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A Heat Shock Protein 70-Based Vaccine with Enhanced Immunogenicity for Clinical Use

Jianlin Gong,* Yunfei Zhang,* John Durfee,† Desheng Weng,* Chunlei Liu,* Shigeo Koido,‡ Baizheng Song,* Vasso Apostolopoulos,§ and Stuart K. Calderwood¶

In previous studies, we have shown that heat shock protein 70-peptide complexes (HSP70.PCs) derived from the fusion of dendritic cells (DCs) to tumor cells (HSP70.PC-F) possess superior properties compared with HSP70.PCs from tumor cells. HSP70.PC-F are more effective in stimulation of DC maturation and induction of CTL that are able to provide protection of mice against challenge with tumor cells. To develop an improved formulation of HSP70.PC-based tumor vaccine for patient use, we extracted HSP70.PC-F from DCs fused to patient-derived ovarian cancer cells or established human breast cancer cells and examined their properties as tumor vaccines. HSP70.PC-F induced T cells that expressed higher levels of IFN-γ and exhibited increased levels of killing of tumor cells, compared with those induced by HSP70.PC derived from tumor cells. Enhanced immunogenicity of HSP70.PC-F was associated with improved composition of the vaccine, including increased content of tumor Ags and their processed intermediates, and the detection of other heat shock proteins (HSPs) such as HSP90 and HSP110. The present study has therefore provided an alternative approach to preparation of HSP-based vaccines using DC/tumor fusion technology and gentle and rapid isolation of HSP peptide complexes. The Journal of Immunology, 2010, 184: 488–496.

Heat shock proteins (HSPs) are members of a number of families of stress-induced proteins, whose main intracellular functions are as molecular chaperones (1–3). The HSPs possess the intrinsic property of recognizing unfolded or disordered sequences in target polypeptides and then aiding in folding or refolding them, targeting them to the proteasome for destruction (4). In addition, after exiting the cell and entering the extracellular environment, HSPs are capable of promoting Ag presentation of chaperoned peptides through interaction with the receptors on APCs (5), a process called Ag cross-presentation. Thus, when HSPs form HSP-peptide complexes (HSP.PCs) that are derived from tumor cells, they possess the qualities of tumor vaccine. In previous animal studies, immunization with HSP.PCs purified from tumor cells provided protection against tumors from which the HSP.PCs were derived (6–9). In addition, using the vaccine to treat mice with established tumors can slow the rate of tumor growth and stabilize disease (10, 11). In clinical trials, autologous tumor-derived HSP96.PC were used to treat a variety of malignancies, including melanoma (12–14), colorectal cancer (15), and renal cell carcinoma (16) with immunological and clinical responses in a subset of patients. Overall, the clinical response was muted in the randomized phase III trial (14, 16). These results suggest a need for enhancement in the potency of such vaccines.

We have attempted to produce an improved HSP70-based vaccine that might have a clinical application. In our previous study, HSP70.PCs derived from dendritic cell (DC)-tumor fusion cells (HSP70.PC-F) contained enriched antigenic peptides compared with HSP70.PC derived from tumor cells (HSP70.PC-Tu) and possessed superior properties over its counterpart from tumor cells (17). To develop an HSP70.PC-based tumor vaccine with enhanced immunogenicity for patient use, we extracted HSP70.PC-F from DCs fused to ovarian carcinoma (OVCA) cells from patients or established human breast cancer cells, respectively, and examined their properties as tumor vaccines. Our studies show that HSP70.PC-F carry increased levels of peptides of tumor Ags that stimulate enhanced T cell responses against tumor cells. In addition, the experiments provide a proof of principle indicating the use of alternative sources of DCs and tumor cells to produce HSP70.PC vaccine.

Materials and Methods

Tumor cells

Human breast carcinoma cells MCF-7 (HLA-A*0201+), BT-20 (HLA-A*0201+/A*1101+), ZR75 (HLA-A*1101+), and SKBR3 (HLA-A*1101+) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in medium according to the manufacturer’s instruction.

OVCA cells were obtained from patients with ovarian cancer (18). The resected tumors were weighed, minced to small pieces (1–3 mm), and mashed through a sterile 50-μm nylon mesh (Sigma-Aldrich, St. Louis, MO) in a tissue culture hood. Single-tumor cell suspensions were obtained by processing a filter and serum column to remove dead tumor cells and other type cells. OVCA cells (~80–95% purity) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated human Ab serum, and used for fusion partner, CTL targets, or extraction of HSP70.PC.

Preparation of human DCs and T cells

PBMCs were isolated from leucopacks obtained from healthy donors using Ficoll density gradient centrifugation (Ficoll-Paque plus, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). PBMCs were collected and plated in a tissue culture dish with 5% human Ab male serum (Sigma-Aldrich) in RPMI...
1640 medium containing 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin for 1 h in a humidified CO₂ incubator. The adherent fraction was cultured in 1000 U/ml GM-CSF (Genzyme, Framingham, MA) and 500 U/ml IL-4 (R&D Systems, Minneapolis, MN) RPMI 1640/AIM-V (1:1) medium with 1% human Ab male serum for 5 d. On day 4, the loosely adherent cells were collected and further enriched through repeated adherence method for fusion use. The nonadherent cells were frozen with 10% DMSO in human Ab serum and used as a source of T cells.

Preparation of DC-tumor fusions and HSP70.PC extraction

Tumor cells were mixed with DC preparations at 1:10 and washed in serum-free, prewarmed RPMI 1640 culture medium. The mixture cell pellet was resuspended in a polyethylene glycol solution (MW1450, Sigma-Aldrich) for 5 min at room temperature, and prewarmed, serum-free RPMI 1640 medium to dilute the polyethylene glycol was progressively added in the next 5 min. After washing, fusion cells were resuspended in RPMI 1640 medium supplemented with 5% human Ab serum and 500 U/ml GM-CSF and then cultured in 5% CO₂ at 37 °C for 5 d.

Tumor and DC-tumor fusion cells (FCs) were collected and incubated with lysis buffer (50 mM Tris-HCl [pH 8.0] containing 50mM NaCl, 1% Nonidet P-40, 1mM PMSF) on ice for 30 min. The lysates were clarified by centrifugation, and the aqueous phase were collected and incubated with mAb against human HSP70 (SC1A12, ProMab Biotechnologies, Albany, CA) at a concentration of 1/100 overnight at 4 °C. Next, 50 µl protein A/G (1/1) agarose were added and incubated at 4 °C for an additional 1.5 h. After extensive wash with lysis buffer, the immunoprecipitates were eluted with PBS with high salt (500 mM NaCl). The eluted of HSP70.PC-F or HSP70.PC-Tu was used to stimulate the fusion cells for an additional 1.5 h. After extensive wash with lysis buffer, the immunoprecipitates were eluted with PBS with high salt (500 mM NaCl). The eluted of HSP70.PC-F or HSP70.PC-Tu was used to stimulate the fusion cells for an additional 1.5 h. After extensive wash with lysis buffer, the immunoprecipitates were eluted with PBS with high salt (500 mM NaCl). The eluted of HSP70.PC-F or HSP70.PC-Tu was used to stimulate the lymphocytes. The HSP.PC preparations were checked by imunoblotting.

Immunoblotting

The proteins from cell lysates or immunoprecipitation with anti-HSP70 Ab (19) were subjected to SDS-PAGE and transferred on nitrocellulose membrane. The membranes were incubated with anti-HSP90 (SPA-400), anti-HSP90 (SPA-800), HSP110 (SPA-1101) Abs (Stressgen, Ann Arbor, MI), anti-HSP70 (5G10; BD Pharmingen, San Diego, CA), anti-MUC1 (HMPV; BD Pharmingen), anti-MUC1 peptide Ab (BCP8, anti-DTR) (20), anti-c-ErbB2/Neu (Ab-3; Calbiochem, San Diego, CA) and β-actin (Sigma-Aldrich). The Ag/Ab complexes were visualized by ECL (ECL Detection System; General Electric, Fairfield, CT). Densitometric analysis of the bands was performed using GelDoc 2000 (Bio-Rad, Hercules, CA).

Phenotype of DCs, tumor cells and FCs

DCs, breast cancer cells, and patient-derived OVCA cells were incubated with anti-human mAbs against MUC1 (HMPV), HLA-DR (TUS6), CD86 (IT2.2, anti-B70/B7-2), anti-HLA-ABC (W6/32; BD Pharmingen), anti-HLA-A2 (0791HA) and HLA-A11 (0264HA; One x, Canoga Park, CA); anti-c-ErbB2/Neu (TA-1; Oncogene Research Products, San Diego, CA) and anti-CA125 (NCL-L-CA125; NovoCastra Laboratories, Newcastle, U.K.). After 1 h incubation with Abs, the cells were washed and incubated with FITC-conjugated anti-mouse IgG (Chemicon International, Temecula, CA). To determine the efficiency of DC-tumor fusions, the fusion cells were dual stained with FITC-conjugated anti-MUC1 (HMPV) and PE-conjugated anti-HLA-DR (TUS6) or anti-CD86 (IT2.2; BD Pharmingen). Cells were fixed in 2% paraformaldehyde and analyzed by flow cytometry using CellQuest software (BD Biosciences, San Diego, CA).

Confocal microscope

Approximately 2 x 10⁶ FCs were spin onto slides by Cytospin (Thermo Shandon, Waltham, MA). The cells were dual stained with FITC-conjugated anti-MUC1 (HMPV) and PE-conjugated anti-CD86 (IT2.2) for 1 h at 4 °C, respectively. The cells were washed, fixed and analyzed using a Laser Scanning Confocal microscope (TE2000-E; Nikon, Melville, NY).

T cell proliferation

T cells (nonadherent cell population, 1 x 10⁵) were resuspended with 2 µg/ml HSP70.PC extracted from tumor or FCs in RPMI 1640 medium containing 10% human Ab serum, 10 U/ml human IL-2, 15 mM HEPES, 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5 x 10⁻⁵ M β-mercaptoethanol. DCs were added at 1:10 ratio into T cells and seeded in 96-well U bottom plates in a volume of 200 µl per well for 5 d cultures. T cell proliferation was assessed by [³H]thymidine incorporation after an additional 12-h incubation with 1 µCi per well of [³H]thymidine. Radioactivity (mean ± SD of triplicates) was measured by liquid scintillation counting.

IFN-γ or tetramer staining

Lymphocytes (nonadherent cell population) were stimulated with 2 µg/ml HSP70.PC extracted from tumor or fusion cells in the presence of 10 U/ml human IL-2 in RPMI 1640 medium containing 10% human Ab serum for 5 d. On day 5, the T cells were collected and purified through a nylon wool column to remove DCs and then stained with anti-human CD4/IFN-γ or anti-human CD8/IFN-γ (BD Pharmingen) according to the manufacturer’s instructions. For tetramer staining, T cells obtained after passing through a nylon wool column were incubated with PE-conjugated MUC1 tetramer 1 (HLA-A*0201, STAPPVHNV) or PE-conjugated HER2/Neu tetramer (HLA-A*0201, ILHNGASYL; PROJIMMUNE, Oxford, U.K.) for 1 h at 4 °C. After washing, the T cells were further stained with FITC-conjugated anti-CD3 mAb for 40 min at 4 °C. Cells were washed and fixed with 2% paraformaldehyde and analyzed by flow cytometry using CellQuest software (BD Biosciences).

CTL assay

T cells (nonadherent cell population) from healthy donors were mixed with autologous DCs (DC/lymphocytes at 1:10 ratio) and placed in a six-well plate with 2 µg/ml HSP70.PC extracted from tumor cells or FCs in RPMI 1640 medium containing 10% human Ab serum and 10 U/ml human IL-2. After 5 d of culture, the cells were collected and T cells were purified through a nylon wool column and used for effector T cells. Breast tumor or OVCA cells isolated from patients with ovarian cancer were labeled with 100 µCi Na₂¹¹⁷¹CrO₄ for 60 min at 37 °C and washed to remove unincorporated isotope. In an Ab-blocking assay, the targets were incubated with indicated Abs for 1 h on ice. The effector T cells or tumor target cells were reconstituted in CTL assay medium at indicated E:T ratios and placed in 96-well V-bottom plates for 5 h at 37 °C. After incubation, the supernatants were collected and radioactivity was quantitated in a well V-bottom plates. Statistical analysis

Statistical significance was analyzed using χ², one-way ANOVA, or Student’s t test. p < 0.05 indicates statistic significance.

Results

Characterization of FCs

Monocyte-derived DCs from donors were generated in the presence of GM-CSF and IL-4 and fused to human breast cancer cells including MCF-7 or BT-20, and analyzed for tumor or DC markers. The breast cancer cells were positive for MUC1, HER2, and CD86 and expressed predictably high levels of HER2/new and MUC1, but minimal levels of HLA-DR and CD86 molecules that were expressed at high levels by DCs (Fig. 1A–C). In contrast, fusions of DC (HLA-A*0201) and breast cancer cells expressed both the tumor-derived Ags MUC1 and the DC-derived cosublimatory molecules CD86 and CD200 molecules that were expressed at high levels by DCs (Fig. 1A–C). In contrast, fusions of DC (HLA-A*0201) and breast cancer cells expressed both the tumor-derived Ags MUC1 and the DC-derived cosublimatory molecules CD86 and CD200 molecules that were expressed at high levels by DCs (Fig. 1A–C). In contrast, fusions of DC (HLA-A*0201) and breast cancer cells expressed both the tumor-derived Ags MUC1 and the DC-derived cosublimatory molecules CD86 and CD200 molecules that were expressed at high levels by DCs (Fig. 1A–C). In contrast, fusions of DC (HLA-A*0201) and breast cancer cells expressed both the tumor-derived Ags MUC1 and the DC-derived cosublimatory molecules CD86 and CD200 molecules that were expressed at high levels by DCs (Fig. 1A–C).
HLA-A*0201* and DC generated from leucopack 10 [DC10], HLA-A*0201*/A*1101* fused to patient-derived OVCA cells by two-color FACS analysis (Fig. 1E). We further characterized the fusion cells by confocal microscopy. Tumor-derived cell surfaces were identified by MUC1 expression (green color) (Fig. 1F, left panel), and DCs were identified as CD86-positive DC stained orange cells (Fig. 1F, middle panel). Overlapping these images resulted in detection a subset of cells with yellow color (Fig. 1F, right panel), indicating DC-tumor fusion.

**Extraction and characterization of HSP70.PCs**

The fusion process introduces abundant tumor Ags into the efficient Ag processing milieu of the DC cytoplasm. It has been demonstrated that HSPs play a role in the processing of these Ags and chaperoning processed peptides (21). We thus hypothesized that we could potentially obtain enriched tumor Ags and their intermediates from human FCs. To test this hypothesis, we first determined the level of expression of HSPs in DCs, OVCA cells, and their counterparts in FCs by immunoblotting with Abs against HSPs. HSP70, HSP90, and MUC1 were detected in the lysates of OVCA and fusion cells, whereas low levels of HSP90 and minimal MUC1 were detected in the lysates of DCs. Notably, the expression of HSP90 was increased in the fusion cells (Fig. 2A). A 3-fold increase of HSP90 was observed in the lysates of fusion cells compared with HSP90 from tumor cells (Fig. 2B). The relative levels of expression of HSP70 between OVCA2 (HLA-A*0201*/A*1101*) and DC/OVCA2 fusions was comparable (Fig. 2A, 2B).

To determine whether HSP70 is associated in the immunoprecipitates with tumor Ags, their processed intermediates, and other HSPs, proteins, lysates from OVCA and DC/OVCA fusion cells were precipitated with anti-HSP70 mAb (19), followed by immunoblotting with a panel of anti-HSP Abs. HSP40, HSP90, and HSP110 were detected in the immunoprecipitates by anti-HSP70 Ab from lysates of DC/OVCA2 fusion cells and OVCA2 cells (Fig. 2C, 2D), suggesting intracellular association of HSP70 with these proteins. Interestingly, the expression of HSP90 was significantly increased in DC/OVCA2 fusions cells. In addition, the recovery of MUC1 and HER2/neu tumor Ags was significantly increased in the immunoprecipitates of HSP70 from DC/OVCA2 fusion cells compared with those from OVCA2 cells (Fig. 2E, 2F). A BCP8-positive band was observed in the immunoprecipitates, suggesting the existence of processed tumor Ag in HSP70.PC. Increased recovery of tumor Ags and their processed intermediates in the immunoprecipitates from FCs may be related to the increased expression of HSP90 in such cells.

**Proliferation and stimulation of T cells by HSP70.PC-F**

The ability of HSP70.PC-F to stimulate T cell proliferation was assessed by the standard [3H]-thymidine incorporation assay. Incubation with HSP70.PC from DC/MCF7 fusion cells in the presence of IL-2 resulted in the proliferation of T cells (Fig. 3A). In contrast, a lower level of T cell proliferation was observed when T cells were incubated with IL-2 and either HSP70.PC-Tu or medium alone. Similar results were obtained in HSP70.PC from DC/BT-20 fusion cells (Fig. 3B). Increased T cell proliferation

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**FIGURE 1.** Characterization of FCs. A, Phenotype of established human breast cancer cell lines. Human breast tumor cell lines MCF7, BT20, SKBR-3, and ZR75 were stained with indicated Abs, including isotype IgG (open histogram) as a control and analyzed by flow cytometry. B–C, Human DCs generated from PBMCs of healthy donors were fused to MCF7 or BT20 tumor cells to produce DC/MCF7 or DC/BT20 fusion cells. The fusion efficiency was determined using double staining with FITC-conjugated anti-MUC1 and PE-conjugated anti-HLA-DR (B) or PE-conjugated anti-CD86 (C) Abs and analyzed by flow cytometry. D, Phenotype of OVCA4 cells derived from patients with ovarian cancer. OVCA cells freshly isolated from patient number 4 were stained with indicated Abs and analyzed by flow cytometry. E, The fusion efficiency of DC9/OVCA4 (left panel) or DC10/OVCA4 (right panel) was determined by staining with Abs against CA125, MUC1, and MUC1 peptide DTR (BCP8) followed by staining with anti-mouse IgG-FITC, and then by double staining with PE-anti-HLA-DR or PE-anti-CD86 Abs and analyzed by flow cytometry. F, Identification of DC/OVCA fusion cells using confocal microscope. Cytocentrifuge preparations of DC/OVCA fusion cells were dually stained with FITC-conjugated anti-MUC1 (green color, left panel) and PE-conjugated CD86 (red color, middle panel) Abs, and examined under confocal microscopy (magnification ×40). The cells with orange color are fusion cells in the merge image (right panel). The data in D–F were representative in four ovarian cancer samples.
was observed in T cells stimulated by HSP70.PC from DC/BT-20 fusion cells compared with those stimulated by HSP70.PC from BT-20 tumor cells (Fig. 3B).

To characterize the T cells stimulated by these HSP70 preparations, they were double stained with Abs against IFN-γ and either CD4 or CD8 prior to analysis by FACS. We found a subset of T cells stimulated by HSP70.PC extracted from MCF7 tumor cells or FC/MCF7 fusion cells expressed IFN-γ (Fig. 3C), suggesting activation of these T cell subpopulations. Furthermore, IFN-γ-positive CD4 or CD8 T cells were significantly increased when stimulated by HSP70.PC-F compared with HSP70.PC-Tu (4.3% ± 1.7 versus 2.03% ± 0.8 in CD4 T cells; 8.6% ± 2.6 versus 3.9% ± 1.9 in CD8 T cells; Fig. 3D). To determine whether tumor Ag-specific T cells were induced, T cells were further analyzed with tetramers specific for either MUC1 peptide (HLA-A*0201, ILHN-GAYSL) or HER2/neu (HLA-A*0201, ILHN-GAYSL). MUC1 peptide-specific or HER2/neu peptide-specific T cells were induced by both HSP70.PC-F and HSP70.PC-Tu (Fig. 3E). Consistent with the findings on IFN-γ expression, MUC1- or HER2/neu-specific T cells were significantly increased in T cells stimulated by HSP70.PC-F, indicating that induction of tumor Ag-specific T cells is enhanced by HSP70.PC-F exposure (10.3% ± 3.9 versus 6.2% ± 4.1; 5.24% ± 0.59 versus 1.23% ± 0.3; Fig. 3F). Thus, stimulation with HSP70.PC-F enhances the quantity and quality of stimulated T cells.

**Induction of Ag-specific CTL by HSP70.PC-F**

To determine the influence of HSP70.PC on lymphocyte killing of tumor targets, the standard Cr-release assay was next used. T cells stimulated by HSP70.PC extracted from FC/BT20 breast carcinoma fusion cells showed higher CTL activity against BT20 tumor cells than those stimulated by HSP70.PC extracted from BT-20 tumor cells (Fig. 4A). Similar findings were observed when T cells stimulated by HSP70.PC extracted from FC/SKBR3 fusion cells were incubated with SKBR3 tumor targets (Fig. 4B). Furthermore, T cells stimulated by HSP70.PC extracted from patient-derived OVCA cells or from DC/OVCA1 or DC/OVCA2 fusion cells lysed OVCA tumor targets from which the HSP70.PC were derived. As with the experiments performed on tumor cell lines, higher levels of CTL activity against OVCA cells were observed in T cells stimulated by HSP70.PC extracted from T cells stimulated by HSP70.PC extracted from fusions of DC/SKBR3 (HLA-A*0201+) and HSP70.PC-F than those stimulated by HSP70.PC extracted from OVCA cells (HLA-A*0201+). The difference in CTL activity induced by HSP70.PC-F or HSP70.PC-Tu was statistically significant (Fig. 4A–D). To assess the Ag specificity of the CTL, multiple tumor targets were used. T cells (HLA-A*1101+) stimulated by HSP70.PC extracted from DC/SKBR3 (HLA-A*1101+) fusion cells were able to kill not only SKBR3 tumor cells but also relevant ZR75 (HLA-A*1101+) tumor cells (Fig. 4E). Interestingly, T cells (HLA-A*0201+) stimulated by HSP70.PC extracted from fusion of DCs and OVCA1 (HLA-A*0201+) were effective in lysis of...
The indicated number of lymphocytes and DCs (10:1 ratio) were incubated with 2 μg/ml HSP70.PCs extracted from DC/MCF7 fusion cells or MCF-7 tumor cells (A) or HSP70.PCs extracted from DC/BT-20 fusion cells or BT-20 tumor cells (B) for 5 d. T cell proliferation was measured using standard [3H]-thymidine incorporation. C and D, Intracellular expression of IFN-γ in CD4 and CD8 cells stimulated by HSP70.PC from FC/MCF7. T cells were purified through nylon wool after 5 d stimulation and stained with mAbs against IFN-γ and CD4 or CD8 and analyzed by FACS. The data in C are representative and the four experiments are summarized in D. E and F, Ag-specific T cells induced by HSP70.PCs derived from DC/MCF7. T cells (HLA-A*0201) were stimulated by HSP70.PCs derived from MCF-7 (HLA-A*0201) or DC/MCF7 cells (HLA-A*0201) for 5 d and then purified by nylon wool for tetramer staining. T cells were stained with PE-conjugated HER2/neu peptide tetramer (HLA-A*0201/IHLNGAYSLS; upper panel) or MUC1 peptide tetramer (HLA-A*0201/STAPPVHNV; lower panel), respectively. After washing with cold PBS, the cells were further stained with FITC-conjugated anti-CD8 Ab and analyzed by flow cytometry. The numbers of CD8 T cells were gated, and the average percentage of tetramer positive cells in CD8 T cells from three experiments was presented. The statistical significance was determined by χ2 test.

FIGURE 3. Proliferation and activation of T cells by HSP70.PCs derived from FCs. A and B, The indicated number of lymphocytes and DCs (10:1 ratio) were incubated with 2 μg/ml HSP70.PCs extracted from DC/MCF7 fusion cells or MCF-7 tumor cells (A) or HSP70.PCs extracted from DC/BT-20 fusion cells or BT-20 tumor cells (B) for 5 d. T cell proliferation was measured using standard [3H]-thymidine incorporation. C and D, Intracellular expression of IFN-γ in CD4 and CD8 cells stimulated by HSP70.PC from FC/MCF7. T cells were purified through nylon wool after 5 d stimulation and stained with mAbs against IFN-γ and CD4 or CD8 and analyzed by FACS. The data in C are representative and the four experiments are summarized in D. E and F, Ag-specific T cells induced by HSP70.PCs derived from DC/MCF7. T cells (HLA-A*0201) were stimulated by HSP70.PCs derived from MCF-7 (HLA-A*0201) or DC/MCF7 cells (HLA-A*0201) for 5 d and then purified by nylon wool for tetramer staining. T cells were stained with PE-conjugated HER2/neu peptide tetramer (HLA-A*0201/IHLNGAYSLS; upper panel) or MUC1 peptide tetramer (HLA-A*0201/STAPPVHNV; lower panel), respectively. After washing with cold PBS, the cells were further stained with FITC-conjugated anti-CD8 Ab and analyzed by flow cytometry. The numbers of CD8 T cells were gated, and the average percentage of tetramer positive cells in CD8 T cells from three experiments was presented. The statistical significance was determined by χ2 test.

OVCA1 cells and, to a lesser extent, MCF7 breast cancer cells (HLA-A*0201). Minimal CTL was observed in the killing of SKBR3 (HLA-A*1101) or BT-20 (HLA-A*0201/1*1101) tumors with different HLA type (Fig. 4F). Similar results were observed in T cells (HLA-A*1101) stimulated by HSP70.PC extracted from fusions of DCs and OVCA2 (HLA-A*0201/1*1101) with highest killing to OVCA2 cells and, to a lesser extent, SKBR3 (HLA-A*1101) tumor cells and minimal killing to K562 cells (HLA-A*0201/1*1101; Fig. 4G). These experiments show that the induction of Ag-specific T cells is enhanced by the stimulation of HSP70.PC-F and that CTL capable of lysing the target from which HSP70.PCs are extracted can also kill other tumor cells, provided they share the expression of tumor Ags and restriction elements.

Broad application of HSP70.PC-F

The data in Fig. 4 show that HSP70.PC extracted from FCs can lys multiple targets with shared tumor Ags, suggesting broad applicability of this vaccine. We next determined whether the source of DCs can be altered in the production of HSP70.PC. In these experiments, OVCA4 cells (HLA-A*0201/1*1101) were fused to DCs derived from PBMCs of two individual donors (DC9, HLA-A*0201/1*1101 and DC10, HLA-A*0201/1*1101) to generate DC9/OVCA4 and DC10/OVCA4 fusions, respectively. Then HSP70.PC were extracted from these fusion cells and OVCA4 tumors, respectively. HSP70.PC from DC9/OVCA4, DC10/OVCA4, or OVCA4 tumor cells were incubated with T cells (HLA-A*0201/1*1101) isolated from the same donor from whom DC9 was generated. Comparable T cell proliferation was observed in T cells stimulated by HSP70.PC from DC9/OVCA4 or DC10/OVCA4 fusion cells (Fig. 5A). In contrast, HSP70.PC from OVCA4 stimulated minimal T cell proliferation. In addition, more CD4 and CD8 T cells stimulated by HSP70.PC from DC9/OVCA4 and, to a lesser extent, DC10/OVCA4 expressed IFN-γ than those stimulated by their counterparts from OVCA4 (Fig. 5B). Similar results were observed in the induction of CTL. HER2/neu and MUC1-specific CTLs were induced by HSP70.PC from DC9/OVCA4 or DC10/OVCA4 fusion cells as demonstrated by tetramer staining (Fig. 5C), and importantly, they were effective in lysis of OVCA4 tumor cells (Fig. 5D). To determine the specificity and restriction elements of CTLs, an Ab blocking assay was performed. As shown in Fig. 5E, CTL activity against OVCA4 was almost completely blocked by mAb against HLA-ABC (upper panel) and partially blocked by mAbs against HLA-A2 or MUC1 (middle and lower panels). These results indicate that CTLs induced by HSP70.PCs from DC9/OVCA4 or DC10/OVCA4 fusion cells are Ag-specific. It is likely that polyclonal CTLs were induced against MUC1 and other tumor Ags such as HER2/neu (Fig. 5C), and multiple restriction elements in addition to HLA-A2 were involved. To confirm these results, parallel experiments were performed using T cells (HLA-A*0201/1*1101) isolated from the same donor from whom DC10s were generated. Comparable results were observed in T cells from donor 9 or donor 10 stimulated by HSP70.PCs. HSP70.PCs from DC10/OVCA4 or DC9/OVCA4 fusion cells induced higher T cell proliferation (Supplemental Fig. 1A), higher level of IFN-γ expression (Supplemental Fig. 1B), frequency of CTLs (Supplemental Fig. 1C), and CTL activity against OVCA4 T cells (Supplemental Fig. 1D) than their counterparts extracted from
OVCA4 tumor cells. In addition, Ab blocking assay shows that CTL activity against OVCA4 was blocked or partially blocked by mAbs against HLA-ABC, HLA-A11, or MUC1 (Supplemental Fig. 1 E).

Together, these experiments indicate that HSP70.PCs from fusion cells can be readily represented by DCs with different HLA backgrounds, suggesting the potentially broad use of the HSP70.PC-F approach in the clinical setting.

**Discussion**

HSP-chaperoned antigenic peptides elicit antitumor immunity when used as a tumor vaccine (10, 22–25). Indeed, HSP-based vaccines (HSP70, HSP90, or GP96) derived from cancer cells have been widely studied in animal experiments (10, 26–29). In these elegant approaches, HSP.PCs derived from cancer cells reproducibly induced CTL responses against the cancer cells from which the HSP.PCs were purified (23, 24, 30, 31). Importantly, immunization of mice with HSP.PCs provides protection against the challenge with the tumor cells from which HSP.PCs are purified or slows the progression of that tumor (7, 10, 31). Based on these results, HSP-based vaccines have been used in human clinical trials (12–16, 32–34).

**FIGURE 4.** Enhanced Ag-specific CTL activity induced by HSP70.PCs derived from FCs. A–D, T cells with indicated HLA type were stimulated with 2 μg/ml HSP70.PC derived from fusion cells (black bar) or tumor cells (hatched bar) in medium containing 10% human Ab serum in the presence of 10 U/ml human IL-2. The PBMCs cultured in medium without HSP70.PC protein (gray bar) were used as control. After 5 d of stimulation, T cells were purified through a nylon wool column and used as effector cells. A, T cells (HLA-A*0201/A*1101+) were stimulated by HSP70.PCs from FC/BT20 (HLA-A*0201/A*1101+) or BT20 cancer cells (HLA-A*0201/A*1101+) and then incubated with 51Cr-labeled BT-20 tumor target. B, T cells (HLA-A*1101+) were stimulated by HSP70.PC from FC/SKBR3 (HLA-A*1101+) or SKBR3 cancer cells (HLA-A*1101+) and then incubated with 51Cr-labeled SKBR3 tumor target. C, T cells (HLA-A*0201+) were stimulated by HSP70.PCs from FC/OVCA1 (HLA-A*0201+) or OVCA1 cells (HLA-A*0201+) and then incubated with 51Cr-labeled OVCA1 target. D, T cells (HLA-A*1101+) were stimulated by HSP70.PCs from FC/OVCA2 (HLA-A*0201+/A*1101+) or OVCA2 cells (HLA-HLA-A*0201+/A*1101+) and then incubated with 51Cr-labeled OVCA2 target. The CTL activity against tumor cells was determined by standard 51Cr release assay in triplicates and presented as the mean ± SD. E–G, Determination of tumor specificity of CTLs induced by HSP70.PCs derived from FCs. T cells stimulated by HSP70.PCs derived from DC/SKBR3 (HLA-A*1101+; E), FC/OVCA1 (HLA-A*0201+; F) or FC/OVCA2 (HLA-A*0201+/A*1101+; G) were incubated with indicated 51Cr-labeled tumor targets. The CTL activity were measured in triplicate and expressed as the mean ± SD. The statistical significance was determined by χ² test.
FIGURE 5. T cells stimulated by HSP70.PC-F extracted fusion cells with altered fusion partners. DCs were generated from DC9 (HLA-A*0201+) or DC10 (HLA-A*0201+/A*1101+) in the presence of GM-CSF/IL-4 medium. The DC9s or DC10s were fused with OVCA4 tumor cells (HLA-A*0201+/A*1101+; Fig. 1D) to generate DC9/OVCA4 or DC10/OVCA4 fusion cells from which HSP70.PC were extracted. A, Indicated number of lymphocytes (HLA-A*0201+) and DCs (10:1 ratio) were incubated with 2 μg/ml HSP70.PC extracted from DC9/OVCA4, DC10/OVCA4 fusion cells, or OVCA4 cells for 5 d. On day 4, cells were added 1 μCi per well of 3H-thymidine and harvested on filters after 18 h of radioactivity (mean ± SD of triplicates) was measured by liquid scintillation counting. B, Intracellular expression of IFN-γ in CD4 and CD8 cells stimulated by HSP70.PC from DC9/OVCA4, DC10/OVCA4 fusion cells or OVCA4 cells. T cells were purified through nylon wool after 5 d stimulation, and then stained with mAbs against IFN-γ and CD4 or CD8 and analyzed by FACS. C, Ag-specific T cells induced by HSP70.PC derived from DC/OVCA4. T cells (HLA-A*0201+) were stimulated by HSP70.PC derived from DC9/OVCA4, DC10/OVCA4 fusion cells, or OVCA4 cells for 5 d and then purified by nylon wool for tetramer staining. T cells were stained with PE-conjugated HER2/neu peptide tetramer or MUC1 peptide tetramer, respectively. The cells were further stained with FITC-conjugated anti-CD8 Ab and analyzed by flow cytometry. D, CTLs (HLA-A*0201+) induced by HSP70.PCs derived from DC9/OVCA4 or DC10/OVCA4 fusion cells (black bar) or OVCA4 cancer cells (hatched bar) were incubated with 51Cr-labeled OVCA4 target cells (50:1 ratio). The CTL activity was measured in triplicate and expressed as the mean ± SD. The statistical significance was determined by χ² test. E, Abs block CTL assay. The OVCA4 targets were incubated with anti-HLA-ABC (top panel), anti-HLA-A2 (middle panel), and anti-MUC1 (lower panel) Abs for 1 h on ice and then incubated with T cells at 50:1 ratio. T cells incubated with OVCA4 target cells with Abs (hatched bar) or with IgG (black bar) as controls. Spontaneous release of 51Cr was measured in triplicate and expressed as the mean ± SD.
were obtained in a subset of patients with malignant tumors (12, 13, 15, 32). The randomized phase III trials, however, showed mixed results (14, 16). In a group of 728 patients with renal cell carcinoma that were enrolled into the trial with evaluable results, 361 patients received vitespen vaccination. No difference in recurrence-free survival was seen between patients given vitespen and the control cohort with no treatment (16). In the treatment of patients with melanoma, 133 patients received vitespen. Although the overall survival in the vitespen arm is statistically indistinguishable from that in the control cohort of 107 patients with physician’s choice of treatment, a subset of patients with early stage disease that received a large number of vitespen injections survived longer (14). In addition, these trials showed that clinical responses were limited by the amount of resected tumor available for HSP isolation, as responses to vaccines are related to the number of HSP injections (14).

We have attempted to produce an enhanced HSP70-based vaccine through rapid isolation of HSP70.PC from FCS. In the animal studies, HSP70.PC-F show superior properties such as enhanced induction of CTL against tumor cells and stimulation of DC maturation over its counterpart from tumor cells (17). In the current study, we aimed to extend the findings from animal to human samples and develop an HSP70-based vaccine for patient use with enhanced immunogenicity. We have shown that: 1) HSP70.PC can rapidly be extracted from DCs fused to either patient-derived OVCA cells or established breast cancer cells, and these extracts carry higher levels of tumor Ags and their processed intermediates; 2) stimulation of T cells with HSP70.PC-F results in proliferation of T cells and induction of HSP70-specific CTLs that are able to lyse relevant tumor cells, thus improving the quality and quantity of T cells stimulated by HSP70.PC-F. Unlike allogenic cellular vaccine, HSP70.PC-F is a peptide vaccine and can be readily taken up by APCs and represented to Ag-specific T cells, thus minimizing activation of allo-reactive T cells; 3) HSP70.PC-F can be extracted from fusion cells with altered fusion partners, and DCs or tumor cells can be interchanged while eliciting comparable CTL activity against tumor cells; and 4) HSP70.PC-F carry multiple antigenic peptides with the ability to induce polyclonal CTLs to kill the tumor cells from which the HSP70 was extracted as well as tumor cells with shared Ag and restriction element.

Most HSP-based vaccines such as vitespen are purified from the resected tumors of patients to whom the vaccine is applied. These customized vaccines possess certain advantages, because they carry the repertoire of peptides of individual cancer without the need to identify them. This approach of vaccine preparation, although beneficial, also has certain limitations. For example, vitespen was prepared in 49% of patients enrolled in the trial after the 13% of patients with fewer than four injections were excluded (14). Studies show that four injections are minimal doses for administration of vitespen (14). To circumvent this problem, we have assessed the feasibility of extracting HSPPC from both patient-derived and established cancer cells as fusion partners and using them to stimulate T cells. Although this approach has the drawback of introducing an extra step to the simple and elegant approach of using autologous vaccines based solely on affinity purified HSP, it does appear to have significant advantages. CTLs induced by HSP70.PC-F kill not only the tumor cells from which they are derived but also other tumor cells with different genotypes but shared tumor Ag (Fig. 4). Thus, HSP70.PC-F may be effective in clinical settings where the resected tumor sample is insufficient for the preparation of multiple doses of customized vaccine. In addition, HSP70.PC-F could be used as a prophylactic vaccine because immunotherapy is most effective in patients with minimal tumors or no tumor. One further downside of this approach is that relatively few shared tumor Ags have been identified at this time. With the advancement of molecular and biologic technology, however, it is likely that more shared tumor Ags will be identified, thus increasing the applicability and potency of the vaccine.

The molecular basis for enhanced immunogenicity in the HSP70.PC-F is not fully elucidated, and our studies are ongoing. However, DCs are the most potent APCs in the body, and their fusion to tumor cells introduces abundant tumor Ags into the efficient Ag processing and presentation of machinery of DC. It is thus likely that the fusion cells sort and produce a large repertoire of antigenic peptides than tumor cells for surface presentation. These antigenic peptides are likely to be chaperoned by HSPs during the Ag processing, thus maximizing the extraction of HSP-chaperoned peptide and potentially increasing immunogenicity of an HSP.PC vaccine. Indeed, the immunoprecipitates from fusion cells contain higher levels of MUC1 antigenic peptides than those from tumor cells (Fig. 2) (17). In addition, the gentle and rapid extraction of HSP70.PC used in the current study may also contribute to the retention of peptides and possible other HSPs or molecules that may facilitate the Ag cross-presentation through interaction with the receptors on APCs.

In summary, we have presented an alternative method and source for the preparation of HSP-based vaccines. This approach relies on the efficient Ag processing and presentation machinery of DCs to produce tumor antigenic peptides after fusion with tumor cells and gentle, rapid isolation of HSP70 complexes enriched in biologically active polypeptides. Enhanced enrichment and preservation of antigenic peptides and proteins associated with HSP70 thus increases the immunogenicity of this HSP-based vaccine.

Disclosures
The authors have no financial conflicts of interest.

References


