Human Heat Shock Protein 70 Peptide Complexes Specifically Activate Antimelanoma T Cells

Chiara Castelli, Anne-Marie T. Ciupitu, Francesca Rini, Licia Rivoltini, Arabella Mazzocchi, Rolf Kiessling, and Giorgio Parmiani

INTRODUCTION

The last years of research in tumor immunology have witnessed an explosion in the identification of T cell-defined human tumor antigens, and several epitopes recognized by patients’ T cells, restricted by class I and class II MHC, have been identified (1). When used in vaccination trials, these epitopes revealed a relatively weak immunogenicity and a limited clinical efficacy (2). This could be attributable to the nature of these epitopes, which derive from self proteins for which tolerance may have occurred (3). However, it is also well accepted that the way in which a given antigen is presented to the immune system is crucial in determining the type of the evoked response (4, 5).

Studies aimed at evaluating the immunogenicity of murine tumors led to the discovery of the HSP. This family of chaperone proteins, the functions of which include sampling intracellular peptides derived from unfolded cytoplasmic proteins, displays a strong immunogenic potential, and by providing an immunogenic context for their complexed peptides, HSP can be considered adjuvant of mammalian origins (6). In addition to their involvement in the degradation pathway of cellular proteins during stress condition, HSP70 assists the new synthesized proteins in their correct folding and is also directly involved in the translocation of proteins across membranes into different cellular compartments (7, 8). This large selection of physiological activities and the ability to traffic among different cell compartments make the repertoire of HSP70 chaperone peptides potentially wide and involving proteins having different subcellular localization.

HSPs obtained from tumors or virus-infected cells have been shown to induce CTL responses in vitro against a variety of antigens expressed in the cells from which HSPs have been purified. The specificity of the induced CTLs relies on the peptides chaperoned by these HSPs (9, 10). Murine studies have carefully assessed the usage of chaperone molecules as cancer vaccines, and their efficacy was demonstrated in tumors of different histologies in a prophylactic as well as in a therapeutic setting (11). The immunogenicity of HSP70 and gp96 has been demonstrated for viral as well as tumor antigens in murine systems, but no data are as yet available for the immunogenic potential of human tumor-derived HSPs.

For human melanoma, a variety of tumor-associated peptides recognized by MHC-restricted T cells have been described recently (12). Exploiting this knowledge and the availability of melanoma-specific T cells with defined peptide specificity, we decided to analyze the capacity of HSP70 melanoma-derived peptides to specifically activate T cells. We show here that HSP70 obtained from melanoma is able to reconstitute the epitope for HLA class I-restricted T-cell clones directed against melanoma differentiation antigens. The HSP70-mediated T-cell activation occurs via recognition of MHC molecules of the matched APCs pulsed with the melanoma-derived HSP70 and is strictly dependent on the presence of HSP chaperoned peptides. In addition, HSP peptide presentation to the antimelanoma-specific T-cell clones is likely to occur via cross-priming because MHC matching between melanoma cell lines used as a source of HSP70 and responding T-cell clones was not required. These findings provide a possible rationale for HSP-based vaccination in human cancer.

ABSTRACT

Members of the heat shock protein 70 (HSP70) family display a broad cellular localization and thus bind a repertoire of chaperoned peptides potentially derived from proteins of different cellular compartments. In this report, we show that HSP70 purified from human melanoma can activate T cells recognizing melanoma differentiation antigens in an antigen- and HLA class I-dependent fashion. HLA class I-restricted antimelanoma T cells were susceptible to MHC-restricted, HSP70-dependent stimulation, indicating that HSP70 complexed peptides were able to gain access to the class I HLA presentation pathway. In addition, MHC matching between the melanoma cells used as a source of HSP and the responding T cells were not required, indicating that HSP70 activation may occur across MHC barriers. Besides the MHC-restricted and peptide-dependent activation pathway, HSP70 with no endogenous complexed peptides or HSP70 purified from antigen-negative cells was also able to induce IFN-γ release by melanoma T cells by a MHC-independent mechanism. In this case, however, higher doses of HSP70 were required. The capacity to activate class I-restricted, antitumor T cells as well as antigen-presenting cells, together with the finding that the HSP70 chaperoned peptide repertoire includes melanoma-shared epitopes, holds promise for a HSP70-based cancer vaccine.

MATERIALS AND METHODS

Cell Lines and Tumor-specific T Cells. The antimelanoma T-cell line DIL15392 was established from peripheral blood lymphocytes, whereas TB254 and TB327 were established from tumor-infiltrating lymphocytes of the melanoma patient 15392 (who typed as HLA-A3, -B60, -B14, -Cw6, -Cw8, -DR1, -DR10; Refs. 13 and 14). The A42 CTL clone was derived from tumor-infiltrating lymphocytes of melanoma patient 501 (typed as HLA-A2, -A24, -B18, -B35, -Cw4, -Cw7, -DR5; Ref. 15). All of these effectors were CD3+, CD8+, CD4−. Analysis of T-cell receptor β variable family transcripts by reverse transcription-PCR showed the DIL15392 line to be monoclonal with the expression of T-cell receptor β variable region 1 and T-cell receptor α variable region 2 (data not shown). The specificity of these effectors has been described previously (13–15) and is summarized in Table 1. Melanoma cultures were maintained in RPMI 1640 with 10% FCS.

Purification of HSP70. The HSP70 used was purified adapting the method from Peng et al. (16). Melanoma tumors or LCL cells were homogenized in hypotonic buffer without detergent (10 mM Tris-As, 10 mM NaCl, and 0.2 mM phenylmethylsulfonyl fluoride, pH 7.2) and centrifuged at 100,000 × g. The supernatant was incubated with Active Blue 2 Sepharose beads (Sigma-Chem-
HSP70 Peptide Complexes Activate Antimelanoma T Cells

Table 1. Specificity of the T-cell clones

<table>
<thead>
<tr>
<th>T-cell clone</th>
<th>Antigen recognized</th>
<th>HLA restriction</th>
</tr>
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<tbody>
<tr>
<td>DIL15392</td>
<td>gp100</td>
<td>A3</td>
</tr>
<tr>
<td>TB 254</td>
<td>gp100</td>
<td>Cw8</td>
</tr>
<tr>
<td>TB 327</td>
<td>TRP-2</td>
<td>Cw8</td>
</tr>
<tr>
<td>A42</td>
<td>MART-1</td>
<td>A2</td>
</tr>
<tr>
<td>Fle</td>
<td>NP265-273</td>
<td>A3</td>
</tr>
</tbody>
</table>

 SDS-PAGE and Immunoblot. Samples of the purified HSP70 were run on SDS-PAGE 12.5% homogenous gels. The gel was silver stained after partial transfer to polyvinyl difluoride membrane (Bio-Rad Laboratories, Richmond, CA). The membrane was blocked in 1% BSA at 4°C overnight, incubated for 1 h at room temperature with the mouse anti-HSP/hsc70 antibody SPA-820 (StressGen Biotechnologies Corp., Victoria, British Columbia, Canada), and washed three times for 5 min each with 0.5% Tween 20 in PBS. It was then incubated with peroxidase-conjugated rabbit antimouse antibody (Dakopatts, Denmark) for 1 h, washed as above, incubated with peroxidase-conjugated swine antirabbit antibody (Dakopatts), and washed again as described above. The blot was developed with 9 mg/ml 3,3'-diaminobenzidine (Sigma) and 0.018% H2O2 in PBS for 10–50 s at room temperature, washed in 0.5% Tween-PBS, and dried.

T-Cell Stimulation Assay. Monocytes were prepared by adherence. Briefly, peripheral blood monocyte cells of HLA-typed healthy donors were seeded in RPMI 1640 medium and let to adhere to plastic for 18 h. The adherent cells were mechanically recovered and resuspended in RPMI 1640 and 10% human serum. As control, the recovered cells were stained with monoclonal antibody recognizing CD14 and HLA class II molecules. Flow cytometric analysis of the stained cells showed that 23% of the cells were CD14+ HLA class II+.

RESULTS

Purification of HSP70. HSP70 were purified from melanoma 15392, melanoma 501, and LCL 15392 as described in “Materials and Methods.” The protein purity was estimated to be >95% as determined by silver staining of the SDS-PAGE. The specificity of the bands seen on the silver-stained gel was checked by Immunoblot using an HSP70-specific antibody recognizing both the constitutive and the inducible forms of HSP70. An example of the HSP70 purification is shown in Fig. 1.

HSP70 Chaperoned Peptides Activate Melanoma-specific Class I, HLA-restricted, T-Cell Clones. Whereas HSP demonstrated a strong capacity to stimulate antiviral or antitumor CTLs in a murine system (6, 18), no data have been provided for HSP as a chaperone for immunogenic peptides in a human setting. In addition, immunization of mice with tumor-derived gp96 and HSP70 was shown to elicit a protective immunity, mainly directed against unique tumor antigens (6). We therefore analyzed whether HSP70 derived from human melanomas would include in its chaperoned peptide repertoire epitopes derived from normal differentiation proteins known to be immunogenic in an autologous setting. To this end, we evaluated the capacity of melanoma-purified HSP70 to functionally activate antimelanoma CTL clones recognizing MART-1, gp100, and TRP-2 melanoma antigens (Table 1). The restriction alleles for these CTL clones were HLA-A2 for MART-1, HLA-A3 and HLA-Cw8 for gp100, and HLA-Cw8 for TRP-2-specific clones, respectively (Table 1). HSP70 was purified from Me15392 autologous to the CTL DIL15392, TB254, and TB327 and from Me501 autologous to the A42 CTLs. Taking advantage of two alternative purification procedures, HSP70 complexed with the endogenous associated peptides HSP70 (ADP) or with no associated peptides HSP70 (ATP) was obtained from each melanoma cell line. Monocytes purified from class I HLA-matched peripheral blood monocyte cells of healthy donors were loaded with HSP70 (ADP) or HSP70 (ATP) and evaluated for the ability to activate melanoma-specific CTLs. As shown in Fig. 2, three of the four CTL clones, i.e., anti-gp100 CTL DIL15392 and TB254 as well as the anti-MART-1 A42, released IFN-γ in response to stimulation with HSP70 (ADP)-loaded monocytes. Moreover, HSP70 (ADP) activation was MHC class I dependent because the IFN-γ released by the stimulated CTLs was significantly inhibited by incubation of APCs with the anti-HLA-class I antibody W6/32. The level of cytokine specifically induced by HSP70 stimulation was significantly different among the CTLs tested, with the lowest release detected for the anti-MART-1-specific CTLs. This different level of CTL triggering could reflect the different binding capacity of the tumor-derived epitopes for the HSP70 binding groove.

The stimulatory activity of an equal amount of HSP70 (ATP) was also evaluated. The results for DIL15392, TB254, and A42 clones indicated that although a stimulatory activity could be detected, the amount of IFN-γ released was low. The HSP70 (ATP)-mediated
activation was diminished by the HLA class I-specific antibody, probably indicating that in the ATP purification procedure, some HSP70 complexed with endogenous peptides was still present as a contaminant. This inhibition, however, failed to reach statistical significance. No peptide-dependent, HSP70-mediated activation could be shown for the clone TB327, because this clone was induced to release similar amounts of IFN-γ by ATP-agarose (ATP, lower panel) or ADP-agarose (ADP, left panel) were loaded on monocytes (APC, 4 x 10^5) matched for HLA class I with the effectors cells and evaluated for the ability to induce IFN-γ release by antigen-specific CTLs (1 x 10^4) in the absence or in the presence of the anti-HLA-class I antibody W6/32. Monocytes incubated with medium alone and autologous melanoma (Auto-Me) were also used as stimulators. IFN-γ was evaluated by ELISA (Mabtech). Values of IFN-γ release significantly inhibited by incubation of the target with the monoclonal antibody W6/32 are indicated (+).

To better define the role of chaperone-assisted peptides and the HMC involvement in the HSP-mediated stimulation of the antitumor CTLs, different doses of ADP- or ATP-purified HSP70 were compared for the ability to induce cytokine production by the HLA-A3-restricted, gp100-specific LCL15392 (Fig. 3) and by the HLA-A2-restricted anti-MART-1 A42 CTLs. A dose-dependent, MHC class I-restricted activation of both CTL clones was achieved by the peptide containing HSP70 (ADP) (Figs. 3A and 4A), whereas the amount of IFN-γ induced by HSP70 (ATP) was not dependent on MHC class I molecules and was clearly detectable only with the highest doses tested (Figs. 3B and 4B).

To confirm that the peptide-dependent, HSP-mediated stimulation of CTL clones required the expression of the correct T-cell receptor-restricting MHC I allele on the presenting cells, different doses of HSP70 (ADP) were loaded on HLA-A2-negative monocytes and used as stimulators for the HLA-A2-restricted anti-MART-1 CTLs (Fig. 4C). No HLA class I-dependent IFN-γ release was induced by clone A42 by MHC-unmatched APCs; the amount of cytokine in the medium was comparable with the level achieved when the same clone was stimulated by HLA-matched APCs loaded with HSP70 with no endogenous peptide HSP70 (ATP) (Figs. 2 and 4B).

HSP70 Derived from Antigen-negative Cell Lines Does Not Activate Antimelanoma CTLs. To further support the notion that CTL activation occurs through recognition of their nominal peptide chaperoned by HSP70 and presented by the MHC molecules of the APCs, we analyzed the stimulatory activity of HSP70 (ADP) obtained from antigen-negative sources. For this purpose, HSP70 purified from LCL15392 not expressing any of the melanoma differentiation antigens was used in a stimulation assay of the anti-gp100, HLA-A3-restricted DIL15392 and HLA-A2-restricted anti-MART-1 A42 CTLs. No specific activation occurred for any of the antimelanoma CTLs tested (Fig. 5, A and B). For DIL15392, the low amount of cytokine detectable in the medium was at least 3 logs lower than the amount achieved by the specific peptide-dependent stimulation mediated by the HSP70 (ADP) purified from the autologous tumor (see Fig. 2A). In addition, the cytokine released in the medium by A42 was not MHC-restricted because similar background levels of IFN-γ were produced when HLA-matched or HLA-unmatched monocytes (Fig. 5B) were used as APCs.

LCL-derived HSP70 was functionally active because it was able to activate a polyclonal anti-Flu, HLA-A3-restricted T-cell line. IFN-γ was released in a dose-dependent fashion, and cytokine production was not inhibited by the incubation of the APCs with the anti-class I...
antibody W6/32 (Fig. 5C). The activation of anti-Flu CTLs was therefore mediated by a MHC-independent, antigen-unrelated stimulation. In fact, HSP70 was purified from LCLs not infected by influenza virus and therefore not expressing the epitope recognized by the effector T cells used in the assay. These results reinforced the notion that HSP70 per se may exert a stimulatory activity for T cells and, in addition, indicate that the HSP70 (ADP) MHC-dependent stimulation of CTLs can occur only when the nominal epitope is included in the HSP70-chaperoned peptide repertoire.

**HSP70-mediated T-Cell Activation Occurs via Cross-Priming.** Because HSP70-mediated T-cell activation was dependent on peptides derived from shared tumor antigens, we evaluated the capacity of HSP70 to stimulate across the MHC barriers. For this purpose, DIL15392 was stimulated with HLA-A3+ monocytes loaded with HSP70 purified by ADP or ATP agarose from an allogeneic, HLA-A3-negative melanoma expressing a high level of gp100 (data not shown). In the same experiment, the stimulating ability of HSP70 purified from the autologous melanoma was also evaluated as control. No difference in the activation pattern of DIL15392 could be detected between the autologous and allogeneic melanoma-derived HSP70, as evaluated by the amount of IFN-γ released (Fig. 6A). Furthermore, the allogeneic HSP70 CTL stimulation was MHC dependent (Fig. 6A) and linked to the presence of chaperoned peptides because peptide-free HSP70 (ATP) preparations failed to significantly stimulate cytokine release by T cells (Fig. 6). The cytokine release by DIL15392 was dependent on the dose of the allogeneic purified HSP70 (ADP) used, and IFN-γ could be detected, even at the lowest dose of 0.6 μg/ml (Fig. 6C). The overall stimulating capacity of allogeneic HSP70 (ADP) was therefore comparable with the stimulating ca-
**DISCUSSION**

In this study, we show that HSP70 peptide complexes purified from human melanoma, when loaded on HLA-matched APCs, specifically activate HLA-class I restricted antimalanoma T cells. This is the first evidence for a role of HSPs as chaperones of immunogenic peptides in a human setting. Our data also imply that the peptide sampling activity of HSP70 includes the association with peptides derived from proteins shared among different tumors. These findings open new perspectives for the clinical application of HSPs in cancer therapy that will potentially include as vaccine HSP-derived from nonautologous tumors.

Here we demonstrate that HSP70-complexes purified from human melanoma include peptides derived from normal differentiation proteins and that these epitopes, via APC presentation, may lead to the activation of the cognate T cells. In our experimental system, we were unable to check the presentation of “unique” peptides because no antimalanoma T cells recognizing unique antigens on the melanoma 15392 were available. Therefore, the relative abundance of shared versus unique epitopes in a human HSP70-chaperoned peptide repertoire could not be compared directly. Peptides derived from normal differentiation antigens should, however, be well represented in the HSP repertoire because four of five CTLs tested and recognizing differentiation antigens were stimulated by HSP70-derived peptides. Among the responding CTLs, the level of cytokine specifically induced by HSP70 stimulation was significantly different with the lower release detected for the anti-MART-1-specific CTLs. Unfortunately, we did not have the possibility to test the susceptibility of HSP70-mediated activation of CTLs expressing different T-cell receptors but recognizing the same epitope and therefore address the question of whether a different susceptibility to HSP70 stimulation could be an intrinsic characteristic of each CTL expressing a particular T-cell receptor.

T-cell clones recognizing peptides derived from MART-1, tyrosinase, and gp100 were activated in a MHC-restricted fashion by melanoma-derived HSP70 loaded on APCs, whereas the anti-TRP-2 clone was not. The melanoma cell lines used as a source for HSP70 were susceptible to lysis by TRP-2-specific CTLs, indicating that the level of expression of this protein was sufficient for the peptide to be presented by the MHC of tumor cells. We cannot, however, rule out the possibility that the abundance of HSP-TRP-2 peptide complexes is inadequate to sensitize monocytes for a T-cell-mediated recognition. Alternatively, the TRP-2 epitope cannot be allocated in the binding pocket of HSP70 because of structural constrains. Arguing against this possibility, however, both TRP-2 and gp100 HLA-Cw8 presented epitopes; the second, recognized by the T-cell clone TB254 susceptible to the HSP70-mediated activation, displays a similar primary structure (13) and is positioned inside an highly hydrophobic region of the respective molecules. Moreover, HSP70 is known to have a broad binding specificity that only requires the presence of a hydrophobic region composed of six or more amino acid residues (18). Alternatively, the regions flanking the epitopes in the TRP-2 and in the gp100 molecules may directly contribute to or negatively influence the peptide/HSP70 binding, implying that the HSP70-chaperoned peptide is indeed a longer precursor of the nominal CTL epitope. Supporting this possibility, two histidines, not present in the corresponding gp100 sequence, are found in the NH$_2$ or COOH terminus extended sequences of the TRP-2 epitope. These histidines may potentially interfere with the hydrophobic milieu required for binding.

This second hypothesis is in agreement with our data indicating the capacity of HSP70 to induce a CTL response across MHC barriers. MHC matching between melanoma cell lines used as source of HSP70 and responding T-cell clones is not required, suggesting that the HSP70-complexed peptide repertoire is not limited to the MHC class I ligands of the HSP70 donor cells. The HSP70-complexed peptides, therefore, should include precursor peptides that are then “trimmed” into the final CTL epitope. Experiments further dissecting the general mechanism involved in the HSP70-mediated activation of melanoma-specific T cells are in progress. The present data, however, show clearly that specific CTL stimulation of antimalanoma T cells takes place only in the presence of MHC-matched APC, whereas no specific activation can occur when unmatched monocytes are used. In addition, in the presence of the correct APC, HSP70 derived from autologous or allogeneic melanoma cells displayed the same efficacy in triggering the cognate CTLs. Such an efficient presentation also for peptides bound to HSP70 isolated from allogeneic melanomas cannot be the result of unspecific trimming of peptides by serum-derived proteases (19) and instead argues for a role of the APCs in the...
processing and presentation of the HSP70-chaperoned peptides to the MHC molecules. This is the first example indicating that HSP70 can elicit CTL responses via “cross-priming,” as shown previously with gp96 (10, 20). The ability of the APCs to “trim” the HSP70-bound peptides to the size associated with MHC class I does not exclude that in some cases HSP70 could preferentially bind the final sized octamer- or nonamer epitope, as shown recently for the Lα-restricted antigens of the RL1o mouse leukemia (21).

Besides the MHC-restricted, peptide-dependent CTL induction, HSP70-mediated activation of bone marrow-derived dendritic cells was dependent on the toll-like receptor-4 and resulted in the activation of the nuclear factor-κB signaling pathway. Also, previous findings obtained with gp96 have demonstrated in vivo and in vitro activation of T cells after administration of antigen-negative HSPs (23).

In conclusion, our data show that HSP70 derived from human melanoma can exert a pleiotropic triggering of antigenpresenting T cells involving class I as well as MHC-independent pathways. We therefore suggest a model where cellular HSP70 is located at the interface between the different subsets of cells in the immune system including T cells and APC. When released by tumors undergoing necrosis, HSP70 may efficiently mediate the transfer of precursor peptides for uptake by APCs and to act as an “intrinsic” adjuvant, is in line with data published recently in a human setting (22) and with our results in a murine system. These peptide-independent activation was more evident with high doses of HSPs, whereas the peptide and MHC-dependent activation generally required lower amounts of HSP70. Furthermore, the peptide-independent, HSP70-mediated activation required the presence of APCs excluding a direct activation of T cells by HSP70. This unique feature of HSP70, both being able to deliver antigenic peptides for uptake by APCs and to act as an “intrinsic” adjuvant, is in line with data published recently in a human setting and with our results in a murine system. These latter results demonstrate that murine HSP70 efficiently induce bone marrow-derived dendritic cells to express high levels of MHC and costimulatory molecules and to secrete proinflammatory cytokines.4 Similar to LPS, HSP70 treatment of bone marrow-derived dendritic cells was dependent on the toll-like receptor-4 and resulted in the activation of the nuclear factor-κB signaling pathway. Also, previous findings obtained with gp96 have demonstrated in vivo and in vitro activation of T cells after administration of antigen-negative HSPs (23).

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REFERENCES


4. Unpublished data.


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