

## RESEARCH ARTICLE

# Heat-Shock Protein 70 as a Tumor Antigen for *in vitro* Dendritic Cell Pulsing in Renal Cell Carcinoma Cases

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### Abstract

Immunological functions of heat shock proteins (HSPs) have long been recognized. In this study we aimed to efficiently purify HSP70 from renal cell carcinoma and test it as a tumor antigen for pulsing dendritic cells *in vitro*. HSP70 was purified from renal cell carcinoma specimens by serial column chromatography on Con A-sepharose, PD-10, ADP-agarose and DEAE-cellulose, and finally subjected to fast protein liquid chromatography (FPLC). Dendritic cells derived from the adherent fraction of peripheral blood mononuclear cells were cultured in the presence of IL-4 and GM-CSF and exposed to tumor HSP70. After 24 hours, dendritic cells were phenotypically characterized by flow cytometry. T cells obtained from the non-adherent fraction of peripheral blood mononuclear cells were then co-cultured with HSP70-pulsed dendritic cells and after 3 days T cell cytotoxicity towards primary cultured renal cell carcinoma cells was examined by Cell Counting Kit-8 assay. Dendritic cells pulsed *in vitro* with tumor-derived HSP70 expressed higher levels of CD83, CD80, CD86 and HLA-DR maturation markers than those pulsed with tumor cell lysate and comparable to that of dendritic cells pulsed with tumor cell lysate plus TNF- $\alpha$ . Concomitantly, cytotoxic T-lymphocytes induced by HSP70-pulsed dendritic cells presented the highest cytotoxic activity. There were no significant differences when using homologous or autologous HSP70 as the tumor antigen. HSP70 can be efficiently purified by chromatography and induces *in vitro* dendritic cell maturation in the absence of TNF- $\alpha$ . Conspecific HSP70 may effectively be used as a tumor antigen to pulse dendritic cells *in vitro*.

**Keywords:** Heat-shock protein 70 - dendritic cells - renal cell carcinoma - tumor antigen

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### Introduction

Heat-shock proteins (HSPs) are important molecular chaperons that play essential roles in the regulation of protein synthesis, folding and vesicular trafficking. They are ubiquitously expressed at a basal level but are specifically induced in response to various stress conditions such as elevated temperature (Srivastava et al., 2002). In recent years, the eponymous member of the 70 kiloDalton family of HSPs, HSP70, has been widely used in tumor immunotherapy as a carrier molecule. HSP70 induces specific cytotoxic T lymphocyte (CTL) responses by delivering chaperoned antigens into dendritic cells (DCs) which are recognized by CD8+ T cells (Srivastava et al., 2002; Calderwood et al., 2005). HSP70-pulsed DCs may allow the induction of polyclonal CTLs against epitopes of multi-types of tumor cells. Since HSP70 is a purified single protein, it would not induce autoimmune responses. HSP70 will be highly significant when used in conspecific tumors of other individuals, especially those inoperable for their non-allotype. Renal cell carcinoma (RCC) is a

life-threatening disease that demonstrates resistance to standard chemotherapeutic agents. In contrast, it has a particular susceptibility to immune-based treatment strategies. A promising area of investigation is the use of cancer vaccines to educate host immunity to specifically target and eliminate malignant cells (Ramsey et al., 2006; Passalacqua et al., 2006). In this study, we improved the purifying method of higher purity immunocompetent HSP70, and we demonstrate the ability of HSP70 from conspecific RCC specimens to be used as a tumor antigen for pulsing DCs *in vitro*.

### Materials and Methods

#### *Purification and identification of HSP70*

Renal cell carcinoma (RCC) tissue samples were obtained from patients submitted to surgery on the First Affiliated Hospital of China Medical University. Tissue cells were homogenized in 4 volumes of hypotonic buffer (30 mM NaHCO<sub>3</sub>, pH 7.2) with an ultrasonic cell disruptor (ULTRA-TURRAX T8, IKA-WERKE, Germany) and

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centrifuged at 100,000 g at 4°C for 2 hours. The resulting supernatant was then added to a ConA-sepharose column (Pharmacia, US) and the unbound material was collected. The cobuffer was changed to buffer A (20 mM Tris-HCl, 20 mM NaCl, 15 mM  $\beta$ -mercaptoethanol, 3 mM MgCl<sub>2</sub>, pH 7.5) with a PD-10 column (Sephadex G-25, Pharmacia Biotech). The sample was next applied to an ADP-agarose column (5mL, Sigma) and the bound material was washed with buffer A containing 3 mM ADP. The buffer of the eluate was again changed using a PD-10 column to buffer B (20 mM Tris-HCl, 20 mM NaCl, pH 7.5). Then, the protein was added to a DEAE-cellulose column (Whatman, UK), resolved on a fast protein liquid chromatography (FPLC) system (Pharmacia, US) and eluted by a 20–500 mM NaCl gradient. Collected fractions were resolved on a 10% SDS-PAGE. Presence and homogeneity of the HSP70-containing fractions were verified by Coomassie blue staining and Western blotting with an anti-HSP70 antibody (Santa Cruz, Canada). Protein concentration was determined with the BCA assay box (Thermo Scientific, US).

#### DC preparation, pulsing and phenotypical characterization

Peripheral blood from healthy volunteers was separated by density gradient centrifugation over Ficoll-Paque Plus (Ameraham Biosciences, US) to obtain peripheral blood mononuclear cells (PBMCs), which were then resuspended in serum-free AIM-V medium (GIBCO, US). PBMCs were incubated in a humidified incubator for 2 hours at 37°C to allow for plastic adherence. The adherent cell fraction was used for dendritic cell (DC) culture by incubation in serum-free AIM-V medium supplemented with recombinant human interleukin-4 (IL-4, 1,000 units/mL) (Peprotech, US) and recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF, 1,000 units/mL) (Peprotech). Resupply of IL-4 and GM-CSF to the culture medium occurred on days 2 and 5 after this DCs were obtained from the suspension fraction. Tumor antigens and TNF- $\alpha$  (Peprotech) were added to the medium on day 7 and DCs were harvested and phenotypically characterized using antibodies for CD83, CD80, CD86 and HLA-DR (BD Biosciences, US) by flow cytometer (BD FACS Calibur, BD Biosciences, US) after 24 hours.

#### Primary RCC culture

RCC tissue specimens were cultured by several different methods as previously described (Besch et al., 1983; Kim et al., 2008; Pittoggi et al., 2008) in DMEM medium (GIBCO, US) containing 1% penicillin, 1% streptomycin, 1% amphotericin B, 0.5% L-Glutamide and 20% fetal cattle serum. A portion of the primary culture and second generation cells were cryopreserved in liquid nitrogen. The third generation cells were used as target cells in T cell cytotoxicity assays.

#### Non-adherent PBMCs preparation and co-culture with DCs

PBMCs obtained by density gradient centrifugation over Ficoll-Paque Plus from peripheral blood of healthy volunteers were cultured in a humidified incubator for

2 hours at 37°C to allow for plastic adherence. The suspension cells were centrifuged at 100 g for 10 min at 18–20°C and frozen at -80°C in cryopreservation medium. PBMCs were recovered at day 7 and co-cultured with antigen-pulsed DCs in a 1 : 10 ratio for 3 days.

#### Cytotoxicity assay

Autologous primary cultured RCC cancer cells were used as target (T) cells. PBMCs co-cultured for 3 days with DCs were used as effector (E) cells. Tumor cells were incubated with PBMCs at an E : T ratio of 20 : 1 for 24 hours. The cytotoxicity of PBMCs was examined with the Cell Counting Kit-8 assay box (Dojindo, Japan).

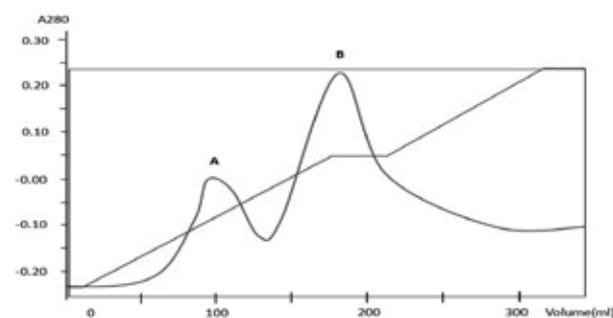
## Results

#### Chromatographic separation and identification of HSP70

After separation by two step affinity chromatography and DEAE cellulose column, we obtained HSP70 from RCC tissue samples with a high purity, as observed on a FPLC system chromatogram (Figure 1) and by Western blotting (Figure 2). Approximately 20–100 $\mu$ g of HSP70 could be obtained per gram of tumor tissue or cells.

#### Dendritic cell maturation induced by HSP70

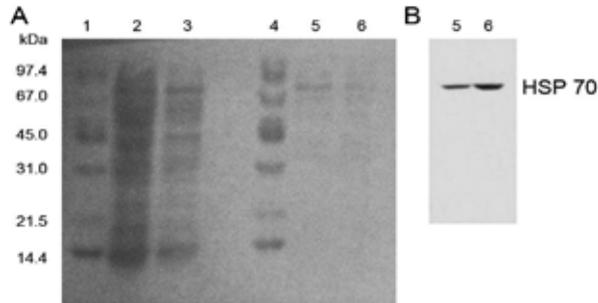
After addition of GM-CSF and IL-4 to the adherent fraction of PBMCs, some cells grew on suspension from the first day. On day 3, the number of suspension cells increased and they exhibited an irregular morphology. On day 5, the number of suspension cells increased further and they exhibited an increased size and extending protuberances. On day 7, most cells were in suspension and DCs formed clusters of large cells with stellate morphology, as observed by inverted microscopy (Figure 3). We obtained a percentage of DCs of about 5–10% of PBMCs. After addition of tumor lysate and TNF- $\alpha$ , expression of the mature DC markers CD83, CD80, CD86 and HLA-DR, increased significantly ( $P < 0.01$ ) when compared to either DCs not exposed to antigen or DCs exposed to tumor lysate in the absence of TNF- $\alpha$  (Table 1). In the absence of cytokines, HSP70-Peptide Complexes (HSP70-PC) induced DCs to express high levels of CD83, CD80, CD86 and HLA-DR, with no significant differences ( $P > 0.05$ ) from DCs pulsed with tumor lysate plus TNF- $\alpha$ . There were also no significant differences between autologous and homologous HSP70 ( $P > 0.05$ ).



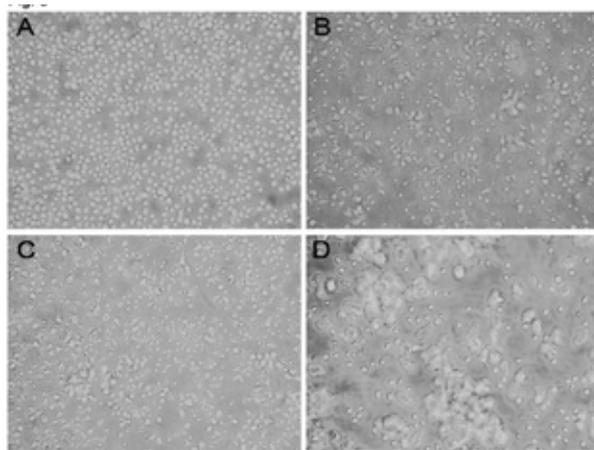
**Figure 1. Chromatogram of RCC Extracts upon DEAE Cellulose Separation on a FPLC System. Peak A) other protein; peak B) HSP70**

### Establishment of primary RCC cultures

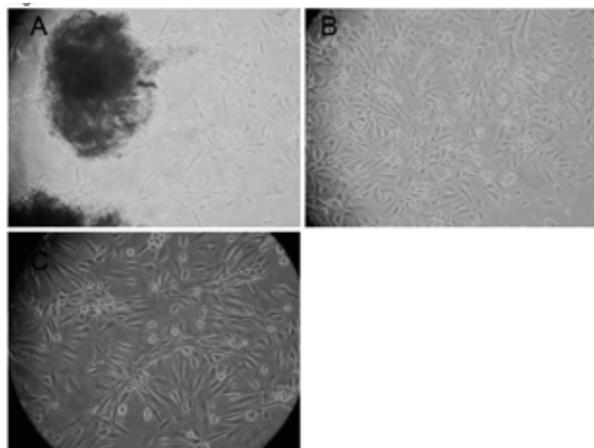
Primary cultures of RCC were established from tissue samples by different methods. Comparing these methods, we found that the enzymatic digestion one was the best for the rapid development of the primary cell culture (Figure 4). The third generation of cells obtained through this primary culture was used in T cell cytotoxicity assays.



**Figure 2. Western blotting analysis of HSP70.** A) Proteins were resolved on a 10% SDS-PAGE and the presence and homogeneity of the HSP70-containing fractions were verified by Coomassie blue staining. B) Western blotting with anti-HSP70 antibody. 1, 4: Molecular weight marker; 2: tumor lysate; 3: unbound material from Con A-sepharose; 5: bound material from Con A-sepharose; 6: Fraction A from DEAE cellulose elution



**Figure 3. Morphology of DCs in Culture Along Time (×200).** A) Day 1; B) Day 3; C) Day 5; D) Day 7



**Figure 4. Primary RCC Cultures Established by Different Methods (×200).** A) tissue adherence method; B) mechanical dispersion method; C) enzymatic digestion method

### Cytotoxicity of T cells induced by HSP70-pulsed DCs

CTLs induced by DCs pulsed with autologous HSP70-PC exhibited a significantly higher ( $P < 0.01$ ) cytotoxic activity towards primary cultured RCC cells when compared to both DCs not exposed to antigen and DCs pulsed with tumor lysate plus TNF- $\alpha$  (Table 2). There were no differences in the cytotoxicity of CTLs induced by DCs pulsed with autologous and homologous HSP70.

## Discussion

RCC has been shown to be susceptible to immunotherapeutic treatment strategies. However, radical nephrectomy assisted with INF- $\alpha$  and IL-2 cytokine therapy could not efficiently decrease recurrency and metastasis (Tanriverdi, 2013). Therefore, investigation of active immunotherapy of a possible DC tumor vaccine plays a crucial role for a future clinical application (Ramsey et al., 2006; Passalacqua et al., 2006). Successful vaccination using DCs loaded with an antigenic protein was preliminary achieved in some tumors such as breast, thyroid and uterine cervix cancers (Pinzon-Charry et al., 2006; Kuwabara et al., 2007; Bellone et al., 2007; Hu et al., 2013). The preparation of a tumor vaccine for RCC has been hampered due to the lack of a defined antigen. There are currently different strategies, such as tumor RNA and tumor lysates, applied in delivering specific antigens into DCs (Heiser et al., 2001; Wierdecky et al., 2006; Amato et al., 2008). Another approach is to use renal tumor lysate as antigen, thus allowing the induction of polyclonal immune responses against epitopes that could be presented by MHC class I and II (Ernstoff et al., 2007; Mayordomo et al., 2007). However, the optimal amount of protein is difficult to determine and there is the potential of autoimmune disease induction (Ludewig et al., 2000). The ideal antigen delivered into DC should induce a polyclonal CD8+ T cell reaction to various tumor epitopes, not induce an autoimmune reaction, not be restricted by MHC class and be easy to prepare and apply. Our findings showed that HSP70-PC extracted from tumor cells could be an ideal antigen delivered into DCs.

How to purify HSP70 has been the focus of considerable attention. Udono originally purified HSP70 through Con A affinity chromatography and ion-exchange chromatography, which resulted in a high efficiency but low purity (Udono et al., 1993). Afterwards, gelatin-affinity chromatography and deoxyspergulin-affinity chromatography have emerged (Nadler et al., 1992; Nandan et al., 1994; Nadeau et al., 1994). Peng confirmed that HSP70 obtained by these methods did not possess immunocompetence for any of the chaperoned antigenic polypeptides, but the one obtained by ADP-affinity chromatography did and could rouse a specific anti-tumor immunoreaction to the chaperoned antigen (Peng et al., 1997). We improved that method by applying Con A-affinity chromatography first and obtained high purity immunocompetent HSP70.

In our study, we cultured autologous tumor cells as targets to investigate the tumoricidal activity of T cells that were exposed to HSP70-PC antigens delivered by DCs. We show that the cytotoxic effect of those T cells is similar

to the one obtained by exposure to tumor lysate plus TNF- $\alpha$ -pulsed DCs and much more efficient than that of the tumor lysate in the absence of cytokines. There was no difference in the activity of CTLs induced by DCs pulsed with autologous and homologous HSP70. Thus, HSP70 can be obtained from other patient's tumor specimen and used to treat advanced tumors using DC vaccine.

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