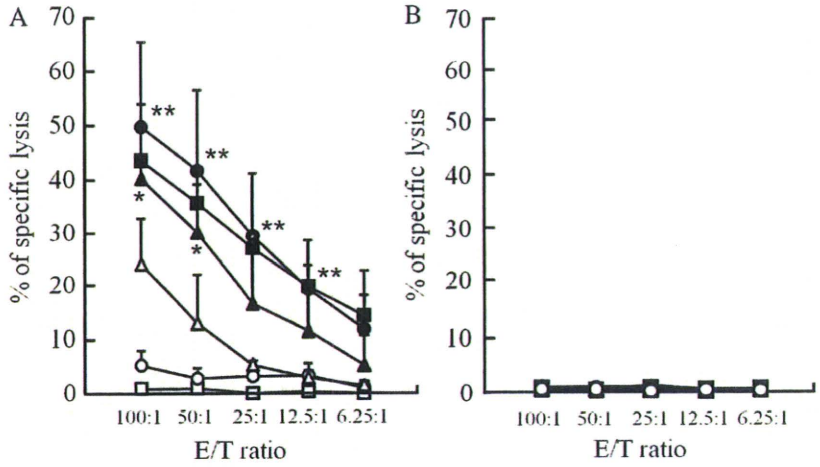

Figure 4. Generation of OVA-specific CTLs after immunization with pCMV-OVA or pPep-ER via the intradermal (id.) or intrasplenic (is.) route. Mice were immunized twice with 100 μg of plasmid DNA followed by electroporation. Seven days after the last immunization, spleen cells isolated from immunized mice were co-cultured with EG7-OVA cells at 37 °C for 5 days. Then, a standard ^{51}Cr release assay was performed ($n = 4$). EG7-OVA (A) and EL4 (B) cells were used as target cells. The results are expressed as the mean ± SD of four mice. □, No treatment; ■, OVA in CFA; open symbols, intrasplenic route; solid symbols, intradermal route: △ ▲, pCMV-OVA; ○ ●, pPep-ER. *$p < 0.05$ compared to pCMV-OVA (is.). **$p < 0.01$ compared to pPep-ER (is.)

intradermal gene transfer produced antigens for a long period of time, and this would be of benefit for the induction of antigen-specific immune responses. Damage caused by the surgery, injection and electroporation could be another factor contributing to the rapid decrease, even though we used optimized parameters for gene transfer.

The characteristics of the host APCs at the administration site of vaccines and their activation state are two critical factors determining the continuation or abolition of the immune response. To estimate which type of cells are involved in the response after injection of plasmid DNA, we selected two types of plasmid DNA in the present study: pCMV-OVA and pPep-ER. In the case of pCMV-OVA, it could undergo both direct-presentation and cross-presentation *in vivo*. The cross-presentation pathway is considered to be the major mechanism for T cell priming by DNA vaccine [27] because the number of APCs, especially dendritic cells, is very limited at the site for DNA vaccination [28]. Therefore, the ability of APCs, especially DCs, to take up antigens expressed in and released from non-APCs, is a key step in priming the T cell response. Although LCs (i.e. the immature DCs in the epidermis) account for only 1–3% of the total epidermal cells, their long and branched processes form a dense network covering almost 20% of the entire skin surface, which favors LC capture and antigen processing [28]. In our preliminary experiments, we observed transgene expression in keratinocytes and dendritic-like cells after intradermal injection. On the other hand, APCs in the spleen appeared to have less ability to take up antigens than LCs in the skin. A previous study [29] suggested that the resident DCs (i.e. the central player among APCs) differ in function between those in the spleen and those in lymph nodes. Although the precise mechanism for the tissue-dependent efficacy of DNA vaccine requires further investigation, the results obtained in the present study suggest that either transport or presentation of antigen by spleen APCs is less effective than that by LCs, as discussed previously [15]. In the case of pPep-ER, the ER signal sequence and the ER retention signal sequence ensures that the translated peptide is targeted and retained in the ER compartment, where the MHC class I molecules are located. Thus, pPep-ER can only undergo direct presentation and only the peptide translated from pPep-ER taken up by APCs has an opportunity to be presented to T cells. The intradermal immunization of pPep-ER induced a strong antigen-specific CTL activity and anti-tumor response, although the intrasplenic immunization of pPep-ER scarcely induced any antigen-specific immune response. Therefore, we consider that the number of DCs that contribute to the direct-presentation of antigens is much fewer in the spleen than that of LCs in the epidermis. Alternatively, the different location of DCs in the organs may also be another factor responsible for the difference in the immune responses after the direct injection of pPep-ER into the spleen and the skin. Tupin *et al.* [30] have found that intrasplenic CMV promoter-bearing plasmid resulted mostly in the transfection of T cells because of a poor expression of CMV promoter in the other spleen cell types, or because of the fact that the electric settings are specifically elective for T cells. This finding may explain the reason why pPep-ER induced hardly any antigen-specific immune response after intrasplenic injection in the present study. Further studies on the distribution of plasmid DNA after intrasplenic injection, as well as the location of splenic APCs, are required to understand the poor immune response after intrasplenic injection of pPep-ER. In addition, Maioli *et al.* [14] suggested that, in localized infections, regional lymph nodes may efficiently replace the immunological role of the spleen in the cellular immune response. In the anti-tumor immune response, the regional lymph nodes may play a key role rather than the spleen, and this remains to be examined further. Moreover, other factors, such as the induction

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and expansion of OVA-specific regulatory T cells, should be investigated in future studies.

It is no doubt that the final goal of DNA vaccination in tumor immunotherapy is to treat patients with tumors, so that the therapeutic effects of DNA vaccine are much more important than its prophylactic effects. In a previous study [20], we demonstrated that the intradermal injections of pPep-ER to EG7-OVA-bearing mice were effective in significantly increasing the survival of mice, which is a promising example of DNA vaccination for tumor immunotherapy. Because the intrasplenic immunization was found to be less effective in inducing antigen-specific immune responses, the therapeutic effects of intrasplenic immunization were not examined in tumor-bearing mice.

In conclusion, we have demonstrated that intrasplenic immunization is not an optimal substitute for intradermal immunization against tumor challenge, which is in contrast to a previous study [31] in which intrasplenic immunization was considered to be an attractive strategy for improving antigen immunogenicity. We also identified that the properties of transgene expression and the type and location of APCs in the spleen and skin are two important factors determining the immune response by DNA vaccines.

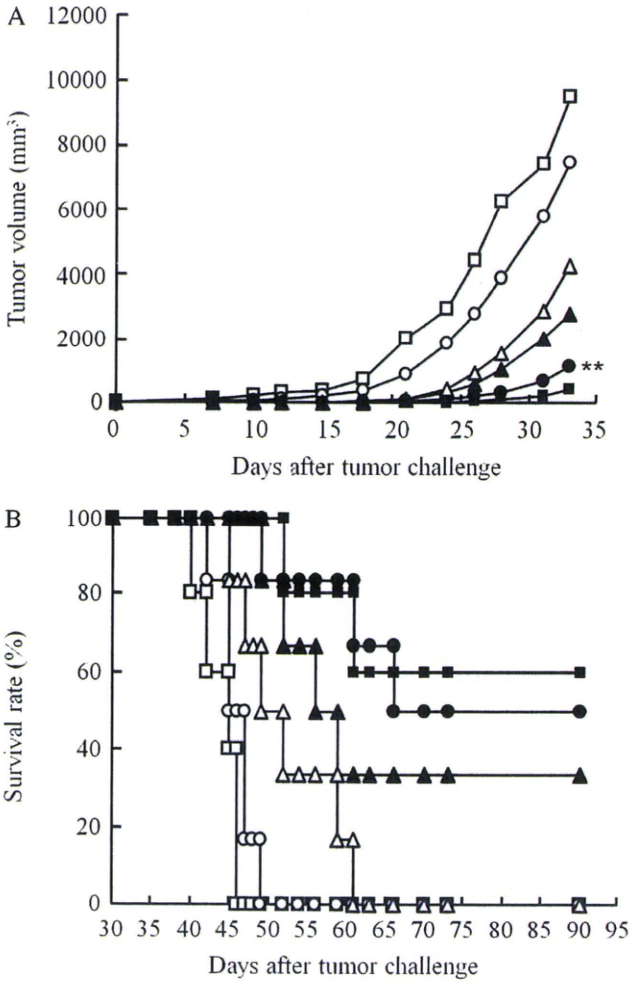

Figure 5. Tumor growth in and survival of EG7-OVA-bearing mice after immunization with pCMV-OVA or pPep-ER via the intradermal (id.) or intrasplenic (is.) route. Mice were immunized twice with 100 μg of plasmid DNA followed by electroporation. Eight days after the last immunization, 1 x 10^6 cells of EG7-OVA were inoculated intradermally ($n = 5$ or 6). The tumor size was measured periodically (A) and the survival of mice was monitored until 90 days after the start of treatment (B). □, No treatment; ■, OVA in CFA; open symbols, intrasplenic route; solid symbols, intradermal route: △ ▲, pCMV-OVA; ○ ●, pPep-ER. **$p < 0.01$ compared to pPep-ER (is.)

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Development of a novel Hsp70-based DNA vaccine as a multifunctional antigen delivery system

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ABSTRACT

DNA vaccination is a simple and effective method to induce immune responses against a variety of tumors as well as infectious diseases. Vaccination with major histocompatibility complex (MHC) class I tumor peptide has been carried out to induce an antigen-specific and tumor-reactive cytotoxic T lymphocytes (CTLs) response *in vivo*. In this study, we describe a novel DNA vaccine based on heat shock protein 70 (Hsp70), which can chaperon antigenic peptides and initiate innate and adaptive immune responses, to induce a more effective immune response. Ovalbumin (OVA) MHC class I epitope peptide ($OVA_{257-264}$: SIINFEKL) was selected as a model antigen and polyhistidine was used to facilitate the cytosolic delivery of the antigen-Hsp70 after endocytic uptake. A novel plasmid DNA vector encoding polyhistidine, Hsp70 and $OVA_{257-264}$ (pHis-Hsp70-pep) was designed. When mice were immunized with pHis-Hsp70-pep by intradermal injection in combination with electroporation, strong antigen-specific CTL responses were generated. pHis-Hsp70-pep also showed a significant protective effect against tumor challenge with an OVA-expression EL4 tumor line. These results indicate that the Hsp70-based DNA vaccine is useful as a multifunctional antigen delivery system to induce the antigen-specific immune response.

1. Introduction

Heat shock protein 70 (Hsp70), is a member of the heat shock protein family, which is induced under stress conditions. Hsp70 also plays important roles in immune responses against tumor, bacterial and viral targets [1–5]. It can form complexes with various antigenic peptides through its polypeptide binding domain [6], to stimulate adaptive and innate immune responses [7]. Recent studies have indicated that the targeting of antigen to APCs through Hsp70 is a useful strategy to induce efficient cross-presentation, and it has been investigated for cancer immunotherapy [8].

The factors favoring the development of immunization strategies with plasmid DNA include the relative easy and inexpensive nature of preparation compared with peptide and protein vaccines, as well as its stability and no-infectious nature compared with viral vaccines [9–11]. In addition, DNA vaccines can also be designed or modified so that antigens encoded by plasmid DNA can be controlled to target specific cellular sites by using additional peptides or proteins, subsequently initiating the desired intensity and type of immune responses, including both humoral and cellular immune responses [12].

Polyhistidine, the imidazole-containing polymers, followed by Hsp70-antigen peptide is capable of escaping from the endosome/lysosome for minimizing degradation under acidic conditions, the so-called 'proton sponge' effect [13–16]. Enhanced cytosolic delivery of Hsp70-associated antigen would increase the entry of antigens into the class I presentation pathway, resulting in improved induction of specific $CD8^+$ T cells. In our previous study, we have designed a novel protein vaccine, Hsp70-based antigen delivery system, Hsp70-associated antigen fused to polyhistidine. The fusion protein of His-Hsp70-pep, which was expressed in *E. coli* and purified, exhibited a significantly improved efficacy of MHC class I-restricted presentation of antigen *in vitro*, and also generated strong antigen-specific CTL responses and antitumor activity in mice [17]. However, based on a number of advantages over protein vaccines, DNA vaccination may be an attractive alternative for delivering His-Hsp70-pep vaccines, which can induce stronger immune responses because of the presence of immunostimulatory unmethylated CpG motifs in the structure of plasmid DNA. There are reliable evidences showing that antigens can be taken up and presented by antigen presenting cells (APCs) through two distinct pathways [18–20]: the direct presentation pathway and the cross-presentation pathway [21–23]. In cross-presentation, professional APCs acquire antigens from other cell types such as keratinocytes or myocytes following intradermal [24] or intramuscular DNA vaccination [25]. It is likely that the cross-presentation pathway is the major mechanism for T cell priming by DNA vaccine [26] because of the relatively low quantity of APCs, especially dendritic cells (DCs), present at the sites for administration [27]. Accordingly, induction of cross-presentation is expected to be more effective for DNA vaccination. Based on this consideration, we have

Abbreviations: Hsp70, heat shock protein 70; OVA, ovalbumin; His, histidine; CTL, cytotoxic T lymphocytes; PAGE, polyacrylamide gel electrophoresis.

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designed a novel plasmid DNA vector expressing the fusion protein, His-Hsp70-pep, as a secretory protein from the cells transfected in order to increase the probability of cross-presentation. Then, we investigated whether the vaccination with this novel plasmid DNA vector could enhance the antigen-specific CTL responses and antitumor activity in mice.

2. Materials and methods

2.1. Cells and animals

DC2.4 cells, a cell line of murine dendritic cells (haplotype H-2^b) [28], 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 [29], were a generous gift from Dr. C. V. Harding (Case Western Reserve University, Cleveland, OH). EL4 cells, C57BL/6 T lymphoma, and EG7 cells, an OVA transfect clone of EL4 [30], 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, 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.

Five-week-old female C57BL/6 mice were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions and all animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences, Kyoto University.

2.2. Plasmid DNA construction

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 pcDNA3.1 vector using pGEM®-T vector (Promega, USA), subcloning vector system.

An MHC class I 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. TEWTS sequence was added to the epitope because it has been reported to facilitate the liberation of SIINFEKL in proteasomes [31]. The oligonucleotide coding the 25 histidines (polyhistidine) was also synthesized and incorporated into the N-terminal of Hsp70. The original Hsp70 is expressed as a cytosolic protein in mammalian cells. In order to render Hsp70 to be expressed as a secretory protein following transfection, an endoplasmic reticulum (ER) signal sequence amplified from pCMV/mic/ER Shooter vector (Invitrogen, USA) was inserted into the upstream region of each fusion gene. The sequences of these recombinant DNAs were confirmed by Kyoto Science Corporation Technical Service (Kyoto, Japan). The schematic structures of the constructs are shown in Fig. 1A.

2.3. Expression of the fusion proteins in COS-7 cells

Plasmid DNA/LipofectAMINE™ 2000 complexes (DNA-Lipoplex) were prepared in polypropylene centrifuged tubes according to the protocol provided by the manufacturer (Molecular Probes, Invitrogen). COS-7 cells were seeded on 6-well culture plates and incubated

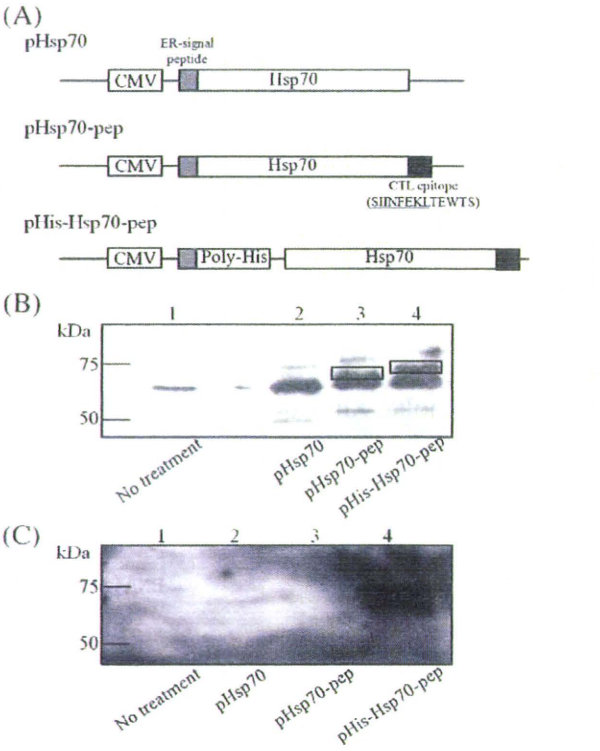

Fig. 1. (A) Schematic representation of Hsp70-CTL epitope fusion constructs. A mini-gene encoding a CTL epitope ($OVA_{257-264}$) was incorporated into the C-terminus of the mouse Hsp70.1 gene; polyhistidine (His_{25}) was further fused to the N-terminus of the Hsp70 gene. These fusion genes were inserted into the pcDNA3.1(+) vector. (B) and (C) Western blot analysis of COS-7 lysates hybridized with monoclonal anti-Hsp70 antibody (B) and anti-histidine antibody (C). The band surrounded by the square indicates fusion protein encoded by pHsp70-pep or pHis-Hsp70-pep, respectively. Lane 1, No treatment; Lane 2, pHsp70; Lane 3, pHsp70-pep; Lane 4, pHis-Hsp70-pep.

with each plasmid DNA/cationic liposome (LipofectAMINE™ 2000, Invitrogen) complexes prepared in Opti-MEM (Invitrogen) for 4 h in 5% CO_2 at 37 °C. Then, the medium was replaced with culture medium. Cells were collected in PBS (300 μl/well) at 24 h after transfection, and lysed by sonication. Then, sonicates were loaded on 8% SDS-PAGE gel for separation. Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon PTM, Millipore) and blocking was carried out overnight (o/n) with 9% skimmed milk, 0.1% Tween-20 in PBS buffer. Then, the membrane was incubated with monoclonal mouse anti-Hsp70 antibody (Santa Cruz Biotechnology, Inc., USA) or anti-histidine antibody followed by anti-mouse IgG horseradish peroxidase-conjugated antibody. The immunoblots were developed with an enhanced chemiluminescence reagent kit and exposed to a film (both from Amersham Biosciences, USA).

2.4. Antigen presentation assay

The efficacy of MHC class I presentation activity of the plasmid DNA 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 interleukin-2 (IL-2). This simple method has been widely used for the evaluation of OVA vaccination systems [32]. Plasmid DNA/LipofectAMINE™ 2000 complexes prepared in Opti-MEM were added to DC2.4 cells (1×10^5/well) which were cultured on 96-well plates overnight. After coincubation for 2 h, medium was replaced with culture medium. Then, 6 h later, CD8OVA1.3 T hybridoma cells (1×10^5/well) were added and coincubated with DC2.4 cells in 5% CO_2 at 37 °C and, 20 h later, the cell culture supernatants were collected and freeze-thawed. The response of CD8OVA1.3 T cells was determined by measuring IL-2 levels in the

supernatants with an enzyme-linked immunosorbent assay (ELISA; OptiEIATM Set Mouse IL-2 and TMB Substrate Reagent Set, BD Biosciences, San Diego, CA).

2.5. CTL assay

C57BL/6 mice were immunized three times at weekly intervals intradermally (i.d.) in the dorsal skin with a total of 50 μl plasmid DNA (2 mg/ml) in normal saline. Electroporation was applied to the injection site (1000 V/cm, 5 ms, 4 Hz, 12 pluses) 30 s after injection. Seven days after the last immunization, splenocytes were isolated from the immunized mice followed by restimulation *in vitro* for 5 days with mitomycin C-treated EG7. Target cells (EG7 or EL4; EL4 was used as a target control) were 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 culture medium for 4 h at 37 °C. Spontaneous release of ^{51}Cr without effector cells and maximal release in the presence of 1% TritonX-100 were also evaluated. Cells were centrifuged (420 *g*) for 5 min, and 100 μl of each supernatant was collected for radioactivity measurements. The cytosolic activity of CTLs was calculated as [33]:

$$\%\,\text{killing} = (\text{observed release} - \text{spontaneous release}) \div (\text{maximal release} - \text{spontaneous release}) \times 100.$$

2.6. Tumor challenge experiments

C57BL/6 mice were intradermally immunized three times at weekly intervals in the back with each plasmid DNA, followed by electroporation. Eight days after the last immunization, 1×10^6 cells per mouse of EG7 were administered intradermally into the back as a challenge. The mice were monitored for tumor growth every three or four days. The tumor size was measured using the longest and the shortest diameters. The tumor volume was calculated from the equation: $(\text{longest}\times\text{shortest})^{3/2}\times\pi/6$. The survival time of the tumor-challenged mice was also recorded.

3. Results

3.1. Characterization of constructed plasmid DNA

Three types of Hsp70 recombinant plasmid DNA: pHsp70, pHsp70-pep and pHis-Hsp70-pep were constructed. The ER signal sequence, which directs proteins synthesized in the cytosol to the endoplasmic reticulum, inserted at the N-terminal of each fusion gene made them become secreted molecules. Each plasmid DNA was transfected into COS-7 cells to characterize the fusion protein. Western blot analyses of COS-7 cell lysates with the anti-Hsp70 antibody (Fig. 1B) and anti-histidine antibody (Fig. 1C) confirmed each plasmid DNA expressed the designed fusion protein. As shown in figure 1B, the band of approximately 70 kDa (predicted molecular weight: 70 kDa) in lane 1 represented endogenous Hsp70 in cells. The band of 70 kDa in lane 2 represented the mixture of endogenous Hsp70 and Hsp70 translated from pHsp70. The bands in lane 3 and 4 just above 70 kDa represented the fusion proteins of Hsp70-peptide and polyhistidine-Hsp70-peptide, respectively, translated from corresponding plasmid DNAs in COS-7 cells.

3.2. MHC class I presentation

In order to assess the efficacy of MHC class I presentation activity of the fusion proteins encoded by constructed plasmid DNAs, we performed an *in vitro* antigen presentation assay using DC2.4 cells and T hybridoma cells against SIINFEKL-K^b based on IL-2 production (Fig. 2). High production of IL-2 by CD8OVA1.3 T hybridoma cells were observed because of the effective presentation of the MHC class I epitope encoded by pHis-Hsp70-pep and pHsp70-pep. Furthermore, pHis-Hsp70-pep was more effective than pHsp70-pep in the production of IL-2, suggesting that the MHC class I presentation activity was increased by fusion of polyhistidine, probably because of facilitated cytosolic delivery of the fusion protein. In our previous antigen presentation assay *in vitro*, similar results were obtained for these fusion proteins [17].

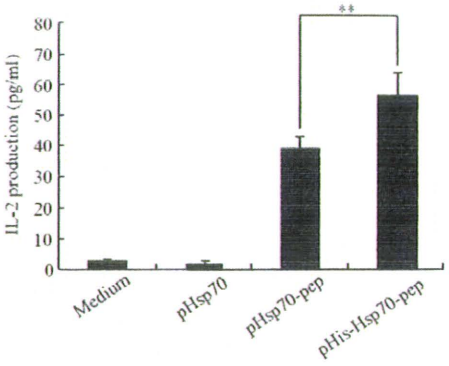

Fig. 2. MHC class I-restricted presentation assay *in vitro*. DC2.4 cells were transfected with each plasmid DNA. At 8 h after transfection, CD8OVA1.3 T cell hybridoma was added to DC2.4 cells. After 24 h, IL-2 production from CD8OVA1.3 cells were measured by ELISA. Results are expressed as mean ± S.D. ($n=3$). Statistically significant differences were assessed using Student's *t*-test against pHsp70-pep (**$p<0.01$).

3.3. Induction of tumor-specific CTLs

To evaluate whether a high level of antigen-specific CTLs was elicited by immunization with pHis-Hsp70-pep, we examined the OVA-specific CTL response using EG7 cells expressing OVA. The splenocytes from the mice immunized with pHis-Hsp70-pep by intradermal injection followed by electroporation showed a higher level of OVA-specific CTL response compared with that from the mice treated with pHsp70-pep (Fig. 3A). No significant CTL activity was seen in the EL4 cells, indicating that the CTL activity was an OVA-specific response (Fig. 3B).

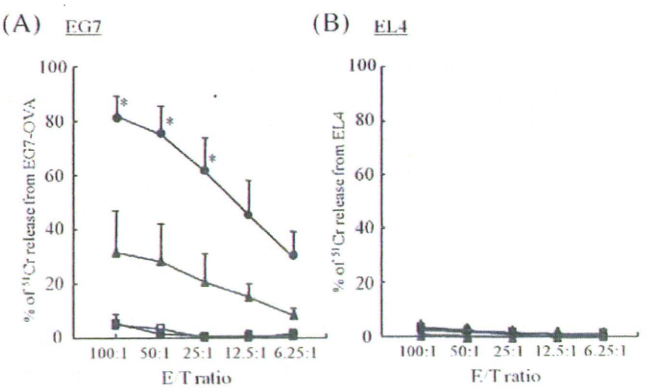

Fig. 3. Generation of OVA-specific CTLs by immunization with pHsp70, pHsp70-pep and pHis-Hsp70-pep. Mice were immunized three times with 100 μg plasmid DNA. 7 days after the last immunization, spleen cells were isolated from the immunized mice followed by restimulation with EG7 cells *in vitro* for 5 days, then standard ^{51}Cr release assay was performed ($n=3$). EG7 (A) and EL4 (B) were used as the target cells. (□) No treatment; (■) pHsp70; (▲) pHsp70-pep; (●) pHis-Hsp70-pep. Results are expressed as mean ± S.D. ($n=4$). Statistically significant differences were assessed using Student's *t*-test against pHsp70-pep (*$p<0.05$).

3.4. In vivo growth inhibitory effect of EG7 tumor cells

We finally examined the effect of immunization with pHis-Hsp70-pep to protect mice from an EG7 tumor challenge. Tumor growth was significantly inhibited in the pHis-Hsp70-pep-treated group, followed by group treated with the pHsp70-pep (Fig. 4A). The survival of mice challenged intradermally with EG7 tumor cells was also prolonged in the mice immunized with pHis-Hsp70-pep. Untreated mice or those treated with pcDNA3.1 (Mock) or pHsp70 all died by day 57, whereas about 30% and more than 80% of the mice immunized with pHsp70-pep or pHis-Hsp70-pep, respectively, survived the first 100 days (Fig. 4B).

4. Discussion

Based on our previous study on protein vaccine [17], the Hsp70-based DNA vaccine, pHis-Hsp70-pep, was designed as a multifunctional antigen delivery system to induce the antigen-specific immune response in our present study. The multiple functions include: the antigen-Hsp70 complex can be taken up by APCs via Hsp receptors, such as CD91 and LOX-1; Hsp70 can also activate the innate immunity through CD40 and Toll-like receptor-2 (TLR-2) and TLR-4 to induce cytokine secretion [7,8]; polyhistidine can enhance the cytosolic delivery to improve MHC class I antigen presentation [13,17]. Moreover, in addition to the functions described above, which also are possessed by the protein vaccine, the bacterial immunostimulatory unmethylated CpG motifs in the structure of plasmid DNA are potent activators of innate immunity and induce cellular response mediated by TLR-9 [34]. Furthermore, plasmid DNA possesses of relative easy and inexpensive nature for preparation, quality stability and long-time storage period, compared with protein vaccines. Therefore, it would be an attractive alternative for delivering His-Hsp70-pep vaccines.

DNA vaccine has shown promise in eliciting an effective CTL response to antigens since Wolff and colleagues first observed that an intramuscular injection of naked DNA led to the expression of encoded genes in myofiber cells [35]. Direct transfection of DCs with DNA is one way to promote antigen presentation by APCs, but only a few DCs are transfected after DNA injection, because of the relatively low amount of APCs, especially dendritic cells (DCs), present at the sites for administration. Another way is to enhance the cross-presentation, in which APCs exogenously take up antigenic protein which is expressed in DNA-transfected non-APCs. It seems that the latter is more effective from the perspective of gene expression. In the present study, we added the endoplasmic reticulum (ER) signal sequence at the upstream region of each fusion gene inserted into pcDNA3.1 vector, which made Hsp70 a secretory protein, in order to increase the probability of cross-presentation. In fact, the vector is different from the one we used for protein vaccine in our previous study in terms of backbone, promoter and form of the expressing protein [17]; pGEX-6P-2. GST-tag expression plasmid was used to express Hsp70 fusion proteins in *E. coli* DH5α cells as a recombinant protein.

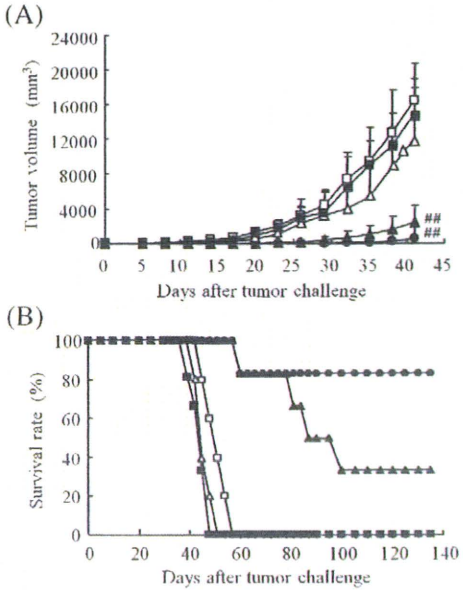

Fig. 4. *In vivo* growth inhibitory effect (A) and prolongation of survival time (B) obtained by immunization with plasmid DNA. Mice were immunized intradermally twice with 100 μg plasmid DNA and 7 days after the last immunization, 1×10^6 cells of EG7 were injected intradermally ($n = 5$ or 6). (□) No treatment; (△) Mock (pcDNA3.1); (■) pHsp70; (▲) pHsp70-pep; (●) pHis-Hsp70-pep. Results are expressed as mean ± S.D. ($n = 5$ or 6). Statistically significant differences were assessed using Student's *t*-test against Mock (## $p < 0.001$).

Because antigen internalized into endosome is degraded by various protease or likely loaded on MHC class II molecules [36], enhancing release of antigen from endosomes into cytoplasm is required to increase antigen-specific CTLs. In our previous study, we successfully used polyhistidine to enable antigen to escape from endosomes into cytoplasm in order to increase the potency of the vaccine based on Hsp70 [17]. Examination of the intracellular location of fusion protein by confocal microscopy showed that His-Hsp70-pep fusion protein was widely distributed in DC2.4 cells, suggesting that the fusion protein was transferred into the cytoplasm. It has been reported that exogenously-administered PEI/DNA particles undergo normal endocytotic trafficking and complexes are distributed to the cytoplasm and enter nuclei in the form of large, discrete structures and imidazole-containing polymers also exhibit these functions [13,37,38]. Therefore, it is considered that His-Hsp70-pep fusion protein follows the same course and escapes from endosomes by the imidazole groups of histidine.

When DC2.4 cells were transfected with plasmid DNA encoding fusion protein to examine the antigen presentation efficiency, pHis-Hsp70-pep was more effective than pHsp70-pep in the production of IL-2 (Fig. 2), suggesting that the MHC class I presentation activity was increased by polyhistidine fusion. We speculate that the presentation procedure is as follows: the proteins translated from the plasmid DNA are directed into the endoplasmic reticulum by the ER signal peptide at the N-terminal of the plasmid DNA, and are then transported into the extracellular space through the Golgi apparatus. Then, the fusion proteins can be taken up again by the DC2.4 cells even including those secreting the fusion protein, where polyhistidine can play its designed role.

Intradermal and intramuscular injections have been often used for DNA vaccination [39]. In this study, we selected intradermal injection to immunize mice for the presence of abundant APCs, such as Langerhans cells and dendritic cells, in the epidermis [40]. Activated Langerhans cells and dendritic cells can traffic to draining lymph nodes where presentation of the encoded antigens to T cells and B cells occur, thereby initiating a variety of immune responses. We also applied electroporation after intradermal injection to increase the level of transgene expression.

In our previous study, we demonstrated that the protein vaccine of His-Hsp70-pep can generate strong antigen-specific CTL responses and antitumor activity in mice [17]. Here, we demonstrate that the DNA vaccine using pHis-Hsp70-pep can also strongly enhance the antigen-specific CTL responses and antitumor activity in mice even compared with its protein vaccines. These results indicate that the control of intracellular trafficking of Hsp70-antigen using polyhistidine is also a useful strategy to enhance the antigen-specific immune response induced by DNA vaccine. The inhibition effect of tumor growth by DNA vaccine in this study was stronger than that by the protein vaccine, although the survival rate was similar between them

(Figs. 3 and 4), suggesting the advantage of the DNA vaccine over the protein vaccine. In addition to the vector encoding the fusion protein having 25 polyhistidine repeats, we also developed the DNA vector encoding the fusion protein with more polyhistidine repeats (50 repeats). In the case of protein vaccine, the fusion protein with 50 repeats was much less effective probably due to inactivation during protein purification [17]. On the other hand, the DNA vaccine expressing the fusion protein with 50 repeats showed antigen presentation activity and antigen-specific CTL activities comparable with the vector the fusion protein with 25 repeats, pHis-Hsp70-pep (data not shown). These results also suggest DNA vaccine encoding the fusion protein is more advantageous than the same fusion protein vaccine.

In summary, this study demonstrates for the first time that the plasmid DNA vector encoding antigen fused to polyhistidine and Hsp70 is a potent vaccine compared with the vaccine encoding antigen only fused to Hsp70. Thus, the usefulness of the novel vector as a multifunctional antigen delivery system has been shown. An advantage of DNA vaccine with plasmid DNA, through altering the antigen sequence inserted into the multiple clone site of a plasmid DNA, is that the vector constructed can also be used to treat other tumors and infectious diseases.

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Analysis of the Complete Open Reading Frame of Hepatitis C Virus in Genotype 2a Infection Reveals Critical Sites Influencing the Response to Peginterferon and Ribavirin Therapy.

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Short title: PEG-IFN/RBV response in HCV-2a

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Abbreviations

EVR: Early Virological response

IFN: Interferon

IRF-1: Interferon Regulatory Factor 1

IRRDR: Interferon Ribavirin Resistance Determinant Region

ISDR: Interferon Sensitivity Determinant Region

ORF: Open Reading Frame

PEG-IFN: Pegylated-Interferon

PePHD: PKR -eIF2 Phosphorylation Homology Domain

PKR-BD: Double-Stranded RNA-activated Protein Kinase Binding Domain

RBV: Ribavirin

RVR: Rapid Virological Response

SVR: Sustained Virological Response

ABSTRACT

Purpose: A proportion of patients infected with genotype 2a hepatitis C virus (HCV) cannot achieve a sustained virological response (SVR) to pegylated-interferon plus ribavirin therapy (PEG-IFN/RBV) but the reason remains unclear. The present study aimed to clarify the possible correlation between viral sequence variations and final outcome.

Methods: The pretreatment complete open reading frame (ORF) sequences of genotype 2a HCV were determined by direct sequencing for two independent groups of patients (43 patients as test; group 1 and 35 as validation; group 2), and the correlation with the final outcome was explored.

Results: Patients with SVR (n=58) and with non-SVR (n=20) differed significantly in pretreatment HCV RNA level ($p=0.002$), fibrosis score ($p=0.047$), and cumulative ribavirin dosage ($p=0.003$). By comparison of all amino acid positions in the complete HCV ORFs, threonine at amino acid (aa) 110 in the core region was remarkably frequent in SVR ($p=0.01$ for group 1, $p=0.004$ for group 2, and $p=5E-05$ for combined). A sliding window analysis revealed that the total numbers of amino acid variations within the NS5A aa 2258 to 2306 region were significantly high in SVR compared to non-SVR patients ($p=0.01$ for group 1, $p=0.006$ for group 2, and $p=0.0006$ for combined). Multivariate analyses revealed that core aa 110 ($p=0.02$), NS5A aa 2258-2306 ($p=0.03$), and cumulative ribavirin dosage ($p=0.02$) were identified as independent variables associated with the final outcome.

Conclusions: The outcome of PEG-IFN/RBV therapy is significantly influenced by variation in the core and NS5A regions in genotype 2a HCV infection.

INTRODUCTION

Worldwide, 180 million of people are estimated to be infected with hepatitis C virus (HCV), and HCV is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (1). In HCV-infected patients with chronic hepatitis, treatment with interferon (IFN) can result in viral clearance and biochemical and histological improvements(2). The response to the therapy varies according to HCV genotype and pretreatment HCV RNA level (3-4).

The currently recommended treatment for patients infected genotype 2a HCV with high viral load is pegylated-interferon (PEG-IFN) plus ribavirin (RBV) for 24 weeks (1). Approximately 80% of patients infected with genotype 2a HCV can achieve a sustained virological response (SVR) with this regimen (5-6), although much lower percentages of patients infected with other genotypes can achieve SVR, especially with genotype 1(1). Because of its high response rate, shorter treatment duration was suggested by some studies, although an agreement has not been reached yet (7-8). On the other hand, about 20% of patients infected with this genotype cannot achieve SVR and it remains elusive which patients show poor responses.

Previous studies have reported that amino acid variations in the NS5A-ISDR (9), NS5A-IRRDR(10), NS5B(11), PKR-eIF2 phosphorylation homology domain (PePHD) of E2(12), and core (13-14) correlate with clinical outcome of IFN-based therapy, including PEG-IFN/RBV therapy in patients infected with genotype 1b HCV. Recent full HCV open reading frame analysis for genotype 1 also have reported that core, NS3, and NS5A were associated with early viral response and the outcome in PEG-IFN/RBV therapy (15-16). However, in genotype 2a infection, only a few studies have investigated the association between HCV sequence variation and treatment

response (17-19), and the role of viral factors has not been established yet, especially in the era of PEG-IFN/RBV therapy. Moreover, these previous studies investigated only several isolated HCV genomic regions, and comprehensive analysis of the full HCV open reading frame (ORF) has not been undertaken so far.

In the present study, to assess comprehensively the influence of viral variations on response to the PEG-IFN/RBV therapy in genotype 2a HCV infection, we determined the complete pretreatment HCV ORFs from Japanese patients and investigated viral amino acid variation and their correlation with the response to the combination therapy of PEG-IFN plus RBV.

PATIENTS & METHODS

Study Population

A total of 103 adult Japanese patients infected with genotype 2a HCV, who received the combination therapy with PEG-IFN (PEGINTRON®, Schering-Plough, Tokyo, Japan) plus RBV (REBETOL®, Schering-Plough) between 2005 and 2008 at University of Yamanashi, Tokyo Medical and Dental University, and related institutions were first included in the study. They all fulfilled following criteria: (1) negative for hepatitis B surface antigen, (2) high viral load (≥100 KIU/ml), (3) absence of hepatocellular carcinoma, (4) no other form of hepatitis, such as primary biliary cirrhosis, autoimmune liver disease, or alcoholic liver disease, (5) free of co-infection with human immunodeficiency virus. Informed consent was obtained from each patient. The study was approved by the ethics committees of all the participating universities and hospitals. The therapy was performed according to the standard treatment protocol of PEG-IFN/RBV therapy for Japanese patients established by a hepatitis study group of the Ministry of Health, Labour, and Welfare, Japan (PEG-IFNα-2b 1.5 μg/kg body weight, once weekly subcutaneously, and RBV 600-800 mg daily per os for 24 weeks). To clearly disclose the non-SVR viral characteristics, we have considered those patients who achieved total drug administration of 60% or more for both PEG-IFN and RBV, with the completion of the standard treatment duration. Moreover, although we excluded the patients with extended therapy to make the studied population uniform, we have included non-SVR patients with extended therapy to clarify the specific characteristics of non-SVR patients, a minor population group. As a result, 25 patients were excluded for the following reasons: 4 patients received insufficient dose, 8 patients were discontinued from the therapy within 12 weeks, and 13 SVR patients received

extended therapy. Finally, 78 patients were considered as eligible for the study. During the combination therapy, blood samples were obtained at least once every month before, during and after treatment and were analyzed for blood count, ALT and HCV RNA levels. Liver biopsy specimens were obtained from most of the patients.

The 78 patients belonging to the different institutions were separately analyzed: 43 patients registered in Y-PERS (Yamanashi Pegintron Ribavirin Study Group) were included in group 1 (test group), and the 35 patients from Tokyo Medical and Dental University and related institutions (Ochanomizu Liver Conference Group) were included in group 2 (validation group). We divided the patients into these two groups in order to exclude the false positives (type I errors) which might arise in successive HCV-ORF study. Since genotype-2a HCV contains as many as 3033 amino acids, it was possible that incorrect amino acids to be judged as significant in full HCV-ORF comparison study as a result of type I errors. Therefore, to guard against false positives, HCV-ORF comparison study was undertaken in group 1, group 2, and combined group.

Complete HCV-ORF Sequence Determination by Direct Sequencing from Pretreatment Sera

HCV RNA was extracted from pretreatment serum samples by the AGPC method using Isogen (Wako, Osaka, Japan) according to the manufacturer's protocol. Complementary DNA was synthesized with Superscript II (Invitrogen, Tokyo, Japan) using random primers (Invitrogen) and then amplified by two-step nested PCR using the primers newly designed for this study. All samples were initially denatured at 95°C for 7 min., followed by 40 cycles with denaturation at 95°C for 15 seconds, annealing at 55°C

for 15 seconds, and extension at 72°C for 45 seconds with BD Advantage™ 2 PCR Enzyme System (BD Biosciences Clontech, CA, USA).

PCR amplicons were sequenced directly by Big Dye Terminator Version 3.1 (ABI, Tokyo, Japan) with universal M13 forward / M13 reverse primers using an ABI prism 3130 sequencer (ABI). Generated sequence files were assembled using Vector NTI software (Invitrogen) and base-calling errors were corrected following inspection of the chromatogram.

Sliding Window Analysis

A sliding window analysis was introduced to search through HCV amino acid "regions", rather than single amino acid positions, related to the final outcome of PEG-IFN/RBV therapy. Briefly, the total number of amino acid substitutions compared to the consensus sequence within a given amino acid length were counted in each amino acid position in each HCV sequence. Then the relation of substitution numbers and the final outcome was compared statistically between the SVR and non-SVR groups by Mann-Whitney's U test for each amino acid position. In this study, we changed the window length from 1 to 50 to search for those HCV regions. To visualize the result, significantly lower p-values were colored in red and non-significant p-values were colored in green to generate a "heat map" appearance using Microsoft Excel software. In the present study, p-value of 1/1000 or lower was colored in the maximum red.

Statistical Analysis

Statistical differences in the parameters, including all available patients'

demographic, biochemical, hematological, and virological data such as sequence variation factors, was determined between the various groups by Student t test or Mann-Whitney's U test for numerical variables and Fisher's exact probability test for categorical variables. To evaluate the optimal threshold of variations for SVR prediction, the receiver operating characteristic curve was constructed. Variables that achieved statistical significance ($p<0.05$) in univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. We also calculated the odds ratios and 95% confidence intervals. All p values of <0.05 by the two-tailed test were considered significant.

RESULTS

Characteristics of the patients studied

Of the patients analyzed, the SVR rate was 78.3% (58/74) with the standard therapy (four non-SVR patients received an extended therapy). The baseline characteristics of the patients (group 1, group2, and combined) classified according to SVR achievement are shown in Table 1. Fibrosis score (*p=0.047*) and HCV RNA levels (*p=0.002*) were significantly higher in non-SVR patients, but the cumulative ribavirin dose ≥80% (*p=0.003*) and rapid virological response (RVR) rate (*p=0.011*) were significantly higher in SVR patients. In addition, patients with non-SVR had a tendency to be older (*p=0.058*). Achievement of RVR reached 61.5% when all patients were included, and this rate was extremely high compared to achievement of RVR in patients with genotype 1b infection (~10%) observed in Yamanashi University Hospital (data not shown). The early virological response (EVR) rate was equally high in the SVR (100%) and non-SVR (89%) groups, showing that relapse to be the characteristic feature of the non-SVR patients with genotype 2a HCV. Actually, 18 patients in non-SVR were relapser, while two patients were null responder.

Comparison of amino acid variations between the SVR and non-SVR in the complete HCV polyprotein and each HCV protein

To determine whether the sequence variations differed between the SVR and non-SVR groups, we first compared amino acid variations that were unique, relative to a population consensus, to either the SVR or non-SVR patients for the complete HCV polyprotein and each HCV protein. The number of amino acid variations in the

sequences from the SVR patients was significantly higher than in those from the non-SVR patients, when the entire HCV polyprotein was analyzed (Fig.1, left). These differences were especially significant in E1 and NS3 (Fig.1, right). This result demonstrated that HCV sequences from patients with SVR comprised a heterogeneous population, while HCV sequences from patients with non-SVR comprised a rather homogeneous population, indicating the existence of unique non-responsive HCV sequences.

Comparison of HCV sequence variation between the SVR and non-SVR patients at each amino acid position

Next, each amino acid position in the HCV ORF was compared to detect any differences between the SVR and non-SVR patients after determination of the consensus sequence from all 78 patients. In Fig.2a, the final differences of the two independent studies combined are shown as dots demonstrating – logP values. As shown in the figure, amino acid usage at amino acid 110 in the core region differed strikingly between the two groups (*p=5E-05*). The site was detected in group 1 (*p=0.01*) and was validated in group 2 *(p=0.004)* (Table 2a), and the final p-value became remarkably high, making the p-value at this site most significantly low. Variations of aa 773 in p7, aa 2099 in the NS5A, and aa 3013 in NS5B were also shown to differ significantly between the SVR and the non-SVR patients when the two studies were combined; however, they were not confirmed by one of the studies (Table 2a). Fig.2b shows the aligned sequences of amino acids 1-120 of the core region. Substitutions at aa 110 from non-T (N/S) to T were significantly more frequent in SVR (32/58, 55.2%) than in non-SVR (1/20, 3.6%, *p=5E-05*). Amino acid 4, the site reported recently to vary