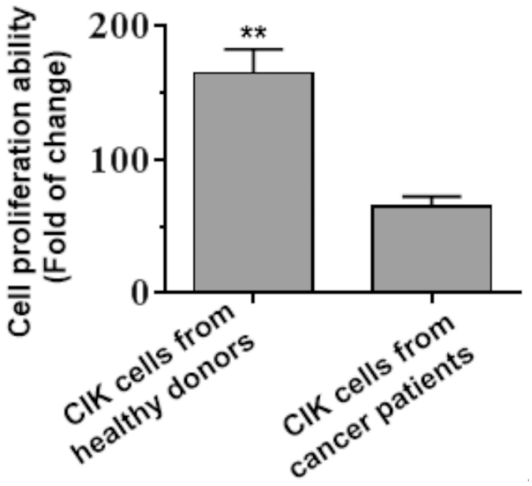

Figure 1. Proliferation ability of CIK cells from healthy donors and cancer patients.

and metastasized while the condition of 1 patient remained undetermined following treatment with traditional therapies. Regarding metastatic locations, cancer had localized to the lymph nodes either under the arm or on the clavicle in 2 patients. In the remaining 8 patients, there were multiple metastases of the lungs, liver, and bones, with brain metastasis in 2 patients.

Reagents and instruments

Culture media GT-T503 (for human lymphocytes) and GT-T603 culture bag were purchased from Beijing BaoRi BioTech. MTT, trypsin, RPMI-1640 culture medium, and fetal bovine serum (FBS) were obtained from Gibco™ (USA). IL-2, IFN-, and CD3 monoclonal antibody were obtained from Cytalab USA. $CD3^+$, $CD8^+$, $CD3^+$ $CD56^+$ double-labeled fluorescent antibody kit, and flow cytometry analysis software, Cellquest, were obtained from BD company (USA). An inverted microscope was purchased from Olympus, Japan while the CO_2 incubator was obtained from Forma Scientific Company, USA.

Preparation of CIK and PBMC cells from healthy donors

Criteria for healthy donors: Ages 18-35 years, regardless of sex or blood type, WBC > 5000/mm3, lymphocytes > 20%, and without hepatitis A, B, C virus, HIV virus, or history of syphilis. Peripheral blood monocytes were collected from 10 patients with advanced breast cancer and 12 healthy donors. CIK cells were prepared from each sample. Preparation and usage guide of cells from healthy donors and cancer patients was described previously [4], with a minor modification: 14-days CIK cells were collected and cultured in RPMI-1640 medium with 10% FBS.

MTT assay

Cytotoxicity of CIK and PBMC cells on killing K562 and LOVO cells were determined by the MTT method, according to manufacturer instructions. Briefly, at the end of cell incubation (72 hours), DMSO 10 μL of the MTT solution (Sigma; St. Louis, MO) was added to each well at a concentration of 5 mg/mL and incubated for an additional 4 hours. Medium was aspirated and formazan crystals were solubilized by the addition of 100 μL of DMSO to each well. OD value was determined at 570 nm using a spectrophotometer (Bio-Tek Instruments; Winooski, VT). Cell viability was calculated using the following formula: cell viability (%) = OD_{570nm} in cells treated with extracts/OD_{570nm} in control cells × 100%. All assays were performed in triplicate with at least 3-5 independent experiments.

In vivo experiment

A total of 30-35 × 10^8 CIK cells from qualified healthy donors were suspended in 100-150 mL saline containing 1% human albumin and applied via intravenous drip to 10 advanced breast cancer patients. It was applied one time per day, for 6 days, a total of three courses.

Tumor status was evaluated every 2-3 months, following RECIST standards: Complete remission (CR) - all lesions disappeared for at least 4 weeks; Partial remission (PR) - the sum of vertical diameter and horizontal diameter of the largest tumor reduced by at least 30% and maintained in that state for at least 4 weeks; Stable Disease (SD) - the sum of vertical diameter and horizontal diameter of the largest tumor changed between PR and CR states; Progressed Disease (PD) - the sum of vertical diameter and horizontal diameter of the largest tumor increased by at least 20%.

Statistical analyses

Data are presented as mean ± SD, unless otherwise indicated. Data was analyzed by Student's *t*-test to determine any differences

Table 1. Cell surface antigens of PBMC and CIK cells ($\bar{x}$ ± S %, n=12)

Different target cell groups	Cases	PBMC		CIK	
		$CD3^+$	$CD3^+CD56^+$	$CD3^+$	$CD3^+CD56^+$
Treatment group (healthy people)	12	72.08±11.30	1.83±0.52	97.08±2.38	34.68±7.12
Control group (Cancer patients)	10	70.67±9.4	1.48±0.14	96.14±2.11	16.28±3.11
T		0.1662	2.342	0.5119	4.102
P		0.8761	0.0622	0.6357	0.0148

Table 2. K562 and LOVO killing activity of PBMC ($\bar{x}$ ± S%, n=12)

Different target cell groups	Cases	K562		LOVO	
		20:1	10:1	20:1	10:1
Healthy people PMBC	12	71.00±4.55	64.51±2.97	52.87±2.29	40.93±2.59
Cancer patients PMBC	10	66.00±1.41	55.89±3.30	35.16±3.05	25.45±3.15
T		2.818	3.363	8.043	6.640
P		0.0432	0.0282	0.0013	0.0027

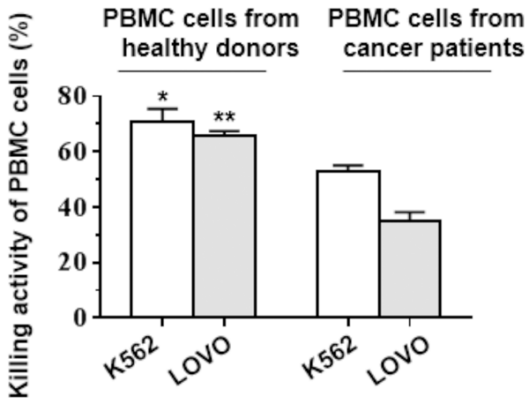

Figure 2. K562 and LOVO killing activity of PBMC. *P < 0.05, **P < 0.01, PBMC cells from healthy donors compared with cancer patients.

between groups, using Prism version 5. Values of $P < 0.05$ were considered statistically significant.

Results

Proliferation ability of CIK cells in the peripheral blood of both healthy donors and cancer patients

CIK cells from healthy donors and cancer patients started to divide at day 4 and reached the highest division rate at day 14, about 165.07-fold and 65.35-fold, respectively, compared to that of day 1. Proliferation ability of CIK cells between the two type sources was statistically significant (P < 0.01) (**Figure 1**).

Cell types and cell surface antigens of CIK and PBMC cells of healthy controls and cancer patients were compared. After 14 days of incubation, a higher percentage of $CD3^+CD56^+$ CIK cells were detected from healthy donors than from cancer patients (**Table 1**).

Killing activity (%) of PBMC cells and CIK cells

Cytotoxicity of CIK and PBMC cells on killing K562 and LOVO cells were determined by the MTT method. Data from K562 and LOVO cell-killing assays showed that killing activity of healthy PBMC against K562 and LOVO cells was significantly higher than cancerous PMBC (P < 0.05) (**Table 2**; **Figure 2**). In addition, the killing activity of healthy CIK against K562 and LOVO cells was significantly higher than cancerous CIK (P < 0.05) (**Table 3**; **Figure 3**).

Evaluation of clinical effects according to RECIST medical imaging standards

Of the 10 advanced breast cancer patients, 9 showed complete remission (CR) and 1 showed partial remission (PR). Total effectiveness, to date, is 100%. All 10 cases were followed up continually. One patient survived for 1 year (received two courses of CIK therapy), 1 patient survived for 3 years (with brain metastasis), 4 patients survived for more than 5 years and were diagnosed as 'tumor-free' (they have resumed normal work and life now), and 4 patients survived for 2-4 years.

Table 3. K562 and LOVO killing activity of CIK ($\bar{x}$ ± S%, n=12)

Different target cell groups	Cases	K562		LOVO	
		20:1	20:1	20:1	20:1
Healthy people PMBC	12	86.25±4.11	76.72±4.44	64.48±3.20	57.53±2.46
Cancer patients PMBC	10	74.18±4.64	65.59±4.40	45.17±2.39	36.31±3.16
T		3.373	3.084	8.374	9.178
P		0.028	0.0368	0.0011	0.0008

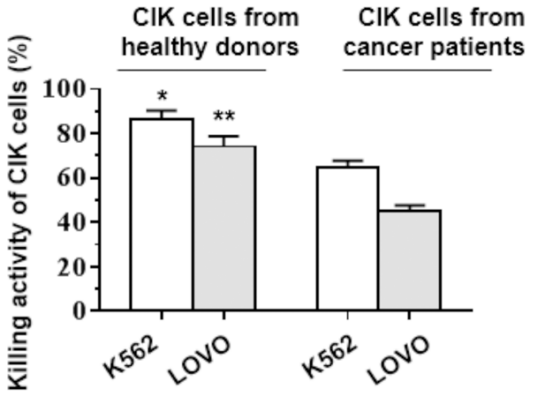

Figure 3. K562 and LOVO killing activity of CIK. *P < 0.05, **P < 0.01, CIK cells from healthy donors compared with cancer patients.

Side effects

None of the patients that received CIK therapy reported significant side effects, except occasional minor fatigue or excitement. These were eased without additional treatment. Occasionally, they had fever or chills. When body temperature reached 39.5°C, medication was used to treat the condition.

Several typical cases

Mrs. Wang, age 36, underwent an infiltration ductal carcinoma surgery on her left breast in October 2006, followed by chemotherapy. The cancer was found to have relapsed in June 2007, with metastasis in both the lobes of the lung and mediastinal lymph nodes, with multiple lesions on the bone. She started receiving CIK therapy from healthy donors in July 2007. After 3 months, cancer lesions on both lobes of the lung and mediastinal lymph nodes disappeared. Pleural effusion was completely absorbed. The situation improved considerably (**Figure 4**). She died 1 year after discontinuing therapy. **Figure 4** shows an image of serious pleural effusion in both lobes of the lung, before therapy, and disappearance of pleural effusion and tumor lesions after therapy.

Mrs. Feng, age 62, underwent surgery on her left breast in October 2007, followed by traditional chemotherapy. The cancer was found to have relapsed in June 2008, with metastasis on the left clavicle lymph nodes. She received chemotherapy and targeted therapy. The cancer was found to have metastasized to the right clavicle lymph nodes in November 2008 and she received local chemotherapy. In February 2009, another metastasis to the lung was suspected. Because of multiple metastases in less than 2 years and repeated surgeries, chemotherapies, radiation therapies, and targeted therapies, her blood parameters declined severely. Her physical condition was too poor to continue with traditional therapies. She turned to CIK cell therapy. It has been 6 years since CIK cell therapy. Her cancer is completely in remission and she has been diagnosed as 'tumor-free'.

Mrs. Zhao, age 61, underwent infiltration ductal carcinoma surgery on her left breast in January 2002, followed by traditional chemotherapy. The cancer was found to have relapsed in 2005, with metastasis on both lobes of the lung. She received chemotherapy again. The cancer was found to have metastasized to the thoracic cavity in 2007. She received another surgery, followed by radiation therapy. Another metastasis was found on the liver in 2011 and she received another round of chemotherapy. There were obvious side effects, including hemoptysis, fatigue, and difficulty breathing. She then turned to CIK cell therapy. It has been 5 years since and all side effects have disappeared. She is still under follow up and observation.

Discussion

Biological tumor therapy has been accepted as the 4th major therapy, following surgery, chemotherapy, and radiation therapy [10-12]. It has been considered increasingly significant in the present-day battle against cancer [13-15]. The

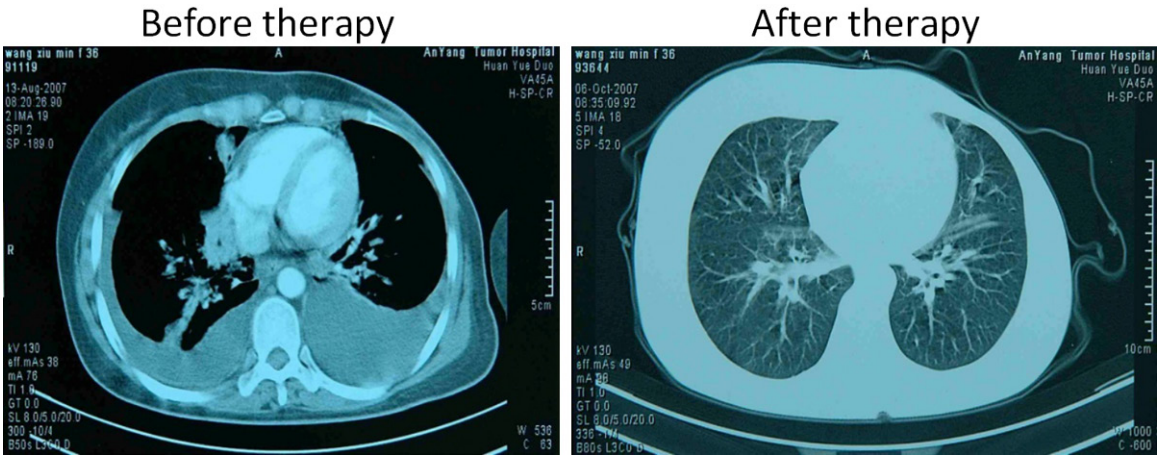

Figure 4. Picture of pleural effusion before and after therapy.

strategy of using self CIK cells (DC-CIK) to treat advanced breast cancer has been widely applied in clinics [9, 15, 16]. There is an obvious benefit of DC-CIK therapy combined with chemotherapy over chemotherapy alone [17]. Quality of life and immune function of cancer patients significantly improves, with clinical efficacies of 42.8%, 43.5%, and 47.2%, and an average of 44.5%. Longevity of cancer patients receiving combined therapies is 4-19 months, with an average of 13 months [18].

However, the effectiveness of using CIK cells from healthy donors in improving survival was 100% [19, 20]. Longevity has increased to 1-6 years, with an average of 48 months, thus far [21]. Average longevity is expected to improve, as the majority of treated patients are still alive.

CIK cells from healthy donors have shown obvious advantages over use of CIK cells from the patients themselves. CIK cells from healthy donors fare better than those from cancer patients, comparing proliferation rates, CD3/CD56 antigen amounts, and killing activity. Proliferation activity of CIK cells isolated from healthy patients (following 14-day culture) was 165-fold, much higher than that of CIK cells of cancer patients, which was 65-fold. Double surface antigens, CD3/CD56, of CIK cells isolated from healthy donors were 34.68%, higher than the 15.28% of CIK cells of patients. Killing activity of CIK cells isolated from healthy donors against K562 was 86%, also much higher than the 74% from patient CIK cells. CIK cell amounts and therapy arrangement: for DC-CIK therapy (using patient CIK cells), 10×10^8 cells were injected into the patient each time, 5-8 times per course, usually daily or every other day, in certain cases once a week. However, CIK therapy with healthy donor cells involved 30-35 × 10^8 cells injected each time, usually daily, with consecutive 6 days as a course. The total cells delivered was 180-200 × 10^8.

The strength of immune shock effect is another reason for better clinical efficacy. Another explanation for higher efficiency of CIK therapy is the possible “immuno-shock”, foreign cell exposure, and fresh immune cell usage associated with this therapy. In summary, CIK cells isolated from healthy donors are better than DC-CIK of patients themselves because of: ① Larger number of CIK cells delivered (3-4 times than that in DC-CIK from patients themselves); ② Greater activity of CIK cells isolated from healthy donors (1.5 times higher than that of CIK cells isolated from patients); and ③ Immune-shock of foreign cell exposure and foreign antigen stimulation.

The number of cases for this present study was limited. Additionally, the relationship between tumors and the body’s immune surveillance is not yet completely clear. It is necessary to keep an eye on the progress of immune response research and development of novel immune therapies.

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Disclosure of conflict of interest

None.

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