The inducible Hsp70 as a marker of tumor immunogenicity

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Abstract Growing evidence indicates that the stress response in general and heat shock proteins (Hsps) in particular have a profound impact on tumor immunogenicity. In this study, we show that tumor cells subjected to a nonlethal heat shock stress are unable to form tumors in syngenic mice, whereas they do so in athymic nude mice. Moreover, heat-shocked MethA immunity is tumor specific. Enhancement of T-cell-mediated immunogenicity correlates with the expression of the inducible Hsp70 but not the constitutive Hsc70. These observations have a bearing on the proposed functional role of Hsp-peptide association in antigen processing and presentation by major histocompatibility complex I molecules under normal and stressful conditions.

INTRODUCTION

The functions of the 70-kDa heat shock proteins (Hsp70s) have recently been extended to their participation in the immunological defense against infectious diseases and cancers (Srivastava et al 1998). It has been shown that virus- and tumor-derived Hsp70s specifically immunize against the cells from which the Hsp70s were derived (Udono and Srivastava 1993; Ciupitu et al 1998). One key property of the Hsp70s, which explains their immunogenicity, is related to their ability to chaperone peptides (Udono and Srivastava 1993; Zhu et al 1996; Peng et al 1997). Remarkably, the Hsp70s lose their specific immunogenicity after elution of bound peptides. This observation demonstrates that the Hsp70-peptide complex is required for a specific immune response. The mechanism by which the Hsp-peptide complexes prime CD8⁺ T lymphocytes has been proposed (Udono and Srivastava 1993; Udono et al 1994; Srivastava et al 1998), tested (Suto and Srivastava 1995), and described in detail elsewhere (Ménoret and Chandawarkar 1998; Srivastava et al 1998). The current view is that antigenic peptides chaperoned by the Hsps can be endocytosed after binding to their receptor, CD91, which is expressed on professional antigen-presenting cells (APCs) (Arnold-Schild 1999; Binder et al 2000a). The APCs, loaded with the Hsp-peptide complexes, home to the lymphatic node (Binder et al 2000b),

where priming of CD8⁺ T lymphocytes occurs. Since Hsps immunize in the absence of other immunological stimuli, they are considered to be natural adjuvants themselves (Blachère et al 1997). This concept has recently been supported by the observation that Hsps can stimulate macrophages and dendritic cells to secrete the proinflammatory cytokines interleukin (IL) 1 β , IL-6, IL-12, and tumor necrosis factor α (TNF- α) (Asea et al 2000; Basu et al 2000; Moroi et al 2000).

We have previously shown that all Hsp70s are not immunologically equal in that the immunogenicity of tumor cells cosegregates with the expression of the inducible Hsp70 but not with the constitutive Hsc70 (Ménoret et al 1995). We observed that among clones derived from a primary tumor, only those having the ability to synthesize the inducible Hsp70 were rejected by the immune system. Conversely, clones that grew progressively did not synthesize the inducible Hsp70. This cosegregation has recently been confirmed by other investigators. Transfection of tumor cell lines with the gene encoding the inducible Hsp70 restores its sensitivity to lysis by specific cytotoxic T lymphocytes (Wells et al 1998) and generates in vivo CD8⁺ T-cell-dependent immunity (Melcher et al 1998). Additionally, inducible Hsp70 expression is induced during nonapoptotic cell death and increases tumor immunogenicity in vivo (Melcher et al 1998). In this study, we confirm the hypothesis that tumor cells that express the inducible Hsp70 following stress become more immunogenic and show that heat-shocked cells stimulate the innate and adaptive immune system.

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Fig. 1. (A) Expression of inducible Hsp70 and constitutive Hsc70 after heat shock. MethA cells were incubated at 37°C or 42°C for 20–60 minutes followed by incubation at 37°C for 16 hours, washed twice in cold phosphate-buffered saline, homogenized by a cold detergent solution (0.5% NP40, 0.5% *N*-octyl- β -D-glucopyranoside, in 50 mM Tris, 150 mM NaCl pH 7.3), and centrifuged ($14\,000 \times g$) for 10 minutes at 4°C. Supernatants (30 μ g of protein per lane) were analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Gels were fixed and Coomassie stained or immunoblotted as previously described (Ménoret and Bell 2000) using monoclonal antibodies specific for the inducible Hsp70 (StressGen, clone C92F3A–5), the constitutive Hsc70 (StressGen, clone 1B5), and actin (SIGMA, clone AC-40). (B) In vitro survival of heat-shocked MethA cells. Tumor cells were incubated at 37°C (no treatment) or 42°C for 20, 40, 60, and 90 minutes, washed, counted, and seeded at 20,000 cells per flask in RPMI supplemented with 5% fetal calf serum. An aliquot of the culture was counted daily for 5 days. (C) Immunogenicity of heat-shocked MethA. Tumor cells were incubated at 37°C (no treatment) or 42°C for 20, 40, and 60 minutes, washed, and diluted to 5×10^5 cells/mL. Viability was determined before and after injection by trypan blue exclusion. Cells (1×10^5 in 200 μ L) in plain RPMI were injected intradermally in either immunocompetent BALB/cJ mice or immunocompromised athymic BALB/cJ nude/nude mice. The viability of the cells was assessed after injection by trypan blue exclusion. The growth of the tumors was recorded every 2–3 days by caliper measurement of the diameter of the tumors. Each line represents the growth of a tumor in a single animal.

Fig. 2. Injection of heat-shocked cells induces TNF- α release in splenocytes. BALB/cJ mice were injected with either culture medium (RPMI), 1×10^5 MethA cells or 1×10^5 MethA cells incubated for 60 minutes at 42°C (MethA 42°C) as described in the legend of Figure 1C. Spleen cells harvested 48 hours after tumor injection were plated on ELISPOT 96-well plates previously coated with anti-TNF- α antibody. The incubation was performed at 37°C for 24 hours without exogenous stimulation (white background) or with MethA cells (black background). After incubation, the plates were washed and incubated with anti-TNF- α antibody conjugated with alkaline phosphatase and developed with 3-amino-g-ethylcarbazole (AEC) substrate. Spots were counted by ELISPOT analyzer (Autoimmune Diagnostica).

RESULTS AND DISCUSSION

A direct prediction of the cosegregation of inducible Hsp70 expression with increased tumor immunogenicity is that stimuli that induce overexpression of Hsp70 in tumor cells should increase their intrinsic immunogenicity. This hypothesis was tested in the chemically induced fibrosarcoma MethA tumor model. We observed that inducible Hsp70 can be overexpressed after heat shock, whereas the expression of constitutive Hsc70 remains stable. Hsp70 was not detectable by immunoblotting cell extracts from untreated MethA control cells (0 minutes at 42°C). On the contrary, Hsp70 expression increased after heat shock treatment and reached a plateau after treatment for 40 minutes at 42°C (Fig 1A). The quantity of constitutive Hsc70 remained constant for all the samples, proportionally similar to the expression of actin and other proteins (Fig 1A, 2 right panels), confirming that Hsc70 is poorly inducible by heat shock treatment as previously reported (Ménoret et al 1995).

The survival of heat-shocked MethA cells was tested. Cells incubated at 37°C (control) and at 42°C for 20–90 minutes were seeded in tissue culture flasks and counted

daily. We observed that the proliferation and cell survival was not impaired by heat treatment for 60 minutes or less (Fig 1B). The same cells that had been heat shocked and tested for viability *in vitro* (Fig 1B) were injected *in vivo* into syngenic BALB/cJ and nude mice (Fig 1C). We observed that the untreated MethA cells grew progressively in both BALB/cJ and nude mice (Fig 1C, top panels). The growth of heat-shocked MethA cells in nude mice confirms that tumorigenicity of MethA tumor cells is not compromised by heat treatment (Fig 1C, right panels). On the contrary, the same cells heated for 20–60 minutes at 42°C and injected into syngenic BALB/cJ mice did not develop tumors or else showed a growth delay (Fig 1C, left panels). The dramatic reduction of tumor growth in immunocompetent mice shows that immunogenicity of tumor cells increased after heat shock treatment. We cannot exclude the theory that the delay in growth of the 60-minute heat-shocked tumors cells injected in nude mice (but not 20 and 40 minutes; Fig 1C, right bottom panel) was due to the marginal decrease of cell survival observed *in vitro* (Fig 1B). Alternatively, the decrease of tumorigenicity in nude mice can be linked to the previous observation that heat shock cells are more susceptible to natural killer-dependent cell lysis (Botzler et al 1996).

The immune response against the heat-shocked MethA (60 minutes at 42°C) was further studied by ELISPOT assay of TNF- α release. Spleen cells derived from mice injected with non-heat-shocked MethA (MethA at 37°C) produce low amounts of TNF- α after stimulation with either culture medium (no stimulation) or MethA cells (Fig 2). On the contrary, more than 70 spots per 10^6 spleen cells were detected in the cultures of spleen cells from mice injected with live heat-shocked MethA and stimulated with either culture medium (no stimulation) or live MethA cells (Fig 2). No TNF- α release was detected from naive spleen cells, showing that secretion of this cytokine was related to the injection of live tumor cells.

Tumor rejection of a syngeneic but distinct tumor, the colon carcinoma CT26-E, was used to test the antigenic specificity of the heat-shocked MethA. These tumors grow aggressively in naive BALB/cJ mice (Fig 3, bottom panels), but only CT26-E-injected mice develop tumors in animals immunized with irradiated MethA cells (Fig 3, center panels). Mice injected with MethA cells heat shocked for 60 minutes are protected against MethA but not CT26-E challenge (Fig 3, top panels), indicating that heat shock MethA immune response is tumor specific. The physiological mechanism by which this immune stimulation occurs is still unclear. The boost of immunogenicity may be related to the increase of stress-inducible Hsps that may stimulate APCs (macrophages, monocytes, dendritic cells) to release proinflammatory cytokines (Basu et al 1998; Asea et al 2000; Moroi et al 2000) and contribute to the development of the adaptive immune

Fig. 3. Heat-shocked MethA immunity is tumor specific. BALB/cJ mice were injected intradermally at day -7 in the right flank, with either culture medium (RPMI) or 1×10^5 MethA cells incubated for 60 minutes at 42°C as described in the legend of Figure 1C. Positive control of immunization was performed by injecting 2×10^7 lethally γ -irradiated MethA cells subcutaneously at day -14 and -7. All mice were challenged intradermally in the left flank at day 0 with 1×10^5 live MethA or with 1×10^5 live CT26-E (a clone derived from the BALB/c colon adenocarcinoma CT26). The growth of the tumors was recorded every 2–3 days by caliper measurement of the diameter of the tumors. Each line represents the growth of a tumor in a single animal.

response. Additionally, it has been recently reported that the activity of other components of the immune system can be regulated by Hsp. For example, natural killer cells can be cytotoxic to tumor cells that express surface Hsp70 (Multhoff et al 1997). Hsp60-antibody complexes can activate complement, and Hsp70 can activate complement independently of antibodies (Prohaszka and Fust 2000). Finally, the heat shock protein gp96 binds specifically to human activated and resting platelets (Hilf et al 2000). However, our study shows that the immunity induced against heat-shocked MethA is tumor specific, suggesting that the innate component of this immune response is insufficient for the rejection of an antigenically distinct tumor. Another explanation for the increase of immunogenicity after heat shock is a modification in quantity

and/or quality of the repertoire of tumor antigenic peptides accessible to the Hsps and to the immune system in general. The heat treatment may unfold usually stable proteins, revealing neo and cryptic antigens. It may also alter the processing of proteins into the antigen presentation pathway to the major histocompatibility complex molecules (Srivastava et al 1994). Indeed, recruitment of tumor antigens under conditions that alter cytosolic homeostasis and the role of the Hsps in immunity have previously been discussed at length (Forsdyke 1999).

Taken together, these observations and the present study suggest that Hsps have a strong influence on the priming, stimulation, and regulation of the immune response and contribute to the idea that the immune system might be extremely sensitive to conditions that enhance

expression of inducible Hsps. The findings also bear on the use of Hsp70- and Hsc70-based vaccines for cancer and infectious diseases (Ménoret and Chandawarkar 1998). One may also predict that human tumors that express inducible Hsp70 should be good candidates for immunotherapeutic interventions.

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