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Enhanced Generation of Cytotoxic T Lymphocytes by Heat Shock Protein 70 Fusion Proteins Harboring Both CD8$^+$ T Cell and CD4$^+$ T Cell Epitopes

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Abstract: Heat shock protein 70 (Hsp70) can be a potent carrier of antigens because it is effectively delivered to antigen presenting cells (APCs) and activates innate immunity. To induce a potent cytotoxic T lymphocyte (CTL) response, a Hsp70 fusion protein harboring both CD8$^+$ and CD4$^+$ T cell epitopes was developed based on the recent understanding of the importance of the role of CD4$^+$ T cells in inducing the CTL response following vaccination. $OVA_{257-264}$ (pepI) and $OVA_{323-339}$ (pepII) were selected as the CD8$^+$ and CD4$^+$ T cell epitope of a model antigen, ovalbumin (OVA), respectively. Hsp70 and its fusion proteins, Hsp70-pepI, pepII-Hsp70, Hsp70-pepII and pepII-Hsp70-pepI, were developed. pepII-Hsp70 and pepII-Hsp70-pepI were effectively presented on MHC class II of macrophages compared with Hsp70-pepII, suggesting that pepII conjugation to the N-terminus of Hsp70 is better than the C-terminus for more effective MHC class II antigen presentation. Immunization with pepII-Hsp70-pepI resulted in a higher CTL activity than immunization with the mixture of Hsp70-pepI and pepII-Hsp70. Furthermore, pepII-Hsp70-pepI exhibited a greater antitumor effect in the mice bearing EG7 tumor cells than the physical mixture of Hsp70-pepI and pepII-Hsp70. In addition, immunization with the DCs pulsed with the fusion proteins also suggested that APCs which present both pepI and pepII can induce the highest CTL generation. These results demonstrated that Hsp70 fusion protein harboring both pepI and pepII is a useful option for Hsp70-based antigen delivery systems.

Keywords: Hsp70; CTL; protein vaccine; antigen delivery; OVA; CD8$^+$ T cell epitope; CD4$^+$ T cell epitope

Introduction

Successful immunotherapy of cancer requires induction of cytotoxic T lymphocyte (CTL)-mediated immune responses. Antigen-specific CTLs are generated when antigen-presenting cells (APCs) present antigen-derived peptides on major histocompatibility complex (MHC) class I molecules to naive CD8$^+$ T cells. There are a variety of approaches to elicit CTL by vaccination with peptides or proteins, such as the use of adjuvants,[1] liposomes[2] and microparticles.[3] Among them, soluble protein formulations[4] without adjuvants are useful, safe, and easy to prepare if they can induce antigen-specific CTL effectively.

Heat shock protein 70 (Hsp70) is a member of a family of molecular chaperones that are induced under stress

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conditions.[5,6] In addition, Hsp70 has been investigated for tumor vaccines with the ability to bind antigenic peptide. Immunization with Hsp70 protein preparations in solution is able to induce cross-priming of T cells without any adjuvant administration. It has been reported that Hsp70 derived from tissues and cells[7] and synthetic peptide reconstituted with Hsp70 in soluble form can elicit strong antigen-specific CTL responses following immunization of mice.[8] It is known that Hsp70 is efficiently taken up by APCs via Hsp receptors, such as CD91 or LOX-1.[9−11] Hsp70 can also activate the innate immunity through CD40 and Toll-like receptor-2 and -4 with cofactor CD14 and induce cytokine secretion from dendritic cells (DCs).[12−17]

Some recent basic studies have demonstrated that optimal $CD8^+$ T cell responses often require concomitant $CD4^+$ helper cells during immune induction.[18−20] It has been also reported that the effects of helper T cells could be substituted by using anti-CD40 antibody to costimulate $CD8^+$ T cells or provide the cytokine products of $CD4^+$ T cells such as interleukin-2 (IL-2).[21−23] These studies suggested that functional efficacy of the $CD8^+$ T cell response induced by immunization with vaccines could be enhanced by concomitant stimulation of $CD4^+$ T cells.

In order to establish a vaccination method for immunotherapy of tumors and viral infections using both $CD8^+$ and $CD4^+$ T cell epitopes, efforts have been made to develop carriers of the antigens for their effective delivery, such as nanobeads,[24] pullulan or mannan[25] and lipopeptide.[26] As far as Hsp70 is concerned, Kumaraguru et al. have reported that immunization by Hsp70 loaded with $CD8^+$ and $CD4^+$ T cell peptide epitopes increases the $CD8^+$ T cell memory response up to 3-fold.[27] In another approach using one of the Hsp families, heat shock cognate protein 70 (Hsc70), fused to both $CD8^+$ and $CD4^+$ T cell epitopes, has exhibited increased CTL activity.[28]

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In a series of studies, we have developed strategies to establish effective antigen delivery systems using Hsp70 systematically from the viewpoint of pharmacokinetics. An in vivo pharmacokinetic study has demonstrated that Hsp70 was effectively distributed to the liver and spleen via CD91 and scavenger receptors in mice after intravenous injection. Hsp70 also accumulated in the regional lymph nodes following subcutaneous injection, indicating that Hsp70 has advantageous characteristics at the whole body level.[29] We have also succeeded in designing a delivery approach to control the intracellular pharmacokinetics of Hsp70. We have developed a fusion protein, $CD8^+$ T cell epitope fused to Hsp70-polyhistidine protein, which can enhance cytosolic delivery and subsequently improve the MHC class I antigen presentation in DCs and increase the CTL activity.[30] This approach has been applied to a DNA vaccine, plasmid DNA (pDNA) encoding polyhistidine-Hsp70-$CD8^+$ T cell epitope fusion protein gene, which showed a higher CTL activity than the corresponding protein vaccine.[31] Furthermore, we have developed a unique Hsp70-based DNA vaccine for induction of antitumor immune responses without delivery of any antigens in tumor-bearing mice.[32] Intratumoral gene transfer with plasmid encoding Hsp70-cell-penetrating peptide fusion protein has resulted in induction of high CTL activity and a prolonged survival rate by effective presentation of putative endogenous tumor antigens through efficient delivery to APCs by the cell-penetrating peptide.

In this study, we have examined the efficacy of a novel approach by concomitant use of the $CD4^+$ T cell epitope of ovalbumin (OVA) (pepII) together the $CD8^+$ T cell epitope of OVA (pepI) for Hsp70 based fusion protein vaccines, although we used the $CD8^+$ epitope alone in our previous studies. In vitro and in vivo characterization of the originally developed fusion protein, pepII-Hsp70-pepI, was carried out to assess the usefulness of the Hsp70-based antigen delivery system. We established a new in vitro experimental system for the assessment of MHC class II restricted antigen presentation using antigen presenting cells (macrophages in primary culture) and T cell hybridoma cells (DO11.10). The usefulness of concomitant delivery of pepI and pepII in one Hsp70 molecule was also evaluated in ex vivo experiments using antigen pulsed DCs.

Materials and Methods

Cells and Animals. DC2.4 cells, a cell line of murine dendritic cells (haplotype H-2^b), were kindly provided by Dr. K. L. Rock (University of Massachusetts Medical School, Worcester, MA). CD8OVA1.3 cells, T hybridoma cells against SIINFEKL-K^b, were a generous gift from Dr. C. V. Harding (Case Western Reserve University, Cleveland, OH). DO11.10, T hybridoma cells against ISQAVHAAHAEINEA-GR-A^d, were a generous gift from Dr. P. Marrack (National Jewish Medical and Research Center, USA). EL4 cells, C57BL/6 T lymphoma, and EG7 cells, an OVA-transfected clone of EL4, were purchased from American Type Culture Collection (Manassas, VA).

DC2.4 cells were cultured in RPMI 1640 medium (Nissui Pharmaceuticals Pharmaceuticals, Tokyo, Japan) supplemented with 10% heat-inactive fetal bovine serum (Equitedh-Bio, Kerrville, TX), 50 μM 2-mercaptoethanol, 2 mM L-glutamine, and antibiotics (all from Invitrogen, Carlsbad, CA). CD8OVA1.3 and EL4 cells were cultured in Dulbecco's modified Eagle medium (Nissui) supplemented as described for RPMI 1640 medium. EG7 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactive fetal bovine serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, glucose, sodium pyruvate, HEPES and G418. DO11.10 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactive fetal bovine serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, HEPES and nonessential amino acids.

Five-week-old female C57BL/6 mice and male BALB/c were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions.

Isolation of Peritoneal Macrophages. Peritoneal macrophages of BALB/c mice were isolated as previously described.[33] After mice were euthanized, their peritoneal cavities were lavaged with RPMI 1640 medium supplemented with 1% L-glutamine, 100U/ml penicillin G and 100 μg/mL streptomycin (all obtained from Invitrogen, Carlsbad, CA). Peritoneal macrophages were allowed to adhere to 24-well tissue culture plates at a density of 1×10^6 cells/mL for 3 h in RPMI 1640 medium supplemented with 10% FBS, 1.2 μg/mL amphotericin B (Sigma, St. Louis, MO), 1%

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L-glutamine, 100 U/mL penicillin G and 100 μg/mL streptomycin before washing to remove nonadherent cells.

Expression Vectors of Hsp70 Fusion Proteins. The pTrc99A expression vector containing the genomic mouse clone *hsp70.1* cDNA was kindly supplied by Dr. Paul Slusarewicz (Mojave Therapeutics, Inc., USA). Murine cDNA of *hsp70.1* was amplified by PCR and inserted into pGEX-6P-2 expression vector (Amersham, Tokyo, Japan). An $CD8^+$ T cell epitope peptide of OVA ($OVA_{257-264}$: SIINFEKL-K^b) was selected as a model antigen, and oligonucleotides corresponding to the amino-acid sequence SIINFEKLTEWTS were purchased from Hokkaido System Science (Hokkaido, Japan), and inserted into the C-terminal of Hsp70. The TEWTS sequence was added to the epitope because it has been reported to facilitate the liberation of SIINFEKL in the proteasome.[34] The oligonucleotide coding the $CD4^+$ T cell epitope peptide of OVA ($OVA_{323-339}$; ISQAVHAAHAEINEAGR) was also synthesized and incorporated into the N or C-terminal of Hsp70. The DNA sequences of the vectors were confirmed using a DYEnamic ET terminator kit Cycle Sequencing Kit (Amersham Pharmacia). The schematic structures of the constructs are shown in Figure 1A.

Purification of Hsp70 Fusion Proteins. The expression of all Hsp70 based fusion proteins in the *Escherichia coli* DH5α cells was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the harvested cells were disrupted. The clarified supernatants of the fusion proteins were loaded onto a glutathion sepharose column (Amersham Pharmacia), and these proteins were eluted by applying prescission protease (Amersham Pharmacia) in 50 mM Tris-HCl solution (pH 7.5). Each eluate was concentrated by ultrafiltration and the purity of the expressed proteins was confirmed by 8% SDS−PAGE and Western blotting. Limulus amebocyte lysate assay (Limulus F single test; Wako, Osaka, Japan) revealed that LPS contamination in all the purified proteins was less than 0.5 EU/μg protein.

Antigen Presentation Assay. The efficacy of MHC class I presentation activity of the fusion proteins was assessed by an in vitro antigen presentation assay using DC2.4 cells and T hybridoma cells that specifically recognize SIINFEKL-K^b and release IL-2. This simple method has been widely used for the evaluation of OVA vaccination systems.[35] DC2.4 cells (10^5/well) were cultured on 96-well plates and directly incubated with CD8OVA1.3 T hybridoma cells (10^5/well) at 37 °C. Then, 24 h later, the cell culture supernatants were collected and freeze−thawed and the response of CD8OVA1.3 T cells was determined by measuring IL-2 levels in the supernatants with an enzyme-linked immunosorbent assay (ELISA; AN'ALYZA mouse IL-2, Genzyme-techne, Minneapolis, MN).

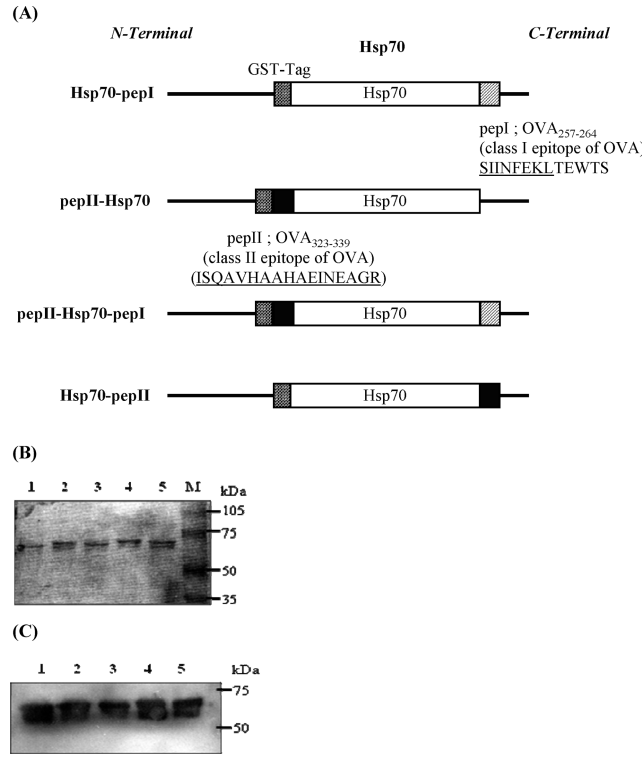

Figure 1. (A) Expression vectors encoding mouse Hsp70 fused to MHC class I and II antigenic peptide. The expression vectors were constructed on pGEX-6P-2. (B) SDS−PAGE of Hsp70-based fusion proteins. Lane 1: Hsp70. Lane 2: Hsp70-pepI. Lane 3: pepII-Hsp70. Lane 4: pepII-Hsp70-pepI. Lane 5: Hsp70-pepII. Lane M: Marker. (C) Western blotting of Hsp70-based fusion proteins. Lane 1: Hsp70. Lane 2: Hsp70-pepI. Lane 3: pepII-Hsp70. Lane 4: pepII-Hsp70-pepI. Lane 5: Hsp70-pepII.

In addition, the efficacy of MHC class II presentation activity of the fusion proteins was assessed by an in vitro antigen presentation assay using peritoneal macrophages cultured after 24 h and T hybridoma cells, DO11.10 cells. Peritoneal macrophages (10^5/well) were cultured on 96-well plates and directly incubated with fusion proteins and DO11.10 T hybridoma cells (10^5/well) at 37 °C. Then, 24 h later, the cell culture supernatants were collected and freeze−thawed. As described above, the response of DO11.10 T cells was determined by measuring IL-2 levels in the supernatants. In the antigen presentation assay, all cells used in this study were cultured in the presence of serum to maintain normal cellular functions during the experiments.

Immunization. *Immunization with Proteins.* C57BL/6 mice were immunized twice at weekly intervals in the back with l0 μg Hsp70 fusion proteins in 50 μL PBS solution.

Immunization with DC2.4 Cells. The ex vivo experiments were carried out. 1×10^6 DC2.4 cells were pulsed with 50 μg of Hsp70 fusion proteins and incubated at 37 °C. Then 6 h later, cells were washed with PBS and treated with mitomycin C for 0.5 h. After washing of the cells with PBS,

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DC2.4 cells were dissolved in HBSS (Hanks' balanced salt solution). C57BL/6 mice were immunized twice at weekly intervals with 1×10^6 treated DC2.4 cells in 100 μL of HBSS intradermally.

CTL Assay. C57BL/6 mice were immunized twice at weekly intervals in the back with Hsp70 fusion proteins or DC2.4 cells. Seven days after the last immunization, splenocytes were isolated from the immunized mice followed by restimulation in vitro for five days with mitomycin C-treated EG7 target cells (EG7 or EL4; EL4 was used as a target control) labeled with ^{51}Cr by incubating with $Na_2{}^{51}CrO_4$ in culture medium for 45 min at 37 °C. After washing, 2×10^4 of the ^{51}Cr-labeled target cells and serially diluted splenocytes were coincubated in 200 μL of culture medium for 4 h at 37 °C. The spontaneous release of ^{51}Cr with no effector cells or the maximal release in the presence of 1% TritonX-100 was also evaluated. Cells were centrifuged (1500 rpm) for 5 min, and 100 μL of each supernatant was collected for the radioactivity measurements. The cytosolic activity of CTLs was calculated as described elsewhere:

$$\% \text{ of killing} = 100 \times (\text{observed release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})$$

Tumor Challenge Experiments. C57BL/6 mice were immunized twice at weekly intervals in the back with each antigen. Eight days after the last immunization, 5×10^6 EG7 cells per mouse were administered intradermally into the back as a challenge. The tumor size was measured using the longest and the shortest diameters. The tumor volume was calculated from this equation: $(\text{longest} \times \text{shortest})^{3/2} \times \pi/6$. The survival time of the tumor-challenged mice was also recorded.

Results

Construction of Plasmid Vectors and Purification of Hsp70 Fusion Proteins. Figure 1A shows the schematic structures of the designed constructs. A typical MHC class I epitope of OVA, SIINFEKL ($OVA_{257-264}$, pepI) and TEWTS sequence, which has been reported to facilitate the degradation of the SIINFEKL peptide in the proteasomes, was fused to the C-terminus of the Hsp70 because the conjugation of pepI to C-terminus was a more efficient design to induce CTL responses in comparison with the N-terminus conjugation.[30,36] Therefore, we did not examine N-terminus conjugation of pepI in this study. For preliminary evaluations to decide the appropriate conjugation of $CD4^+$ T cell epitope with Hsp70, a typical $CD4^+$ T cell epitope of OVA, ISQAVHAAHAEINEAGR ($OVA_{323-339}$, pepII) was fused to the N or C-terminal of Hsp70. Furthermore, Hsp70 fusion protein with both pepI and pepII was constructed. These fusion gene constructs were inserted into the pGEX-6P-2 expression vector and expressed in *E. coli* DH5α cells. The expressed fusion proteins were purified from the cells.

To confirm whether the target fusion proteins were obtained and to examine the purity of the fusion proteins, SDS−PAGE was conducted. Figure 1B shows the results of SDS−PAGE of the purified Hsp70 fusion proteins, demonstrating that Hsp70 and the designed fusion proteins, Hsp70, Hsp70-pepI, pepII-Hsp70, pepII-Hsp70-pepI and Hsp70-pepII, had been successfully obtained (Figure 1B). The purity of all the fusion proteins was also confirmed on the basis of the image. Western blot analysis of purified Hsp70 fusion proteins was also performed with anti-Hsp70 antibody (Figure 1C), confirming the molecular weight and purity of each fusion protein.

MHC Class II and Class I Restricted Presentation of Hsp70 Fusion Proteins or OVA. In order to examine the efficacy of MHC class-II and -I presentation activity of the fusion proteins, an in vitro antigen presentation assay using primary cultured peritoneal MΦ and DC2.4 cells and T hybridoma cells, DO11.10 and CD8OVA1.3, was conducted based on IL-2 production under 6 μM antigen concentrations (Figure 2). As shown in Figure 2A, OVA showed a minimal activity whereas Hsp70 fused to pepII induced a high level of IL-2 production when the cells were pulsed with the antigens, Hsp70 fusion proteins and OVA, at the same concentration. This result indicated that Hsp70 is a useful carrier for pepII. Although the differences between the absolute values were not so large, pepII-Hsp70 and pepII-Hsp70-pepI induced a significantly higher level of IL-2 production than Hsp70-pepII, suggesting that more effective MHC class II antigen presentation would take place when pepII was fused to the N-terminus of Hsp70 rather than the C-terminus. Taking into consideration that pepI should be fused to the C-terminus Hsp70, we decided to fuse pepII to the N-terminus of Hsp70.

Figure 2B shows the MHC class I restricted presentation of Hsp70 based fusion proteins. Both Hsp70-pepI and pepII-Hsp70-pepI showed the same level of IL-2 production (statistically no significant difference), indicating that additional conjugation of pepII to the N-terminus of Hsp70-pepI did not influence the MHC class I antigen presentation.

Generation of CTL by Hsp70 Fusion Proteins. To evaluate whether the antigen specific CTLs were enhanced by immunization with Hsp70 fused pepI and pepII, we examined the OVA-specific CTL response using EG7 cells expressing OVA as targets and EL4 cells as a control (Figure 3). The splenocytes from the mice treated with pepII-Hsp70-pepI showed a higher level of OVA-specific CTL activity compared with that observed for the physical mixture and Hsp70-pepI alone (Figure 3A). The CTL activity of the physical mixture and Hsp70-pepI alone was almost identical, suggesting that the coadministration of pepII-Hsp70 could not promote CTL activity induced by Hsp70-pepI unlike the immunization with pepII-Hsp70-pepI, which harbored both pepI and pepII in a Hsp70 molecule. No CTL activities were seen in EL4 cells, indicating that the CTL activities were OVA-specific responses (Figure 3B).

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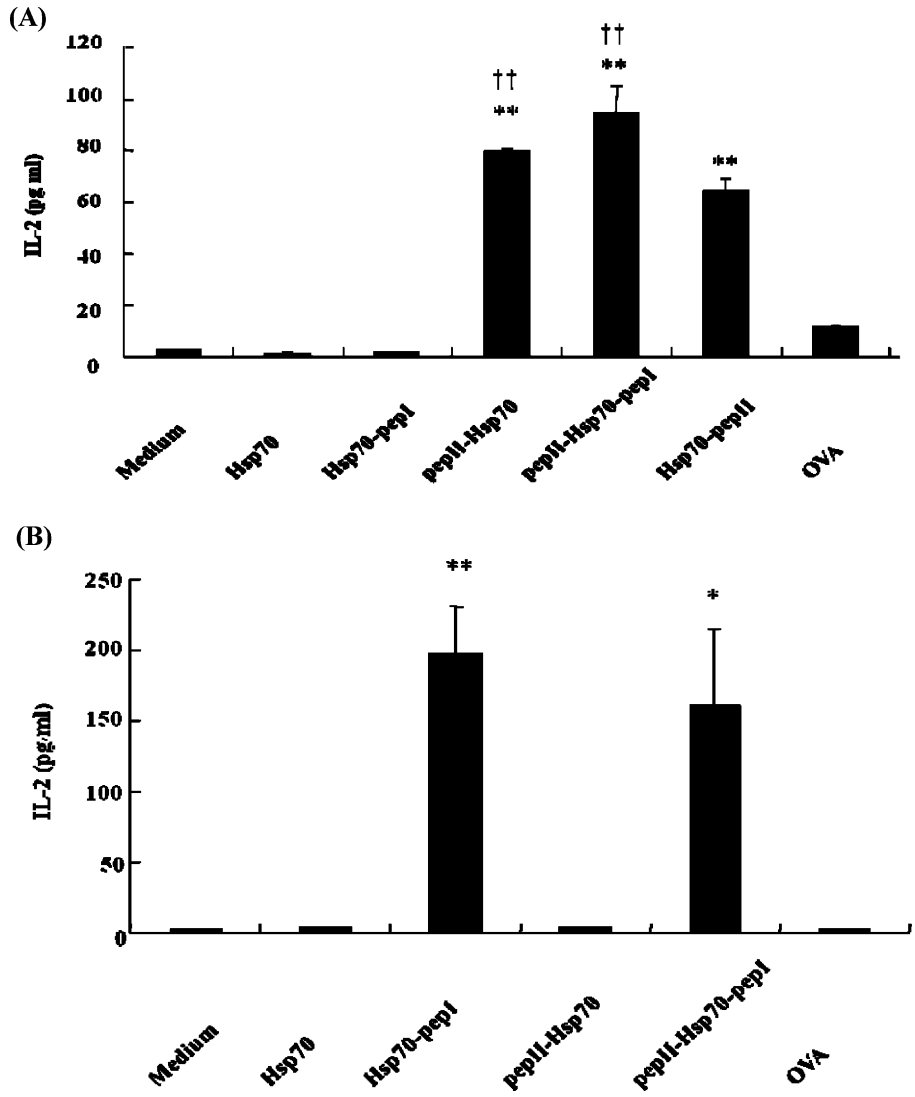

Figure 2. (A) MHC class II-restricted presentation of Hsp70-based fusion proteins and OVA. Peritoneal macrophages isolated from BALB/c mice and DO11.10 T hybridoma cells were incubated with either 6 μM Hsp70-based fusion protein or OVA at 37 °C. After 24 h, IL-2 production from DO11.10 T hybridoma cells was measured by ELISA (n = 3). Statistically significant differences were assessed using Student's t test compared with Hsp70-pepII ($^{\dagger\dagger}P < 0.01$) or OVA ($^{**}P < 0.01$). (B) MHC class I-restricted presentation of Hsp70-based fusion proteins and OVA. DC2.4 cells and CD8OVA1.3 T hybridoma cells were incubated with either 6 μM Hsp70-based fusion protein or OVA at 37 °C. After 24 h, IL-2 production from CD8OVA1.3 T hybridoma cells was measured by ELISA. Statistically significant differences were assessed using Student's t test compared with OVA ($^{*}P < 0.05$, $^{**}P < 0.01$).

In Vivo Growth Inhibitory Effect on EG7 Tumor Cells and Survival Rates of Mice Following Immunization with Hsp70 Fusion Proteins. To investigate the in vivo efficiency of fusion proteins by using another experimental method, we examined the effect of immunization with Hsp70 fusion protein on the protection of mice from an EG7 tumor challenge. Figure 4A illustrates the mean tumor volumes as a function of the days after tumor challenge. Rapid tumor growth was observed for the groups treated with Hsp70-pepI and pepII-Hsp70, as the untreated group. The physical mixture group showed a partial inhibitory effect whereas tumor growth was significantly inhibited in the groups treated with pepII-Hsp70-pepI. As shown in Figure 4B, only the mice immunized with pepII-Hsp70-pepI exhibited prolonged survival following challenge with EG7 tumor cells, although a partial inhibitory effect was observed in the physical mixture group. These results in the tumor challenge experiments are corresponded relatively well with the results of CTL assay (Figure 3A).

Generation of OVA-Specific CTL after Administration of DC2.4 Cells Pulsed with Hsp70−Peptide Fusion Proteins. To elucidate the mechanisms by which the vaccination with pepII-Hsp70-pepI enhanced the CTL activity, we investigated the OVA-specific CTL response by ex vivo experiments using DCs pulsed with Hsp70−antigenic peptide fusion proteins (Figure 5). DC2.4 cells were pulsed with (1) Hsp70-pepI, (2) pepII-Hsp70-pepI, (3) a physical mixture of both Hsp70-pepI and pepII-Hsp70 (protein mixture) and (4) a

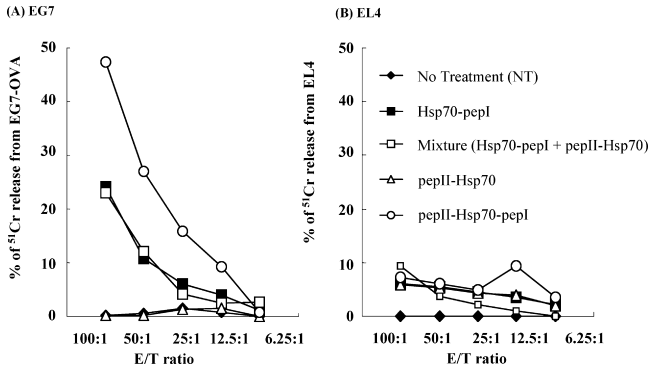

Figure 3. Generation of OVA-specific CTL by immunization with Hsp70-based fusion proteins. Mice were immunized twice with 10 μg of Hsp70 fusion proteins intradermally (mixture: 10 μg of pepII-Hsp70 and 10 μg of Hsp70-pepI). Seven days after the last immunization, spleen cells were isolated and five days after the isolation of spleen cells, standard ^{51}Cr release assay was performed ($n = 3$). (A) EG7, (B) EL4.

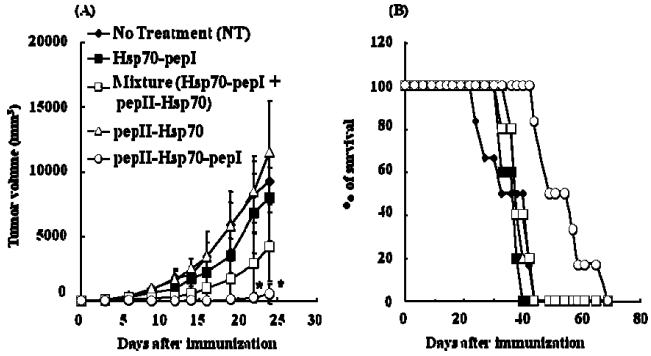

Figure 4. Effect of immunization with Hsp70-based fusion proteins on the growth of intradermal tumor (A) and survival rate (B). Mice were immunized twice with 10 μg of Hsp70 fusion proteins (mixture: 10 μg of pepII-Hsp70 and 10 μg of Hsp70-pepI) and eight days after the last immunization, 5×10^6 EG7 cells were injected intradermally ($n = 6-7$). Statistically significant differences were assessed using Student's t test compared with the mixture ($^*P < 0.05$).

mixture of both pepI-Hsp70 pulsed DCs and pepII-Hsp70 pulsed DCs (cell mixture). We assumed that most of the DCs pulsed with protein mixture would present either MHC class I or II peptide, and only a part of the cells would present both MHC class I and II peptide. On the other hand, all the DCs in the cell mixture group should present either MHC class I or II peptide separately, without a cell population that presents both MHC class I and II peptide. Most probably, DCs pulsed with pepII-Hsp70-pepI would have the highest probability that the individual cells would present both MHC class I and II peptide following uptake of the fusion protein. As shown in Figure 5, the group treated with pepII-Hsp70-pepI showed the highest CTL activity, and the group treated with protein mixture expressed the second highest activity, although the percentages of the target cell lysis were not significantly high. The level of CTL activity of the pepII-Hsp70-pepI group was significantly higher than the cell

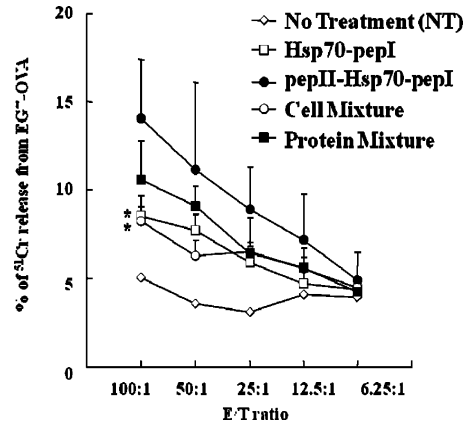

Figure 5. Generation of OVA-specific CTL by 2-fold immunization of antigen pulsed DC2.4 cells. Mice were immunized twice with DC2.4 cells (1×10^6 cells) pulsed with 50 μg of Hsp70 fusion proteins intradermally (protein mixture, 50 μg of pepII-Hsp70 and 50 μg of Hsp70-pepI per 1×10^6 cells; cell mixture: 50 μg of pepII-Hsp70 per 1×10^6 cells and 50 μg of Hsp70-pepI per 1×10^6 cells). Seven days after the last immunization, spleen cells were isolated, and five days after this, a standard ^{51}Cr release assay against EG7 cells was performed in each treated group ($n = 3-4$) and the no treatment group ($n = 1$). Statistically significant differences were assessed using Student's t test compared with the pepII-Hps70-pepI group ($^*P < 0.05$).

mixture and Hsp70-pepI groups. The CTL activity of the cell mixture group reached almost the same level as the group treated with Hsp70-pepI alone. These results suggest that the DC population presenting both MHC class I and II epitopes on the same cells might induce a higher CTL activity compared with the DC population presenting MHC class I and II epitopes on different cells.

Discussion

Previous studies have shown that synthetic peptide reconstituted with Hsp70[8] and the purification of Hsp70 from tumor cells[7] could elicit strong antigen-specific CTL responses because Hsp70 can bind the tumor antigens in its own peptide binding domain. Another vaccination strategy used Hsps, fusion of genes encoding CTL epitope to Hsp70 or heat shock cognate protein 70 (Hsc70), cDNA to construct a fusion protein were applied to induce efficient CTL generation[36,37] because a rational design of the designated proteins is easy using gene-recombination technologies. However, the approaches using a Hsp−peptide complex have limitations regarding the quality of tumor antigens bound to Hsps if the length and amino acid sequence of the epitope are not always suitable for Hsp binding activity.

(37) Suzue, K.; Zhou, X.; Eisen, H. N.; Young, R. A. Heat shock fusion proteins as vehicles for antigen delivery into the major histocompatibility complex class I presentation pathway. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 13146–13151.

Some basic immunological studies have demonstrated that the CTL activity was enhanced by $CD4^+$ T cells, and by the production of cytokines such as IL-2.[21] Production of IL-2 by $CD4^+$ T cells markedly activates the APCs and CTLs in the vicinity of the $CD4^+$ T cells.[38] The importance of the role of $CD4^+$ T cells in vaccine strategies has also been reported.[24−26] Very recently, Mizukami et al. reported that vaccination with a Hsc70 fused to a CTL epitope, $OVA_{257-264}$, and $CD4^+$ T cell epitope, $OVA_{265-280}$, resulted in enhanced CTL generation and a significant protective effect against in vivo tumor growth compared with treatment with Hsc70-CTL epitope fusion protein.[28]

In our series of studies, we have developed a variety of Hsp70-based antigen delivery systems. With regard to our previous antigen delivery system, we have focused solely on the $CD8^+$ T cell epitope and developed a protein vaccine of the epitope fused to polyhistidine-Hsp70 fusion protein[30] and a DNA vaccine encoding the gene of the protein vaccine.[31] The $CD4^+$ T cell epitope could be used in our antigen delivery systems.

In the present study, we have shown that an OVA class II antigenic peptide ($OVA_{323-339}$; ISQAVHAAHAEINEAGR) fused to Hsp70, a novel design for Hsp70 based tumor vaccines, was effectively presented on primary cultured mouse MΦ cells compared with OVA, and would be taken up by MΦ cells probably via mannose receptors (Figure 2). As shown in pepI fusion proteins,[30] Hsp70 could also be a useful carrier for pepII via CD91 or LOX-1 receptors in MHC class II antigen presentation. Regarding the site for conjugation of pepII, the N-terminus of Hsp70 was slightly but significantly better than the C-terminus in terms of the activity of MHC class II antigen presentation. Although other designs of fusion proteins (e.g., both epitopes in tandem on one terminus) might be possible, we considered that the fusion protein, pepII-Hsp70-pepI, would be the most appropriate for an efficient presentation of both epitopes. This design of Hsp70 fusion protein would be applicable to other tumor-associated class I and class II epitopes; however, further studies are required.

The endocytosis efficacy of all fusion proteins is considered to be similar. The effect of steric hindrance due to the epitope conjugation on the receptor recognition and subsequent internalization would not be significant because the molecular weight of pepI (M_w approximately 1 kDa) and pepII (M_w approximately 2 kDa) were small enough compared with Hsp70 (M_w approximately 70 kDa). In addition, we demonstrated that the uptake of Hsp70 fusion proteins, Hsp70-pepI and Hsp70-pepI fused to plolyhistidine (M_w approximately 4 kDa) at the N-terminus (His_{25}-Hsp70-pepI) by DCs were almost identical to native Hsp70.[30]

In vivo experiments in the present study have demonstrated that pepII-Hsp70-pepI is a more potent vaccine than Hsp70-pepI in terms of CTL induction (Figure 3), the inhibitory effect on tumor growth and the prolongation of survival of tumor-bearing mice (Figure 4), indicating that concomitant delivery of the MHC class II epitope enhances the antigen-specific CTL responses induced by the MHC class I epitope. These results are in clear contrast to those of pepII-Hsp70-pepI in in vitro experiments (Figure 2) where no enhancement occurred because only one T cell type ($CD8^+$ or $CD4^+$) activation should take place even if APCs present both epitopes. More importantly, the effect of pepII-Hsp70-pepI was greater than that of the physical mixture of Hsp70-pepI and pepII-Hsp70 in these in vivo experiments, suggesting that efficient simultaneous delivery of both MHC class I and II epitopes to the individual APCs would be necessary for the enhanced effect in vivo. In order to confirm the hypothesis that both $CD8^+$ T cell and $CD4^+$ T cell epitopes are required in a single Hsp70 molecule to enhance the CTL generation, we conducted another immunization experiment using DC2.4 cells pulsed with the same fusion protein preparations used for the in vivo experiments (Figure 5). Again, the splenocytes isolated from the mice injected with the DC preparation pulsed with pepII-Hsp70-pepI showed the highest CTL activity compared with other groups, suggesting that the DC population presenting both MHC class I and II epitopes on the same cells might induce a higher CTL activity compared with the DC population presenting MHC class I and II epitopes on different cells. In this ex vivo experiment, the DCs in the protein mixture group were pulsed with 100 μg of protein in total (the mixture of 50 μg of pepII-Hsp70 and 50 μg of Hsp70-pepI) whereas the DCs in the pepII-Hsp70-pepI group were pulsed with only 50 μg of pepII-Hsp70-pepI. This experimental design was due to adjustment of the dose in terms of epitopes (PepI and PepII); however, the dose of Hsp70 was almost 2-fold (or more). It is well-known that Hsp70 can activate DCs through CD40 and Toll-like receptor-2 and -4. Therefore, activation of the innate immunity in the protein mixture group could be higher than that of the pepII-Hsp70-pepI group. This could be a factor for the insignificant difference between two groups. Furthermore, CTL activities induced in the ex vivo experiments were not so high, which might be another factor. Although we cannot draw a definitive conclusion, the results in Figure 5 could support our conclusion that concomitant delivery of $CD4^+$ T cell and $CD8^+$ T cell epitopes by Hsp70 would be useful.

In a DNA vaccination study using pDNA encoding tumor antigen or SEREX-derived antigen, Nishikawa et al. suggested that both pDNA encoding $CD8^+$ T cell antigen and $CD4^+$ T cell antigen must be taken up and presented together by the same APCs.[39] The authors showed that CTL enhancements were observed when the gold particles coated with a mixture of pDNA encoding $CD8^+$ T cell epitope and pDNA encoding $CD4^+$ T cell epitope were administered at the same site by the gene gun, although the injection of the mixture of gold particles coated separately with each pDNA was ineffective. The results of our present study are in good agreement with those of this DNA vaccination study, although we should pay attention to the distinct differences between two approaches.

(38) Ridge, J. P.; Di Rosa, F.; Matzinger, P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* **1998**, *393*, 474–478.

In this study, we have demonstrated that a novel Hsp70 fusion protein harboring both pepII and pepI is a useful Hsp70-based antigen delivery system. Recently, Mizukami et al. also have demonstrated that fusion of both $CD4^+$ and $CD8^+$ T cell epitopes to Hsc70, another Hsp family, enhances tumor immunity by extensive in vivo experiments without in vitro evaluations of the fusion proteins. We have attempted to develop Hsp70 fusion proteins through a rational design based on in vitro antigen presentation assay for both MHC class I and II epitopes. In addition, we have provided a direct evidence that concomitant delivery of both MHC class I and class II by Hsp70 to the same APCs might be important for enhanced CTL induction by ex vivo experiments using the DCs pulsed with several types of antigens.

Conclusions

The present study has demonstrated that pepII-Hsp70-pepI, a Hsp70-based vaccine in which not only the $CD8^+$ T cell epitope but also the $CD4^+$ T cell epitope is incorporated into a Hsp70 molecule, could be a useful option for Hsp70-based antigen delivery systems. The findings in this study provide useful information for the rational design of Hsp70-based antigen delivery systems. This approach using both $CD4^+$ T cell and $CD8^+$ T cell epitopes in Hsp70 fusion protein could be applied to the design of DNA vaccines to induce stronger antitumor immunity.

Abbreviations Used

Hsp70, heat shock protein 70; CTL, cytotoxic T lymphocyte; OVA, ovalbumin; PAGE, polyacrylamide gel electrophoresis; IL-2, interleukin-2; pDNA, plasmid DNA; APC, antigen presenting cell; MΦ, macrophage; DC, dendritic cell; pepI, $CD8^+$ T cell epitope of OVA (SIINFEKL, $OVA_{257-264}$); pepII, $CD4^+$ T cell epitope of OVA (ISQAVHAAHAEINEAGR, $OVA_{323-339}$).

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(39) Nishikawa, H.; Tanida, K.; Ikeda, H.; Sakakura, M.; Miyahara, Y.; Aota, T.; Mukai, K.; Watanabe, M.; Kuribayashi, K.; Old, L. J.; Shiku, H. Role of SEREX-defined immunogenic wild-type cellular molecules in the development of tumor-specific immunity. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 14571–14576.