Article

Response to Hepatocarcinoma Hca-F of Mice Immunized with Heat Shock Protein 70 from Elemene Combo Tumor Cell Vaccine

Lianying Guo[1], Guangxia Shi[1, 2], Zhihong Gao[1], Jie Shen[1], Rong Xing[1] and Zhenchao Qian[1]

To analyze immune response to murine hepatocarcinoma Hca-F of mice immunized with heat shock protein 70 (HSP70) derived from elemene combo tumor cell vaccine (EC-TCV) of Hca-F, HSP70 was isolated from EC-TCV by ADP affinity chromatography. Mice were immunized with HSP70 intraperitoneally three times and spleen cells were sampled. For cells, their proliferation and cytotoxicity against Hca-F were measured with MTT assay and their phenotypes were analyzed with flow cytometry. Spleen cells of immunized mice with HSP70 exhibited more potent cytotoxicity against Hca-F and proliferation than that of normal control mice, but less potent than that of mice immunized with EC-TCV. Among three groups, the percent of γδ T lymphocytes in the mice immunized with HSP70 (35.5%) was the highest compared with 6.25% in normal mice, and 28.4% in the mice immunized with EC-TCV. Immunization of HSP70 derived from EC-TCV could elicit potent immune response to Hca-F. HSP70 is one of elements inducing anti-tumor immune responses against Hca-F. *Cellular & Molecular Immunology*. 2006; 3(4):291-295.

Key Words: HSP70, EC-TCV, immune response, Hca-F

Introduction

Heat shock protein 70 (HSP70) is a member of a highly conserved superfamily of intracellular chaperones called stress proteins (1). Constitutively expressed stress proteins participate in protein synthesis, folding, trafficking, and degradation, while inducible stress proteins typically protect cells from environmental damage resulting from heat shock, free oxygen radicals, and other forms of stress (2). Immunization with HSP-peptide complexes, whether derived endogenously or reconstituted *in vitro*, elicits potent T cell responses against the chaperoned peptides and hence against the cells from which the HSPs are purified (3). HSP70 upregulation in tumor cells or HSP70-rich cell lysates, through heat- or drug-induced stress or by gene transfection, increases tumor immunogenicity and protects animal models from challenge by wild-type tumor (4, 5). Elemene is an effective anti-tumor monomer isolated from *curcuma aromatica* (6). It has been proved that elemene exhibits inhibiting and killing effects on and inducing apoptosis of various tumor cells both *in vivo* and *in vitro* (7). When tumor cells were treated with elemene, they expressed heat shock protein (8). Tumor cells treated with elemene, mitomycin C and glutaraldehyde may be used as a tumor cell vaccine, named elemene combo tumor cell vaccine (EC- TCV), to induce immune response against L615 leukemia and Hca-F hepatocarcinoma etc, such as cytotoxicity to tumor cells, IL-12 secretion, and protective effects from tumor challenges (9, 10). HSP70-peptide complexes derived from EC-TCV of Hca-F immunization elicited potent protective effects from Hca-F challenge (11). The present study aimed to detect anti-tumor mechanisms of HSP70-peptide complexes derived from EC-TCV and compare with that of EC-TCV.

[1]Institute for Cancer Biotherapy, Dalian Medical University, Dalian 116027, China;

[2]Corresponding to: Dr. Guangxia Shi, Institute for Cancer Biotherapy, Dalian Medical University, Dalian 116027, China. Tel: +86-411-8472-0142, E-mail: shiguangxia@hotmail.com.

Received Jul 18, 2006. Accepted Aug 26, 2006.

Copyright © 2006 by The Chinese Society of Immunology

Materials and Methods

Animals and tumor strain

BALB/c mice (H-2^d) were 8-12 weeks old and weighting 18-24 g. Hca-F (12), a high lymphatic metastatic subline of murine ascitic hapatoma H22 (non-MHC-I class molecule expression) was gifted by Professor Maoying Ling of the Department of Pathology in Dalian Medical University, L929 fibroblastoma was gifted by Professor Youhui Zhang of the Institute for Cancer of Chinese Academy of Medical Science.

Reagents

ADP, ADP-agarose were from Sigma Corp. RPMI 1640 and fetal calf serum was purchased from GIBCO Corp. MTT was purchased from Fluca Corp. FITC-labeled rat anti-mouse

CD4, CD8 and γδTCR monoclonal antibodies were from Pharmingen Corp. Elemene solution were from Dalian Institute for Medicine and Pharmacy. Mitomycin C was product of Japanese United Corp. All other reagents were of analytic grade.

Preparation of EC-TCV

The ascites of mice, which had been inoculated with Hca-F intraperitoneally, were collected and washed with PBS. Red blood cells of ascites were lysed with distilled water and recovered to isotonic state with salt solution. Harvested Hca-F cells were washed three times with PBS and suspended with elemene/MMC solution and incubated in 37^{o}C water for 1 h (9).

Isolation of HSP-peptide complexes

EC-TCV cells were washed twice with PBS and cell pellets were homogenerated with hypotonic buffer (10 mM $NaHCO_3$, 0.05 mM PMSF, pH 7.1) and centrifuged at 500× g, the supernatant was centrifuged at 100,000× g for 90 min at 4^{o}C. The supernatant was added to ADP-agarose column equilibrated previously with buffer D at rate of 12 ml/h. Unbounded proteins were eluted with buffer D. Then the column was eluted with buffer D containing 3 mM ADP, eluted proteins were collected in detection of low-pressure liquid chromatographer and identified as HSP70 with SDS-PAGE electrophoresis and Western blot assay (11, 13).

Protocol for active immunoprotection against Hca-F

BALB/c mice were immunized three times at weekly intervals subcutaneously with HSP70-peptide complexes (50 μg/mouse) derived from EC-TCV or EC-TCV cells (3 × 10^6/mouse). They were challenged with fresh untreated Hca-F cells (4 × 10^5/mouse) at day 7 after last immunization. Survival rate (percentage of mice living over 60 days after challenge) and mean survival day (MSD) of dead mice were used as standard for immunoprotective effects.

In vivo immunization with HSP70-peptide complexes for detection of immune response to Hca-F

BALB/c mice were immunized three times at weekly intervals intraperitoneally with HSP70-peptide complexes (50 μg/mouse) derived from EC-TCV or EC-TCV cells (3 × 10^6/mouse). They were inoculated with polyformaldehyde-treated Hca-F at day10 after last immunization. After 3 days, spleen cells were sampled to analyze their proliferation, phenotype, and cytotoxicity against Hca-F.

In vitro repeated sensitization with polyformaldehyde-treated Hca-F and cell culture

Mice were killed by cutting neck. Spleen cell suspensions were made from normal mice, EC-TCV-immunized mice and HSP70-peptide complexes-immunized mice. The red blood cells were lysed with distilled water and recovered to isotonic state with salt solution. Harvested spleen cells were washed three times with Hanks' solution and suspended with RPMI 1640 supplemented with 10% heat-inactivated fatal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Spleen cells were plated at 1 × 10^5/100 μl in 96 well plates. For proliferation assay 1 × 10^4/100 μl polyformaldehyde-treated Hca-F were added into spleen cell culture wells in 96-well plates. Cells were cultured in 37^{o}C, 5% CO_2 for 8 h. Proliferation reaction was measured with MTT assay. Proliferation index (PI) = A (mixed spleen cells and polyformaldehyde-treated Hca-F) / [A (spleen cells) + A (polyformaldehyde-treated Hca-F)]. For cytotoxicity 1 × 10^4 /100 μl untreated Hca-F or L929 cells were added. Cells were cultured at 37^{o}C, in 5% CO_2 for 4 h. Cytotoxicity was measured with MTT assay. Cytotoxicity = {1 – [A (mixed spleen cells and Hca-F) – A (spleen cells)] / A (Hca-F)} × 100%.

Cell cycle assay

Fresh spleen cells were washed three times with PBS, and cell pellets were stained with pyridine iodide and assayed with flow cytometry.

Phenotype assay

Fresh spleen cells were incubated with FITC-labeled rat anti-mouse CD8α, CD4 or γδTCR monoclonal antibodies for 20 minutes at 4^{o}C, and cells were washed three times with PBS containing 1% beef serum albumin, 0.1% NaN_3. Cells were fixed with PBS containing 1% polyformaldehyde, 2% glucose and 0.1% NaN_3. Percents of CD8, or CD4 or γδ T lymphocytes were measured with flow cytometry.

Statistical analysis

Data are presented as the mean ± SD per group. Statistical analysis was made for multiple comparisons using analysis of variance and Student *t*-test. A p-value < 0.05 was considered to be statistically significant.

Results

Effect of HSP70-peptide complex immunization on survival of mice challenged with Hca-F

All of control mice injected with PBS died of challenge with Hca-F, MSD is 20.8 ± 4.6 days. Mice immunized with EC-TCV cells survived free Hca-F over 60 days. Half of mice immunized with HSP70-peptide complexes derived EC-TCV survived free over 60 days, others died of challenge

Table 1. Effect of HSP70-peptide complex immunization on survival of mice challenged with Hca-F

Treatment	n	Dose	Survival rate (%) (NO. of survival mice)	MSD ± SD of dead mice
PBS	10	0.1 ml/mouse	0	20.8 ± 4.6
HSP70	8	50 μg/mouse	50 (4)* #	21.6 ± 3.9
EC-TCV	8	3 × 10^6/mouse	100 (8)*	

**$p < 0.01$, *vs* mice injected with PBS; ##$p < 0.01$, *vs* mice immunized with EC-TCV cells.

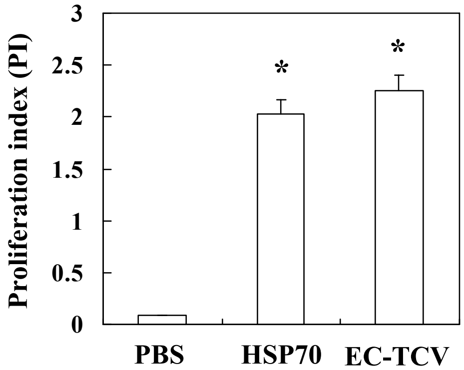

Figure 1. Assay of the proliferation of the total spleen cells cultured for 8 h *in vitro*. Spleen cells (1×10^5/100 µl) were mixed with 1×10^4/100 µl polyformaldehyde-treated Hca-F in 96-well plates for 8 h. Proliferation response was measured with MTT. *$p < 0.01$ *vs* PBS.

with Hca-F. The result showed that HSP70-peptide complexes could induce immunoprotective effect on Hca-F, but the effect is weaker than that of EC-TCV from which the HSP70-peptide complexes were purified (Table 1).

Proliferation response of spleen cells in vitro to repeated sensitization of Hca-F

Mice immunized three times with HSP70-peptide complexes derived from EC-TCV or EC-TCV cells were inoculated with polyformaldehyde-treated Hca-F cells. Their spleen cells were sampled to analyze proliferation response to Hca-F repeated sensitization. Results from MTT assay showed that proliferation index (PI) of spleen cells in the mice immunized with HSP70-peptide complexes derived from EC-TCV was 2.03 ± 0.11, 2.25 ± 0.10 in the mice immunized with EC-TCV, and 0.085 ± 0.02 in the mice injected with PBS (Figure 1). The G2-M and S phase of spleen cells in the mice immunized with HSP70-peptide complexes derived from EC-TCV was higher than that in the mice injected with PBS, but lower than that in the mice immunized with EC-TCV cells (Table 2).

In vitro cytotoxicity of spleen cells against Hca-F and L929 cells

The mice were immunized three times with HSP70-peptide complexes derived from EC-TCV or EC-TCV cells and inoculated with polyformaldehyde-treated Hca-F cells. Their spleen cells mediated cytotoxicity against Hca-F cells from which EC-TCV were prepared. They have non-specific cytotoxicity against L929 cells. The result showed that Hsp70-peptide complexes induced more potent cytotoxicity against Hca-F than EC-TCV cells.

Table 2. Assay of cell cycle of the total spleen cells

Treatment	Cell cycle phase (%)			
	G1	S	G2-M	S + G2-M
PBS	72.05	25.11	2.84	27.95
HSP70	45.32	47.33	7.35	54.68
EC-TCV	40.36	51.32	8.32	59.64

Fresh spleen cells washed with PBS were stained with pyridine iodide and cell cycles were assayed by flow cytometry.

Table 3. *In vitro* cytotoxicity of spleen cells against Hca-F cells or L929 cells

Treatment	Cytotoxicity (%)	
	Hca-F	L929
PBS	22.18 ± 3.39	43.21 ± 3.21
HSP70	50.27 ± 2.42**#	68.11 ± 3.37*#
EC-TCV	45.10 ± 2.56**	59.12 ± 3.25*

*$p < 0.01$, ** $p < 0.05$, *vs* spleen cells from normal mice; #$p < 0.05$, *vs* spleen cells from EC-TCV cell immunized mice.

Phenotypes of spleen cells

The percent of γδ T cells in spleen cells of the mice immunized with HSP70-peptide complexes derived from EC-TCV was higher than that of mice immunized with EC-TCV cells and that of mice injected with PBS. But the percent of $CD4^+$ or $CD8^+$ cells was lower than that of the others. The result showed that immunization with HSP70-peptide complexes derived from EC-TCV has potent ability to induce γδ T lymphocytes (Figure 2).

Discussion

Tumor immunotherapy has exploited the dual roles of heat

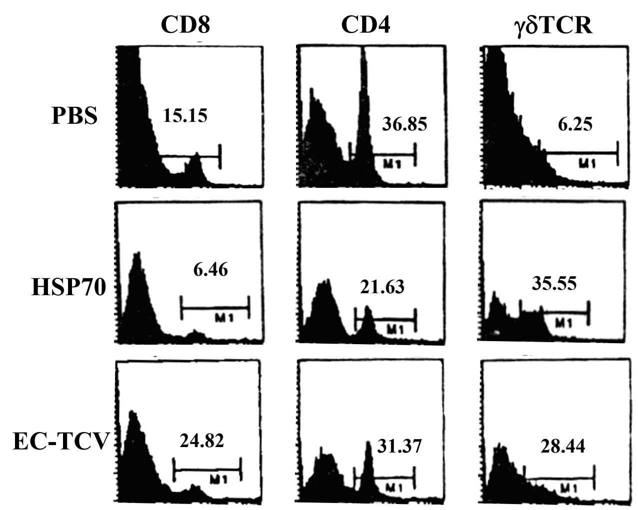

Figure 2. **Phenotypes of spleen cells.** Fresh spleen cells were incubated with FITC-labeled rat anti-mouse CD8α, CD4 or γδTCR monoclonal antibodies and assayed by flow cytometry.

shock proteins as molecular vehicles for antigen cross-priming and as activation signals for the innate immune system cells that promote T cell priming. The most common strategy is based on the purification of heat shock protein-peptide complexes from tumor cell lines or from tumor surgical samples for *in vivo* administration. The experiment showed that HSP70-complexes derived from EC-TCV had potent immuno-protective effects on Hca-F, a kind of MHC class I-deficient tumors. Survival rate of immunized mice was higher than that of control mice. It could induce spleen cells to proliferate and mediate cytotoxicity against Hca-F and L929 cells. *In vivo* EC-TCV exhibited more potent anti-tumor protective efficacy than that of HSP70-complexes derived from EC-TCV. The results suggested that HSP70 was one of effectors that mediate protective immune responses of EC-TCV against Hca-F. Hsp70-peptide complexes induce its protective effects by different mechanisms from EC-TCV cells.

The present results showed that responses induced by HSP70-peptide complexes derived from EC-TCV are different from that of EC-TCV. Effects in inducing γδ T lymphocyte and cytotoxicity against Hca-F and L929 cells were stronger than that of EC-TCV cells in spite of the fact that effects in immuno-protection and inducing proliferation of HSP70-peptide complexes derived from EC-TCV was weaker than that of EC-TCV cells. A possible reason for this is resistance of Hca-F to lysis mediated by $CD8^+$ cytotoxic T lymphocytes due to absence of MHC class I molecules. The lysis of tumor targets by the γδ T cells is brought about *via* recognition of heat-shock proteins expressed on the surface of tumor cells and is not restricted by MHC class I molecule (14), it is possible that Hca-F is sensitive to lysis mediated by γδ T cells and that cytotoxicity mediated by γδ T is less specific. HSP70-peptide complexes have more potent activity to induce γδ T than EC-TCV. It is conceivable that γδ T cells are major effectors of lysis of Hca-F *in vitro* assay. Some authors have claimed that gene deletion and/or mutational inactivation in the HLA region are common mechanisms of tumor immune evasion (15). HSP70-peptide complexes derived EC-TCV and EC-TCV are advisable to immuno-therapy of tumors deficient in expression of MHC class I molecule due to inducing MHC class I molecule non-restricted anti-tumor immune response.

In our previous studies we found that elemene and MMC treatment induced tumor cells to express heat shock proteins on their plasma membranes and happened to die by apoptosis and/or necrosis (7, 8). EC-TCV had enhanced immuno-genicity compared with ^{60}Co treated tumor cells of the same origin (9). Vaccination with dendritic cells pulsed with EC-TCV lysates of Hca-F could elicit protective effects on subsequent Hca-F challenge (16). Two mechanisms are proposed to explain what roles these HSP70-peptide complexes play in immune effects of EC-TCV. One possible hypothesis is that HSP70-peptide complexes of EC-TCV chaperones more and changed peptides than that of untreated tumor cells, thereby supply more potent antigen signals to the immune system (such as $CD8^+$ and γδ T lymphocytes) and induce immunity to tumors of origin. Another hypothesis is that EC-TCV cells expressing inducible HSP70 are stressed apoptotic cells capable of supplying danger signals to and activating dendritic cells. Inducible HSP70 is one of important endogenous danger signals needed to activate local antigen presenting cells (APCs), potentiating the immune responses against the antigens (17). Masse D et al. reported that increased expression of inducible HSP70 in apoptotic cells is correlated with their efficacy for anti-tumor vaccine therapy (18). Our results support the hypothesis that HSP70-peptide complexes are source of tumor-associated antigens in cellular therapy against cancer.

Acknowledgements

This work was supported by Key Programs of Science and Technology Research of Education Ministry of P. R. China and the National Science Foundation of China (39730440-II).

References

1. Elfriede Noessner, Robert Gastpar, Valeria Milani, et al. Tumor-derived heat shock protein 70 peptide complexes are cross-presented by human dendritic cells. J Immunol. 2002;169:5424-5432.
2. Blachere NE, Li Z, Chandawarkar RY, et al. Heat shock protein-peptide complexes, reconstituted *in vitro*, elicit peptide-specific cytotoxic T lymphocyte response and tumor immunity. J Exp Med. 1997;186:1315-1322.
3. Castelli C, Rivoltini L, Rini F, et al. Heat shock proteins: biological functions and clinical application as personalized vaccines for human cancer. Cancer Immunol Immunother. 2004; 53:227-233.
4. Granner MW, Zeng Y, Feng H, et al. Tumor-derived chaperone-rich cell lysates are effective therapeutic vaccine against a variety of cancers. Cancer Immunol Immunother. 2003;52:323-330.
5. Melcher A, Todryk S, Hardwick N, et al. Tumor immunogenicity is determined by mechanism of cell death *via* induction of heat shock protein expression. Nat Med. 1998;4:581-587.
6. Guo YT. Isolation and identification of elemene from the essential oil of Curcuma wenyujin. Zhong Yao Tong Bao. 1983; 8:31.
7. Yang H, Wang X, Yu L. The antitumor activity of elemene is associated with apoptosis. Zhonghua Zhong Liu Za Zhi. 1996; 18:169-172.
8. Gao ZH, Guo LY, Shen J, et al. Influence of elemene or heat shock treatment upon the expression of membrane HSP70 and HSPs genes in HepG2 cells. Zhongguo Mian Yi Xue Za Zhi. 2002;18:790-794.
9. Qian ZC, Wang DQ, Wei WH. Experimental investigation on immunoprotective effect of active immunization with curcuma aromatica and β-elemene treated tumor cells. Biotherapy. 1989; 3:310-317.
10. Zhao WH, Shi GX, Yuan XL, et al. Immunotherapeutic effects of EC-TCV on Hca-F and its influence on secretion of IL-10 and IL-12. Zhongguo Zhong Liu Sheng Wu Zhi Liao Za Zhi. 2001;8:126-128.
11. Piao H, Jin M, Guo LY, et al. Expression and immunoprotective effect of HSP70 of elemene combo tumor cell vaccine. Shanghai Mian Yi Xue Za Zhi. 2002;22:230-233.
12. Ling MY, Wang MH, Guo LL, et al. Comparison of metastasis

phenotype between two sub-lines of murine hepatocarcinoma. Dalian Yi Xue Yuan Xue Bao. 1994;16:124-127.
13. Peng P, Menoret A, Srivastava PK. Purification of immunogenic heat shock protein 70-peptide complexes by ADP-affinity chromatography. J Immunol Methods. 1997;204:13-21.
14. Thomas ML, Samant UC, Deshpande RK, et al. γδ T cells lyse autologous and allogenic oesophageal tumors: involvement of heat-shock proteins in the tumor cell lysis. Cancer Immunol Immunother. 2000;48:653-659.
15. Seliger B, Maeurer MJ, Ferrone S. Antigen-processing machinery breakdown and tumor growth. Immunol Today. 2000;21:455-464.
16. Xue B, Shi GX, Guo LY, et al. Antitumor effects of dendritic cells pulsed with tumor antigens of Hca-F hepatic carcinoma cells treated with elemene. Shanghai Mian Yi Xue Za Zhi. 2000; 20:293-295.
17. Matzinger P. The danger model: a renewed sense of self. Science. 2002;296:301-305.
18. Masse D, Ebstein F, Bougras G, Harb J, Meflah K, Gregoire M. Increased expression of inducible HSP70 in apoptotic cells is correlated with their efficacy for antitumor vaccine therapy. Int J Cancer. 2004;111:575-583.