HSP70 peptide-bearing and peptide-negative preparations act as chaperokines

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Abstract We recently elucidated a novel function for the 70-kDa heat shock protein (HSP70) as a chaperone and a cytokine, a chaperokine in human monocytes. Here we show that peptide-bearing and peptide-negative HSP70 preparations isolated from EMT6 mammary adenocarcinoma cells (EMT6-HSP70) act as chaperokines when admixed with murine splenocytes. EMT6-HSP70 bound with high affinity to the surface of splenocytes recovered from naive BALB/c mice. The $[Ca^{2+}]_i$ inhibitor BAPTA dose dependently inhibited HSP70- but not LPS-induced NF- κ B activity and subsequent augmentation of proinflammatory cytokine TNF- α , IL-1 β , and IL-6 production. Taken together, these results suggest that presence of peptide in the HSP70 preparation is not required for spontaneous activation of cells of the innate immune system.

INTRODUCTION

Until recently, the primary function ascribed to heat shock proteins (HSP) was as intracellular molecular chaperones of naive, aberrantly folded, or mutated proteins as well as in cytoprotection following a wide range of stressful stimuli. However, recent reports have ascribed novel roles for HSPs found in the extracellular milieu or expressed on the surface of cells. For instance, immunization of tumor-derived HSP-peptide complexes have been shown to elicit potent CTL (CD8⁺) and T-helper (CD4⁺) cell-mediated responses that result in the drastic reduction of tumor burden (Tamura et al 1997). Moreover, treatment of antigen-presenting cells with preparations of HSP70, HSP90, and gp96 was shown to induce potent cytokine production in macrophages (Basu et al 1998). In addition, the correlation between HSP70 expressed on the surface of tumor cells with an increased sensitivity to allogeneic natural killer (NK) cell-mediated killing (Botzler et al 1996a, 1996b; Multhoff et al 1997) suggest the presence of a specific HSP-bound receptor. Evidence for this has recently been advanced by several laboratories (Arnold-Schild et al 1999; Wassenberg et al 1999; Asea et al 2000; Singh-Jasuja et al 2000).

The functional consequence of exogenously added HSP70 includes the inhibition of TNF- α -induced apoptosis of human promonocytes (Guzhova et al 1998) and protection of axotomy-induced death of neurons (Houenou et al 1996). In addition, work by Todryk and colleagues shows that tumor lysate containing HSP70 augments immature dendritic cells to enhanced phagocytosis in vitro and that expression of HSP70 in B16 tumors results in the accumulation of T lymphocytes, macrophages, and dendritic cells into the tumor microenvironment (Todryk et al 1999). Exposure of HSP70 to human monocytes elicits proinflammatory cytokine production via a CD14-dependent, $[Ca^{2+}]_i$ -dependent, NF- κ B-mediated signaling pathway (Asea et al 2000). Taken together, these findings indicate that HSPs found in the extracellular milieu act as both chaperone and cytokine, a chaperokine, with the ability to activate the proinflammatory cytokine-producing mechanism of antigen-presenting cells. The physiological relevance of extracellular HSP70's ability to function as a chaperokine helps elucidate recent novel findings indicating that HSPs can be potent adjuvants for eliciting immune responses and are powerful inducers of antitumor immunity (Multhoff et al 1997; Suzue et al 1997; Tamura et al 1997). The present study shows that the presence of peptide in the HSP70 preparations derived from tumors is not required for spontaneous acti-

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vation of an NF- κ B-dependent proinflammatory cytokine production in cells of the innate immune system.

MATERIALS AND METHODS

Animals and isolation of splenocytes

Eight- to 10-week-old female BALB/c mice were purchased from Taconic Farms (Germantown, NY, USA) and housed in laminar flow isolation units in the Dana-Farber Cancer Institute's vivarium under alternate dark and light cycles. The animals were housed 5 per cage in a pathogen-free environment with air filter tops in filtered laminar flow hoods. Animals were maintained on food and water ad libitum and screened regularly for the presence of pathogens and consistently tested negative. All animals were treated humanely and in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Animal Resources, National Research Council and Dana-Farber Cancer Institute.

Isolation of splenocytes was performed as previously described (Asea and Stein-Streilein, 1998). Briefly, animals were killed following inhalation of an excess of CO₂. The spleen was aseptically removed and gently tapped through a sterile nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Debris was allowed to sediment for 3 to 5 minutes at room temperature before transferring the cell suspension and collecting cells after pelleting at 250 $\times$ g for 10 minutes. Viable cells counts were accomplished by trypan blue exclusion and phase contrast microscopy of single cell suspension. Dead cell counts were consistently <5%.

Tumor cells and growth conditions

EMT6 is a mouse mammary adenocarcinoma cell line (ATCC, Rockville, MD, USA) that was maintained in MEM (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 units/mL penicillin G, 100 μ g/mL streptomycin, and 2 mM L-glutamine. Cells were cultured at 37°C in 5% CO₂/95% air humidified incubator. At approximately 70% confluence, cells were harvested using trypsin-EDTA, washed, and counted using a hemocytometer. Cells were grown in this fashion for no more than 5 passages before inoculation into BALB/c mice.

Isolation and purification of EMT6-HSP70

EMT6-HSP70 was purified according to the protocol previously described (Peng et al 1997) with minor modifications. Briefly, a 10-mL pellet of EMT6 tumor cells from BALB/c were homogenized in 40 mL hypotonic buffer

(10 mM NaHCO₃, 0.5 mM PMSE, pH 7.1) by Dounce homogenization. The mixture was centrifuged at 100 000 $\times$ g. The pellet was discarded, and the supernatant was then applied to an ADP-agarose column (Sigma, St. Louis, MO, USA) that had been buffered with buffer D (20 mM Tris-acetate, 20 mM NaCl, 15 mM β -mercaptoethanol, 3 mM MgCl₂, 0.5 mM PMSE, pH 7.5). The column was washed with buffer D containing 0.5 M NaCl and then buffer D alone until no more protein could be detected by the Bradford protein assay (BioRad, Richmond, CA, USA). The column was then incubated with buffer D containing 3 mM ADP (Sigma) at room temperature for 30 minutes and then eluted with 25 mL of buffer D (3 mM ADP). The buffer of the eluate was then changed with PD-10 to FPLC buffer (20 mM sodium phosphate, 20 mM NaCl, pH 7.0). The protein was then resolved on an FPLC system (Mono Q, Pharmacia Biotech, Piscataway, NJ) and eluted by a 20- to 600-mM NaCl gradient. All proteins were quantified using the Bradford assay, and BSA was used as the standard. The HSP70-peptide complex (HSP70-EMT6) was then aliquoted at 100 μ g/mL and stored at -80°C. As a peptide control for purified HSP70 devoid of antigenic peptide EMT6[-HSP70], the same procedure as mentioned previously with an ATP (instead of ADP)-agarose column (Fluka Chemika-BioChemika, Trenton, NJ).

Confocal microscopy

EMT6-HSP70 and OVA (Sigma) were biotinylated using an Immunoprobe™ Biotinylation Kit from Sigma, according to the manufacturer's directions. Splenocytes were then stained with 70 nM biotinylated-EMT6-HSP70 or 70 nM biotinylated-OVA or 70 nM streptavidin-conjugated-FITC (Sigma) for 15 minutes at 4°C (surface staining) or 37°C (intracellular staining). For surface staining, cells were washed twice and stained with streptavidin-FITC, fixed using 2% paraformaldehyde in PBS for 30 minutes, then washed twice in PBS alone. For intracellular staining, cells were simultaneously fixed and permeabilized using PermeaFix buffer (OrthoDiagnostics) for 40 minutes at room temperature in the dark, as previously described (Asea et al 1996; Hansson et al 1996). Cells were then washed twice in PBS and stained with streptavidin-FITC for 15 minutes at room temperature in the dark. Following both treatment regimes, 5 μ L of cell pellet was mixed with an equal volume of SlowFade (Molecular Probes, Eugene, OR, USA) and placed onto a glass slide, and the coverslip was sealed with nail polish. Fluorescence distribution was analyzed using a Zeiss model LSM410 confocal laser scanning microscope (Zeiss, New York, NY, USA) equipped with an external argon-krypton laser (488 and 568 nm). Optical sections of 512 $\times$ 512 pixels were digitally recorded within 2 seconds. Images were printed

with a Fujix Pictography 3000 printer (Fujii, Japan) using Adobe Photoshop software (Adobe Systems, Mountain View, CA, USA) on an Apple Macintosh computer.

Flow cytometric analysis

Following various treatment protocols, 2×10^6 cells were simultaneously fixed and permeabilized using 2 mL PermeaFix (OrthoDiagnostics, Raritan, NJ, USA) for 40 minutes at room temperature in the dark, as previously described. Cells were then washed 3 times in PBS. Non-specific binding was inhibited by treating cells with 5.5% normal goat serum (NGS) in PBS for an additional 1 hour at room temperature with gentle rocking. For the measurement of intracellular cytokine expression, cells were treated with anti-human TNF- α -PE or anti-mouse IL-1 β -PE (1 μ L/ 10^6 cells; Pharmingen) for 40 minutes at room temperature in the dark. Cells were then washed twice in PBS and analyzed by flow cytometry. For the measurement of the phosphorylation state of I- κ B α , phospho-specific I- κ B α (NEN Biolabs, Beverly, MA, USA) at a 1:500 dilution was admixed to the cells for 1 hour at room temperature. Cells were then washed 3 times with PBS (5.5% NGS) and treated with anti-rabbit-FITC for 30 minutes. The cells were then washed twice in PBS and analyzed by flow cytometry.

Flow cytometric analysis was performed on a FACScan with a Lysis II software program (Becton Dickinson). Individual cells were gated on the basis of forward (FSC) and orthogonal scatter (SSC). The photomultiplier (PMT) for PE (FL2-height) was set on a logarithmic scale. Cell debris was excluded by raising the FSC-height PMT threshold. The flow rate was adjusted to <200 cells/s, and at least 10 000 cells were analyzed for each sample.

Western blot analysis

Proteins were extracted from cells following treatment with ice-cold RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 2.5% deoxycholate, 2 mM EGTA, 1 mM leupeptin, 1 mM aprotinin, 10 mM NaF, and 1 mM PMSF) and samples cleaned by centrifugation at $15\,000 \times g$ for 20 minutes at 4°C. For immunoprecipitation experiments, supernatant was carefully removed and incubated with primary antibody for 1 to 2 hours on ice, and immunoprecipitates were collected with Protein A-Sepharose beads (Pharmacia Biotech, Piscataway, NJ, USA) for 30 minutes at 4°C. The precipitate was washed 3 times with phosphate-buffered saline (PBS) and boiled in SDS-PAGE sample buffer prior to electrophoresis on 12% SDS-polyacrylamide gels and transferred to Immobilon PVDF membranes (Millipore Corp., Bedford, MA, USA). The membrane was blocked by incubation in TBS buffer supplemented with 5% nonfat dry milk (Bio-Rad Laborato-

ries, Hercules, CA, USA) and 0.1% Tween-20 for 1 hour at room temperature, washed 3 times in TBS buffer, and incubated overnight with appropriate primary antibody at 4°C. Membranes were washed 3 times with TBS buffer and incubated with appropriate alkaline phosphate-conjugated secondary antibody for 1 hour at room temperature. Detection of proteins was achieved by the ECL system.

Statistical analysis

The double-tailed Student's *t*-test (Nycomed, As, Oslo, Norway) was used to analyze the significance of the difference between control and experimental groups. Differences were considered significant when $P \leq 0.05$.

RESULTS

Exogenous EMT6-HSP70 specifically binds to splenocytes

Immunization with HSP-peptide complexes isolated from cancer cells has been shown to elicit protective anti-tumor immune responses (Udono and Srivastava 1993, 1994; Feldweg and Srivastava 1995; Srivastava 1996). Indeed, the immunizing capacity of HSP preparations is thought to be because the HSP molecule is associated with peptides generated in the cells from which the HSP is isolated (Srivastava and Maki 1991). We recently demonstrated that highly purified HSP70 (devoid of peptide) binds with high affinity to the plasma membrane of human monocytes (Asea et al 2000). In this study, we test the ability of HSP70 preparation derived from EMT6 mammary adenocarcinoma cells to elicit a similar NF- κ B-dependent proinflammatory cytokine response in naive splenocytes.

EMT6 tumors recovered from EMT6-bearing BALB/c mice were enriched for HSP70 using ADP affinity chromatography (Fig 1, lanes 2–7), a purification method previously shown to result in HSP70 protein preparations containing antigenic peptides (Peng et al 1997), (EMT6[+]HSP70) or by ATP affinity chromatography, a method shown to result in HSP70 protein preparations devoid of peptide (Peng et al 1997), (EMT6[–]HSP70) (data not shown). The single band in lane 7 attests to the purity of the EMT6(+)HSP70 preparation.

The binding capability of peptide-bearing and peptide-negative HSP70 preparations on mouse splenocytes was tested by treatment of splenocytes freshly recovered from naive BALB/c mice with 70 nM biotinylated-EMT6(+)HSP70 on ice (to inhibit internalization or phagocytosis), resulting in specific surface binding as revealed by laser confocal microscopy (Fig 2A) and fluorescence microscopic analysis (data not shown). Cross-sectional analysis through the center of mouse spleno-

Fig 1. A typical purification profile of tumor-derived HSP70-peptide complex using ADP affinity chromatography. Results show Coomassie Blue staining of different proteins fractions depicting the various stages of purification; lane 1, molecular weight marker; lane 2, wash following first PD10 column; lane 3, total protein following Dounce homogenization; lane 4, wash following high salt solution; lane 5, eluate following ADP-agarose column; lane 6, wash following second PD10 column; 7 final eluate (EMT6-HSP70).

cytes demonstrated that binding was localized to the plasma membrane when binding was performed at 4°C (Fig 2A). EMT6(–)HSP70 used at similar concentrations revealed a similar staining profile (data not shown). Control cells treated with biotinylated-OVA (ovalbumin) (Fig 2B) or streptavidin-FITC (Fig 2C) resulted in only weak, nonspecific binding.

Exogenous EMT6-HSP70 induces proinflammatory cytokine production

In order to test the functional consequence of EMT6(+)HSP70 binding to murine splenocytes, we treated freshly recovered mouse splenocytes with either 70 nM EMT6(+)HSP70 (peptide-bearing) or 70 nM EMT6(–)HSP70 (peptide-negative) for various time periods. EMT6(+)HSP70 treatment resulted in enhanced

up-regulation in expression of the proinflammatory cytokine TNF- α from a baseline level to 38.9% at 2 hours (Fig 3, second panel from top) to 80.9% at 4 hours posttreatment (Fig 3, fourth panel from top). EMT6(–)HSP70 treatment resulted in enhanced up-regulation in expression of the proinflammatory cytokine TNF- α from a baseline level to 37.7% at 2 hours (Fig 3, third panel from top) to 76.5 at 4 hours posttreatment (Fig 3, bottom panel), as judged by flow cytometric analysis of the percentage of cells expressing respective intracellular cytokines. Splenocytes treated with control protein (OVA) did not up-regulate the expression of TNF- α significantly above background values, 6.5% (Fig 3, top panel). Pretreatment of the splenocytes with either Polymyxin B or Lipid IVa (potent LPS inhibitors) (Duff and Atkins 1982; Golenbock et al 1991) did not significantly alter the cytokine production profile, indicating that the effects of EMT6-HSP70 were not due to contamination with bacterial products (data not shown). In a separate experiment, the ability of EMT6(+)HSP70 to induce IL-1 β was tested. EMT6(+)HSP70 augmented the expression of IL-1 β from baseline levels to 19.9% at 2 hours to 38.9% at 4 hours posttreatment (Fig 4).

The endotoxin content of EMT6-HSP70 was routinely measured by *Limulus* ameocyte lysate assay kit (Bio-Whittaker, Walkersville, MD, USA) and found to be consistently less than 0.01 ng/mL. Further, heat treatment (100°C, 20 minutes) before incubation with cells completely abrogated EMT6-HSP70-induced but not LPS-induced cytokine production. Trypan blue exclusion analysis performed at the end of each experiment to test for cell viability revealed that none of the compounds used at the concentrations stated had a toxic effect on the cells (data not shown). Taken together these data show that peptide-bearing EMT6(+)HSP70 and peptide-negative EMT6(–)HSP70 are potent activators of mouse splenocytes.

Fig 2. EMT6-HSP70 specifically binds to surface membrane of murine splenocytes. Laser confocal microscopy of a section through the center of a murine splenocyte incubated with (A) biotinylated-EMT6-HSP70, (B) biotinylated-OVA, or (C) streptavidin conjugated-FITC only. Bar = 10 μ m. Results are representative of 2 independently performed experiments with similar results.

Fig 3. Exogenous EMT6-HSP70 augments the expression of TNF- α . Murine splenocytes treated at 37°C for 2 hours with 100 μ g/mL OVA (top panel), 70 nM EMT6(+)-HSP70 (second panel from top), or 70 nM EMT6(-)-HSP70 (third panel from top) or at 37°C for 4 hours with 70 nM EMT6(+)-HSP70 (fourth panel from top) or 70 nM EMT6(-)-HSP70 (bottom panel). Cells were then simultaneously fixed and permeabilized using PermeaFix (OrthoDiagnostics), stained with TNF- α -PE (Pharmingen), and analyzed by flow cytometry. Results represent the relative number of murine splenocytes staining positive for TNF- α and are representative of 22 independently performed experiments with similar results.

Role of NF- κ B in EMT6-HSP70-induced signaling

The activation of NF- κ B is a key step in the up-regulation in multiple proinflammatory cytokines by human monocytes (Ghosh et al 1998). The activation of NF- κ B is regulated by its cytoplasmic inhibitor, I- κ B α via phosphorylation at Serine 32 (Ser-32) and 36 (Ser-36), which targets it for degradation by the proteasome and releases NF- κ B to migrate to the nucleus and activate the promoter of target genes (Baeuerle and Baltimore 1988). To further address the signal transduction cascade activated by exogenous EMT6-HSP70-peptide preparations that contain peptide or are deficient in peptide, we next examined the phosphorylation state of I- κ B α at Ser-32 (Beg and Baldwin 1993; Baeuerle and Baltimore 1988) using antibodies specific for Ser-32 followed by flow cytometric analysis. Treatment of splenocytes with EMT6-HSP70 induced the

Fig 4. Exogenous EMT6-HSP70 induces IL-1 β production in murine splenocytes. Murine splenocytes treated with 100 μ g/mL OVA (top panel), 70 nM EMT6(+)-HSP70 for 2 hours (middle panel), or 70 nM EMT6(+)-HSP70 for 4 hours (bottom panel). Cells were then simultaneously fixed and permeabilized using PermeaFix (OrthoDiagnostics), then stained with IL-1 β -PE (Pharmingen) and analyzed by flow cytometry. Results represent the relative number of murine splenocytes staining for IL-1 β and are representative of 2 independently performed experiments with similar results.

phosphorylation of I- κ B α at Ser-32 (Fig 5). Pretreatment with the Ca^{2+} inhibitor BAPTA inhibited EMT6-HSP70-induced phosphorylation of I- κ B α at Ser-32 in a dose-dependent manner (Fig 5, filled circles). LPS-induced phosphorylation of I- κ B α was not inhibited by pretreatment with BAPTA-AM (Fig 5, filled squares). BAPTA, which had no effect on control protein OVA, did not induce the phosphorylation of I- κ B α (data not shown).

DISCUSSION

Our present studies present a novel function of the 70-kD heat shock protein as a chaperone and cytokine, a chaperokine. We show that the chaperokine effect is present in tumor-derived peptide-bearing and peptide-negative HSP70 preparations. We further show that in the mouse model, the chaperokine effect is transduced via a Ca^{2+} and NF- κ B-dependent pathway.

The findings that EMT6-HSP70 bind to mouse splenocytes are in agreement with recent observations showing

Fig 5. Role of $[Ca^{2+}]_i$ in EMT6-HSP70-induced phosphorylation of I- κ B α . Murine splenocytes were pretreated for 30 minutes at 37°C with various concentrations of intracellular Ca^{2+} chelator, BAPTA-AM. Cells were then treated with either 70 nM HSP70 (filled circles) or 100 μ g/mL LPS (filled squares). Thirty minutes posttreatment, cells were immediately fixed and permeabilized using PermeaFix (OrthoDiagnostics), then treated with phospho-specific I- κ B α antibody (New England BioLabs, Beverly, MA, USA), stained with anti-rabbit conjugated-FITC, and analyzed by flow cytometry. Results are the mean inhibition of I- κ B α (%) $\pm$ SD. * P < 0.01 vs respective controls (Student's t -test).

that monocytic cell line P388D and the dendritic cell line D2SC/1 stained with gold-labeled HSC70 and gp96 stains within clathrin-coated pits (Arnold-Schild et al 1999). In addition, binding of gp96 was shown to be cell specific, binding to peritoneal macrophages but not CHO or COS cells (Wassenberg et al 1999). Further studies by Schild and coworkers later revealed that endocytosed gp96 colocalizes with recycled MHC class I in dendritic cells (Singh-Jasuja et al 2000). Here, we show that both peptide-bearing and peptide-negative HSP70 preparations bind to the surface of splenocytes with equal affinity. These results suggest that the "docking" region that HSP70 uses to bind to the plasma membrane-bound receptors on the cell is distinct from the peptide-binding pocket. Support for this comes from recent experiments

showing the presence of a distinct peptide-binding pocket in the bacterial HSP70 molecule (Zhu et al 1996). In addition, recent studies using fluorescent probes point to the presence of a hydrophobic peptide-binding pocket in the gp96 molecule (Wearsch and Nicchitta 1997; Wearsch et al 1998; Linderoth et al 2000).

Seminal work by Srivastava and coworkers established the ability of HSP-peptide preparations to confer immunity against autologous tumor preparations from which these HSPs had been isolated (for review, see Srivastava et al 1998; Schild et al 1999). The mechanism by which HSP-peptide preparations elicit protective anti-tumor immunity was shown to be dependant on antigen-specific activation of CTL and T-helper cell responses, cells crucial for activation of the adaptive immune response (Udono et al 1994). Our studies do not contradict these findings. The data presented in this study analyze the innate immune response to HSP70-peptide preparations. Thus, during the first few hours of the immune response, we show that cells of the innate immune system recognize both peptide-bearing and peptide-negative HSP70 preparations as a "danger signal." This nonspecific effect most likely helps reduce the tumor burden until the adaptive immune response is ready to take over. Here, the presence of peptide will result in augmentation of the immune response and activation of CTL and T helper functions.

The possibility exists that minuscule amounts of peptide might still be present on the peptide-negative HSP70 preparation. Coworkers of Srivastava have elegantly shown that HSP preparations are effective at immunizing against tumors and inducing potent CTL in vivo at nanogram rather than microgram amounts (Srivastava and Udono 1994; Srivastava et al 1994; Tamura et al 1997; Srivastava et al 1998). Therefore, even minuscule amounts of peptide on HSP preparation could trigger a response. Experiments are under way to test whether our peptide-negative HSP70 preparation loses its capacity to activate CTL and T-helper cell tumor regression.

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