Efficient CTL productivity of modified fusion cells by increase of heat shock protein 70

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Abstract. In various loading methods of whole tumor cells or their derivatives, cells fusion of dendritic cells (DCs) and tumor cells have some advantages for antigen presentation, and could up-regulate cytotoxic T cells (CTL) for multiple tumor antigens, including unknown antigens. However, the mechanisms of strong CTL productivity of fusion cells (FCs) are still unknown. Heat shock proteins (HSPs) are molecular chaperones that cross-present chaperoned antigenic peptides with MHC class I molecules. Herein, we focused on clarifying the potential of FCs for CTL production from the comparison of DCs pulsed with two kinds of tumor cell lysates, such as soluble tumor cell lysates or freeze-thawed tumor cell lysates. DCs, CD8+ T cells and tumor cells were prepared from ten patients with gastric cancer, and paired autologous tumor cells were used in all experiments. FCs of OK432-treated DCs and heat-stressed tumor cells (modified FCs) showed significant up-regulation of tumor-associated CEA and HER-2 antigen, and DC-related HLA-DR and co-stimulatory molecules (CD83 and CD86). FCs showed significantly higher IFN-γ and CTL productivity of CD8+ T cells than DCs pulsed with soluble or freeze-thawed tumor cell lysates. IFN-γ and CTL productivity of FCs was significantly increased by the heat stress of tumor cells. HSP70 mRNA levels and production of HSP70 protein of modified FCs increased significantly as compared with those of DCs pulsed with soluble or freeze-thawed tumor cell lysates. DCs pulsed with HSP70.PC extracted from modified FCs could enhance CTL productivity significantly more than that of DCs pulsed with HSP70.PC from soluble or freeze-thawed tumor cell lysate pulsed-DCs. The significant up-regulation of HSP70 mRNA and protein levels of modified FCs was related to the potential of CTL productivity. These results suggest that modified FCs possess stronger ability for MHC-restricted CTL production than DCs loaded with soluble or freeze-thawed tumor cell lysates.

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Introduction

Dendritic cells (DCs) are specialized antigen-presenting cells with the ability to initiate a strong primary immune response (1). DCs loaded with tumor-associated antigen can effectively stimulate host immune responses to syngeneic tumor cells. Although many methods for the preparation of tumor-associated antigen have been examined, there are two general approaches for loading tumor-associated antigens: the use of a defined single tumor-associated antigen, such as a peptide, and the use of whole tumor cells or their derivatives for the induction of polyclonal CTLs to multiple antigens, including the unknown antigens. The loading of DCs with defined peptides seems to be a logical approach in the design of specific tumor vaccines; however, because of its dependence on the efficacy of antigen loading and availability of chemically defined antigens, peptides have significant limitations, such as the human leukocyte antigen (HLA) restriction as a source of tumor antigens. In contrast, whole tumor cells or their derivatives are interesting because of no restriction by HLA typing to increase patients receiving a DC-based tumor vaccine. Various methods of antigen preparation using whole tumor cells have been attempted, such as fusion cells (FCs) of DCs and tumor cells, irradiated tumor cells, tumor lysates, RNA extracted from tumor cells (2,3). Using the mouse model, a few studies reported the comparison of the antigen loading method (4,5). However, there is still considerable controversy as to which antigen-loading methods represent the most immunogenic forms of vaccines that are capable of eliciting clinical relevant therapeutic immunity.

FCs made from DCs and tumor cells possess both cell components of tumor antigens and accessory molecules necessary for antigen processing and presentation. The protective and therapeutic effects of an FC vaccine have already been reported in animal models (6-9). In clinical trials, partial therapeutic effects have been demonstrated in patients with melanoma, renal carcinoma, and gastrointestinal cancer; however, the mechanisms of strong antigen presentation and the induction of anti-tumor immunity of FCs have not been defined (10-17). In a mouse model, it has been reported that heat shock protein 70 (HSP70) with peptide complex (HSP70.PC) derived from FCs possess strong immuno-

genicity as a tumor vaccine (18). HSPs are molecular chaperones and play a significant role in the immune response when released from cells in complex with chaperoned antigenic peptides (17-20). DCs have a take-up process, and present exogenous antigens such as HSPs in association with MHC class I molecules. This process is termed cross-presentation. It has been demonstrated that HSPs gain access to the MHC class I-processing pathway and initiate cytotoxic T lymphocyte (CTL) responses (21,22).

In this study, we compared the CTL productivities of FCs and DCs pulsed with tumor lysates, and clarified the potential of modified FCs of OK-432-stimulated DCs and heat-stressed tumor cells. In addition, the mechanism of CTL productivity was investigated focusing on the expression of HSP70.

Materials and methods

Cell lines and autologous tumor cells. The human gastric cancer cell lines MKN28 (HLA-A*31012, HER-2+) and leukemia cell line K562 (NK sensitive) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin and streptomycin (Invitrogen Corp., CA, USA) at 37˚C in 5% CO_2 atmosphere. Surgical specimens of ten patients with gastric cancer were obtained with informed consent and the approval of our institutional review board. Cell suspension of gastric cancer tissues and normal gastric tissues were prepared by treatment of enzyme mixture of collagenase (0.02%), DNase (0.02%) and Pronase (0.05%), and maintained in AIM-V medium (Invitrogen Corp., NY, USA) supplemented with 10% heat-inactivated autologous serum, 2 mmol/l L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. For heat shock, cancer cells were warmed to 40˚C for 3 h and incubated for 24 h at 37˚C in 5% CO_2 for recovery (23). Then live cells were collected and used for cell fusion.

Preparation of DCs. Blood samples were obtained with the informed consent and approval of our institutional review board. Immature DCs were generated from peripheral blood mononuclear cells of patients with gastric cancer as described previously (24). For maturation, immature DCs were stimulated with 0.1 KE/ml (0.01 mg of dried streptococci) OK-432 (Chugai Pharmaceutical Co., Tokyo, Japan) for 24 h (OK-DC) (24).

DCs pulsed with tumor soluble lysate or freeze-thawed tumor cells. Two kinds of tumor lysate, soluble and freeze-thawed tumor cell lysates were prepared. Soluble autologous tumor lysate was prepared as described previously (4). Briefly, heated or unheated tumor cells were homogenized and the extracts were centrifuged at 50,000 x g for 30 min. The supernatant was dialyzed in phosphate-buffered saline and prepared as soluble lysate. For preparation of freeze-thawed tumor cell lysate, autologous heated or unheated tumor cells were stressed in four cycles of freezing and thawing. Soluble (Sol-Lysate) or freeze-thawed tumor cell lysate (FT-Lysate) was cultured with immature DCs at 37˚C for 18 h (tumor cell: DC=2:1); then, stimulated with 0.1 KE/ml OK-432 for 24 h for DC maturation.

Preparation of fusion cells of DCs and autologous tumor cells. OK-432 stimulated DCs were fused with heated or unheated irradiated (100 Gy) autologous tumor cells at a ratio of 2:1 by the two-step fusion method described previously (7). To determine fusion efficiency, DCs and tumor cells were pre-stained with the fluorescent dyes DiO and DiI (Invitrogen), respectively, and analyzed with FACSCalibur flow cytometer and Cell Quest software (BD Biosciences, CA, USA).

Phenotype analysis. Cells were stained with FITC-conjugated monoclonal antibodies (mAbs) against CEA (MBL, Gunma, Japan), HER-2 (BD PharMingen, CA, USA), HLA-ABC, HLA-DR, CD80, CD83 and CD86 (Beckman Coulter Inc., CA, USA). CEA mAb were used after the treatment of IntraPrep (Beckman Coulter Inc.) to increase the membrane permeability. The expression of surface molecules was quantified using FACSCalibur and Cell Quest software.

Enzyme-linked immunospot (ELISPOT) assay. For the study of IFN-γ productivities of FCs and tumor lysate-pulsed DCs, eight experimental groups were examined as follows: 1) OK-432-treated DCs and unheated autologous tumor cells (FC), 2) FCs of OK-432-treated DCs and heated autologous tumor cells (HS/FC), 3) DCs pulsed with freeze-thawed autologous tumor lysate (FT-Lysate), 4) DCs pulsed with heat-stressed freeze-thawed autologous tumor lysate (HS/FT-Lysate), 5) DCs pulsed with autologous soluble tumor lysate (Sol-Lysate), 6) DCs pulsed with heat-stressed autologous soluble tumor lysate (HS/Sol-Lysate), 7) Mixture of OK-432 treated DCs and heat-stressed autologous tumor cells (Mix/HS-Tumor/DC) and 8) medium. CD8 T cells were stimulated every 5 days by autologous FC or DC pulsed with autologous tumor lysate in complete medium (RPMI-1640 medium supplemented with at 25 mM HEPES, 10% heat-inactivated FCS, 2 mM l-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 5x10^{-5} M 2 ME) with 10 units of IL-2. After once or twice stimulation, CD8+ T cells that secrete IFN-γ were detected by ELISPOT assay kits (BD Pharmingen), and viable spots were enumerated using the ELISPOT reader (Carl Zeiss, Berlin, Germany).

Real-time RT-PCR of HSP70 mRNA. Total RNA was isolated using RNeasy spin column kits (Qiagen, Valencia, CA, USA), and cDNA was synthesized by SuperScript II RNase H reverse transcriptase (Invitrogen Corp., CA, USA). Real-time quantitative RT-PCR of HSP70 mRNA and glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) mRNA was performed using a StepOne (ABI, CA, USA). Primers and probes of HSP70 (Hs00382884) and GAPDH (Hs02786624) were purchased from Applied Biosystems (ABI). For amplification, initial denaturation at 95˚C for 10 min was followed by 40 cycles of 15 sec at 95˚C and 1 min at 60˚C. All samples were measured in triplicate. The level of HSP was normalized by GAPDH, and the ratio of HSP to GAPDH was calculated.

ELISA for HSP70 protein mesurements. FCs, tumor lysate-pulsed DCs and mixture of DC and tumor cells were cultured for 3 days. HSP70 protein levels of supernatants of these

Table I. Characteristics of gastric carcinoma and percentage of DC/tumor FCs.

Patient list	HLA-ABC	HLA-A*24	HLA-A*2	HLA-DR	CEA	HER-2	Fusion efficiency (%)	
							FC	HS/FC
1	+	+ (2402101)	-	-	+	-	50.2	61.2
2	+	+ (2402101)	-	-	+	-	47.3	53.1
3	+	+ (2402101)	-	-	+	-	43.8	50.7
4	+	+ (2402101)	-	-	+	+	57.4	51.4
5	+	+ (2402101)	-	-	+	-	55.2	47.6
6	+	+ (2402101)	-	-	+	+	42.1	57.7
7	+	+ (2402101)	-	-	+	-	38.7	60.1
8	+	-	+ (0201)	-	+	+	57.1	62.5
9	+	-	+ (0207)	-	+	+	46.2	55.7
10	+	-	+ (0207)	-	+	+	48.4	55.8
Mean ± SD							48.6±6.4	55.6±4.9

cells were tested by ELISA assay (StressXpress, Stressgen Biosciences, MI, USA).

Preparation of HSP70 by immunoprecipitation. The cells were incubated in lysis buffer (PBS with 1% Triton X-100, 1 mg/ml BSA, 0.2 U/ml aprotinin, 1 mM PMSF) with 20 U/ml apyrase (Sigma-Aldrich Japan, Tokyo, Japan) for 1 h on ice. The lysates were clarified by centrifugation and aqueous phases were collected. Anti-HSP70 mAb was added and incubated overnight at 4˚C, and then protein A/G agarose was added and incubated at 4˚C for an additional 1 h. After extensive washing with lysis buffer, peptide complex with HSP70 was extracted (HSP70.PC), and a portion of sample was analyzed by Western blotting described previously (7). Then peptide complex with HSP70 was purified by gel filtration chromatography (GE Healthcare UK Ltd., Buckinghamshire, UK) for CTL assay. A portion of sample was analyzed by 12.5% SDS-PAGE and silver staining (EzStain Silver, ATTO Co., Tokyo, Japan) (23).

CTL assay. CTL assays were examined for two purposes of the experiment. The first purpose was examination of CTL productivity by stimulation with DC-based vaccines as follows: HS/FC, FC, Mix/HS-Tumor/DC and DCs pulsed with HS/FT-Lysate, FT-Lysate, HS/Sol-Lysate or Sol-Lysate. The second purpose was examination of CTL productivities by DCs pulsed with HSP70.PC purified from HS/FC, FC, HS/Tumor, Tumor, HS/FT-Lysate or HS/Sol-Lysate. In this study, immature DCs were pulsed with 1.25, 2.5 or 5 μg/ml of purified HSP70.PC for 24 h, and then matured with 0.1 KE/ml OK-432 for 24 h. CD8+ T cells were stimulated every 7 days by autologous DCs pulsed with HSP70.PC. After two times of stimulation, CD8+ T cells were mixed with Na2^{51}CrO$_4$-labeled target cells at various ratios in a 96-well microplate. As target cells, autologous gastric cancer cells, autologous gastric normal cells, allogeneic MKN28 cells and K562 cells were used. In the mAb-blocking assay, target cells were incubated with anti-MHC class I or class II mAb before the addition of effector cells. After incubation for 6 h at 37˚C with 5% CO$_2$, radioactivity levels of supernatants were

quantitated in a gamma-counter and specific cytotoxicity was calculated described previously (7). Each sample was performed in triplicate.

Statistical analysis. Multiple groups were evaluated by ANOVA and the post-hoc Scheffe multirange test. $p < 0.05$ was considered significant. Values are presented as the mean ± standard deviation (SD).

Results

Comparison of phenotypes of tumor cells, DCs, lysate-pulsed DCs and FCs. Tumor cells isolated from gastric cancer patients showed high expression levels of CEA, HER-2 and HLA-ABC molecules, but not HLA-DR, CD80, CD83 or CD86 molecules (Table I and Fig. 1A). Heat-stressed tumor cells (HS/Tumor) resulted in a significant up-regulation of CEA and HER-2 antigens. DCs stimulated with OK-432 expressed significantly higher levels of HLA-DR, CD83 and CD86 molecules as compared with those of immature DCs (Imm-DC) (Fig. 1B). Next, we examined the phenotypes of DCs pulsed with four types of cell lysate as follows: freeze-thawed tumor lysate (FT-Lysate), heat-stressed FT-Lysate (HS/FT-Lysate), soluble tumor lysate (Sol-Lysate) and heat-stressed Sol-Lysate (HS/Sol-Lysate). DCs pulsed with four types of tumor lysates showed high levels of HLA-ABC, HLA-DR, and CD80, CD83, CD86 molecules and medium levels of CEA and HER-2 (Fig. 1C). There were no significant differences between DCs pulsed with Sol-Lysate and FT-Lysate. In contrast, FC of DC and tumor cells showed expressions of HLA-ABC, HLA-DR and co-stimulatory molecules from DCs, and CEA and HER-2 molecules from tumor cells (Fig. 1D). By OK432 stimulation of DC, expression levels of HLA-DR, CD83 and CD86 of FC increased significantly. Furthermore, FC of OK-treated DC and heat-stressed tumor cells (HS/FC) showed a significant up-regulation of CEA and HER-2 molecules as compared with those of non-heat-stressed FC. These findings suggest that the tumor antigen and co-stimulator molecules which are necessary for antigen presentation are expressed in FC, and FC modified by heat
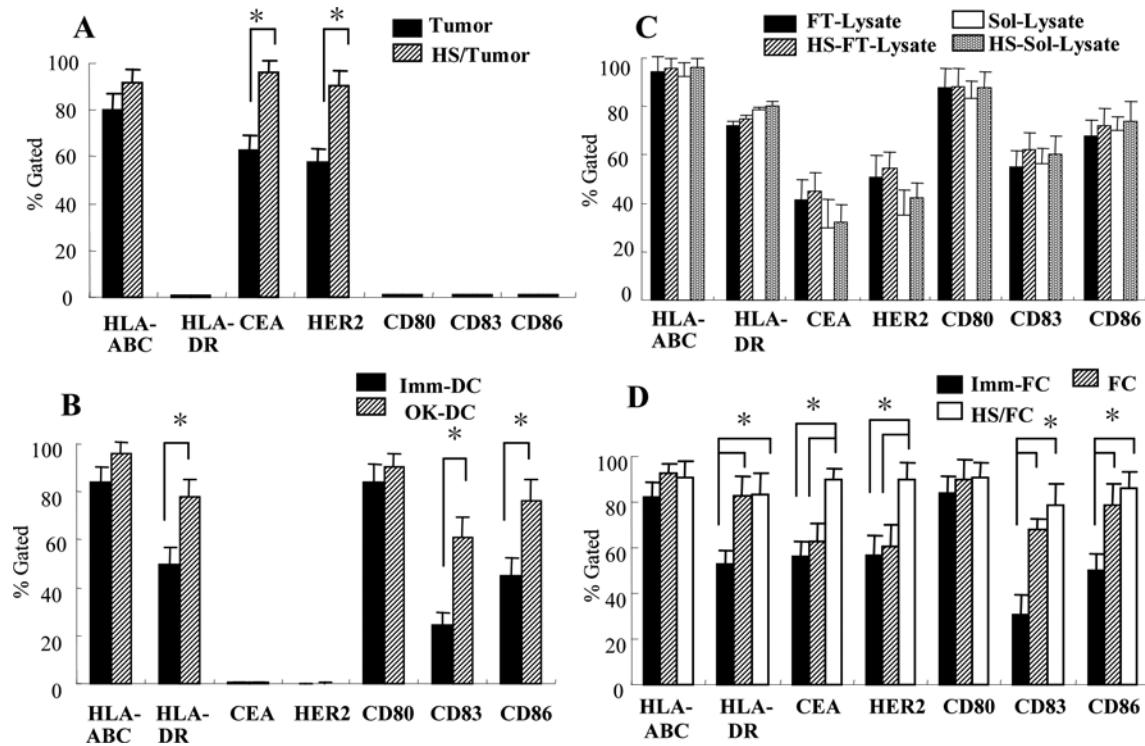
Figure 1. Characteristics of cell-surface phenotypes. (A) Cell-surface markers of unheated-autologous tumor cells (Tumor) and heated autologous tumor cells (HS/Tumor) were analyzed. (B) Cell-surface markers of immature DCs (Imm-DC) and OK432-treated DCs (OK-DC) were analyzed. (C) Cell-surface markers of DCs pulsed with freeze-thawed autologous tumor lysate (FT-Lysate), heat-stressed freeze-thawed autologous tumor lysate (HS/FT-Lysate), soluble autologous tumor lysate (Sol-Lystae) or heat-stressed soluble autologous tumor lysate (HS/Sol-Lysate), were analyzed. (D) Cell-surface markers of FC of immature DC and unheated autologous tumor cells (Imm-FC), FCs of OK432-treated DC and unheat-stressed autologous tumor cells (FC) and FC of OK432-treated DC and heat-stressed autologous tumor cells (HS/FC). Cells were incubated with FITC-conjugated mAbs against HLA-ABC, HLA-DR, CD80, CD83, CD86, CEA or HER-2 and analyzed by flow cytometry. Mean ± SD (n=10), *p<0.05.
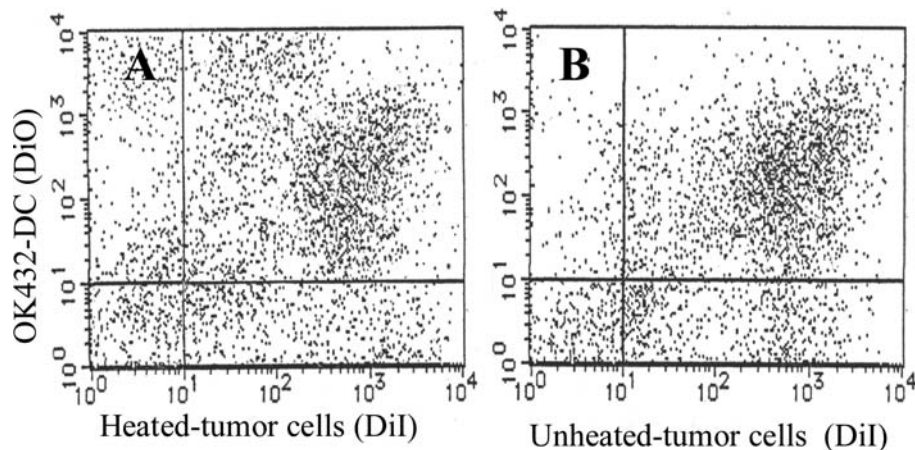

Figure 2. Fusion efficiency of DC and heated or unheated autologous tumor cells. Fusion efficiency of HS/FC (A) and FC (B) was examined by FACS. The presented FACS profile is representative of ten patients.

stress show increased expression levels of tumor antigens molecules.

Fusion efficiency. The average fusion efficiency of non-heat-stressed autologous tumor cells and DC (FC) and heat-stressed autologous tumors and DCs (HS/FC) was 48.6±6.4% and 55.6±4.9%, respectively with no significant differences between them (Table I). Fig. 2 shows the FACS profile of a representative patient.

IFN-γ productivity of FC and tumor lysate-pulsed DC. IFN-γ productivities of autologous CD8+ T cells by stimulation with FC (HS/FC and FC), mixture of autologous heat-stressed tumor cells and OK-stimulated DCs (Mix/HS-Tumor/DC), DC pulsed with two types of autologous tumor lysates, freeze-thawed tumor cells (HS/FT-Lysate, FT-Lysate) and soluble lysate (HS/Sol-Lysate, Sol-Lysate) (Fig. 3). On the first stimulation of DC loaded with tumor antigens, CD8+ T cells stimulated with HS/FC or FC showed significantly high
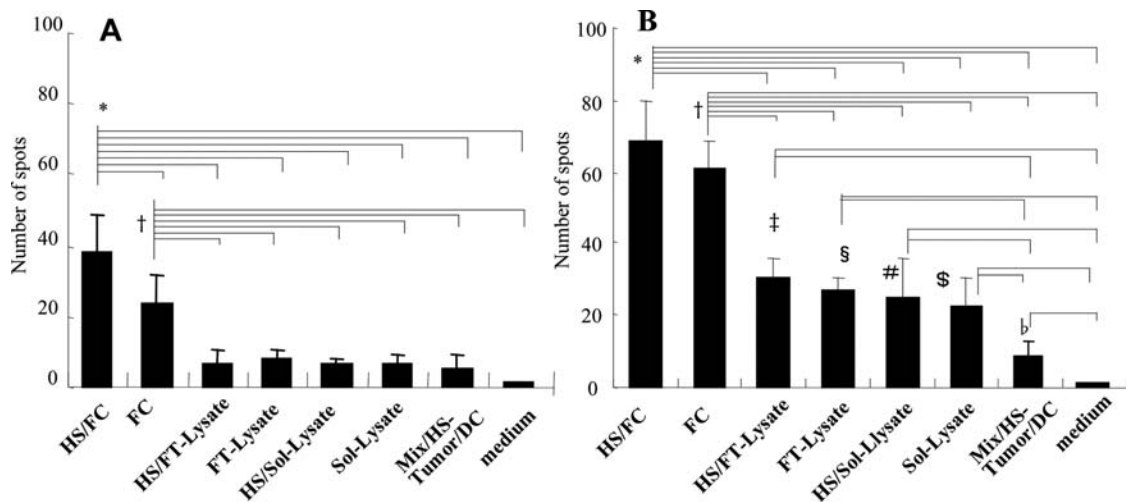
Figure 3. Comparison of IFN-γ productivity by tumor antigen-loading methods. CD8⁺ T cells were stimulated once (A) or twice (B) with autologous HS/FC, FC, Mixture if HS-Tumor and OK-DC (Mix/HS-Tumor/DC) or autologous DC pulsed with HS/FT-Lysate, FT-Lysate, HS/Sol-Lysate or Sol-Lysate. The number of IFN-γ production cells was examined by ELISpot assay. (A) *p<0.05 (HS/FC vs. all other groups), †p<0.05 (FC vs. all other groups). (B) *p<0.05 (HS/FC vs. all other groups), †p<0.05 (FC vs. all other groups), ‡p<0.05 (HS/FT-Lysate vs. Mix/HS-Tumor/DC and medium), §p<0.05 (FT-Lysate vs. Mix/HS-Tumor/DC and medium), #p<0.05 (HS/Sol-Lysate vs. Mix/HS-Tumor/DC and medium), $p<0.05 (Sol-lysate vs. Mix/HS-Tumor/DC and medium), ᵇp<0.05 (Mix/HS-Tumor/DC vs. medium). Mean ± SD (n=10).
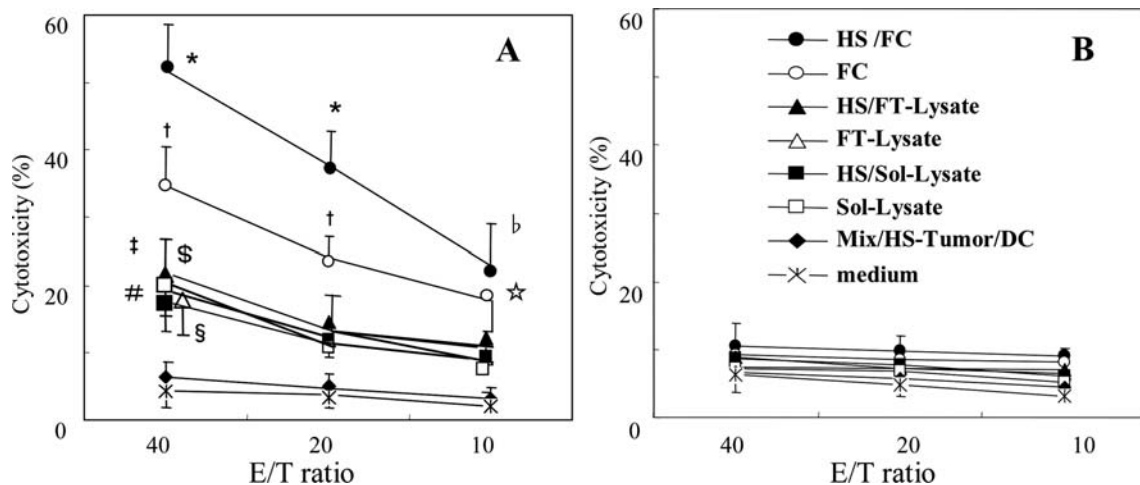

Figure 4. Comparison of CTL productivity by different tumor antigen-loading methods. CD8⁺ T cells were stimulated twice with autologous HS/FC, FC or autologous DCs pulsed with HS/FT-Lysate, FT-Lysate, HS/Sol-Lysate, Sol-Lysate, Mix/HS-Tumor/DC and medium. CTL activities against autologous tumor cells (A) or allogeneic MKN28 (B) were examined by ⁵¹Cr release assay. *p<0.05 (HS/FC vs. all other groups). †p<0.05 (FC vs. all other groups), ‡p<0.05 (HS/FT-Lysate vs. Mix/HS-Tumor/DC and medium), §p<0.05 (FT-Lysate vs. Mix/HS-Tumor/DC and medium), #p<0.05 (HS/Sol-Lysate vs. Mix/HS-Tumor/DC and medium), $p<0.05 (Sol-Lysate vs. Mix/HS-Tumor/DC and medium), ᵇ(HS/FC vs. all groups except FC), ☆(FC vs. Mix/HS-Tumor/DC and medium). Mean ± SD (n=10).

levels of IFN-γ-positive spots as compared with those of other groups (Fig. 3A). In contrast, autologous CD8⁺ T cells stimulated with DC pulsed with freeze-thawed lysates or soluble lysates did not show significant levels of spots. By the second stimulation, spots of IFN-γ-positive CD8⁺ T cells stimulated with HS/FC or FC were increased significantly more than those of HS/FT-Lysate, FT-Lysate, HS/Sol-Lysate, Sol-Lysate, Mix/ HS-Tumor/DC and medium. CD8⁺ T cells stimulated with DC pulsed with freeze-thawed lysate or soluble lysate showed significantly higher levels of IFN-γ-positive spots than those of Mix/HS-Tumor/DC and medium. Heat-stressed tumor cells enhanced productivity of IFN-γ, and the HS/FC-stimulated group showed the highest number

of spots in all groups. These results indicate that FC possess enhanced stimulatory ability of autologous CD8⁺ T cell IFN-γ production as compared with tumor lysate-pulsed DC, and modifications of FC by heat stress could enhance this ability.

CTL productivity of FCs and tumor lysate-pulsed DCs. CD8⁺ T cells from patients were stimulated twice with autologous DCs loaded with autologous tumor cells or tumor lysate, and their cytotoxicity against autologous tumor cells was examined (Fig. 4A). CTL activities induced by HS/FC were significantly higher than those of FC, HS/FT-Lysate, FT-Lysate, HS/Sol-Lysate, Sol-Lysate, Mix/HS-Tumor/DC and medium. The HS/FC group showed the highest cytotoxicity
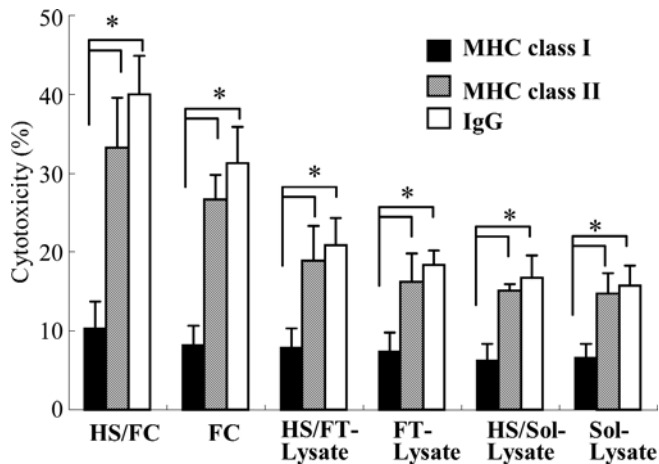
Figure 5. MHC restriction of CTL induced by FC or tumor lysate-pulsed DCs. CD8^{+} T cells from patients were co-cultured with autologous FC or tumor lysate-pulsed DCs (HS/FT-lysate, FT-lysate, HS/Sol-lysate, Sol-Lysate) and cytotoxicity against autologous tumor cell was examined by ^{51}Cr release assay. Target cells were pretreated with anti-MHC class I mAb, anti-MHC class II mAb or IgG. The ratio of E:T cells were 50:1. *p<0.05 (anti-MHC Class I vs. anti-MHC class II, IgG). Mean ± SD (n=10).

Figure 7. Production of HSP70 protein levels by ELISA. FCs (HS/FC, FC), tumor lysate-pulsed DCs (HS/FT-Lysate, FT-Lysate, HS/Sol-Lysate, Sol-Lysate), Mix/HS-Tumor/DC, HS/Tumor and Tumor were cultured for 3 days and HSP70 protein levels of supernatants were examined by ELISA. *p<0.05 (HS/FC vs. all groups except FC). †p<0.05 (FC vs. all groups except HS/FC). Mean ± SD (n=10).
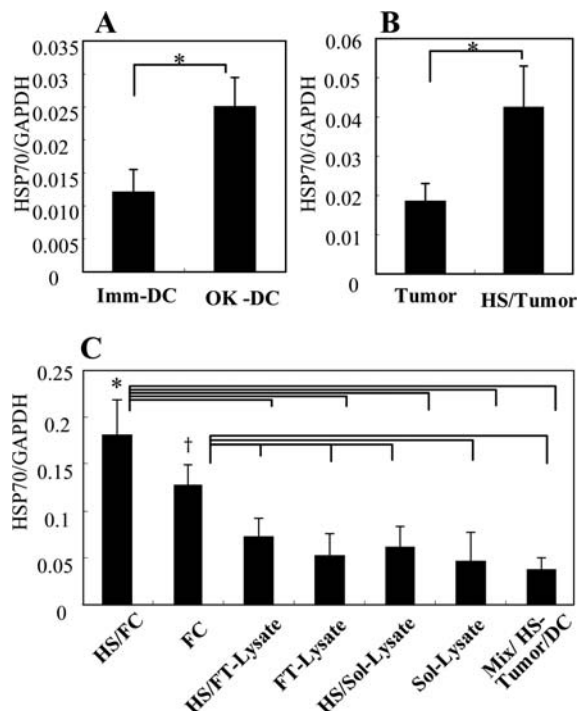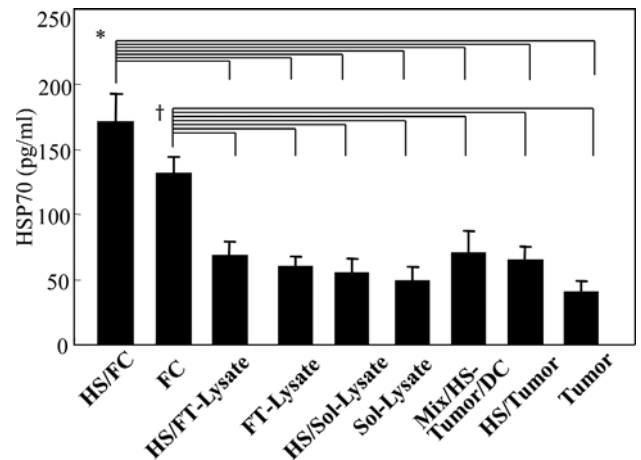

Figure 6. Comparison of HSP70 mRNA expression. (A) The ratios of copy numbers of HSP70 mRNA to GAPDH mRNA (HSP70/GAPDH) of OK432-treated DC and immature DC were examined using real-time RT-PCR. *p<0.05 (OK-DC vs. Imm-DC). (B) HSP70/GAPDH of HS/Tumor and Tumor was examined. *p<0.05 (HS/Tumor vs. Tumor). (C) HSP70/GAPDH of HS/FC, FC, HS/FT-Lysate, FT-Lysate, HS/Sol-Lysate, Sol-Lysate and Mix/HS-Tumor/DC was examined. *p<0.05 (HS/FC vs. all other groups except FC). †p<0.05 (FC vs. all other groups). Mean ± SD (n=10).
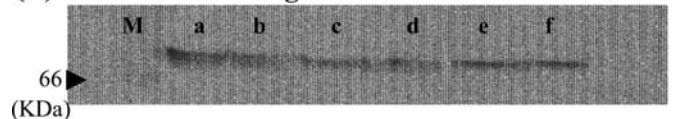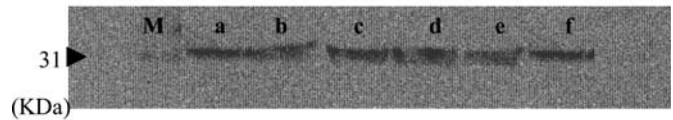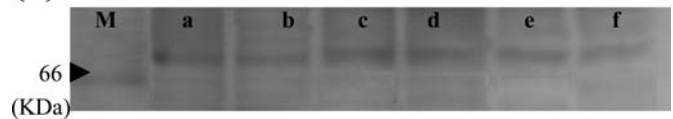

Figure 8. Detection of HSP70.PC by Western blotting and SDS-PAGE. The expressions of HSP70 with peptide complex (HSP70.PC) and ß-actin of immunoprecipitation samples in 8 groups were examined by Western blot analysis (A). Each lane show the molecule marker (M), HS/FC (a), FC (b), HS/Tumor (c), Tumor (d), HS/FT-Lysate (e), and HS/Sol-Lysate (f). After purification of immunoprecipitation samples by gel filtration chromatography, a portion was determined by SDS-PAGE (B). Each lane shows the molecule marker (M), HS/FC (a), FC (b), HS/Tumor (c), Tumor (d), HS/FT-Lysate (e), and HS/Sol-Lysate (f).

in all groups. CD8^{+} T cells stimulated with FC indicated significantly higher cytotoxicity than those of HS/FT-Lysate, FT-Lysate, HS/Sol-Lysate, Sol-Lysate, Mix/HS-Tumor/DC

and medium. In contrast, all lysate groups (HS/FT-Lysate, FT-Lysate, HS/Sol-Lysate and Sol-Lysate groups) showed significant lower levels of CTL activities than Mix/HS-Tumor/DC and medium groups. Next, we examined MHC restriction of CTL induced by FCs and DCs pulsed with tumor lysates. Cytotoxicity against allogeneic MKN28 (HLA-A*31012, HER-2^{+}) was not detected in any groups (Fig. 4B). Furthermore, CTL activities induced by HS/FC, FC, HS/FT-Lysate, FT-Lysate, HS/Sol-Lysate and Sol-Lysate groups were decreased by the addition of anti-MHC class I mAb but not anti-MHC class II mAb (Fig. 5). These results suggest that
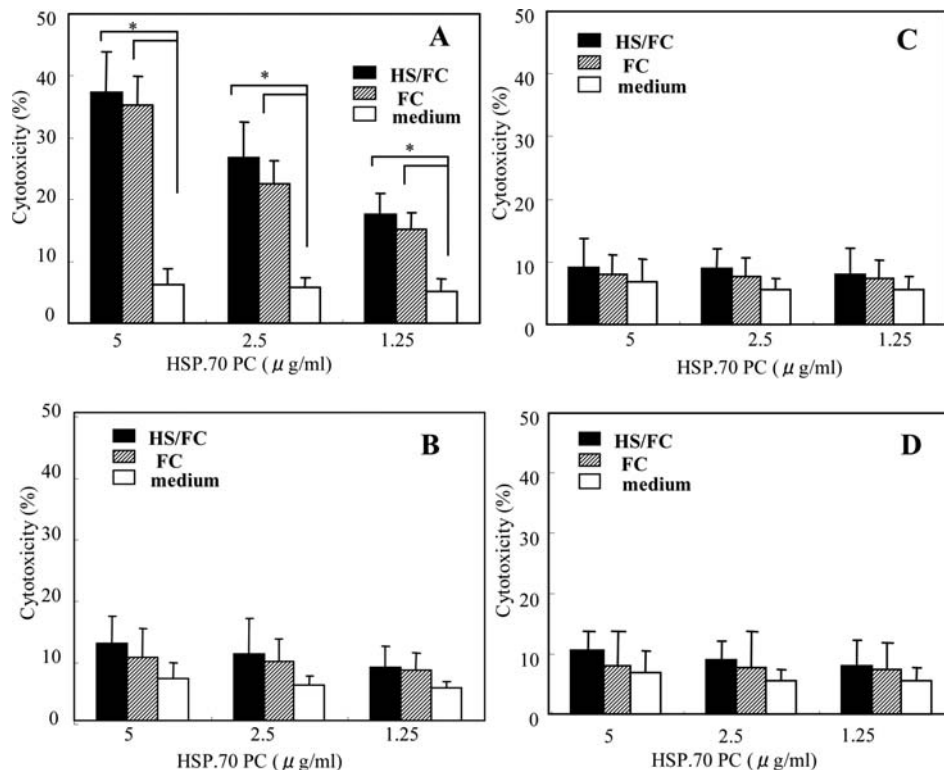
Figure 9. CTLs activity depend on the dose of HSP70.PC. CD8+ T cells from 5 patients were stimulated with autologous DCs pulsed with 5, 2.5 or 1.25 µg/ml of purified HSP70.PC extracted from HS/FC or FC. CTL activity against autologous tumor cells (A), allogeneic MKN28 tumor cells (B), autologous normal cells (C) and K562 cells (D) were examined by ^{51}Cr release assay. *p<0.05 (HS/FC, FC vs. medium). Mean ± SD (n=5).

FC possess stronger ability for MHC-restricted CTL production than DC loaded with soluble lysate or freeze-thawed tumor cell lysate. The modification of FC by heat stress could enhance the ability of CTL production.

Expression of HSP70 mRNA. The ratio of the HSP70 mRNA level to GAPDH level (HSP70/GAPDH) of OK-DC showed a significant increase compared to that of Imm-DC (Fig. 6A). HS-Tumor showed a significant increase of HSP70/GAPDH mRNA levels compared to the Tumor (Fig. 6B). Next, we examined the 8 groups of HSP70/GAPDH mRNA levels. HS-FC showed significantly the highest HSP70/GAPDH levels among the groups (Fig. 6C). HSP70/GAPDH levels of HS-FC and FC were significantly higher than those of HS/Sol-Lysate, Sol-Lysate, HS/FT-Lysate, FT-Lysate, DC/HS-Mixture and OK-DC. Our results suggest that FCs increase HSP70 mRNA levels as compared with DCs pulsed with tumor-soluble lysate and freeze-thawed lysate, and modification of FCs by heat stressing tumor cells increased the expression of HSP70 mRNA.

HSP70 protein levels by ELISA. HSP70 protein levels of HS/FC and FC were significantly higher than those of HS/FT-Lysate, FT-Lysate, HS/Sol-Lysate, Sol-Lysate, Mix/HS-Tumor/DC, HT/Tumor and Tumor (Fig. 7); HS/FC showed the highest protein levels in all groups. These results suggest that FCs increase HSP70 protein production more than DCs pulsed with tumor-soluble lysate and freeze-thawed lysate, and heat stressing tumor cells increased the production of HSP70 protein.

HSP70.PC levels by Western blotting. HSP70-peptide complex (HSP70-PC) separated by immunoprecipitation was purified by gel filtration chromatography, the expressions of HSP70.PC of HS-FC, FC, HS/Tumor, Tumor, HS/FT-Lysate and HS/Sol-Lysate were examined by Western blot analysis (Fig. 8A). All samples showed a clear band of HSP70.PC and ß-actin. Furthermore, immunoprecipitation samples were purified by gel filtration chromatography for CTL assay, and a portion of samples was examined for SDS-PAGE. In SDS-PAGE, clear band of HSP70.PC were detected in all samples.

Production of CTL activities by HSP70.PC-pulsed DCs. To examine the potential of HSP70.PC for CTL production, CD8+ T cells from five patients with gastric cancer were stimulated with autologous DCs pulsed with three different doses of HSP70.PC (5, 2.5, 1.25 µg/ml) purified by gel filtration chromatography. HSP70.PC were prepared from autologous HS/FC and FC, and CTL activities were examined against autologous tumor cells, allogeneic MKN28 tumor cells, autologous normal cell and K562 (Fig. 9). CTL activities against autologous tumor cells increased in a dose-dependent manner of HSP.70 PC (Fig. 9A). In contrast, allogeneic MKN28 and K562 cells did not show any CTL activities (Fig. 9B and C), and autologous normal cells also did not show cytotoxic activities (Fig. 9D).

Next, using the same volume of HSP70.PCs (5 µg/ml), CTL productivity of DCs pulsed with HSP70.PC was compared. HSP70.PC were prepared from 7 groups (Fig. 10). CTL activities against autologous tumor cells induced by DCs pulsed with HSP70.PC of HS/FC were significantly higher
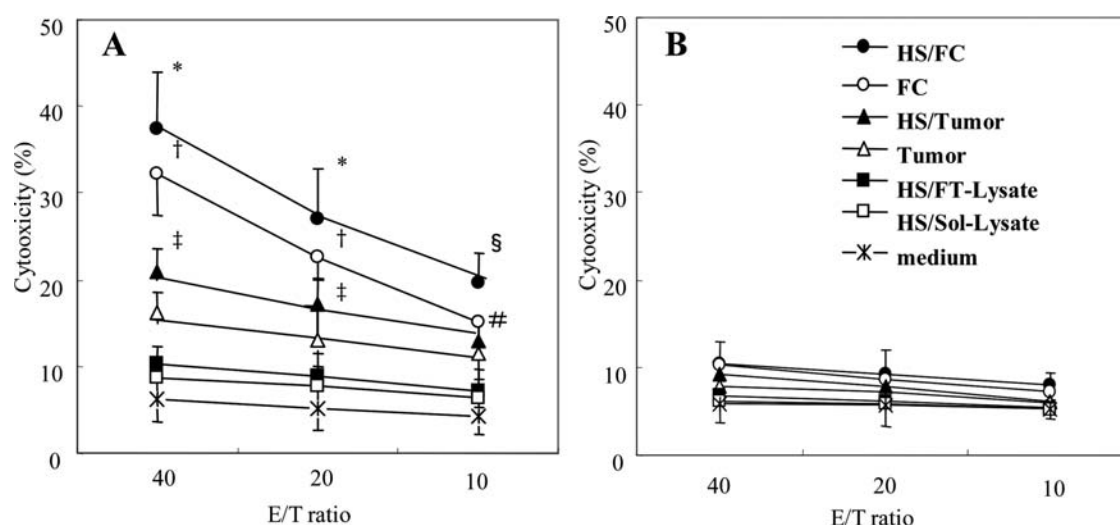
Figure 10. CTL activity by HSP70.PC-pulsed DC. To evaluate the potential of HSP70.PC for CTL production, CD8+ T cells from 5 patients were co-cultured with DC pulsed with 5 μg/ml of HSP70PC, and cytotoxicities against autologous tumor cells (A) and allogeneic MKN28 cells (B) were examined by ^{51}Cr release assay. HSP70.PC were extracted from HS/FC, FC, HS/Tumor, Tumor, HS/FT-Lysate, HS/Sol-Lysate. *p<0.05 (HS/FC vs. HS/Tumor, Tumor, HS/FT-Lysate, HS/Sol-Lysate and medium), †p<0.05 (FC vs. Tumor, HS/FT-Lysate, HS/Sol-Lysate and medium), ‡p<0.05 (HS/Tumor vs. HS/FT-Lysate, HS/Sol-Lysate and medium), §p<0.05 (HS/FC vs. Tumor, HS/FT-Lysate, HS/Sol-Lysate and medium) #p<0.05 (FC vs HS/FT-Lysate, HS/Sol-Lysate and medium). Mean ± SD (n=5).

than those of HS/Tumor, Tumor, HS/FT-Lysate, HS/Sol-Lysate and medium. DC pulsed with HSP70.PC of FC also showed significant higher CTL productivities than those of Tumor, HS/FT-Lysate, HS/Sol-Lysate and medium. HS/FC showed the highest cytotoxicity in all groups. HS/Tumor group induced significant CTL activities; however, these CTL levels were lower than those of FC groups. In contrast, DCs pulsed with HSP70.PC of HS/FT-Lysate or HS/Sol-Lysate did not induce CTL activity. Next, CTL activity against allogeneic MKN28 cells was examined, and it was clarified that CD8+ T cells stimulated with DCs pulsed with HSP70.PC in all 7 groups did not show distinct CTL activity (Fig. 10B). The present results suggest that DCs pulsed with HSP70.PC extracted from HS/FC and FC possess strong ability for CTL production but not NK and autoimmune responses; however, DCs pulsed with HSP70.PC of tumor lysate-loaded DCs did not induce CTL activity.

Discussion

The present studies demonstrated that CTL productivity of FCs is superior to that of DCs pulsed with soluble and freeze-thawed tumor lysates, and it is suggested that up-regulation of HSP70.PC was related to the excellent CTL productivity of FCs.

Abundant experimental evidence indicates that DCs loaded with tumor-associated antigens stimulate anti-tumor immune responses; however, a significant percentage of patients with advanced or metastatic tumors respond poorly to DC-based immunotherapy, as well as adjuvant therapy such as chemotherapy. To increase the efficacy of DC-based immunotherapy, improvement and optimization of vaccine, including the antigen-loading method, are desired. Using the mouse model, a few studies compared the immunotherapeutic reactivity of DCs loaded with a variety of antigen preparation, and demonstrated that the FCs of DCs with tumor cells generated a

superior anti-tumor effect to other antigen loading methods (4,5). However, in human model, there is considerable controversy as to which antigen-loading methods represent the most immunogenic forms of vaccines that are capable of eliciting clinically relevant therapeutic immunity. In our study using autologous DCs and tumor cells from patients with gastric cancer, we compared FCs and DCs pulsed with two types of tumor lysate, because these methods are one of the most popular methods and is used in clinical trials. Our present results demonstrated that FCs possess significantly higher ability for MHC class I restricted CTL production than those of DCs loaded with soluble or freeze-thawed lysate and modification of FCs by heat stress enhanced the ability of CTL production. To the best of our knowledge, the present study is the first to compare the modified cell fusion and tumor lysate using autologous DCs and tumor cells in patients with gastric cancer.

FCs from tumor cells and DCs possess both the cell components of tumor antigens and the accessory molecules necessary for antigen processing and presentation. Previous studies have demonstrated that FCs of tumor cells and DCs create an immunogenic cell by cytoplasm integration between the respective cells. Such integration makes it possible to exchange cytoplasmic contents and share cellular compartments. Our own and other studies have shown the co-expression of tumor-associated antigens and cell-surface molecules of DCs on FCs (4-7). In the present study, we modified FCs by heat stress of tumor cells and maturation of DC by OK-432. The streptococcal preparation OK432, which is a TLR4 agonist, is a commercially available good manufacturing practice grade immunomodulatory agent which has been widely used for cancer patients. We have reported that OK432 possesses strong ability for DC maturation (24). It is known that heat treatment of tumor cells could increase the expression of tumor antigens such as MUC1 (23). Our present data demonstrated that FCs of heat-stressed tumor cells and

ulated DC significantly increased CEA and HER-2 tigens and co-stimulated molecules such as CD80, CD83 and CD88, suggesting the enhanced immunogenicity of these modified FCs.

Next, we examined in more detail the superior CTL production of FCs. It was thought that there are several potential mechanisms for FCs to induce an effective CTL response to tumor antigens. Direct antigen presentation is consistent with DC characteristics; however, other mechanisms, such as cross-presentation, adjuvant activity and bystander effects, may also have important roles. It is known that HSPs can induce the cross-presentation of immunocomplex of tumor antigens. The promising potential of a molecular-chaperone-based tumor vaccine using the immunocomplex combined with HSP has been the focus of much attention; however, research into the mechanisms underlying the effect of HSP on immune cells is only at an early stage. By differences in molecule weight, HSPs are classified into families, such as HSP40, 60, 70, 90 and 110. In these HSP families, HSP70 is one of the important molecules because of its strong binding of the tumor antigen peptide, efficient uptake of HSP70 through the receptors, and activation of an immune response (18-22). HSP70 binds effectively to LOX-1, a member of C-type lectin receptors which is found on the DC cell surface, and signaling through LOX-1, including the activation of TLR-2 and innate immunity (25-27). It is known that at least 12 HSP70 genes are contained within human cells, and three (HSP-70-1a, HSP70-1b, HSP70-HOM) of these genes are encoded by genes within the MHC locus, suggesting a connection to immune function (28-32). Our results showed a significant up-regulation of HSP.70 mRNA and protein levels in FCs as compared with those of DCs pulsed with soluble tumor cell lysates or freeze-thawed tumor cell lysates. Furthermore, mRNA and the protein level of HSP70 of FCs increased significantly by heat stressing tumor cells. A separate study in colorectal cancer, has also demonstrated an increase of HSP70 protein levels in HS/FC, thereby supporting our results (23).

To identify the potential of HSP70.PC for CTL production, we isolated HSP70.PC from FCs by immunoprecipitation and purified them with fluid chromatography. Our present results demonstrate that DCs pulsed with HSP70.PC of FCs induced significantly higher levels of CTL activities than DCs pulsed with HSP70.PC or autologous tumor cell lysates. Using the mouse model, it has been reported that HSP70.PC of FCs stimulated T cell proliferation *in vitro* (18). However, to our knowledge, the present study is the first to demonstrate the superior CTL productivity of HSP70.PC prepared from heat-stressed FCs of gastric cancer patients.

In conclusion, we demonstrated the strong potential of an FC vaccine for CTL production, and this process may be related to the up-regulation of HSP70. The molecular mechanism of FCs that promotes the formation of immuno-genic complexes of HSP with tumor antigenic peptides is complex, and we need further study to clarify the process.

Acknowledgements

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