N-terminally fusion of Her2/neu to HSP70 decreases efficiency of Her2/neu DNA vaccine

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Abstract DNA vaccines consisted of tumor-associated antigen (TAA) are well suited for immunotherapy against tumor. The construct can contain TAA fused to an appropriate molecule (biologic adjuvant) to improve the efficacy of anti-tumor immune response. Heat shock protein 70 (HSP70) has been shown to be an excellent candidate, capable of cross-priming TAA by antigen presenting cells leading to a robust T-cell response. However, the relationship between strong T-cell responses and tumor rejection is not always mutually exclusive, for which TAA loss or activation of suppressive mechanisms may occur. HSP70 fused to downstream of Her2/neu as DNA vaccine has been shown to be efficient against Her2-expressing tumors. In this study, we examined if N-terminally fusion of Her2/neu to HSP70 could also improve efficiency of Her2/neu DNA vaccine. Therefore, mice with an established Her2/neu expressing tumor were immunized with DNA vaccine consisting of extracellular and trans-membrane domain (EC+TM) of rat Her2/neu alone or N-terminally fused to HSP70 and immune response was evaluated. Administration of rat Her2/neu led to partial control of tumor progression. Surprisingly, fusion of HSP70 to N-terminal of rat Her2/neu led to tumor progression. Our result proposes that fusion direction of biologic adjuvant is an important consideration when Her2/neu is used.

Keywords HSP70 · Her2 · Biologic adjuvant ·
Regulatory T cells

Introduction

The discovery of tumor-associated antigens (TAA) was once assumed to be a great success to apply for an effective cancer immunotherapy. This assumption did not last long, since the results of immunotherapy with TAA were not satisfactory. Indeed, immune response to TAA is under the burden of strict regulatory mechanism of immune system (Jiang and Chess 2009). One of these TAAs is Her2 which has been extensively studied (Baxevanis et al. 2004).

Her2, like many other TAAs, is a self-tolerated antigen. To overcome the tolerance, many considerations should be taken into account to elicit an appropriate immune response. It has been demonstrated that rejection of tumor is mainly based on antibodies, cytotoxic T lymphocyte (CTL) response, and decrease in CD4⁺CD25⁺ regulatory T cells (Curcio et al. 2003; Lin et al. 2004; Quaglino et al. 2005). However, the role of Th2 antibodies, even in the absence of CTL activity, has been shown to be a major one (Piechocki et al. 2001). Therefore, determining the effective response could be a promiscuous and yet devastating battle. To elicit the appropriate response and bypass the regulatory burden, many studies have attempted to use adjuvants such as heat shock proteins (HSPs).

HSPs are highly conserved and abundant intracellular proteins, over-expressed under a variety of stresses (Lindquist 1985). Reports have shown the effect of HSPs, including Hsp70, Hsp90, and glucose-regulated protein 96, on immune response (Srivastava 2002). Immunization of mice with tumor-derived HSP70 preparations (not normal cell-derived) has been shown to be effective against the original tumor cells (Udono and Srivastava 1993; Wang et al 2001). Universally applicable novel HSP-mediated strategies have been devised because of limitations with isolation of HSPs from tumor as autologous vaccine (Castelli et al. 2004; Gordon and Clark

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2004). One of the strategies taken so far is DNA vaccination using HSP70 gene fused to or co-administered with a candidate antigen (Chen et al. 2000; Geng et al. 2006; Hsu et al. 2001; Kim et al. 2005; Lan et al. 2007; Li et al. 2006, 2007; Liso et al. 2005; Ma et al. 2005; Pavlenko et al. 2004; Wang et al. 2004; Ye et al. 2004; Zhang and Huang 2006; Zhang et al. 2007). One study demonstrated that DNA vaccine containing HSP70 fused to C-terminal domain of Her2/neu could combat an aggressive Her2-expressing tumor (Kim et al. 2005). The aim of our study was to determine if N-terminally fusion of Her2/neu to HSP70 could also improve efficiency of Her2/neu DNA vaccine. In this study, we applied a DNA vaccine containing HSP70 fused to upstream or N-terminally to extracellular and transmembrane domain (EC+TM) of the rat Her2/neu and investigated its effect on immune response.

Materials and methods

Mice

Female BALB/c mice at the age of 8–10 weeks were purchased from Pasteur Institute, Tehran, Iran. Given free access to food and water, mice were housed for 4 weeks before experiments and maintained in a good standard condition. All experiments were done according to the Animal Care and Use Protocol of Tarbiat Modares University.

Cell line

TUBO is a cloned cell line (kindly provided by Professor Federica Cavallo, University of Turin, Turin, Italy) derived from a lobular carcinoma that arose in a female BALB/c mouse transgenic for the rat HER2/neu oncogene (BALB-NeuT mouse). TUBO cells are poorly immunogenic in normal BALB/c mice; they do not induce anti-rat HER2/neu antibodies or any detectable CTL response (Rovero et al. 2000). The cells were cultured in DMEM (CytoGen, Germany) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (BioSera Ltd, UK) and 20% heat-inactivated fetal bovine serum (Gibco BRL) and maintained at 37°C, 5% CO₂.

A suspension of 0.15 ml containing 3×10^5 of TUBO cells was injected subcutaneously.

The cages were coded, and neoplastic masses were measured with calipers and the volume was calculated using $\text{length} \times \text{width}^2 \times \pi/6$ (Hashemi et al. 2007). Vaccination commenced 13 days after tumor implantation.

DNA expression vectors and vaccination

ECD+TM of Her2/neu (kindly provided by Professor Federica Cavallo; GenBank accession no. NM_017003)

was located between Hind III/Eco RI within pcDNA3* plasmid (pHer2). pcDNA3* was a derivation of the pcDNA3 plasmid (Invitrogen) by deleting the SV40 promoter, neomycin resistance gene, and SV40 poly(A) (Rovero et al. 2000). Subsequently, human HSP70 gene type HSPA1A (kindly provided by Dr. Sue Fox, Northwestern University, IL, USA, GenBank accession no. NM_005345) was cloned into pHer2, upstream of Her2/neu (pHSP70/Her2) between Hind III/Nar I (Nar I was located just at the beginning of Her2/neu). Stop codon was deleted using linker (synthesized by Metabion, Germany) coding for the last 80 bp of the sequence without stop codon. To ensure rotational freedom of the fusion genes, a flexible sequence encoding for glycine was located between HSP70 and Her2/neu.

Expression of each construct was evaluated by cloning of green fluorescent gene at the end of gene. All of the constructs were confirmed by sequencing.

Each construct was amplified using DH5α strain of *Escherichia coli* in Luria–Bertani medium. Large-scale preparation of the plasmid was performed according to standard polyethylene glycol (PEG) precipitation method (Sambrook and Russell 2001). The amount of lipopolysaccharide (LPS) was determined to be less than 20 U/mg using the limulus amebocyte lysate (LAL) assay. The tumor-bearing animals were grouped into four cages (five in each group) and at day 0 the tumor was implanted. Starting 12 days after tumor challenge, the animals were intramuscularly vaccinated with 100 µg of DNA vaccine three times at weekly intervals. The treatment groups were: saline, empty plasmids, pHer2, or pHSP70/Her2. Mice were sacrificed 2 weeks after the last vaccination. The mean diameter of tumor was recorded every other day from the beginning of vaccination until the animals were sacrificed.

Proliferation assay of spleen cells

An extract of TUBO cells was prepared by making a suspension of one single tumor mass, passing through mesh, freeze/thawing, sonication, and finally filtering the extract. The concentration of the extract was then determined at 595 nm and stored frozen at −20°C until use.

Two weeks after the last DNA vaccination, spleen cells of tumor-bearing and vaccinated animals in various groups (five in each group) were harvested and cultured for 24 h at 37°C in 5% CO₂ with 20 µg/ml of the extract of TUBO cells (*T*=test) or 5 µg/ml phytohemagglutinin (PHA) (*P*=positive control) and cells with no treatment (*N*=negative control). The cells were then pulsed for 48 h with 5-bromo-2'-deoxyuridine (BrdU) labeling solution. Uptake of BrdU was detected using the cell proliferation ELISA BrdU kit (Roche Diagnostic GmbH, Mannheim, Germany) and expressed as stimulation index (SI):

$$SI = [(T - N)/(P - N)] \times 100$$

Cytotoxic T lymphocyte assay

The CTL activity of the spleen cells of the tumor-bearing and variously treated mice was assayed immediately by lactate dehydrogenase release (LDH) assay according to the manufacturer's instructions (Takara, Japan). Various concentrations of spleen mononuclear with 1×10^4 target TUBO cells were mixed at 100:1, 50:1, and 25:1 effector/target ratios in round-bottom 96-well plates in triplicate. After 4 h of incubation at 37°C in an atmosphere containing 5% CO₂ followed by centrifugation at 250×g for 10 min, activity of LDH released from the cells to the medium was measured. The OD_{492 nm} of the supernatants was measured with the Universal Microplate Reader. The percent cytotoxicity was calculated as follows:

% Cytotoxicity

$$= \frac{OD_{\text{test}} - [OD_{\text{effector spontaneous}} + OD_{\text{target spontaneous}}]}{OD_{\text{target maximum}} - OD_{\text{target spontaneous}}} \times 100$$

OD_{test} is activity of LDH released from co-cultures of target and effector cells. OD_{effector spontaneous} and OD_{target spontaneous} are activity of LDH released by cultures of effector cells and target cells, respectively. OD_{target maximum} is activity of LDH released from target cells lysed by 2% Triton X-100 (Pharmacia).

Interferon-γ (IFN-γ) and interleukin-4 (IL-4) production

Spleen cells (1×10^6) of tumor-bearing and variously vaccinated mice were re-suspended in 1 ml of fresh RPMI-1640 containing 20% fetal bovine serum (FBS) and antibiotics and cultured at 37°C and 5% CO₂ for 90 h with 20 μg/ml TUBO cell extract in 24-well plates. The plates were then centrifuged at 250×g for 10 min. Supernatants were then collected and frozen at −70°C until the samples were analyzed to detect the presence of IFN-γ and IL-4 using a DuoSet ELISA system (R&D Systems) according to the manufacturer's instructions.

Flow cytometric analysis of the tumor CD4⁺/CD8⁺ and spleen/tumor CD4⁺CD25⁺foxp3⁺ subpopulations

The solid tumors were cut into small pieces, minced with forceps and scalpel, rinsed twice with phosphate buffer saline (PBS), and passed through a 150-μm stainless steel mesh. The mononuclear cells (MNCs) of the suspension were isolated by density centrifugation (700×g, 15 min, and 20°C) using Ficoll-Hypaque (Sigma, UK). Then the layer containing MNCs was removed, washed twice with PBS for 10 min in 360×g and 4°C, and the precipitated cells

were re-suspended in PBS containing 2% fetal calf serum (Gibco, UK). The viability of the cells was determined using trypan blue (90%). Then 1×10^5 of the cells were labeled for CD4 and CD8 markers using fluorescein isothiocyanate (FITC)-conjugated anti-CD4 or anti-CD8 monoclonal antibodies or a fluorescein-conjugated anti-mouse IgG2a as negative control (Becton Dickinson). Flow cytometric analysis was performed with a FACS Calibur (BD Bioscience).

Spleen and tumor MNCs were labeled for CD4⁺, CD25⁺, and foxp3⁺ markers using Mouse Regulatory T Cells kit, according to the manufacturer's instructions (eBioscience). The cells were fixed by 4% paraformaldehyde. Three-color flow cytometric analysis was employed with a FACS Calibur (BD Bioscience) and results were analyzed using WinMID 2.9 software (<http://scripps.edu/software.html>; Scripps Institute, La Jolla, CA, USA).

Statistics

The significance of statistical comparisons was calculated using one-way ANOVA and two-tailed Student's *t* test. Values of *P* < 0.05 were considered to represent statistically significant differences. Experiments were performed in triplicate for each mouse. Results are displayed as means ± standard deviations.

Results

Effect of DNA vaccines on established tumors

There is less than 6% difference of the amino residues between rat Her2/neu and its surrogate in mice. It can be regarded as a xenogeneic resource of TAA (Nagata et al. 1997). TUBO cells grew progressively in BALB/c mice, as they are weakly immunogenic and the animal does not react against Her2/neu expressed by tumor cells. The tumor growth does not differ from those observed in BALB-neuT. In this study, we assessed the effect of DNA vaccination with pHer2 or pHSP70/Her2 on the progression of established tumor in BALB/c mice induced by TUBO cells. The protocol of the study from the beginning of subcutaneous tumor implantation and then inoculation of DNA vaccine is shown in Fig. 1. We did not observe control of tumor progression in mice vaccinated with pHSP70/Her2 (*P*=0.5) in comparison with negative control. In contrast, pHer2 appeared to control tumor progression (*P*=0.017) in comparison to negative control, as shown in Fig. 2. The efficacy of DNA vaccination appeared to be in order of pHer2 > pHSP70/Her2 > control.

Fig. 1 Protocol of DNA vaccination. Thirteen days after tumor implantation, the animals were injected with DNA vaccine intramuscularly three times at weekly intervals. Two weeks after the last vaccination, immunological evaluation was performed

Fig. 2 Rate of tumor growth registered from 1 day before beginning of vaccination until 2 weeks after the last vaccination. Tumor masses were measured every other day with calipers in the two perpendicular diameters

pHSP70/Her2 induced significantly higher IL-4-producing cellular immune responses in comparison to negative control. The level produced by pHSP70/Her2-vaccinated animals was also significantly higher in comparison with pHer2-vaccinated animals ($P<0.001$), as shown in Fig. 4.

Spleen and tumor $CD4^+CD25^+foxp3^+$ regulatory T cells (Tregs) and tumor $CD4^+$ and $CD8^+$ cells level

To detect Tregs, we applied the criteria that Tregs must be $CD4^+CD25^{high}foxp3^+$. The use of $CD25^{high}$ (versus low) and $foxp3^+$ as a Treg marker provides better distinction between activated $CD4^+$ T cells and Tregs. We also independently measured percentage of $CD4^+$ and $CD8^+$ cells by flow cytometry.

Cellular reactivity

The enhancement of the cell-mediated mechanisms associated with the administration of pHer2 or pHSP70/Her2 was evaluated using in vitro proliferation test, CTL assay, and $IFN-\gamma/IL-4$ level.

Spleen cells were collected 40 days after tumor implantation and re-stimulated with the TUBO cell-raised tumor extract. The proliferative response of mice vaccinated with pHSP70/Her2 was markedly enhanced ($P=0.004$). The index was also marginally increased in the pHer2-vaccinated animals (Fig. 3).

We did not observe cytotoxicity of spleen cells against TUBO cells (target) in mice receiving pHSP70/Her2.

To further elucidate the state of Th1/Th2 balance, $IFN-\gamma/IL-4$ level was measured. Vaccination with pHSP70/Her2 induced significantly higher $IFN-\gamma$ level in comparison to pHer2-vaccinated animals ($P<0.01$) and negative control ($P<0.01$). In contrast, animals vaccinated with pHer2 or

Fig. 3 Proliferative response. Spleen cells were re-stimulated with the TUBO cell-raised tumor extract in the presence of BrdU. Results are shown as SI and represent the mean $\pm$ SD

Fig. 4 IFN- γ and IL-4 level in DNA vaccinated mice. The level of responses was determined by a DuoSet R&D ELISA system after 2 weeks of the last immunization. Spleen cells were stimulated with TUBO cell-raised tumor extract in RPMI 1640 10% FBS for 90 h. Vaccination with pHSP70/Her2 led to a significant increase in IFN- γ level (*black columns*). In addition, IL-4 level (*white columns*) was significantly different between pHer2- or pHSP70/Her2-vaccinated animals. Results are represented as the mean $\pm$ SD

There was no statistically significant difference among the Treg percentage of various groups at the spleen. However, our results revealed that vaccination with pHer2 led to no change at Treg percentage at the tumor site, in comparison with the control mice. In contrast, we observed a statistically significant decline in Tregs percentage at the tumor site of pHSP70/Her2-vaccinated animals ($P < 0.01$; Fig. 5a). There was also a decrease in Treg/T effector, as shown in Fig 5b. It indicates that the increase in the Treg/T effector should be due to T effector cells increase. Determination of CD4⁺ cells percentage revealed that there was a significant increase in CD4⁺ cells of pHSP7/Her2-vaccinated ($P = 0.03$) animals and marginally in pHer2-vaccinated animals, in comparison with the negative control, as shown in Fig. 5c. CD8⁺ cells percentage of pHer2- and pHSP7/Her2-vaccinated animals was not significantly increased.

Discussion

Elicitation of appropriate effector immune response is required for eradication of tumor masses. Some of these responses include secretion of inflammatory mediators and CTL response. However, some studies have emphasized the critical role of suppressor mechanisms accompanied by other escape mechanisms of tumors. It follows the complications with the development of robust immunotherapeutic vaccines, since a tumor cannot be just eliminated by the activated immune cells. This is because of the tumor

microenvironment effect on immune cells and evasion mechanisms employed by tumors (Blank et al. 2004; Curier et al. 2004; Hirano et al. 2005). Hence, the lack of vaccine effectiveness should be analyzed locally and systemically, i.e., systemic effect of vaccine and the tumor–host mutual talk at the tumor foci (He et al. 2005).

In this study, we evaluated some of the immunological responses triggered by the DNA vaccine containing EC+TM domain of rat Her2/neu when administered intramuscularly to established tumor-bearing BALB/c mice. The immune response could not be due to endotoxin contamination, as firstly, we used the PEG method for purification of the constructs (Sambrook and Russell 2001), which is believed to be the most suitable method for endotoxin removal and DNA

Fig. 5 Freshly isolated spleen cells and tumor MNCs were stained with anti-CD4 FITC, anti-CD25 PE, and anti-foxp3 PECy5. After gating on CD4⁺CD25⁺ cells, Tregs were defined as CD25^{high} and foxp3⁺. Percentage of Tregs (a) and Treg/T effector ratio were determined (b). Tumor MNCs were labeled with FITC-conjugated anti-CD4 or -CD8, freshly. CD4⁺ cell percentage was observed as the marginal increase in the pHSP70/Her2- and pHer2-vaccinated animals (c)

immunization studies (Boyle et al. 1998). Secondly, endotoxin contamination should have induced a nonspecific immune response with all constructs equally and the GP96-minus Her2/*neu* construct in particular. Thirdly, it has been demonstrated that the effect of endotoxin on immune response is route dependent. Endotoxin has no effect on CTL activity and exerts its effect on antibody raise upon intra-dermal administration, but not intramuscularly. Fourthly, PEG precipitation method makes LPS contamination to be reduced by >95% as compared to QIAGEN (at least 10-fold lower than that achieved with QIAGEN-purified DNA; Boyle et al. 1998).

From the systemic view, in this study DNA vaccination with pHer2 could control tumor growth, while fusion with HSP70 gene could not further enhance the therapeutic efficacy. DNA vaccination with pHer2 led to inducement of IL-4 secretion. It neither caused CTL activity nor increased CD8⁺/decreased Tregs percentage at the tumor site. Apparently, it acted via effective antibody-mediated mechanisms (Curcio et al. 2003; Piechocki et al. 2001; Rovero et al. 2000). In contrast, pHSP70/Her2 administration led to higher IFN- γ and IL-4 level. Increase of IL-4 level in the animals vaccinated with pHer2 or pHSP70/Her2 is consistent with the previous results demonstrating the dependency on IL-4 in Her2/*neu* tumor rejection (Rovero et al. 2000; Amici et al. 1998). Such dependency on IL-4 may be related to previous results demonstrating that Th1 anti-tumor immunity required IL-4 during the priming phase (Schuler et al. 1999). It seems that higher level of IL-4 secretion in pHSP70/Her2-vaccinated animals accelerated and enhanced IFN- γ secretion. It is in contrast to pHer2 animals with lower IL-4 level. Animals vaccinated with pHSP70/Her2 also demonstrated a decrease in Tregs/increase in T effector cell percentage at the tumor site.

All these immune responses did not coincide with control of tumor progression. It indicates that the resultant immune response elicited by pHSP70/Her2 vaccination was not effective against the tumor cells. Two reasons could be presumed. First, the immune responses were not potent enough to overcome tumor cells and finally failed (equivalent of tumor tolerance). Second, higher level of IFN- γ secretion led to tumor escape by Her2/*neu* antigen down-regulation (equivalent of tumor evasion model) (Kmieciak et al. 2007). Tumor escape might be the case, since decreased percentage of Tregs at the tumor site could be an indication of Her2/*neu* antigen down-regulation. This is based on the observation that continual antigenic stimulation generates Tregs (Chakraborty et al. 1999), and pHSP70/Her2 vaccination resulted in decreased Tregs percentage at the tumor site. Antigen loss due to higher level of IFN- γ production has been previously described (Kmieciak et al. 2007; Worschech et al. 2008), which could be due to immune pressure. Antigen down-regulation has been frequently reported (Kmieciak et al. 2007; Kominsky et al. 2000a, b; Yoshino et al. 1994). On

the other hand, antibody response did not attain control of tumor progression, which might be due to Her2/*neu* antigen down-regulation.

In this study, decreased percentage of Tregs was not accompanied by an increased percentage of CD8⁺ cells and CTL activity at the tumor sites of pHSP70/Her2-vaccinated animals. Inefficiency of pHSP70/Her2 might be due to the absence of CTL response, which is presumed to play an important role against tumor.

DNA vaccination with pHSP70/Her2 could not decorate the microenvironment in a way that facilitates anti-tumor immune cells activity. In addition, higher IL-4 production and presence of IFN- γ might have led to the expression of molecules, attraction and activation of immune cells (such as tumor-associated macrophages), or other robust immune mechanisms which favored tumor growth (Lepique et al. 2009; Shevach 2006; Spoelstra et al. 1999; Thornhill et al. 1991). Our results show that fusion of HSP70 to N-terminal domain of Her2/*neu* led to tumor progression. This is in contrast to the previous study (Kim et al. 2005) indicating that efficiency of DNA vaccine encoding HSP70 fused to C-terminal of Her2/*neu* prevented tumor progression. The result by Kim et al. (2005) showed that DNA vaccine containing C-terminally fusion of Her2 to HSP70 could inhibit growth of two Her2-expressing tumor induced by TUBO or 4T1.2Neu cell lines. In contrast, our study showed that fusion of HSP70 to N-terminal domain of Her2/*neu* abrogated the inhibitory effect on the growth of tumor, induced using TUBO cell line. N-terminally fusion of Her2/*neu* to HSP70 might have caused conformational change or steric hindrance, which resulted in the loss or decrease of antigenicity of Her/*neu*. It was previously shown that the signal sequence at the beginning of Her2 sequence have a critical role in the potency of Her2 DNA vaccine (Wei et al. 1999). Our results propose that N-terminally fusion of HSP70 to Her2/*neu* masked or decreased the effect of the signal sequence. Moreover, it has been demonstrated that 70 N-terminal residues of Her2/*neu* play an important role in effective protection against Her2-expressing tumors (Rolla et al. 2008). Therefore, it is also likely that the effect of N-terminal residues of Her2/*neu* is affected by fusion of HSP70. The inefficiency of pHSP70/Her2 was not due to fusion to C-terminal domain of HSP70, as fusion of TAA to C-terminal of HSP70 was previously shown to be successful in tumor immunotherapy (Zhang and Huang 2006).

The significance of our result can be briefly described as follows:

- N-terminally fusion of biologic adjuvant to Her2/*neu* could affect Her2/*neu* DNA vaccine efficiency. N-terminal of Her2/*neu* plays a pivotal role in Her2 DNA vaccines and must be left free, while C-terminal of Her2/*neu* could be used for fusion of biologic adjuvant. This is supported by findings from the

previous study in which IL-2, GM-CSF cytokine genes (as biologic adjuvants) were C-terminally fused to Her2 gene and could inhibit Her2-expressing tumors (Lin et al. 2004)

- Higher immune response could sometimes be regarded as immune pressure and acts in favor of tumor growth, via tumor escape and/or activating inhibitory/suppressive mechanisms.

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