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Tumor-Derived Heat Shock Protein 70 Peptide Complexes Are Cross-Presented by Human Dendritic Cells

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Our study demonstrates that tumor-derived heat shock protein (HSP70) chaperones a tyrosinase peptide and mediates its transfer to human immature dendritic cells (DCs) by receptor-dependent uptake. Human tumor-derived HSP70 peptide complexes (HSP70-PC) thus have the immunogenic potential to instruct DCs to cross-present endogenously expressed, nonmutated, and tumor antigenic peptides that are shared among tumors of the melanocytic lineage for T cell recognition. T cell stimulation by HSP70-instructed DCs is dependent on the Ag bound to HSP70 in that only DCs incubated with HSP70-PC purified from tyrosinase-positive (HSP70-PC) but not from tyrosinase-negative (HSP70-PC/tyr−) melanoma cells resulted in the specific activation of the HLA-A*0201-restricted tyrosinase peptide-specific cytotoxic T cell clone. HSP70-PC-mediated T cell stimulation is very efficient, delivering the tyrosinase peptide at concentrations as low as 30 ng/ml of HSP70-PC for T cell recognition. Receptor-dependent binding of HSP70-PC and active cell metabolism are prerequisites for MHC class I-restricted cross-presentation and T cell stimulation. T cell stimulation does not require external DC maturation signals (e.g., exogenously added TNF-α), suggesting that signaling DC maturation is an intrinsic property of the HSP70-PC itself and related to receptor-mediated binding. The cross-presentation of a shared human tumor Ag together with the exquisite efficacy are important new aspects for HSP70-based immunotherapy in clinical anti-cancer vaccination strategies, and suggest a potential extension of HSP70-based vaccination protocols from a patient-individual treatment modality to its use in an allogeneic setting. The Journal of Immunology, 2002, 169: 5424–5432.

In murine systems, vaccination with heat shock proteins (HSPs) such as glucose-regulated protein (GP)96, HSP70, and HSP90 from cancer tissues but not from normal tissues induces specific immunity and CTL activation (1). The specificity of the induced CTLs relies on the peptides chaperoned by these HSPs (2, 3). This property allows CTL activation without the need to characterize the corresponding Ag, and provides the basis for a new type of vaccine against cancer (4–7). HSPs are classified into several families of sequence-related proteins. Among them, based on protein expression levels, the HSP70 family consists of the constitutively expressed HSP (HSC70) and the heat-inducible HSP (HSP70) 70-kDa proteins.

Immunization with HSP peptide complexes (HSP-PC) is exquisitely dependent on the presence of functional APCs in the immunized host, since depletion of such cells renders the host incapable of mounting immune responses after injection of HSP-PC preparations (8). Dendritic cells (DCs) are very effective activators of CTLs, a process that requires the presentation of Ag bound to MHC molecules, together with expression of adhesion and co-stimulatory molecules (9, 10). In particular, the ability to present exogenous Ags through “cross-presentation” is a key feature of DCs. This term was introduced to describe the representation of exogenous cell-associated Ags by MHC class I (11) and II molecules (12). It became evident that GP96- and HSP70-chaperoned peptides can be presented to CTLs by DCs in the context of MHC class I molecules, and uptake of GP96 or HSP70 requires receptor-mediated endocytosis (2, 13, 14). Recent data provide evidence for the existence of distinct receptors for HSPs (e.g., GP96, HSP90, HSP70) on murine APCs (15), and one of them has been identified as CD91 (16, 17).

The mechanisms involved in the stimulation of T cell responses via HSP70 and GP96 were studied in murine systems using induced tumors and model Ags (18–20), OVA (21), and viral Ags (22, 23). More recently, immunization with tumor-derived HSPs has also been demonstrated for spontaneous tumors (24). These analyses provided the principle knowledge of HSP-mediated cross-presentation and the involvement of APCs and have been the basis for the first clinical trials involving tumor-derived GP96 (5, 6, 25).

Different from most of the studies described so far, we investigated the role of HSP70 family members in a system that more
closely resembles the patient situation. Most studies investigating mechanistic events related to HSP-mediated cross-presentation involved highly immunogenic Ags either induced by mutagenesis or overexpressed by transfection, a situation that does not reflect most human cancers. We selected a human melanoma system and investigated the role of HSP70 in the cross-presentation of the tyrosinase Ag, an endogenously expressed, nonmutated tumor-associated differentiation Ag of low immunogenicity (26) that is shared among tumors of the melanocytic lineage.

We demonstrate that HSP70-PC purified from tyrosinase-positive (HSP70-PC/tyr+) but not from tyrosinase-negative (HSP70-PC/tyr−) melanoma cells delivers the tyrosinase Ag to immature DCs for MHC class I-restricted T cell recognition. Activation of the tyrosinase-specific T cell clone was inhibited when binding of HSP70-PC/tyr− to DCs was competitively blocked by HSP70-PC not carrying the tyrosinase Ag. T cell stimulation by immature DCs incubated with HSP70-PC/tyr− was very efficient even without additional DC maturation signals (e.g., exogenous TNF-α), demonstrating the ability of tumor-derived HSP70-PC to act as a chaperone for peptides and a signal for DC maturation. Using a human tumor system, it can be concluded that endogenously expressed nonmutated and shared tumor Ags with low immunogenicity (26) are chaperoned by HSP70 and efficiently presented by DCs for T cell recognition.

Materials and Methods

Reagents

SPA-810 mAb (clone C92F3A-5, specific for the inducible protein HSP70) and rat mAb SPA-815 (clone 1B5, specific for the protein HSC70) were purchased from StressGen Biotechnologies (Victoria, British Columbia, Canada). Anti-HSP70 mAb (clone BM-22, specific for both HSP70 and HSC70) and rat mAb (clone FL-66) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Rat mAb HSP-6B3, specific for the inducible isoform of HSP70 was generated by our in-house facility. Approximately 50 μg of recombinant human (rh) HSP70 expressed in Escherichia coli (protein SPP-755; StressGen Biotechnologies) were injected i.p. and s.c. into LOUC rats. After a 2-mo interval, a final boost was given i.p. and s.c. 3 days before fusion. Fusion of the myeloma cell line P3X63-Ag8.653 with the rat immune spleen cells were performed according to standard procedure. Hybridoma supernatants were tested in a solid-phase immunoassay using recombinant HSP70 adsorbed to polystyrene microtiter plates. Following incubation with culture supernatants for 1 h, bound mAbs were detected using peroxidase-labeled goat anti-rat IgG1 and a peroxidase-labeled p-nitrophenyl substrate. Color detection was performed by the addition of p-nitrophenyl substrate and absorbance was measured at 405 nm.

The C-19 goat polyclonal Ab specific for tyrosinase was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-tubulin mAb (Santa Cruz Biotechnology) was used in Western blotting procedures to control loading. Abs used for FACS analysis included PE-conjugated anti-CD14, anti-CD83, and goat anti-mouse IgG (Beckman Coulter, for protein loading. Abs used for FACS analysis included PE-conjugated anti-CD14, anti-CD83, and goat anti-mouse IgG (Beckman Coulter, Germany). Anti-HSP70 mAb (clone BRM-22) were pooled. Sephadex G-25, ADP–exchange chromatography using MonoQ Sepharose and eluted over a 20–500 mM NaCl gradient. Fractions containing HSP70-PC as a single protein, as determined by SDS-PAGE silver stain and by Western blot with anti-HSP70 mAb (clone BM-22) were pooled. SDS-PAGE, two-dimensional (2D) isoelectric focusing (IEF)/SDS-PAGE, and Western blot analysis

For analysis of protein content in various fractions of the HSP70-PC isolation steps, samples were denatured by boiling for 5 min in SDS sample buffer and separated on 10% SDS-PAGE. After electrophoresis, proteins were either silver stained or transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) and probed with appropriate Abs as described in the figure legends. Detection of proteins was achieved by the ECL system (Amersham Life Science, Karlsruhe, Germany).

The natural expression levels for HSC70, HSP70, and tyrosinase in cultured cells were determined using cell lysates (lysate buffer; 2% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate in 50 mM HEPES and 200 mM NaCl (pH 7.5), and protease inhibitors). Equal amounts of protein (40 μg/lane measured with the Bio-Rad assay kit) were boiled for 5 min in SDS-sample buffer and separated on a 10% SDS-PAGE. After electrophoresis, Western blot analysis was performed as described above. For detection of tyrosinase, the polyclonal goat serum C-19 was used. HSP70 and HSC70 were detected using the rat mAbs HSP-6B3 and SPA-815, respectively. Equal protein loading was verified by reprobing the blots with anti-β-tubulin Ab (Santa Cruz Biotechnology, data not shown).
For 2D-IEF/SDS-PAGE, the first dimension used IEF using a Multiphor II electrophoresis unit and ImmunobilineDryStrip (IPG) (pH 3–10 NL), all from Amersham Pharmacia Biotech. A total of 100 ng of purified HSP70-PC were solubilized in lysis buffer (10 M urea, 1% DTT, 4% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate, 2.5 mM EDTA, 2.5 mM EGTA) supplemented with 0.05% bromophenol blue and IPG buffer (pH 3–10 NL; Amersham Pharmacia Biotech), loaded onto the IPG (pH 3–10 NL), and covered with parafilm. The running conditions were 91 kVh. IEF was followed by a vertical 10% SDS-PAGE. The IPG strip was equilibrated and placed on top of the vertical 10% SDS-PAGE. After separation, Western blotting and immune detection using the anti-HSP70 and HSC70 mAb (BRM-22) and the ECL kit were performed.

Fluorescence labeling of proteins

Purified HSP70-PC (from A375-MEL melanoma cells) or BSA (1 mg/ml) was incubated with FITC (50 μg; Sigma-Aldrich) or FluoroLink Cy5 (Amersham Pharmacia Biotech) in carbonate-bicarbonate buffer (0.1 M; pH 9.5) overnight at 4°C, or 5 h at room temperature for Cy5. Free unconjugated FITC was removed by passing the mixture over a gel filtration column (Sephadex G-25; Amersham Pharmacia Biotech). For Cy5-labeling, protein was dialyzed (12–14 kDa dialysis membrane) for 12–14 h. FITC-conjugated proteins were analyzed by SDS-PAGE and immunoblotting using anti-HSP70 mAb (clone BRM-22) and anti-FITC Ab. Labeled proteins were centrifuged at 100,000 × g before use to remove any particulate matter.

FACS analysis

For phenotypic characterization, 2 × 10⁵ cells were stained with PE-labeled mAb to CD14 and CD83, FITC-labeled BSA, and FITC-labeled HSP70-PC/tyr⁻¹ or FITC-HSP70-PC/tyr⁻¹ (each 100 ng/ml) in PBS supplemented with 5% FCS for 30 min at 4°C. For competition studies, labeled (100 ng/ml) and unlabeled proteins (in concentrations to reach indicated ratios) were mixed and added to the cells. Cells were washed with ice-cold PBS and analyzed by flow cytometry using a FACScan with CellQuest software (BD Biosciences, Mountain View, CA). The analysis gate was set on propidium iodide negative cells and cell type characteristic forward (forward light scatter) and orthogonal scatter (side light scatter). For intracellular staining of HSC70 and HSP70, cells were permeabilized using the FIX and PERM kit (Dianova). Then, mAb SPA-815 (HSC70) and SPA-810 (HSP70), respectively, in combination with FITC-labeled secondary reagents were applied.

Confocal microscopy

Immature DCs were incubated with 5 μg Cy5-labeled HSP70-PC or Cy5-labeled BSA for 30 min at 4°C (surface staining) or at 37°C (uptake studies). After washing, cells were settled on poly-L-lysine-coated glass slides, labeled mAb to CD14 and CD83, FITC-labeled BSA, and FITC-labeled HSP70-PC buffer (pH 3–10 NL; Amersham Pharmacia Biotech), loaded onto the IPG (pH 3–10 NL), and covered with parafilm. The running conditions were 91 kVh. IEF was followed by a vertical 10% SDS-PAGE. The IPG strip was equilibrated and placed on top of the vertical 10% SDS-PAGE. After separation, Western blotting and immune detection using the anti-HSP70 and HSC70 mAb (BRM-22) and the ECL kit were performed.

Cross-presentation assay

Activation of the T cell clone TyrF8 was assessed by measurement of the amount of IFN-γ released into the culture supernatant. DCs derived from monocyte precursors were seeded on day 7 at a concentration of 10³ cells/well in 96-well round bottom plates in 100 μl of DC culture medium, and indicated concentrations of the HSP70-PC were added. After 24 h, DCs were induced to mature by addition of 200 U/ml TNF-α and incubated for a further 24 h. As a control for the T cell stimulation capacity of the DCs, tyrosinase peptide (aa 368–376; YMNGTMSQV) was added exogenously at concentrations ranging from 1–10 μg/ml to TNF-α-matured DCs. Before T cell addition, the DCs were irradiated (5,000 rad). TyrF8 cells were added (2 × 10⁵ cells/well) in 100 μl of medium to give final concentrations of 25 U/ml IL-2, 5% FCS, and 5% human serum. After 24 h, culture supernatants were harvested and the content of IFN-γ was measured by OpEIA (BD Biosciences). When the cross-presentation assay was performed to investigate the ability of HSP70-PC to mature DCs, we added polymyxin B (Sigma-Aldrich) at a concentration of 1 μg/ml together with HSP70-PC to rule out the possible influence of LPS contamination. For Ab-blocking experiments, before adding the T cells, DCs were incubated for 1 h at 37°C with the mAb HB54 (20 μg/ml) recognizing an antigenic determinant shared by HLA-A*02 and B17 (29). In some experiments, immature DCs were fixed with PFA (1%) for 10 min at room temperature, then they were washed three times with excess of VLE medium before being used in cross-presentation assays (32).

Results

Differential surface binding of tumor-derived HSP70-PC to human monocyte-derived APCs

We previously showed that rh-HSP70 binds to human monocytes (33) and DCs (34). In this study, we demonstrate that HSP70-PC isolated from human melanoma cell lines exhibit a differential binding pattern to human APCs derived from the monocytic lineage. Monocytes (CD14⁺, CD83⁻), immature DCs (CD14⁺, CD83⁻) and mature DCs (CD14⁺, CD83⁺) were coincubated with FITC-conjugated HSP70-PC at 4°C to exclude endocytosis, and surface binding characteristics were analyzed by flow cytometry (Fig. 1A). BSA-FITC used as a control did not result in significant binding to any of the APCs tested. HSP70-PC bound to immature and mature DCs with similar high intensity (mean fluorescence of 40), whereas monocytes bound HSP70-PC at a low intensity (mean fluorescence of 8). The binding characteristics of HSP70-PC thus differed from our previous results with rh-HSP70, which did not bind to mature DCs (34).

2D-IEF/SDS-PAGE analysis of HSP70-PC revealed a heterogeneous composition of melanoma-derived HSP70-PC consisting of both the HSC70 and the inducible HSP70 proteins (Fig. 2A). This composition was found to reflect the natural HSP70 expression pattern of the melanoma cell lines, 624.38-MEL, SK23-MEL, and A375-MEL, that were used for HSP70-PC isolation. As demonstrated by intracellular FACS analysis and Western blot, these cell lines expressed the HSC70 and HSP70 isoforms at physiological growth conditions (Fig. 2B).

To negate the possibility that binding of HSP70-PC to DCs was a consequence of nonspecific events, competition assays between unlabeled HSP70-PC and FITC-labeled HSP70-PC were performed (Fig. 1B). Unlabeled HSP70-PC competitively inhibited the binding of FITC-labeled HSP70-PC to immature DCs. Inhibition was shown to be dependent on the ratio of FITC-labeled to nonlabeled HSP70-PC and displayed a saturation kinetics characteristic for receptor-mediated binding. The inhibition of binding was highly significant at a 10-fold excess of unlabeled HSP70-PC (p < 0.005). In contrast, the surface binding was not affected using a 1:10 ratio of HSP70-PC-FITC to unlabeled BSA.

HSP70 from melanoma cells chaperones the tyrosinase peptide and delivers it to DCs for MHC class I-restricted cross-presentation

To determine the functional consequence of HSP70-PC binding to APCs, experiments were performed in which we tested whether peptides chaperoned by HSP70-PC are effectively cross-presented by MHC class I molecules to T cells. HSP70-PC were isolated from two tyrosinase-positive (HSP70-PC/tyr⁻¹) and one tyrosinase-negative (HSP70-PC/tyr⁺) melanoma cell lines, 624.38-MEL, SK23-
TyrF8 activation was Ag-dependent, since HSP70-PC purified cell lines 624.38-MEL and SK23-MEL (data not shown). Monocytes, immature DCs, and mature DCs were stained with FITC-labeled BSA or FITC-HSP70-PC (from A375 melanoma cells) together with PE-labeled anti-CD14 or anti-CD83 Ab. The gate was set on propidium iodide-negative cells. Percentage of cells is shown for each quadrant. Data are representative of three independent experiments with similar results.

A. Inhibition of surface binding by unlabeled HSP70-PC. Immature monocyte-derived DCs were labeled with FITC-BSA ( ), HSP70-PC-FITC alone or indicated mixtures of labeled and unlabeled HSP70-PC ( ), or a mixture of HSP70-PC and unlabeled BSA, ratio 1:10 ( ), and analyzed by flow cytometry. Data represent mean fluorescence intensity (mean ± SEM) of results from three independent experiments. Statistical significance of data was calculated compared with that of FITC-HSP70-PC alone. *p < 0.5; and ***p < 0.005.

Receptor-dependent binding of HSP70-PC, uptake, and active cell metabolism are required for cross-presentation of chaperoned peptides and T cell stimulation

To test that receptor-dependent binding of HSP70-PC is required for T cell activation, the T cell stimulation capacity of DCs pulsed with HSP70-PC/tyr was measured in the presence of increasing amounts of HSP70-PC/tyr . IFN-γ secretion induced by HSP70-PC/tyr was inhibited by 80% when DCs were coincubated with a 10-fold excess of HSP70-PC/tyr .

FIGURE 1. A. Differential surface binding of tumor-derived HSP70-PC to various monocyte-derived human APCs. Monocytes, immature DCs, and mature DCs were stained with FITC-labeled BSA or FITC-HSP70-PC (from A375 melanoma cells) together with PE-labeled anti-CD14 or anti-CD83 Ab. The gate was set on propidium iodide-negative cells. Percentage of cells is shown for each quadrant. Data are representative of three independent experiments with similar results. B. Inhibition of surface binding by unlabeled HSP70-PC. Immature monocyte-derived DCs were labeled with FITC-BSA alone ( ), HSP70-PC-FITC alone or indicated mixtures of labeled and unlabeled HSP70-PC ( ), or a mixture of HSP70-PC and unlabeled BSA, ratio 1:10 ( ), and analyzed by flow cytometry. Data represent mean fluorescence intensity (mean ± SEM) of results from three independent experiments. Statistical significance of data was calculated compared with that of FITC-HSP70-PC alone. *p < 0.5; and ***p < 0.005.

FIGURE 2. A. Isolation of HSP70-PC from melanoma lines. Left panel. Aliquots from each purification step were resolved by SDS-PAGE (10%) and silver stained. Lane 1, Crude lysate; lane 2, desalted lysate; lane 3, ADP-binding proteins; lane 4, ADP-nonbinding proteins; lanes 5 and 6, desalted ADP-binding proteins; lane 7, HSP70-PC after anion exchange chromatography (pooled fractions). Purity of the last fraction was estimated to be >95%. Right panel, 2D-IEF/SDS-PAGE of HSP70-PC from A375-MEL cells followed by Western blotting with anti-HSP70 mAb (BRM-22), recognizing both the HSP70 and HSC70 isoforms of the 70-kDa HSP family. Data are those obtained from the preparation of A375 cells and are representative of three independent experiments with similar results. HSP70-PC from 624.38-MEL and SK23-MEL cells displayed similar composition.

B. Natural expression (at 37°C) of HSP70, HSC70, and tyrosinase by melanoma cell lines 624.38-MEL, SK23-MEL, and A375-MEL. Intracellular FACS staining was done for 624.38-MEL (BRM-22), recognizing both the HSP70 and HSC70 isoforms of the 70-kDa HSP family. Data are those obtained from the preparation of A375 cells and are representative of three independent experiments with similar results. HSP70-PC from 624.38-MEL and SK23-MEL cells displayed similar composition. HSP70, HSC70, and tyrosinase were also analyzed by Western blotting. Gels were run in parallel and probed with Abs to HSC70 (SPA-815), HSP70 (HP-6B3), and tyrosinase (C-19).
FIGURE 3. HSP70-PC from melanoma cells chaperone the tyrosinase peptide for MHC class I-restricted cross-presentation by human DCs. A, Immature DCs (HLA-A*02-positive) were incubated with indicated amounts of HSP70-PC/tyr\(^{+}\) (from 624.38-MEL cells, ■) or HSP70-PC/tyr\(^{-}\) (A375-MEL cells, □), matured with exogenous TNF-\(\alpha\) and cocultured with the HLA-A2-restricted tyrosinase-specific T cell clone TyrF8. Ag-specific stimulation of TyrF8 is demonstrated by the amount of IFN-\(\gamma\) secreted. \(Y_{50}\) indicates the amount of HSP70-PC/tyr\(^{+}\) (74.76 ng/ml) required for half maximal stimulation of TyrF8. T cell stimulation requires coculture of T cells with HSP70-PC loaded DCs. Neither DCs alone (B) nor TyrF8 alone (C) secrete IFN-\(\gamma\) after incubation with HSP70-PC/tyr\(^{+}\) (■) or HSP70-PC/tyr\(^{-}\) (□). Five independent preparations of the HSP70-PC from the tyrosinase-positive 624.38 cell line and one preparation of tyrosinase-positive cell line SK23-MEL (data not shown) were tested for cross-presentation. All preparations were tested repeatedly and induced IFN-\(\gamma\) secretion with experimental variations ranging between 500 and 80 pg/ml of IFN-\(\gamma\) at 100 ng/ml of HSP70-PC (data not shown). The tyrosinase-negative cell line A375-MEL and tyrosinase-negative B-LCL were also repeatedly tested and never found to induce significant amounts of IFN-\(\gamma\) (data not shown). Variations in dose dependency of individual HSP70-PC preparations from tyrosinase-positive melanoma cell lines might be related to different purities of the preparations. As determined by silver staining, some HSP70-PC preparations contain other, yet undefined proteins, different from HSC70 or HSP90 (data not shown). B, Stimulation of TyrF8 by HSP70-PC-loaded DCs is HLA-A*02-restricted. IFN-\(\gamma\) secretion of TyrF8 was measured after coculture with HSP70-PC/tyr\(^{+}\) (from 624.38-MEL)-loaded DCs in the absence (■) or presence (□) of anti-HLA-A2 Ab (HB54). Values of \(p\) were calculated for all data, comparing IFN-\(\gamma\) values in the presence of HB54 to that in the absence of HB54. **, \(p < 0.05\); and ***, \(p < 0.005\). Data represent the mean IFN-\(\gamma\) concentration in picograms per milliliter (mean ± SEM) of results from four independent experiments.

(Fig. 4A, \(p < 0.005\)). However, excess of BSA did not influence IFN-\(\gamma\) secretion.

The binding of HSP70-PC to DCs and early downstream consequences of binding were analyzed by confocal laser scanning microscopy. Immature DCs were incubated for 30 min with Cy5-conjugated HSP70-PC at 4°C to exclude endocytosis, or at 37°C to induce uptake. Cy5-labeled BSA was used as a negative control. After staining, cells were settled on poly-L-lysine-coated glass slides, fixed and analyzed for transmission and fluorescence. Consistent with the observed FACS analysis (see Fig. 1), BSA did not result in detectable staining of DCs (Fig. 4B, c, d, g, h, k, and l), while all DCs were stained strongly positive with HSP70-PC (Fig. 4B, a, b, e, f, i, and j). Different staining patterns were observed at 4°C and 37°C. At 4°C (Fig. 4B, a, b, e, and f), the fluorescence signal was localized to the cell surface. In contrast, at 37°C (Fig. 4B, i and j) this surface staining was replaced by a vesicular staining at two distinct subcellular locations. Fluorescent signals were localized to perinuclear areas and clusters of focal staining near the cell surface, presumably early endosomes.

These staining patterns suggested that HSP70-PC, after binding to the cell surface, was translocated into the cell interior, a process requiring active cell metabolism. To prove that HSP70-PC does not deliver its peptide cargo to cell surface MHC directly but chaperones it through an intracellular pathway for loading onto newly synthesized MHC class I molecules, immature DCs were fixed with PFA before being used in cross-presentation assays. PFA fixation completely abrogated T cell stimulation, while exogenously added tyrosinase peptide was still efficiently presented (Fig. 4C).

HSP70-PC-dependent cross-presentation and T cell stimulation do not require additional external DC maturation signals

For our cross-presentation assays, we used immature DCs because they demonstrated strongest binding for HSP70-PC and because they are highly efficient in Ag uptake and processing (10). Based on the rationale that for the process of T cell stimulation mature DCs are the most efficient, we added TNF-\(\alpha\) exogenously after HSP70-PC had bound to immature DCs and had delivered the peptide cargo. In the meantime, others and we had observed that rh-HSP70 stimulates secretion of inflammatory cytokines, including TNF-\(\alpha\), from monocytes and DCs (33, 35, 36), and induces maturation of DCs (34, 37, 38). Therefore, we reasoned that DCs through binding HSP70-PC might be stimulated to release TNF-\(\alpha\).
FIGURE 4. Receptor-dependent binding of HSP70-PC, uptake, and active cell metabolism are required for T cell stimulation. A, Inhibition of HSP70-PC/tyr⁻ surface binding to DCs by HSP70-PC/tyr⁻ blocks T cell stimulation. Immature DCs were pulsed with HSP70-PC/tyr⁻ (from 624.38-MEL) alone and various mixtures of HSP70-PC/tyr⁻ with HSP70-PC/tyr⁺ (from A375-MEL) or with a mixture of BSA and HSP70-PC/tyr⁻ (10:1). Maturation of DC was induced by TNF-α, and TyrF8 stimulation was measured by determining the amount of IFN-γ released. Data represent mean IFN-γ concentration in picograms per milliliter (mean ± SEM) of results from two independent experiments. ***, p < 0.005, comparing the IFN-γ value of the 10:1 ratio of HSP70-PC/tyr⁻ and HSP70-PC/tyr⁺ with that of the 10:1 ratio of BSA and HSP70-PC/tyr⁻.

B, Confocal microscopy of immature DCs stained with Cy5-labeled HSP70-PC or Cy5-labeld BSA at 4°C (a–h, surface binding) and 37°C (i–l, uptake). Cy5-labeled BSA was used for control staining (c, d, g, h, k, and l). Cells were analyzed for fluorescence (shown in yellow) and transmission (shown as overlay with the fluorescence signal). Scale bars indicate respective magnifications. a–d, An overview for the staining at 4°C demonstrating that all cells stain positive for Cy5-HSP70-PC and none are positive for Cy5-BSA. For presentation purpose, fluorescence images for Cy5-BSA stainings (c, d, g, h, k, and l) are digitally enhanced twice to allow detection of residual fluorescence. Individual cells are depicted at higher magnification (e–l) to visualize discrete staining patterns at 4°C (surface) and 37°C (perinuclear and vesicular). C, PFA-fixation of cells abrogates T cell stimulation. Left panel, DCs fixed with PFA (1% for 10 min at room temperature) were unable to perform HSP70-PC-mediated cross-presentation, but retained the ability to present exogenously added tyrosinase peptide. Untreated DCs are shown in the left panel.
Tumor-derived HSP70-PC mediate cross-presentation

FIGURE 5. HSP70-PC-mediated cross-presentation and T cell stimulation do not require external (i.e., TNF-α-induced) DC maturation. Immature DCs were incubated with indicated amounts of HSP70-PC/tyr and either treated with HSP70-PC/tyr and either left untreated (intrinsc DC maturation by HSP70-PC) or gave TNF-α (external DC maturation) before addition of the T cells. As shown in Fig. 5, cross-presentation by HSP70-PC-treated DCs without exogenously added TNF-α was even stronger than that with additional TNF-α (p < 0.005). Polymyxin B, a potent inhibitor of LPS, was included in the cross-presentation assay. No inhibitory effect on IFN-γ secretion by the T cells was observed, ruling out the possibility that endotoxin contamination within the HSP70-PC preparations was responsible for DC maturation and their ability to efficiently stimulate the T cells. Immature DCs treated with HSP70-PC only consistently performed better in Ag-specific and allogeneic T cell stimulation assays than those treated with HSP70-PC and TNF-α (data not shown).

Discussion

In the last years, HSPs have been proposed as a tool for cancer therapy. They can function as tumor-associated activation structures (i.e., HSP70) if detected on the surface of tumor cells, thereby activating NK cells (39, 40), or as Ag-presenting molecules, eliciting a specific T cell response through peptides associated in HSP-PC (1-7).

Using HSP70-PC/tyr and HSP70-PC/tyr, and by focusing on the nature of the APCs that mediate cross-presentation as well as the biochemical composition of the tumor-derived HSP70-PC, we were able to demonstrate that the immunogenic potential of HSP70 as a tool to induce anti-tumor immune responses can be extended to naturally expressed nonmutated human tumor Ags of low immunogenicity (26). Binding of HSP70-PC to DCs and intracellular events are required for HSP70-mediated cross-presentation. Although HSP70-PC bound to immature and mature DCs with similar intensity, immature DCs were more efficient in cross-presentation than mature DCs (data not shown). This makes sense from an immunological point of view, since immature DCs are better in Ag uptake than monocytes or mature DCs and have a strong capacity to process Ag (10, 41). Ag processing ability is potentially useful if peptides bind to HSP70 as longer precursors.

Our results demonstrate further that HSP-PC-mediated cross-presentation by immature DCs does not require external maturation signals, such as TNF-α. This finding is consistent with previous observations that HSPs represent natural danger signals to the immune system. When released by stressed cells (42, 43), they stimulate monocytes and DCs to secrete proinflammatory cytokines (TNF-α, IL-12) (33, 35, 36, 44), and are maturation signals for immature DCs (34, 37, 38, 45, 46). Our results demonstrating efficient cross-presentation without external DC maturation signals indicate that the two properties—the chaperoning of antigenic peptides and the induction of DC maturation—are intimately linked within tumor-derived HSP70-preparations. A similar conclusion can be reached for GP96 (47). The significance for the clinical use of tumor-derived HSP preparations in stimulating anti-tumor immune responses is discussed below.

The biochemical analysis of our HSP70 preparations revealed that they consisted of both the HSC70 and the HSP70. This was found to reflect the natural expression pattern of HSP70 and HSC70 in the melanoma cell lines used for HSP70-PC isolation. Similar constitutive expression of HSP70 has also been described for surgical specimens of primary and metastatic human melanoma (48). Our previous findings that rh-HSP70 but not recombinant HSC70 is able to deliver the DC maturation signal (44) indicate that the heterogeneous composition of tumor-derived HSP70-PC might be of functional relevance for the cross-presentation.

The ability to cross-present HSP-bound Ag has also been shown for APCs other than DCs, including monocytes (49) and macrophages (50). Using blood monocytes, Castelli et al. (49) demonstrated HSP70-mediated cross-presentation for melanoma Ags other than tyrosinase. However, in their system a much higher number of APCs and significantly more HSP70-PC were required for T cell stimulation than in our system. Possibly, the poor binding of HSP70 to monocytes is one explanation for this difference (see Fig. 1A). Furthermore, it is our own experience that experimental variations in APCs, i.e., DCs grown in FCS vs autologous serum, not fully differentiated DCs still expressing residual CD14, or matured DCs expressing CD83, influence the efficiency of cross-presentation (data not shown).

The studies presented in this report are of special clinical interest for HSP70-based vaccinations. First, HSP70-PC-mediated Ag presentation by DCs is very efficient, requiring low amounts (in the nanogram range) of HSP70-PC (Fig. 3; and Ref. 6). This can be explained by the receptor-mediated uptake for HSP70-PC vs fluid phase uptake and surface peptide exchange mechanisms for exogenous peptides, respectively. In addition, Binder et al. (51) described that HSP70 positively influences cross-presentation of chaperoned peptides by efficiently directing them to an endoplasmic reticum/Golgi compartment where loading onto the MHC class I molecules occurs. The observation that an endoplasmic reticulum/Golgi localization is important for efficient cross-presentation is consistent with our confocal microscopic data that show a perinuclear staining of immature DCs incubated with Cy5-labeled HSP70-PC at 37°C (see Fig. 4B). An additional explanation for the efficacy of HSP preparations to induce anti-tumor immune responses might be related to the dual function of HSPs delivering Ag and inducing DC maturation. Linking these two properties is one possibility to ensure that Ag presentation occurs in an environment optimal for T cell stimulation. Second, initial vaccination studies using murine tumor models demonstrated that the anti-tumor immunity achieved by vaccination with GP96 or HSP70 preparations is restricted to the tumor from which the GP96/HSP70 was isolated (1, 6). Therefore, HSP-based vaccination strategies are considered patient-individual treatment modalities. This view is challenged by our observation, and that of Castelli et al. (49), demonstrating that HSP70 isolated from human melanoma cells chaperone naturally expressed nonmutated and shared human melanoma Ags and transfer them to APCs for T cell recognition.
If cross-presentation of shared human tumor Ags by HSP70-PC followed by efficient T cell stimulation is routinely achieved with HSP70-PC, the clinical application of HSP70-based vaccines may be extended from a patient-individual treatment to use in an allogeneic vaccination setting. Third, our novel insights into the mechanism of stress protein binding to class I MHC molecules suggest that the clinical application of HSP70-based vaccines may be extended from a patient-individual treatment to use in an allogeneic vaccination setting.

Clinical hyperthermia has been found to be effective as a locoregional treatment for certain solid tumors (52, 53). For melanoma in particular, a randomized phase III study in humans has been completed showing improvement of local tumor control and survival benefits in patients with multiple lesions after hyperthermia treatment (54). During clinical hyperthermia, peak temperatures of up to 42°C can be achieved in the tumor tissue, and at this temperature the de novo synthesis of heat inducible HSP70 is up-regulated (55). Within this temperature range, local necrosis occurs which might result in the release of HSP (56, 57) and at this temperature the de novo synthesis of heat inducible HSP70 and gp170; Ref. 58) and by causing local necrosis (57) in tumor tissue, has the potential to directly activate the immune system against tumors.

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A journal article discussing the effectiveness of heat shock protein-based vaccines in cancer immunotherapy. The article highlights the role of HSP70 in enhancing immune responses and discusses the clinical implications of using these vaccines.

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