RESEARCH ARTICLE

Continuous DC-CIK Infusions Restore CD8+ Cellular Immunity, Physical Activity and Improve Clinical Efficacy in Advanced Cancer Patients Unresponsive to Conventional Treatments

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Abstract

Background: There are few choices for treatment of advanced cancer patients who do not respond to or tolerate conventional anti-cancer treatments. Therefore this study aimed to deploy the benefits and clinical efficacy of continuous dendritic cell-cytokine induced killer cell infusions in such patients. Materials and Methods: A total of 381 infusions (from 67 advanced cases recruited) were included in this study. All patients underwent peripheral blood mononuclear cell apheresis for the following cellular therapy and dendritic cells-cytokine induced killer cells were expanded *in vitro*. Peripheral blood T lymphocyte subsets were quantified through flow cytometry to address the cellular immunity status. Clinical efficacy and physical activities were evaluated by RECIST criteria and Eastern Cooperative Oncology Group scores respectively. Logistic regression model was used to estimate the association between cellular infusions and clinical benefits. Results: An average of $5.7\pm2.94\times10^9$ induced cells were infused each time and patients were exposed to 6 infusions. Cellular immunity was improved in that cytotoxic $CD8^+CD28^+$T lymphocytes were increased by 74% and suppressive $CD8^+CD28^-$T lymphocytes were elevated by 16% ($p<0.05$). Continuous infusion of dendritic cells-cytokine induced killer cells was associated with improvement of both patient status and cellular immunity. A median of six infusions were capable of reducing risk of progression by 70% (95%CI 0.10-0.91). Every elevation of one ECOG score corresponded to a 3.90-fold higher progression risk ($p<0.05$) and 1% increase of $CD8^+CD28^-$ T cell proportion reflecting a 5% higher risk of progression ($p<0.05$). Conclusions: In advanced cancer patients, continuous dendritic cell-cytokine induced killer cell infusions are capable of recovering cellular immunity, improving patient status and quality of life in those who are unresponsive to conventional cancer treatment.

Keywords: DC-CIKs - ECOG scores - cell immunity - clinical efficacy

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Introduction

Cancer has become the leading cause of death in the world. According to the data of IARC (Ferlay et al., 2013), there were 14.1 million new cancer cases and 8.2 million cancer deaths in worldwide in 2012. Among them, 21.8% (3.1 million) of new cases and 26.9% (2.2 million) of deaths occurred in China. The ratio of mortality/ incidence in China is higher than the other countries (72% *vs* 58.2%). These are increasingly demands for more treatment choices for those who failed and/or untolerated to anti-cancer treatments. More and more clinical evidence showed that cancer immunotherapy is becoming one of the promising approaches addressing Eastern Cooperative Oncology Group (ECOG) score improvements and psychological stress (Danielle et al., 2003). Our previous study indicated that for advanced cancer patients, DC-CIK immunotherapy can restore the immune status effectively (Zhao et al., 2014). The purpose of this study is to explore that whether DC-CIK treatment and the improvement of immune status can raise the clinical efficacy.

The mechanism of immunotherapies included stimulation and redirection of the cellular immune responses among cancer patients and leading to cell lysis (Choudhury et al., 2008). Dendritic cells (DCs) are the most potent antigen-presenting cells and able to promote the generation of helper T cells and cytotoxic T cells (CTLs). DC acts as effective T-cell stimulators and

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induces a tumor-specific immune response. Cytokine induced killer cells (CIKs) are ex vivo-expanded T lymphocytes-a subset of T lymphocytes with a natural killer T-cell phenotype expressing both the CD56 and the CD3 markers, present powerful non-histocompatibility complex (MHC)cytotoxicity against many tumor target cells in many trials (Heusel et al., 1994; Kagi et al., 1994; Schmidt-Wolf et al., 1996; Bradley et al., 1998).Increasing evidences suggest that dendritic cells-cytokine induced killer cells (DC-CIKs) can increase the PFS/OS and QoL of lung cancer, breast cancer and colon cancer patients (Chen et al., 2014; Wang et al., 2014b; 2014c). Our previous study also indicated that high-dose chemotherapy combined with DC-CIKs improved both progression-free and overall survival in patients with metastatic breast cancers (Ren et al., 2013).

There were some remained issues that what was the minimum amount of DC-CIKs infusions required to restore the cellular immunity and subsequently improve the clinical benefits. Whether these infusions could be adopted to those advanced patients who failed and/or untolerated to conventional treatments.

Materials and Methods

Patients

From July 2012 to July 2013, 67 patients with advanced cancer of stage IV receiving 381 DC-CIKs infusions were recruited into the study from Beijing Shijitan Hospital, Capital Medical University Cancer Center, Beijing, China. All patients failed to and could not tolerate to any conventional anti-cancer treatments due to poor life status, elderly. The inclusion criteria was included ECOG>2 and chemo-radiotherapy-free at least 3 months. All patients signed the informed consent form. The study was approved by the ethical committee of Beijing Shijitan Hospital. Prior and post one month of DC-CIKs infusions, peripheral blood T lymphocytes were cytometrilized with the contrast of CT or MRI imaging. RECIST criteria was used to evaluated the clinical efficacy. Meantime ECOG status was recorded periodically for physical activity assessment.

ECOG and RECIST criteria

ECOG: 0-Fully active, no restrictions on activities; 1-Unable to do strenuous activities, but able to carry out light housework and sedentary activities;2 -Able to walk and manage self-care, but unable to work. Out of bed more than 50% of waking hours; 3 -Confined to bed or a chair more than 50% of waking hours. Capable of limited self-cares; 4-Completely disabled. Totally confined to a bed or chair. Unable to do any self-care; 5-Death.

RECIST criteria: Complete response (CR): Disappearance of all target lesions. Any pathological lymph nodes (whether target or non-target) must have reduction in short axis to <10 mm. Partial response (PR): At least a 30% decrease in the sum of diameters of target lesions, taking as reference the baseline sum diameters. Progressive disease (PD): At least a 20% increase in the sum of diameters of target lesions, taking as reference the smallest sum on study (this Includes the baseline sum if that is the smallest on study). In addition to the relative increase of 20%, the sum must also demonstrate an absolute increase of at least 5 mm. (the appearance of one or more new lesions is also considered progression). Stable disease (SD): Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD.

Cell isolation and antibodies reagents

2ml of heparinized peripheral blood was drawn from each patient. Then 100 μl blood was incubated in the dark with primary antibody at 4°C for 15 min. After hemolysis for 10 min, samples were centrifuged for 10 min at 1500 rpm at room temperature, and then washed twice in PBS and subjected to flowcytometric analysis (Becton-Dickinson, Franklin Lakes, NJ). Primary antibodies included: anti-CD4-FITC, anti-CD8-PE, anti-CD3-PerCP (Becton-Dickinson), anti-CD4-FITC (Beckman-Coulter), anti-CD25-PE (Beckman-Coulter), anti-CD28-FITC (Beckman-Coulter), anti-CD8-PE (Beckman-Coulter), anti-CD3-FITC .

Flow cytometric analysis

Flow cytometric analysis was performed to determine cell phenotypes. Lymphocyte subset levels were reported as percentages of the total population. Flow cytometry was performed using an FC500 (Beckman-Coulter), and CXP analysis software (Beckman-Coulter) was used for analysis.

DC-CIK induction, generation and infusion

For the induction of DC-CIKs, peripheral blood

Table 1. Multi-variate Analysis of Factors to the Change of ECOG and Immune Function

	ECOG		$CD8^+CD28^+$ T cell		$CD8^+CD28^-$ T cell	
	B	*p*	B	*p*	B	*p*
DC-CIK cycles	-0.36	0.024	0.55	0.89	-13.75	0.001
age	-0.12	0.432	-6.34	0.121	1.22	0.75
sex	0.002	0.65	-0.255	0.06	0.03	0.83

Table 2. Basic Characteristics of Controlled and Progressed Cases

	Controlled cases	Progressed cases	*p*
Age	63.3±15.9	60.1±14.4	0.397
Sex			
Male	14 (50)	19 (48.7)	0.918
Female	14 (50)	20 (51.3)	
BMI	22.7±3.95	22.95±3.15	0.78
Cancer type			0.108
Breast	3 (25)	9 (75)	
Lung	5 (31.3)	11 (68.8)	
Gastric	3 (30)	7 (70)	
Others	17 (58.6)	12 (41.4)	
Surgery			0.44
yes	17 (45.9)	20 (54.1)	
no	11 (36.7)	19 (63.3)	
Chemotherapy			0.33
yes	23 (45.1)	28 (54.9)	
no	5 (31.3)	11 (68.7)	
Radiotherapy			0.95
yes	7 (41.2)	10 (58.8)	
no	21 (42)	29 (58)	

Table 3. Therapy Managements between Controlled and Progressed Cases

	Controlled cases	Progressed cases	*p*	RR*	95% CI
DC-CIK cycles			<0.05	0.3	0.10-0.91
1	7(10.4)	20(29.9)			
>1	21(31.3)	19(28.4)			
Change of ECOG	-0.79±0.63	-0.33±0.58	<0.05	3.9	1.50-10.06
Change of $CD8^+CD28^-$	-9.64±14.08	-0.22±16.98	<0.05	1.05	1.01-1.09
Change of $CD8^+CD28^+$	9.34±17.56	8.76±15.53	0.888	-	

*Cox Hazard Proportional Model adjusting age, sex, BMI and previous treatment (surgery, chemotherapy and radiotherapy)

mononuclear cells (PBMCs) were mobilized by G-CSF. Apheresis was performed using the COBE Spectra cell separator (COBE BCT, Lakewood, CO, USA) and repeated until≥4.5×10^6/kg $CD34^+$ was collected. The mean yield of $CD34^+$ cells was $1.22\pm1.13\times10^6$/kg. 25-50ml of the apheresis product was cultured *in vitro* to generate autologous DCs by culture in the presence of IL-4,TNF-αand GM-CSF.

Mononuclear cells prepared from peripheral blood were separated by Ficoll-Hypaque centrifugation method and activated *in vitro* with the recombinant cytokines IL-2 at 1000U/ml (Boehringer Mannheim, Germany), IFN-γ at 1000U/ml (Boehringer Mannheim, Germany) and CD3 antibody at 1.7μl/ml (Boehringer Mannheim, Germany) for 7-10 days.

Cells growth were observed under the microscope, and DCs phenotypes were determined by FCM of CD80, CD86, HLA-DR, CD1a and CD11c. DCs suspension contained more than 80% of CD80+CD86+ cells before infusion. CIKs express CD3 and CD56. After culture *in vitro* for 7-10 days DCs and CIKs were harvested and administered intravenously QOD, three times composed 1 cycle. The interval of two cycles was 1 month.

Statistical analysis

The software of SPSS 17.0 version was used in the statistical analyses. The difference of cellular immunity and ECOG score were analyzed by paired t-test between pre- and post-immunotherapy. Multiple linear regression was used to estimate the relationship between DC-CIKs infusion cycles and change of ECOG and immunity, with the adjustments of age and sex. Between controlled and progressed cases, the difference of age, BMI, change of ECOG and change of T cells was estimated by t-test, and the other categorical variables were estimated by chi-square test. The significant variables were further analyzed by Cox-Hazard Proportional Regression model with adjustments of age, sex, BMI and conventional treatments. All tests were 2-sided and the significant level was set as 0.05.

Results

Cellular immunity improvement

Average $5.7\pm2.94\times10^9$ of induced cells were infused each time and patients were exposed to 6 infusions. After DC-CIKs infusions, cellular immunity predominant of T lymphocyte was restored: the proportion of $CD3^+$ T cell increased by 18.3%, whereas cytotoxic $CD8^+CD28^+$ T cell increased significantly from 12.2% to 21.2% with the contrast to suppressive $CD8^+CD28^-$ T cell reduced by 15.8%. Every addititional three infusions were related with reduction of $CD8^+CD28^-$ T cell proportion by 13.75% (Table 1, $p<0.05$).

ECOG score improvement

With DC-CIKs infusions, patients' physical status was also improved significantly and ECOG score decreased by 15.6% ($p<0.05$, Table 1). Every additional three infusions were associated with the reduction of ECOG score by 0.36 (Table 1, $p<0.05$).

Clinical efficacy improvement

All subjects were exposure to an average of 6 infusions of DC-CIKs. 2 patients (3.0%) reached PR, 26 patients (38.8%) remained stable, 39 cases (58.2%) progressed, and the disease control rate (DCR) was 41.8%. The celluar immunity recovery induced by DC-CIKs infusions were not related with patients' age, gender, BMI, cancer subtype and previous treatments ($p>0.05$, Table 2).

DC-CIKs infusions were inversely related with disease progression , six and above infusions might reduce the risk by 70% (HR=0.30, 95%CI 0.10-0.91) (Table 3). Elevation of ECOG score and $CD8^+CD28^-$T cells proportion were associated with higher risk of disease progression: every elevation of one ECOG score corresponded to a 3.90-fold higher progression risk ($p<0.05$) and 1% increase of $CD8^+CD28^-$ T cell proportion reflecting a 5% higher risk of progression ($p<0.05$) (Table 3).

Adverse reactions

There were no obvious side effects occurred during the entire infusions except that two patients presented a transient mild fever and chill.

Discussion

Cancer immunotherapy has become a frontier approach in the last 5 years, convincing evidences come out of several clinical trials. The clinical benefits were seen from the previous studies, regarding to lung cancer (Han et al., 2014; Jin et al., 2014; Shi et al., 2014), breast cancer (Ren et al., 2013), renal cancer (Wang et al., 2014a) and gastrointestinal cancer (Shi et al., 2012; Gao et al., 2014; Wang et al., 2014b) etc. DCs have been proven as the most effective antigen-presenting cells, which can digest by endocytose antigens including circulating tumor-associated antigens to elicit the general immune responses and diminish T cell exhausting (Liu et al., 2009; Zhao et al., 2014). Due to cancer heterogeneity and randomized mutiple gene mutations, tumor microenvironment limited some anti-cancer activities induced by chemo and radio

therapy. DC-CIKs infusions were capable of removing immuno suppression conditions. Meanwhile, some cytokines including gamma interferon and IL-12 were also increased (Marten et al., 2001).

Peripheral blood different lymphocyties distribution could represent humoral celluar immunity, even though pathological analysis was not eligible. Our previous study has showed that an increase in the percentage of the $CD8^+CD28^-$ lymphocyte subset from the peripheral blood of metastatic breast cancer patients accompanied by increased IL-6 and IL-10, the elevated $CD8^+CD28^-$ associated with shorten PFS (Song et al., 2013). Clinical results showed that with continous DC-CIK infusions alone, the ratio of $CD8^+CD28^-$ T cells were reduced and the immune status were improved for advanced cancer patients (Zhao et al., 2014). Even in the situation of chemotherapy exposure, DC-CIKs infusions could synchronize chemotherapy ability (Ren et al., 2013). In this study, we explored the relationship of DC-CIKs infusions with the restoring of celluar immuity through quantitative analysis. Our data showed that with the addition of DC-CIKs infusions, the overall ECOG status was dramatically improved, every additional 3 infusions were associated with the reduction of ECOG score by 0.36 (see Table 2). Interestingly, either ECOG score or $CD8^+CD28^-$ T cell proportion was increaed, the patients underwent a higher risk of disease progression.

The amount of DC-CIKs cells plays a key role in restoring cellular immunity. The removal of suppressive activities resulted from cancer cell and microenvironment has become the major interesting when anti-cancer treatment employed *in vivo*. Inadequate cell infusion was not capable of diminishing immunity suppression. We postulated that provision of minimum DC-CIKs was clinically required to conduct the effective immune responses. In this study, we calculated the average infusion cells number of 5.7×10^9 as the basic requirement. Othewise some invalid infusions may occur, which explain the bias of incapability of immunotherapy in some trials. We have not observed the hyper immune reactivity which might be a potential risk when exposure to repetitive DC-CIKs infusions. The validation of monitoring peripheral blood cellular immunity network *in vitro* through T lymphocytes has also be useful for those patients who undertaken the secondary cancer or immunosuppressive reagent exposure.

In conclusion, for patients failed and untolerated to conventional anti-cancer treatments, continuous DC-CIKs infusions could restore cellular immunity, improve the patients physical status and reduce the risk of disease progression. The essential amount of DC-CIK cells was addressed.

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