Heat Shock Protein 70-associated Peptides
Elicit Specific Cancer Immunity
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Summary
Vaccination of mice with heat shock protein 70 (hsp70) preparations derived from the Meth A sarcoma, but not from normal tissues, renders the mice immune to a substantial challenge with Meth A sarcoma. The immunogenicity is dose dependent and tumor specific. Treatment of an antigenically active hsp70 preparation with ATP followed by removal of low-molecular weight material leaves hsp70 intact, as judged by SDS-PAGE but results in loss of antigenicity, as judged by tumor rejection assays. Separation of this low-molecular weight material on a C18 reverse-phase column shows a diverse array of peptides with molecular mass between 1,000 and 5,000 daltons. Our data indicate that antigenicity of hsp70 preparations derives, not from hsp70 per se, but from associated peptides. These observations may suggest a novel method of using the peptide-binding property of hsp70 for specific vaccination against cancer and infectious diseases.

Materials and Methods

I mmune response to heat shock proteins (HSP) has been the focus of considerable attention (1). There are three major paradigms of this response: one, where HSP are processed and presented like other antigens by MHC class I or II molecules and recognized by α/β T cells (2-4); second, where γ/δ T cells recognize HSP by a poorly understood mechanism (5, 6); and third, where an immune response to an HSP leads to autoimmunity because of cross-reactivity between the HSP and a non-HSP self-antigen (7, 8). A new fourth paradigm of immune response to HSP, in which immunization with an HSP elicits immunity not against the HSP itself, but against antigenic peptides chaperoned by the HSP, has emerged. The prototype of this fourth paradigm is the HSP gp96. It has been demonstrated that mice or rats immunized with gp96 preparations from a given tumor develop immunological resistance to the tumor from which gp96 is isolated, but not to antigenically distinct tumors (9-12, Janetzki, S., N. E. Blachere, and P. K. Srivastava, unpublished results). gp96 preparations isolated from normal tissues do not elicit resistance to any tumors tested (13). Examination of the structural basis of the immunological specificity of gp96 from normal tissues and tumors has suggested that gp96 molecules are not immunogenic per se, but are carriers (chaperones) of antigenic peptides (14-16). Heat-shock protein 90 (hsp90), the cytosolic counterpart of gp96, has also been shown to elicit tumor-specific immunity against the tumors from which it is isolated, but not to antigenically distinct tumors (17); in this case, too, the antigenicity does not appear to reside in the hsp90 itself (18).

In this article, we extend this paradigm to one of the most extensively studied HSP, hsp70, and demonstrate that hsp70 preparations isolated from a methylcholanthrene-induced BALB/c fibrosarcoma, Meth A, but not from normal tissues, elicit tumor-specific immunity. This immunity appears to be elicited, not by the hsp70 molecules themselves, but by the ATP-dissociable peptides associated with hsp70.

Materials and Methods

Mice and Antibodies. BALB/cj mice (viral antigen free) were obtained from The Jackson Laboratory (Bar Harbor, ME) and were maintained in the virus-free mouse facilities at Mount Sinai School of Medicine. Antibodies to hsp70 (anti-HSP72/73, SPA-820, clone N27F3-4) were purchased from StressGen Biotechnologies Corp. (Victoria, BC, Canada).

Purification of hsp70. A 40-ml Meth A cell pellet was homogenized in 120 ml hypotonic buffer (30 mM NaHCO3, 0.5 mM PMSF, pH 7.1) and a 100,000-g supernatant obtained. This was applied to a Con A-Sepharose column in presence of 2 mM Ca2+ and 2 mM Mg2+; the Con A unbound material was dialyzed against 10 mM Tris acetate, pH 7.5, 10 mM NaCl, and 0.1 mM EDTA. This fraction was resolved on an FPLC system (Mono Q, Pharmacia Inc., Piscataway, NJ) equilibrated with 20 mM Tris acetate, pH 7.5, 20 mM NaCl, 0.1 mM EDTA, and 15 mM 2-ME. The proteins were eluted by a 20-500-mM NaCl gradient. Fractions (1 ml) were collected, tested by SDS-PAGE, and immunoblotted with a group anti-hsp70 mAb N27F3-4 (StressGen Biotechnologies Corp.). The hsp70-containing fractions were pooled and...
precipitated with increasing saturation levels of ammonium sulfate. hsp70 was precipitated at 50–70% ammonium sulfate saturation. The later fractions in this process were homogeneous by silver staining and were used for immunization of mice. (For purification of hsp70 from liver and spleen, the 100,000-g supernatant was first applied to a blue Sepharose column to remove albumin.)

Isolation and Analysis of Peptides Associated with hsp70. A purified hsp70 preparation was centrifuged through an assembly (Centricon 10, Amicon Corp., Danvers, MA), to remove any low–molecular weight material loosely associated with it. The large–molecular weight fraction was recovered and analyzed by SDS-PAGE, whereas the low–molecular weight material was analyzed by HPLC, as described later. The hsp70 preparation was incubated with ATP at a final concentration of 10 nM at room temperature for 30 min and centrifuged through Centricon 10 as before. The two fractions were recovered and the ATP treatment was repeated two more times. The high–molecular weight fraction was analyzed by SDS-PAGE; the lower molecular weight fractions were pooled, concentrated by evaporation in Speed Vac (Savant Instruments, Inc., Farmingdale, NY), and dissolved in 0.1% TFA. This material was applied to a C18 reverse–phase HPLC column (Vydac, Hesperia, CA) preequilibrated with 0.1% TFA. The bound material was eluted at a flow rate of 0.8 ml/min by a linear gradient of 79.9% acetonitrile, 20% water, and 0.1% TFA. The absorbance at 210 nm was monitored and fractions (0.2 ml) were collected.

Metabolic Labeling of Meth A Cells. Meth A ascites cells were collected and suspended in DMEM without methionine containing 10% dialyzed FCS at a density of 10^6 cells/ml. Cells were deprived of methionine in this manner to deplete the internal methionine pools for 4 h. They were then resuspended in fresh methionine–free medium containing 100 µCi/ml Trans 35S–Label (ICN Biomedicals Inc., Costa Mesa, CA) for 1 h and were harvested and processed for purification of hsp70.

SDS–PAGE and Protein Blotting. Proteins were resolved on SDS–PAGE, subjected to electrophoresis, blotted to nitrocellulose, and probed with appropriate antibodies, as described (10).

Tumor Rejection Assays. Mice (6–8-wk-old female) were immunized with hsp70 or PBS twice at weekly intervals and challenged by intradermal injection of live tumor cells 1 wk after the last immunization (10). A high viability of tumor cells (>98%) is an important prerequisite for reproducible results.

Results

Tumor–specific Immunogenicity of Tumor–derived hsp70 Preparations. Apparently homogeneous hsp70 preparations from the BALB/cJ Meth A fibrosarcoma (Fig. 1, a and b) were obtained as described in Materials and Methods. Typically, 1.5 mg hsp70 is obtained from a 40-ml cell pellet (8 × 10^6 cells). This purification protocol results in a relatively modest recovery compared with the traditional method of hsp70 purification (19). However, for reasons discussed in the next section, the hsp70 purified by the traditional protocol does not elicit immunity. BALB/cJ mice were immunized twice at weekly intervals with three different doses of purified hsp70 and were challenged with 80,000 live Meth A cells 1 wk after the second immunization. Kinetics of tumor growth was monitored (Fig. 2). Tumors grew progressively in all unimmunized mice, but there was significant protection from tumor growth in hsp70-immunized mice. This effect was dose dependent: two injections of 3 µg hsp70 each did not immunize mice against Meth A, whereas two injections of 9 µg each conferred complete protection to all vaccinated mice. The tumor–protective effect of Meth A hsp70 was seen reproducibly in several experiments (Table 1). Protection was observed at challenges of up to 100,000 Meth A cells. Challenges larger than this have not been tested. Furthermore, the protective effect has been determined to be long lasting in that mice challenged up to 6 wk after the last immunization have been observed to resist a challenge with Meth A sarcoma. Longer time intervals between the last immunization and challenge have not been tested.

Because hsp70 is a ubiquitous protein present in normal tissues as well as in tumors, hsp70 preparations from liver and spleen of normal BALB/c mice were tested for immunogenicity as described above. No protective effect of hsp70 derived from normal tissues was observed at any of the three comparable doses tested (Fig. 2). Immunity elicited by vaccination with Meth A–derived hsp70 was observed to be tumor specific: mice immunized with Meth A hsp70 remained sensitive to challenges with antigenically distinct, Meth A–induced BALB/cJ sarcomas CMS4 and CMS5 (Table 2).

Immunogenicity of Tumor–derived hsp70 Is Sensitive to Exposure to ATP. hsp70 preparations from normal tissues and Meth A sarcoma were observed to be antigenically distinct, as shown in Fig. 2. However, proteins of the hsp70 family are highly conserved and nonpolymorphic. It was considered unlikely that the hsp70 genes are mutated specifically in tumor cells. Two members of the hsp70 family have been shown to bind peptides (20–23); the possibility that antigenicity is elicited, not by hsp70 per se but by the peptides chaperoned by it, was considered. The presence of HSP-bound peptides has been shown in gp96 by acid treatment of purified gp96 (14). However, Flynn et al. (22, 23) have demonstrated that binding of peptides by members of the hsp70 family is sensitive to ATP. We used this gentle, nondenaturating method to elute peptides from the immunogenic hsp70 preparation. Purified Meth A hsp70 was applied to an ATP–agarose column and was eluted by ATP, as described in the legend to Fig. 1. The ATP–eluted preparation (Fig. 1 a, right lane) was applied to a Centricon 10 filtration system to remove the peptides and ATP. The retentate was analyzed by SDS-PAGE and appeared indistinguishable from the pre–ATP–exposed preparation, both in quantity and apparent homogeneity (Fig. 1 a, right lane). The ATP–eluted preparation was used to vaccinate mice, as described. Mice vaccinated with Meth A–derived hsp70 be-
fore exposure to ATP (Fig. 1 a, left lane) were significantly protected against tumor challenge, but the mice vaccinated with the ATP-eluted Meth A hsp70 (Fig. 1 a, right lane) were not (Fig. 3). It was noted in an earlier section that in order to isolate immunologically active preparations of hsp70, it should be purified by the method described by us, which does not use ATP-agarose chromatography, and not with the traditional method, which does. The experiments shown in Fig. 3 form the basis of that suggestion.

The low–molecular weight material eluted from hsp70 by treatment with ATP was applied to a C18 reverse-phase column and eluted by an acetonitrile gradient. A number of distinct peaks absorbing at 210 nm were observed (Fig. 4 a, solid line). When the same hsp70 preparation was exposed to 350 mM NaCl and the resulting low–molecular weight fraction analyzed, a distinctly different and much simpler pattern of peaks was observed (Fig. 4 a, dotted line). A salt concentration >350 mM was not used so as not to denature the hsp70 preparation. To demonstrate that the ATP-eluted peaks truly represent peptides and that the peptides are derived from cellular proteins, Meth A cells were metabolically labeled for 1 h with [35S]methionine, as described in Materials and Methods, and hsp70 preparations obtained. The low–molecular weight fraction was derived by Centricon 10 centrifugation, resolved on a C18 reverse-phase column, and fractions collected and counted. A number of distinct 35S-labeled peaks were ob-

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**Figure 2.** Mice were immunized with 9, 6, or 3 µg hsp70 derived from Meth A sarcoma (Fig. 1 a, left lane) or normal liver and spleen of BALB/c mice, as indicated. Immunization was carried out in 200-µl volume subcutaneously twice at a weekly interval. Mice were challenged with 80,000 Meth A cells intradermally 1 wk after the second immunization. Each line represents the kinetics of tumor growth in a single mouse.

**Figure 3.** hsp70 purified through an ATP-agarose column loses its immunogenicity. Mice were (a) not immunized or (b) immunized with Meth A–derived hsp70 purified by conventional chromatography (Fig. 1 a, left lane), or (c) immunized with Meth A–derived hsp70 purified by ATP-affinity chromatography (Fig. 1 a, right lane). Mice were immunized and challenged as described in legend to Fig. 2.

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1393 Udono and Srivastava
tained, indicating that the radioactivity indeed represents peptides derived from newly synthesized cellular proteins (Fig. 4 b). Preliminary mass analysis of these peptides by tandem mass spectroscopy indicates that they constitute a heterogeneous group ranging in molecular mass from 1,000 to 5,000 daltons, the bulk of the peptides being between 1,600 and 3,200 daltons (Hunt, D., personal communication).

Discussion

Our observations suggest that immunogenicity of Meth A hsp70 derives from the antigenic peptides chaperoned by it. The peptides are derived from cellular proteins by proteolytic degradation, presumably during antigen processing for presentation by MHC class I and class II proteins. Our ability to detect a number of radioactive hsp70-associated peptide peaks from cells metabolically pulsed for only 1 h (Fig. 4 b) indicates that this time period is enough for newly synthesized proteins to be processed and for the resulting peptides to associate with hsp70. The repertoire of peptides generated in tumor cells must differ from those generated in normal tissues because of the tumor-associated mutations, hence the difference in antigenicity of tumor-versus normal tissue-derived hsp70 (16). The hsp70 preparations are recognized by antigroup-hsp70 antibody N27F3-4 (StressGen; Fig. 1 b). However, the endoplasmic reticular hspT0, grp78 immunoglobulin binding protein (BiP), is not present in our preparations; it is easily distinguished from the cytosolic hsp70 on SDS-PAGE by its size.

The mechanism whereby the HSP-peptide complexes elicit a cellular immune response is not clear. The tumor immunogenicity elicited by immunization with gp96 HSP is mediated by CD8+ T lymphocytes (our unpublished observations). It is possible that hsp70–peptide complexes also elicit immunity in a similar manner. A class I–restricted response usually requires presentation of antigens through the endogenous pathway; it would appear that the HSP–peptide complexes, even though provided exogenously, are able to channel the peptides into the class I presentation pathway. A clue into this mechanism may emerge from the observation that depletion/functional inactivation of macrophages completely abrogates the tumor-specific immunogenicity of gp96; in contrast, immunization with whole tumor cells is not sensitive to depletion of macrophages (our unpublished observations). It is conceivable that macrophages possess receptors for gp96 and other HSP, allowing them to bind HSP–peptide complexes, which are then directed to the endogenous presentation pathway through a compartment distinct from the lysosomal compartment. The role of macrophages in channeling exogenous antigens into the endogenous presentation pathway has recently been suggested in other systems as well (25–28).

Identification of HSP as chaperones of antigenic peptides may have a direct bearing on immunotherapy of human cancer. One of the major conceptual difficulties in cancer immunotherapy has been the possibility that human cancers, like cancers of experimental animals, are antigenically distinct (29–31). The prospect of identifying immunogenic antigens of individual tumors from cancer patients is daunting to the extent of being impractical. The observation that HSP chaperone antigenic peptides of the cells from which they are derived may circumvent this extraordinary hurdle to specific immunotherapy of human cancer. Furthermore, immunization with a biochemically defined pure protein preparation may avoid some of the significant risks associated with other methods of vaccination such as whole-cell vaccination, e.g., inoculation of patients with immunosuppressive factors (32–34), transforming DNA, or infectious viruses.
Figure 4. Peptides associated with hsp70 preparations. (a) hsp70 was purified from Meth A cells as described in Materials and Methods and was incubated with 350 mM NaCl (dotted line) or 10 mM ATP and 3 mM MgCl₂ (solid line) at room temperature for 30 min. The preparations were centrifuged through Centricon 10 and the eluates applied to a C18 reverse-phase column as described in Materials and Methods. The x-axis represents elution time and the y-axis represents the absorbance at 210 nm. A number of specific peaks are observed to elute from hsp70 by exposure to ATP. (b) Same as in a, except that hsp70 was prepared from metabolically ³⁵S-labeled Meth A cells. The radioactivity peaks represent peptides.

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