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Pseudomonas aeruginosa-mannose sensitive hemagglutinin injection treated cytokine-induced killer cells combined with chemotherapy in the treatment of malignancies

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ABSTRACT

Pseudomonas aeruginosa-mannose sensitive hemagglutinin (PA-MSHA) injection serves as immunological adjuvant in clinical treatment of cancer patients. In present study, we investigated whether PA-MSHA injection enhanced the anti-tumor efficacy of CIK cells. Twenty patients with malignancies were enrolled in this retrospective clinical trial. They were divided into two groups: 10 patients received PA-MSHA treated CIK cells transfusion combined with chemotherapy, and other patients accepted CIK cells and chemotherapy. The efficacy of PA-MSHA treated CIK cells was also observed *in vitro* and *in vivo*. With PA-MSHA treatment CIK cells exhibited enhanced proliferation but decreased expression of inhibitory cell surface markers such as Tim-3 and PD-1. Particularly in CIK cells, PA-MSHA promoted the extrusion of pro-inflammatory cytokines like IFN-γ. Of 10 patients with PA-MSHA treated CIK cells and chemotherapy, two patients reached partial remissions, 7 patients had stable disease and the other one had progressive disease. Some of these patients experienced fever after cell infusion. 8 patients with CIK cells showed stable disease and 2 patients had progressive disease. Moreover, the side effects were small in patients with CIK treatment. Our data indicated that PA-MSHA improves the functions of CIK cells and shed new light on developing more potent therapeutic approaches for malignancies.

1. Introduction

Adoptive cell transfer (ACT) therapy is immune cell-based treatment for cancer patients [1]. The main aim of immune cell-based treatment is to kill tumor cells through the infusion of autologous immune cells to patients with cancer [2]. A variety of immune cells have been adopted, including tumor-infiltrating lymphocytes (TILs), natural killer (NK) cells and cytokine-induced killer (CIK) cells [3,4]. However, the functions of these transferred cells could be suppressed in tumor microenvironment, which were associated with immune evasion [5]. Immune evasion involves multiple molecules. The most important factor is programmed death receptor ligand 1 (PD-L1) [6,7]. PD-L1 is a well-described B7 family member expressed on tumor cells and inhibits T cell activation by interacting with PD-1 on T cells [8]. Acquiring immune cells with enhanced anti-tumor efficacy, and low PD-1 expression *in vivo* is critical for successful ACT therapy.

CIK cells are a heterogeneous population of *ex vivo* expanded T lymphocytes with different phenotypes: $CD3^-CD56^+$, $CD3^+CD56^-$ and $CD3^+CD56^+$ [9]. CIK cells are generated *ex vivo* by stimulating mononuclear cells with interferon-γ (IFN-γ), anti-CD3 monoclonal antibody (mAb) and interleukin-2 (IL-2) for a few weeks as initially described by Schmidt-Wolf et al. [10]. CIK cells have exhibited anti-tumor efficacy against various malignancies in preclinical models [11]. Also CIK cells demonstrate effectiveness and feasibility in clinical studies [12–14]. However, the optimization for the anti-tumor activity has posed a challenge for CIK cell-based immunotherapy. We approached this challenge and investigated whether PA-MSHA adding into the culture system could improve anti-tumor effects of CIK cells.

Abbreviations: CIK, cytokine-induced killer cells; PA-MSHA, *Pseudomonas aeruginosa*-mannose sensitive hemagglutinin; TILs, tumor-infiltrating lymphocytes; NK, natural killer cell; PD-L1, programmed death receptor ligand 1; DCs, dendritic cells; PRs, partial remissions; SD, stable disease; PD, progress disease; TLR, Toll like receptor

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Pseudomonas aeruginosa-mannose sensitive hemagglutinin (PA-MSHA) is a kind of peritrichous MSHA fimbria *P. aeruginosa*, which is around the mycelium and many tenuous and upright fimbriae [15]. Several studies showed that heat-inactivated PA-MSHA can serve as a safe adjuvant and the efficacy is attributed to the fimbriae [16,17]. The PA-MSHA fimbriae are able to activate Th1-type immune responses, stimulate natural killer cells, macrophages, dendritic cells (DCs), and promote DC maturation and migration. Furthermore, some studies indicated that Toll-like receptors (TLR) are important for PA-MSHA to induce DC maturations [18,19]. The use of the PA-MSHA vaccine for adjuvant therapy of cancer and malignant lymphoma has also been reported [20]. However, the influence of PA-MSHA on CIK cells is much less explored. More importantly, the clinical application of PA-MSHA-treated CIK cells has never been reported. In this study, we dissected how CIK cells were influenced by PA-MSHA addition during *in vitro* expansion. We also observed the efficacy and side effects of PA-MSHA-treated CIK cells or CIK cells combined with chemotherapy in treating patients with cancer.

2. Materials and methods

2.1. Ethic statement

We conducted the retrospective study with patients who were admitted in the First Affiliated Hospital of Zhengzhou University from June 2013 to July 2015. All the participants signed the informed consent form before this study. The whole consent procedure was in accordance with the ethical guidelines of the Declaration of Helsinki and approved by Ethics Committee of the First Affiliated Hospital of Zhengzhou University. Enrolling criteria for patients included age between 18 and 80 years, survival duration of > 3 months, and Karnofsky Performance Status (KPS) > 40%. Information collected at baseline included World Health Organization performance status and type, histology, stage and duration of cancer. The sex, tumor type and stage of patients were consistent in PA-MSHA treated CIK cell group and CIK group. Clinical parameters of these patients are summarized in Table 1.

2.2. Co-culture of PA-MSHA and CIK cells

Peripheral blood mononuclear cells (PBMCs) were obtained from each patient after the centrifugation of peripheral blood (PB) on a density gradient, using the Ficoll-Hypaque technique. Then cells were washed twice with PBS and resuspended at a density of 2×10^6/mL in GT-551 serum-free medium (Takara, Japan). Recombinant IFN-γ was added at 1000 U/mL (Beijing SL, China) for 24 h incubation in the atmosphere with 5% CO_2 at 37 °C. The next day, PA-MSHA, 100 ng/mL mAb against human CD3 (Boehringer Mannheim, Germany) and 1000 U/mL IL-2 (Beijing SL, China) were added. To validate the proper dose of PA-MSHA, a small pieces of PBMCs (2×10^6 cells in each group) were added with different concentration of PA-MSHA (4.5×10^6/mL, 9.0×10^6/mL, 13.5×10^6/mL, 18.0×10^6/mL and 22.5×10^6/mL), respectively. The PBMCs without the PA-MSHA work as control group. Fresh medium with IL-2 was added every 2–3 days. After 14 days culture, the PA-MSHA treated CIK cells and CIK cells were ready for further analysis.

2.3. Trypan blue exclusion assay

The cell number and viability were measured using trypan blue (TB) exclusion as previously described [21]. CIK cells with or without PA-MSHA were collected every 2 days, stained with TB (0.4%), and then viable cells were counted using a hemocytometer.

2.4. Flow cytometry

The level of activated or inhibitory cell surface markers was evaluated by six-color flow cytometry. Briefly, a total of 5×10^5 cells were collected by centrifugation and washed twice with PBS on 14 day. And then, cells were stained with fluorescence-conjugated mAbs against CD3, CD4, CD8, CD28, Tim-3 and PD-1 respectively for 20 min in the dark at 4 °C. Nonspecific binding was determined using appropriate isotype controls. After incubation, the cells were washed and resuspended in 500 μL PBS. Data were acquired on a FACSCanto II flow cytometer (BD, USA) and analyzed using Diva software (BD, USA). When detecting the expression of CD28, Tim-3 and PD-1, we gated the population of $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells.

2.5. Phenotypic assay of T lymphocytes in the peripheral blood

The peripheral blood from patients with cancer was obtained before the first cell infusion of CIK cells with or without PA-MSHA treated and 2 weeks after final cycle treatment. The whole blood was incubated

Table 1
Clinical characteristics of patients.

Patient no.	Age (years)	Sex	Weight (kg)	Tumor	Histologic differentiation	Surgery	Chemotherapy
1[a]	61	Male	70	Lung cancer	Poorly differentiation	No	Pemetrexed + carboplatin
2[a]	55	Female	58	Lung cancer	Poorly differentiation	No	Pemetrexeddisodium + nedaplain
3[a]	56	Female	59	Lung cancer	Poorly differentiation	No	Pemetrexed + oxaliplatin
4[a]	55	Female	65	Lung cancer	Poorly differentiation	No	Paclitaxel + nedaplain
5[a]	45	Female	60	Ovarian cancer	Poorly differentiation	Yes	Paclitaxel + carboplatin
6[a]	48	Female	66	Ovarian cancer	Poorly differentiation	Yes	Paclitaxel + oxaliplatin
7[a]	38	Male	86	Colon cancer	Moderate differentiation	Yes	CPT-11 + L-OHP + S1
8[a]	63	Female	51	Colon cancer	Moderate differentiation	Yes	Folofox6
9[a]	51	Male	81	Rectal cancer	Poorly differentiation	Yes	Gemcitabine + oxaliplatin
10[a]	68	Female	49	Esophagus cancer	Poorly differentiation	Yes	Docetaxel + oxaliplatin
11[b]	64	Male	68	Lung cancer	Poorly differentiation	No	Pemetrexed + oxaliplatin
12[b]	53	Female	72	Lung cancer	Poorly differentiation	Yes	Docetaxelum + oxaliplatin
13[b]	59	Female	48	Lung cancer	Poorly differentiation	Yes	Docetaxelum + oxaliplatin
14[b]	58	Female	70	Lung cancer	Poorly differentiation	No	Paclitaxel + nedaplain
15[b]	48	Female	56	Ovarian cancer	Poorly differentiation	Yes	Paclitaxel + carboplatin
16[b]	57	Female	60	Ovarian cancer	Poorly differentiation	Yes	Docetaxel + oxaliplatin
17[b]	38	Male	75	Colon cancer	Moderate differentiation	Yes	Folofox6
18[b]	61	Female	54	Colon cancer	Moderate differentiation	Yes	Folofox6
19[b]	58	Male	80	Rectal cancer	Poorly differentiation	Yes	Gemcitabine + oxaliplatin
20[b]	59	Female	53	Esophagus cancer	Poorly differentiation	Yes	Docetaxel + oxaliplatin

[a] PA-MSHA treated CIK group.
[b] CIK group.