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ORIGINAL ARTICLE

# A study on melanoma treatment using dendritic cells loaded with antigens purified from melanoma cell lines*

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## Abstract

**Objective** The aim of this study was to purify effective tumor peptide complexes from human melanoma cell lines to enhance the treatment effects on melanoma.

**Methods** We purified heat shock protein 70 (HSP70)-peptide complexes (PCs) from human melanoma cell lines A375, A875, M21, M14, WM-35, and SK-HEL-1. We named the purified product as M-HSP70-PCs and determined its immunological activities. Autologous HSP70-PCs purified from primary tumor cells of melanoma patients (9 cases) were used as controls. These two tumor antigenic complexes were loaded into dendritic cells (DCs) and used to stimulate an antitumor response against tumor cells in the corresponding patients.

**Results** Mature DCs pulsed with M-HSP70-PCs stimulated autologous T cells to secrete the same levels of type I cytokines as the autologous HSP70-PCs. Moreover, DCs pulsed with M-HSP70-PCs endued CIK cells with an equal ability as autologous HSP70-PCs to kill melanoma cells in the patients.

**Conclusion** M-HSP70-PCs may be used as an efficient and generalized tumor antigen in the treatment of DC-based malignant melanoma.

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**Key words:** heat shock protein 70 peptide complexes; dendritic cells; CIK cells; melanoma; cellular immunotherapy

For melanoma, dendritic cell (DC) and CIK cell therapy is the earliest and most effective treatment method. At home and abroad, a combination of DC-CIK cells and immune reconstruction therapy has benefited a large number of patients with melanoma. It is well known that the effectiveness of DC-based cellular immunotherapy depends on the tumor antigens it carries, and obtaining comprehensive and effective autologous tumor antigens through fresh tumor tissue cells after surgery is considered the best choice. However, for advanced patients and most tumor patients who have undergone surgery, because of the inability to operate or postoperative specimens soaked in formalin, autoantigens cannot be obtained from fresh tumor tissue, which greatly reduces the therapeutic effect. How to make such patients get better cellular immunotherapy has become an urgent problem.

Our previous research results showed that human-derived malignant tumor cell lines contain a large number of tumor-associated antigen peptides. At the same time, tumor antigen peptides can be isolated and purified by virtue of some characteristics of heat shock protein 70 (HSP70). This study will investigate whether HSP70-peptide complexes (PCs) purified from human melanoma cell lines can enhance the effect of adoptive immunotherapy for melanoma based on DCs.

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## Materials and methods

### Cell culture

Human melanoma cell lines A375, A875, M21, M14, WM-35, and SK-HEL-1 were purchased from Peking Union Medical College. Cells were cultured according to the manufacturer's instructions.

### Purification of M-HSP70-PCs

M-HSP70-PCs were obtained from the above six cell lines by using the separation and purification scheme established previously[1]. The six melanoma cell lines were processed according to the following steps:

(1) Heat shock treatment. Cells were heated in 42 °C water bath for 12 h, and at 37 °C for 2 h.

(2) After incubation with a lysis solution on ice for 15 min, the cells were scraped with sterile cell scrapers to obtain a cell suspension. After counting, $5 \times 10^6$ cells were taken from each group and then crushed with an ultrasonic crusher until there was no complete cell structure under the microscope.

(3) The crude cell extract was obtained through centrifugation at 10000 × *g* and 4 °C for 90 min.

(4) After dialyzing the cells in buffer at 4 °C overnight, the unconjugated parts were collected by ConA Sepharose affinity chromatography at 12 mL/h at room temperature.

(5) The unconjugated part of ConA Sepharose affinity chromatography column was dialyzed overnight at 4 °C. After the dialysis, the cell suspension was passed through ADP agarose affinity chromatography column at the rate of 10 mL/h at room temperature. The column was then washed with three steaming water and buffer solution in turns to the A280 value and returned to the baseline, and then the eluent was discarded.

(6) The column was eluted with buffer containing 3 mmol/L ADP until no protein was washed out. The eluate was then collected to obtain M-HSP70-PCs.

(7) Total protein was detected using the Lowry method.

(8) Endotoxin levels were detected with Limulus amebocyte lysate (LAL) assay.

### Primary melanoma cell culture

Surgical specimens were obtained from 15 patients who underwent resection of skin melanoma tumors at the Oncology Department, Inner Mongolia People's Hospital, in 2018. No patient received preoperative chemotherapy or radiotherapy.

(1) Fresh tumor tissue ($1 \times 1$ cm$^2$ or more) was taken after the operation and immediately put into a 4 °C RPMI 1640 medium containing 100 μg/mL penicillin G and 100 μg/mL streptomycin, within half an hour and in a sterile sealed environment, and delivered to the laboratory. The following processes were performed on the tissue:

(2) Blood clots, skin, and fibrous tissues around the specimen were removed, and the tumor tissue was washed twice with PBS. The specimen was cut into several small pieces ($0.1 \times 0.1 \times 0.1$ cm) with sterile scissors and then put into a sterile culture dish. Next, 1% collagenase was added and shaken in a water bath at 37 °C for 1 h.

(3) The cell suspension was obtained after filtration with a 38 μm mesh. After dilution, the cells were inoculated in a 25 cm$^2$ cell culture flask and cultured at 37 °C in 5% $CO_2$ until the total number of cells reached more than $1 \times 10^7$.

### Purification of autologous HSP70-PCs

The autologous HSP70-PCs of patients were purified from $5 \times 10^6$ primary cells according to the method described in "Purification of M-HSP70-PCs."

### Preparation of DCs and CIK cells

We extracted 50 mL of peripheral blood from the upper arms of healthy volunteers, diluted it twice with sterile heparinized normal saline, and slowly injected it into a centrifuge tube with a preset lymphocyte separation solution (relative density 1.077 g/mL). After density gradient centrifugation, peripheral blood mononuclear cells (PBMCs) were aspirated with a sterile bar pipette. The density of the PBMCs was adjusted to $5 \times 10^6$ g/mL with RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 g/mL streptomycin. After placing it in a cell culture bottle for adherence for 2 h, non-adherent cells were washed away (for inducing CIK cells). Adherent cells were induced with RPMI 1640 medium containing 800 U/mL GM-CSF and 500 U/mL IL-4; the medium and cytokines were replaced every 3 days. DCs were obtained by adding 50 U/mL TNF-a on the 6th day of culture.

The non-adherent cells were resuspended in RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin G, and 100 μg/mL streptomycin, and the density was adjusted to $1 \times 10^6$ g/mL. On the day of culture, 1000 U/mL human recombinant IFN-γ, anti-CD3 monoclonal antibody 50 ng/mL, IL1-β 100 U/mL, and IL-2 300 U/mL were added. The cell density was adjusted every 3 days to $2 \times 10^5$ g/mL. At the same time, new culture medium and 300 U/mL of IL-2 were added and cultured *in vitro* to obtain CIK cells.

### Preparation of tumor specific DC-CIK cells

At the sixth day of culture, the self-immature DCs of melanoma patients were divided into three groups ($1 \times 10^5$ in each group). The following antigen complexes were added and incubated at room temperature for 12 h to prepare specific DC tumor vaccines. Group A received GM-CSF and IL-4 only, group B received 10 μg M-HSP70-PCs, and group C received 10 μg autologous HSP70-PCs purified from primary cells of each melanoma patient.

Similarly, CIK cells were divided into three groups ($1 \times 10^6$ cells in each group) and mixed with the above three groups of DC tumor vaccines for one week to prepare tumor specific DC-CIK cells.

### Detection of cytokine secretion

The secretion of IFN-γ is an important way for DC-CIK cells to kill tumor cells. Therefore, the level of IFN-γ secretion indirectly reflects the antitumor activity of DC-CIK cells. In this study, the supernatant of DC-CIK cells in each group cultured together for 3 days was detected by IFN-γ enzyme-linked immunosorbent kit to determine the level of IFN-γ secreted by the cells in each group.

### In vitro cytotoxicity testing

The DC-CIK cells of the three groups were used as the effector cells, and the patient's primary cells were used as the target cells. The specific cytotoxic activity was detected by using LDH release method. The effector cells and target cells were incubated in RPMI 1640 medium containing 10% FBS at 37 °C for 4 h. The mixture was centrifuged at $300 \times g$ for 10 min and the supernatant was collected. LDH release level of the cells in each group was detected by using an ELISA kit, and the target cell lysis rate was calculated.

### Statistical analysis

All values are expressed as mean ± SD or percent (%). All analyses were conducted using the SPSS 17.0 software. The results were considered statistically significant when $P < 0.05$.

## Results

### Primary melanoma cell culture

The ages of 15 patients (10 males and 5 females) with melanoma ranged from 35–61 years. There were 12 patients with cutaneous melanoma and 3 patients with mucosal melanoma (MM). All patients did not receive any form of radiotherapy, chemotherapy, or targeted treatment before the operation. Part of the tissue was sent to the pathology department of our hospital for immunohistochemical detection to diagnose the disease. Another part of the tumor tissue was used to culture primary tumor cells. In the process of culture, four cells were polluted and two cells stopped growing for unknown reasons. In the other nine cases, the success rate was 60%.

### Antigen-specific IFN-γ production induced by DC-CIK cells

DC and CIK cells co-culture can stimulate IFN-γ secretion and kill tumor cells. The three groups of DC-CIK cells co-cultured for 3 days were tested for IFN-γ secretion level using the IFN-γ ELISA kit. The results showed that the levels of IFN-γ secreted by DC-CIK cells in groups B and C were significantly higher than those in group A ($P < 0.01$), and there was no significant difference between the two groups ($P > 0.05$) (Fig.1).

### In vitro cytotoxicity

The above three groups of tumor-specific DC-CIK cells co-cultured for one week were used as effector cells, and the primary tumor cells of MM patients were used as target cells. After 4 h of mixed culture at different target ratios (5:1, 10:1, 20:1), the LDH levels in the supernatants of each group were measured by the LDH release method to calculate the target cell lysis rate. The results showed that under different target-effector ratio conditions, the lysis rates of target cells in groups B and C were significantly higher than those in group A ($P < 0.01$), and there was no significant difference between the two groups ($P > 0.05$) (Fig. 2).

## Discussion

Melanoma is a kind of malignant tumor derived from the melanocytes of neural crest, which is often found in the skin and mucous membrane. In recent years, the incidence of malignant melanoma has been increasing year after year, and it has become one of the fastest-growing malignant tumors. The disease is characterized by high malignancy, occult incidence, insensitivity to radiotherapy and chemotherapy, and easy to have distant metastasis; therefore the mortality rate remains high. It has been found that the survival rate of patients with malignant melanoma is closely related to the degree of disease progression. Early patients can be completely controlled by surgery, while for late patients with

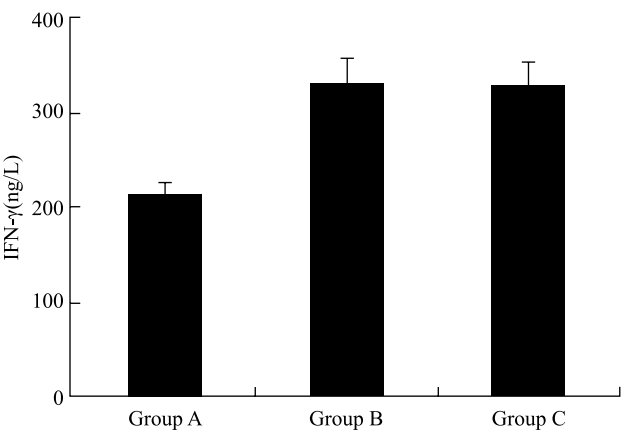


**Fig. 1** Secretion of IFN-γ by T cells induced with autologous DCs. Group A is autologous DCs that received only GM-CSF and IL-4; Group B is autologous DCs pulsed with 10 μg M-HSP70-PCs; Group C is autologous DCs pulsed with 10 μg autologous HSP70-PCs. Assays were performed in triplicate. The results are expressed as the mean ± SD

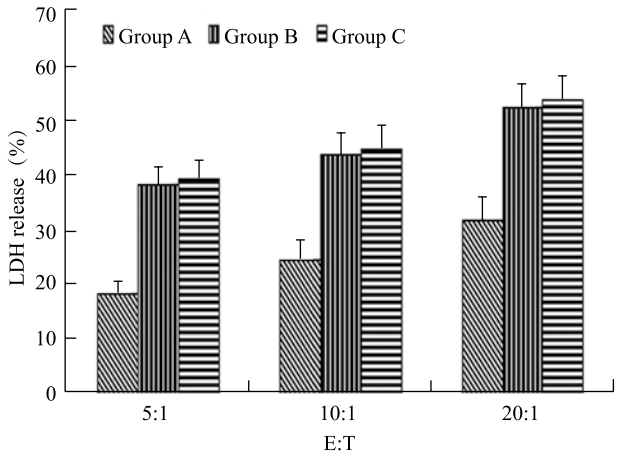


**Fig. 2** LDH release by CD8+ T cells induced with autologous DCs. Primary tumor cells of melanoma patients were used as target cells, and three groups of effector cells were used. Group A is autologous DCs that received only GM-CSF and IL-4; Group B is autologous DCs pulsed with M-HSP70-PCs; Group C is autologous DCs pulsed with autologous HSP70-PCs. Assays were performed in triplicate. Results are expressed as the mean ± SD

metastasis, the five-year survival rate is very low [2–4].

With the rapid development of tumor molecular biology and immunology, the emergence of some new treatment methods has gradually changed the treatment prospects of advanced malignant melanoma. A large number of studies have shown that the new immunotherapy and targeted therapy drugs alone or in combination can significantly improve the treatment effect of patients with advanced malignant melanoma and prolong the total survival period and progression free survival period [5–7]. However, a large number of late melanoma patients in China have not benefited from this. The reason is, firstly, the vast majority of patients are unable to afford the high cost of treatment. Secondly, the circulation channels of drugs are limited, and they cannot be obtained all the time, which affects the treatment cycle. Therefore, it is imperative to continue to develop cheap and efficient treatment methods. Cell immunotherapy based on DC and CIK cells is undoubtedly the best choice [8].

Studies have shown that the immune activity of tumor antigen peptide is the key to DC-CIK cell therapy [9]. Therefore, how to obtain the most comprehensive and effective autoantigen peptide of tumor patients, to achieve individualized treatment has become a research interest of DC based cellular immunotherapy in recent years. It is generally believed that the primary cells cultured in fresh tissue of tumor patients after operation are the best source for obtaining autoantigens. Unfortunately, this ideal treatment is difficult to implement and promote, for the following reasons: (1) Most patients with advanced cancer do not need or cannot tolerate surgical treatment and cannot get tumor tissue. (2) Pathological diagnosis is needed in the postoperative tumor tissue routine, and primary cell culture cannot be carried out after formalin treatment. (3) Fresh tumor tissue should be preserved and transported in strict aseptic condition, and cell culture should be started immediately after *in vitro*. Most medical institutions do not meet this requirement. (4) The primary cell culture technology requires high level of efficiency and the success rate is low.

Subsequently, people continue to explore new antigen preparation methods. Because of the simple preparation method, the freeze-thaw of fresh tumor tissue of patients has become a common antigen source in clinic. However, the autogenous tumor tissue freeze-thaw contains high concentration of cytokines and normal tissue cell components, which has poor immunogenicity and is easy to induce autoimmune response, thus seriously affecting the treatment effect.

The heat shock protein (HSP) family is a group of highly conserved polypeptide protein molecular family, which widely exists in various cells. In normal condition, the content is low, and the expression is obvious in stress. It has been shown that HSP and tumor-related antigen peptide can form complexes by noncovalent bond in tumor cells. HSP tumor peptide complex can be obtained by separating and purifying HSP from tumor cells. By binding with HSP receptor on the surface of DC, it can effectively ingest tumor peptide and stimulate the body to produce antitumor response. Therefore, the separation and purification of HSP tumor peptide complex from tumor cells is an effective way to obtain tumor-associated antigens. In the HSP family, HSP70 has been studied the most and is the best candidate for the preparation of antigen complex [10–11].

In this study, we purified M-HSP70-PCs from six melanoma cell lines. We cultured primary tumor cells of melanoma patients and purified HSP70 tumor peptide complexes of patients. In addition, autologous DC-CIK cells were induced by the two complexes respectively. The results were exciting; there was no significant difference in IFN-γ secretion level and the killing ability between M-HSP70-PCs and the autologous HSP70 tumor peptide complex of patients induced DC-CIK cells ($P > 0.05$). It is suggested that M-HSP70-PCs may be used as an efficient and generalized tumor antigen in the treatment of malignant melanoma.

In the future, we will continue to focus on the study of tumor antigens. In the preparation of melanoma antigen, firstly, we will clarify the identity of tumor antigen peptides in the M-HSP70-PCs. Secondly, we will continue to carry out clinical trials, increase the number of patients, and evaluate the clinical treatment effect and side effects. At the same time, the clinical study of cellular immunotherapy combined with other treatment schemes for melanoma will be carried out. Finally, to increase the

types of human melanoma cell lines as antigen sources, and to explore the possibility of further enhancing the antitumor immune activity of melanoma antigen peptide complex.

### Conflicts of interest

The authors indicated no potential conflicts of interest.

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