Differential Effect of Acute and Permanent Heat Shock Protein 70 Overexpression in Tumor Cells on Lysability by Cytotoxic T Lymphocytes

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ABSTRACT

We have shown previously that acute heat shock protein (Hsp) 70 induction in a human melanoma cell line containing a doxycycline-inducible Hsp70 expression construct increases lysability of these tumor cells by cytotoxic T lymphocyte (CTL) without interfering with MHC class I expression and antigen presentation. The same parental melanoma cell line has now been transduced retrovirally to overexpress Hsp70 permanently. Here we demonstrate that MHC class I cell surface expression is again not altered and that these cells, in contrast with acutely Hsp70 overexpressing cells, do not show augmentation of CTL-mediated apoptosis. Also, long-term induction of Hsp770 in cells with the doxycycline-inducible Hsp70 construct leads to abrogation of increased lysability. Because, furthermore, after heat shock the same permanently Hsp70 overexpressing cells show Hsp70 induction and increased lysability, it is hypothesized that acutely available Hsp70 is able to chaperone proteins that are involved in CTL-mediated apoptosis of target cells and to thereby improve their lysability. We also observed that permanent but not acute Hsp70 overexpression resulted in decreased levels of Hsc70, the constitutively expressed member of the Hsp70 family. Down-regulation of Hsc70 occurs at the post-transcriptional level and can be observed also after long-term induction of Hsp70 in cells containing the doxycycline-inducible expression system. Hsc70 down-regulation might reflect a functional integration of the overexpressed Hsp70 on the basis of a chaperone network so that only acute induction will provide Hsp70 that can improve tumor cell lysability. The implications of the differential effect of acute versus permanent Hsp70 overexpression for tumor therapy are discussed.

INTRODUCTION

Hsp70-mediated protection against apoptosis has been shown in a broad variety of model systems (1). These include examples of stress-induced as well as constitutive Hsp70 overexpression. Hsp70-mediated resistance can be obtained against apoptosis induced, e.g., by heat shock, cytostatic drugs, or tumor necrosis factor α. Hsp70 prevents the association of Apaf-1 with procaspase-9 (2, 3) and blocks the assembly of a functional apoptosome. Furthermore, Hsp70 is able to antagonize AIF, the apoptosis-inducing factor (4). Overexpression of Hsp70 has been described in various tumors, and associated with enhanced tumorigenicity and resistance to therapy (5–7). Conversely, down-regulation of Hsp70 in tumor cells was found in certain animal models to enhance tumor regression (8, 9).

A second remarkable feature of Hsp70, beside its antiapoptotic function, is its role as endogenous adjuvant (10) and immunological danger signal (11, 12). Hsp70 preparations from tumor, virus-infected, or allogeneic cells can be used for vaccination against tumor, virus, or minor histocompatibility antigens (10). Hsp70 chaperones antigenic peptides and channels them in a receptor-mediated manner (13, 14) efficiently into the MHC class I presentation pathway of professional antigen-presenting cells, which are then able to prime peptide-specific CTL. The ability of Hsp70 to facilitate this cross-priming is related to a putative role of Hsp70 in the processing of endogenous antigens. It has been suggested that endogenous antigenic peptides generated by the proteasome are chaperoned in the cytoplasm by Hsc70, Hsp70, or Hsp90 and protected from additional degradation before being delivered by the transporter associated with antigen processing to the ER for association with MHC class I molecules (15). Antigenic peptides have indeed been found associated with chaperones including Hsp70 (16, 17). Interestingly, in a B16 mouse melanoma model stable transfection of Hsp70 increased MHC class I cell surface expression (18).

A third remarkable property of Hsp70, is their ability to activate innate immune responses (10, 19). Thus, for example Hsp70 that is released from dying tumor cells can contribute to an antitumor immune response in vivo (20).

To study the role of Hsp70 in resistance of tumor cells to CTL, we transfected the human melanoma cell line Ge with a Hsp70 gene under control of a tetracycline-dependent promoter (21). Ge cells, like many human tumor cells, express significant amounts of the inducible Hsp70 already constitutively (22). After induction of the transgene by doxycycline, a 2–3-fold increase of Hsp70 was observed within 24 h (21). Contrary to the expected protective effect, this acute overexpression of Hsp70 enhanced the susceptibility of the Ge target cells to CTL without interfering with antigen processing and presentation (21). We hypothesized that Hsp70, as a molecular chaperone, might improve the function of proteins that are involved in executing CTL-induced apoptosis.

To investigate whether increased lysability of target cells depends on the acuteness of Hsp70 induction we transduced Ge cells retrovirally with the Hsp70 gene to obtain cell clones with strong permanent Hsp70 overexpression. Thus, within the same cell line, effects of permanent Hsp70 overexpression in the retrovirally transduced clones can be compared with acute Hsp70 overexpression in the Tet-on system. Notably, in both systems Hsp70 expression is achieved without applying a cellular stress that would lead to an increased demand of molecular chaperones in the cell and numerous additional cellular changes.

We show here that permanent Hsp70 overexpression, in contrast with acute Hsp70 induction, does not alter lysability of Ge target cells by CTL. Interestingly, permanent in contrast with acute Hsp70 overexpression leads to a reduced expression of the normally constitutively and abundantly expressed Hsc70. Thus, the different effect of acute and permanent Hsp70 overexpression might be explained by the adaptation of a chaperone network due to permanent Hsp70 overexpression. This might also have important implications for the tumor biology of Hsp70.
MATERIALS AND METHODS

Cell Culture and Hsp70 Induction. The human melanoma cell line Ge and the sublines derived therefrom were maintained in NaHCO3-buffered DMEM containing 10% FCS (Biochrom, Berlin, Germany) in Petri dishes for tissue culture (Sarstedt, Nürnbrecht, Germany) at 37°C in a 10% CO2 atmosphere. The Ge-tet cells that overexpress Hsp70 conditionally due to the Tet-on system were described previously (21). For Hsp70 induction 5 μg/ml doxycycline (Sigma, Deisenhofen, Germany) were added to these cultures. For long-term induction of Hsp70 medium containing doxycycline was changed every day. MHC class I cell surface expression was stimulated by supplementing the medium with 5000 units/ml recombinant human IFNα (S-P Brinny Company, Innishannon, Ireland and Essex Pharma GmbH, München, Germany). For heat shock treatment melanoma cells were harvested in 1 ml EDTA in PBS, transferred to 12-ml polypropylene tubes (Sarstedt), washed in HEPES-buffered DMEM, and resuspended in this medium containing 10% FCS. Cells were exposed for 1 h to 42°C in a water bath or kept at 37°C as controls. After a recovery period of 4 h at 37°C the cells were used for additional experiments.

Retroviral Transduction of Ge Cells. The rat Hsp70–1 gene (Genebank accession no. X77207) has been cloned previously into the pUHC10–3 vector (21). This construct was used for recloning of Hsp70–1 into the retroviral expression vector pcVGwt (25), and the corresponding retroviral vector

Flow Cytometry. Flow cytometry was done on a FACSscan flow cytometer (Becton Dickinson, Heidelberg, Germany) using CellQuest software. GFP expression was analyzed after washing cells twice with PBS and resuspending them in 500 μl PBS containing 2 μg/ml PI. PI-positive dead cells, always below 8%, were excluded from analysis. For determining cell surface expression of MHC class I molecules a mouse anti-MHC class I (A, B, C) mAb (clone W6/32, IgG2a, MCA81; Serotec, Biozol, Eching, Germany) was used as described (21). Cell surface expression of Hsp70 was examined by mAb RPN1197 (Amersham Pharmacia, Freiburg, Germany) that has been reported to detect Hsp70 on the cell surface (28). Intracellular Hsp70 expression was tested after cytoplasmic staining with a mouse mAb specific for the inducible form of Hsp70 (clone C92F3A–5, IgG1, SAP-810; StressGen, Biomol, Hamburg, Germany) as described previously (21). To determine cytoplasmic Hsp70 expression a specific rat mAb (clone 1B5, IgG2a, SAP-815; StressGen) was used. Secondary reagents were a polyclonal FITC-conjugated goat antigoal IgG Ab (115–095-062; The Jackson Laboratory, Dianova, Hamburg, Germany) or goat antirat IgG Ab (112–095-062; The Jackson Laboratory, respectively). When GFP-transduced cell lines were analyzed, RPE-Cy5-conjugated F(ab)2 fragments of rabbit antimonizumum immunoglobulins were chosen as secondary reagent (C 0090; Dako, Hamburg, Germany). Cell surface and cytoplasmic TCR-β chain gene was cloned directly by reverse transcription-PCR as a water bath or kept at 37°C as controls. After a recovery period of 4 h at 37°C the cells were used for additional experiments.

Northern Blots and Real-Time PCR. RNA preparation and Northern blot analysis using gene probes for rat Hsp70–1 and β-actin were done as described previously (27). Before cDNA synthesis RNA was incubated with RNase-free DNase (Promega, Mannheim, Germany) according to the manufacturer’s instructions to exclude contamination of cDNA with genomic DNA. For subsequent cDNA synthesis 2.5 μg of DNA-free total RNA were reverse transcribed using 100 pmol oligo(dT)18 primer (GACGTAAGTGTAGCTGCTGGATCC, 40 units RNase inhibitor (Promega) and 300 units reverse transcriptase (Promega). Aliquots of this reaction product were subjected to real-time PCR using the manufacturer’s instructions to exclude contamination of cDNA with genomic DNA. For subsequent cDNA synthesis 2.5 μg of DNA-free total RNA were reverse transcribed using 100 pmol oligo(dT)18 primer (GACGTAAGTGTAGCTGCTGGATCC, 40 units RNase inhibitor (Promega) and 300 units reverse transcriptase (Promega). Aliquots of this reaction product were subjected to real-time PCR using the manufacturer’s instructions to exclude contamination of cDNA with genomic DNA.

Western Blot and Metabolic Labeling. Cells were exposed for 1 h to 42°C in a water bath or kept at 37°C as controls. After a recovery period of 4 h at 37°C 6 × 105 cells were cultured for 1 h at 37°C in 100 μl methionine-free HEPES-buffered DMEM containing 1.5 μCi [35S]methionine plus [35S]cysteine (Amersham Pharmacia). Cells were washed twice with PBS and resuspended in 100 μl sample buffer [0.0625 m Tris-HCl (pH 6.75), containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue] per 106 cells and incubated at 100°C for 5 min. The supernatant obtained after a centrifugation (10,000 × g for 5 min at 4°C) was separated by SDS-PAGE using equivalents of 2 × 105 cells per lane. For separation of cytosolic and noncytosolic proteins a pellet of 3 × 106 cells was resuspended in 180 μl lysis buffer [25 mM Tris-HCl (pH 7.4), 140 mM NaCl, 5 mM KCl, 3 mM MgCl2, and 0.5% w/v NP40] and incubated for 40 min on ice followed by a centrifugation (10,000 × g for 5 min at 4°C). The supernatant representing the cytoplasmic protein fraction was transferred into a new tube, and 150 μl sample buffer were added. The pellet including the nuclear fraction was resuspended in 300 μl sample buffer. Samples were incubated at 100°C for 5 min, and 15 μl were loaded then onto a SDS gel. Proteins were transferred to nitrocellulose (Schleicher and Schuell, Dassel, Germany) before Ab staining. The following mAbs were used at dilutions of 1:2,000 in PBS/0.05% Tween 20: anti-Hsp70 (clone C92F3A–5), anti-Hsp70 (clone 1B5), anti-Hsp70/Hsc70 (clone N27F3–3, mouse IgG1, SAP-820; StressGen, Biomol), and anti-β-actin (clone AC-15, mouse IgG1, A-5441; Sigma). Subsequently, blots were incubated with goat antimonizumum IgG Ab (115–005-003; The Jackson Laboratory, Dianova) or goat antirat IgG Ab (112–005-068; The Jackson Laboratory, Dianova) and peroxidase-conjugated rabbit antigoal IgG Ab (305–035–045; The Jackson Laboratory, Dianova) at a dilution of 1:10,000. The substrate reaction was carried out with 0.05% 3,3’-diaminobenzidine/0.003% H2O2 in PBS/0.05% Tween 20. For autoradiography, filters were exposed to Hyperfilm MP (Amersham Pharmacia) for usually 3 days. Densiometric analysis of autoradiograms was performed with an Epson GT-8000 scanner and ScanPack software (Biometra, Göttingen, Germany). For quantification of Hsp70 induction Hsp70:β-actin ratios were determined.

Immunoprecipitation. For coimmunoprecipitation of Hsp70-bound protein, pellets of 10 × 105 cells were lysed in NP40 lysis buffer containing 4 A. Asmuth and T. Herrmann, unpublished observations.

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protease inhibitors leupeptin, aprotinin, pepstatin, and phenylmethylsulfonyl fluoride. The cytosolic fraction was precleared by incubation with 0.1 volumes of protein G Sepharose slurry (Protein G Sepharose 4 Fast Flow; Amersham Pharmacia) for 30 min on ice. The lysate was then incubated for 1.5 h at 4°C with 5 μg anti-Hsp70 mAb C92 followed by addition of 0.2 volumes of protein G Sepharose slurry for another 1.5 h incubation. Unbound protein was removed by centrifugation (10,000 × g for 1 min at 4°C) and three washing steps with 500 μl NP40 lysis buffer. Finally the Sepharose pellet was resuspended in 60 μl sample buffer and incubated at 100°C for 5 min. The supernatant was loaded onto SDS gels that were Coomassie stained.

MALDI-TOF Mass Spectrometry. For the identification of coprecipitated proteins Coomassie-stained protein bands were in-gel digested with trypsin (29), desalted on C18 ZipTip, and analyzed by MALDI-TOF mass spectrometry (Reflex III; Bruker) using dihydrobenzoic acid as matrix and two autolytic peptides from trypsin as internal standards. The peptide mass fingerprint data were used by Mascot search algorithm for protein identification in the NCBI nr protein database.

Cytotoxic Cells and Cytotoxicity Assay. Generation of alloreactive CTL and the 51Cr release assay including blocking studies with anti-MHC class I-specific mAb W6/32 and EGTA were described in detail elsewhere (21). Briefly, alloreactive CTLs obtained from peripheral blood mononuclear cells of blood donors in a mixed lymphocyte culture of 5 days were cocultured with 51Cr-labeled melanoma target cells at 80:1 to 2.5:1 ratios in round-bottomed microtiter plates for 4 h at 37°C. Radioactivity released into the supernatant and radioactivity retained in the cells was determined with a Wallac MicroBeta Trilux counter (Perkin-Elmer Life Sciences, Köln, Germany) to calculate specific lysis.

RESULTS

Permanent Overexpression of Hsp70 in a Human Melanoma Cell Line. Cells of the same parental human melanoma cell line Ge that had been chosen previously for transfection with the rat Hsp70–1 gene using the Tet-On system for conditional overexpression of Hsp70 (21) were transduced retrovirally with a Hsp70 expression construct to obtain cell clones with constitutive overexpression of the normally stress-inducible rat Hsp70–1 gene. Strong overexpression of Hsp70 could be demonstrated by flow cytometry after cytoplasmic staining (Fig. 1A) and by immunoblot (Fig. 1B) in four clones (Ge-Hsp70–A, -B, -C, and -D) that were selected for additional experiments. Notably, the level of permanently expressed Hsp70 is 4 to 6 times higher in these cells than the maximal Hsp70 induction by doxycycline in the Ge-tet cells (Ref. 21; compare also Fig. 7B with Fig. 8). The corresponding rat and human Hsp70 proteins, which are encoded in the MHC, show 97% sequence identity (30), and, therefore, are not distinguishable by the available Hsp70-specific mAbs. Thus, expression of the rat Hsp70–1 gene was confirmed at the mRNA level by Northern blot using a probe specific for the 3′untranslated region of rat Hsp70–1 (Fig. 1C). To generate control cell lines overexpressing an irrelevant protein, Ge cells were transduced with a rat TCR-β or GFP expression construct derived from the same vector (23). Overexpression of TCR-β and GFP was analyzed by flow cytometry and is exemplified in Fig. 1, D and E. From both control lines (Ge-TCR and Ge-GFP) four clones (A, B, C, and D) that express the respective transgene in the same range (compare Fig. 1, D and E) were selected for additional experiments. Hsp70 expression was not affected in Ge-TCR and Ge-GFP clones (Fig. 1, B and C). As expected TCR-β chains were not detected by a specific mAb (R73) at the cell surface of the transfected cells (data not shown). The expression of the transgenes Hsp70, TCR-β, and GFP was controlled in all of the additional experiments and found to be stable in each clone.

Permanent Hsp70 Overexpression Does Not Change MHC Class I Cell Surface Expression. Constitutive Hsp70 overexpression has been reported by Wells et al. (18) to increase MHC class I cell surface expression on mouse B16 melanoma cells and their lysability by CTL. In previous experiments we had not found a change of MHC class I cell surface density after acute Hsp70 overexpression in Ge cells (21). One explanation of these discrepant results could be that permanent but not acute Hsp70 overexpression might affect MHC class I expression. Therefore, the Ge
sublines overexpressing Hsp70 or TCR-β constitutively were analyzed by flow cytometry for MHC class I cell surface expression using the pan-MHC class I mAb W6/32. The summary of three independent experiments is shown in Fig. 2. Also under conditions of permanent Hsp70 overexpression no change of MHC class I expression was found.

Hsp70 was suggested to participate in the transport of peptides to the ER, where suitable peptides are loaded onto MHC class I molecules (15). Wells et al. (18) have argued that Hsp70 overexpression could correct a relative deficiency of peptides that limits expression of MHC class I molecules because their stability on the cell surface depends on peptide loading. One could argue that in the Ge cells we have used, other factors than the delivery of peptides to the ER, e.g., the amount of peptides themselves, are limiting MHC class I cell surface expression. Therefore, we treated Ge cells with IFN-α (5000 units/ml for 72 h) that is known to enhance MHC class I gene transcription (31, 32). An increase of MHC class I cell surface expression could be demonstrated in Ge cells (Fig. 2) that is only possible if these class I molecules carry peptides. Hsp70 itself was not induced by IFN-α (data not shown). Therefore, MHC class I cell surface expression cannot be restricted by a general shortage of peptides in Ge cells. Importantly, the magnitude of MHC class I cell surface augmentation by IFN-α was not different between Ge cells overexpressing Hsp70 or TCR-β (Fig. 2).

Permanent Hsp70 Overexpression in Target Cells Does Not Alter Their Lysability by CTL. Acute overexpression of Hsp70 increased lysability of Ge cells by CTL despite unchanged MHC class I expression (21). To test whether the same effect occurs after strong and permanent Hsp70 overexpression the various transductants were used as target cells for alloreactive CTL in a standard 51Cr release assay. Permanent (Fig. 3A) in contrast with acute Hsp70 overexpression (Fig. 3B) did not alter lysability compared with GFP or TCR-β transductants or parental Ge cells. The same result was obtained with each of the four cell clones of each line (data not shown). The cytotoxic cells used were identified as CTL by effective blocking with anti-MHC class I-specific mAb W6/32 (data not shown). The CTL used the granule-exocytosis killing pathway because lysis was calcium-dependent (data not shown; see Ref. 21).

Enhanced Lysability After Acute Hsp70 Overexpression Disappears during Long-Term Expression. To test whether indeed the effects of acute and permanent Hsp70 overexpression on lysability of Ge cells by CTL are different we tried to mimic permanent Hsp70 overexpression in the Tet-on system by long-term doxycycline treatment. Lysability of Ge-tet cells was tested after long-term (8 days) and acute, short-term (24 h) induction of Hsp70. As reported previously (21), short-term induction of Hsp70 leads to increased lysability by CTL (Fig. 3B). After long-term induction, however, lysability was not changed any longer compared with control cells (Fig. 3B). Thus, increased lysability was dependent on the acuteness of Hsp70 increase in the Ge-tet system.

Effect of Heat Shock on Lysability of Target Cells by CTL. In contrast with the stress-free Hsp70 induction by doxycycline in the Ge-tet system heat shock does not only induce Hsp70 expression but increases dramatically the demand for additional chaperones in the cells. Therefore, the effect of acute Hsp70 induction by heat shock (1 h at 42°C and 6 h recovery at 37°C) on lysability of Ge cells

Fig. 2. Permanent as well as acute Hsp70 overexpression do not increase MHC class I cell surface expression. Summary of three independent experiments comparing cell surface expression of MHC class I molecules and the effect of IFN-α (5000 units/ml, 72 h) on parental Ge cells, four Ge-TCR, and four Ge-Hsp70 clones that overexpress Hsp70 permanently. Mean of the MFI obtained with pan class I mAb W6/32 are given; bars, ±SD.
Acute and Permanent Overexpression. Heat shock was observed in five of eight independent experiments as summarized in Fig. 6A. Importantly, heat shock was able to increase the CTL-mediated lysis also of permanently Hsp70-overexpressing cells (Fig. 6C). Thus, acute Hsp70 induction appears to be able to augment the susceptibility of target cells to CTL irrespective of constitutive Hsp70 expression levels.

Permanent Hsp70 Overexpression Leads to Reduced Hsp70 Expression at the Protein Level. To explain the different effects of acute and permanent Hsp70 expression we analyzed whether permanent in contrast with acute Hsp70 overexpression might lead to down-regulation of other chaperones. The most likely candidate to be affected by Hsp70 overexpression appeared to be the constitutively expressed Hsc70, which is closely related and shares its cytoplasmic and nuclear localization with Hsp70. Thus, the expression of Hsc70 was found exclusively in the cytosolic protein preparations (Fig. 5A). Heat shock (1 h at 42°C without recovery time to avoid strong Hsp70 induction) leads to appearance of Hsp70 in the noncytosolic fraction, compatible with a translocation of Hsp70 into the nucleus (Fig. 5A). In Ge-Hsp70 cell clones that overexpress Hsp70 constitutively a fraction of Hsp70 was found in noncytosolic protein preparations in the absence of cellular stress. In the Ge-TCR control cell lines Hsp70 was found exclusively in the cytosolic protein preparations (Fig. 5A). We analyzed Hsp70 localization also in Ge-tet-1 cells that were treated with doxycycline for 1 day for acute Hsp70 overexpression and in cells treated for 5–8 days with doxycycline to mimic permanent Hsp70 overexpression in the Tet-on system. Ge-tra cells that were transfected with the transactivator of the Tet-on system only (21) served as controls. In both cell lines traces of Hsp70 were found in noncytosolic protein preparations of untreated cells. Hsp70 increased in both fractions after acute (1-day) and long-term (5-day and 8-day) Hsp70 induction by doxycycline in Ge-tet-1 cells (Fig. 5B). Because this noncytosolic localization occurred after acute as well as permanent Hsp70 overexpression it does not offer an explanation for the observed differences in lysis by CTL.

Because Hsp70 has been reported to function as recognition structure for natural killer cells (35) when expressed at the cell surface of target cells, we also tested cell surface expression by flow cytometry. As reported previously (21) no Hsp70 was detectable on the cell surface of parental Ge cells as well as on Ge-tet cells before and after acute Hsp70 induction. In addition, permanent Hsp70 overexpression also did not lead to a cell surface localization of Hsp70 in the four Ge-Hsp70 clones (data not shown).

Heat Shock-Induced Hsp70 Expression and Increased Lysability by CTL Is Not Impaired by Permanent Hsp70 Overexpression. Interestingly, despite the strong overexpression of Hsp70 heat shock inducibility of Hsp70 was in the normal range in permanently Hsp70 overexpressing transductants as demonstrated by metabolic labeling that allows to assess the synthesis of new proteins after heat shock (Fig. 6A). Thus, the demand for additional Hsp70 chaperones after stress exists also in Hsp70-overexpressing cells. The same result was obtained with each clone of each cell type as summarized in Fig. 6B. Importantly, heat shock was able to increase the CTL-mediated lysis also of permanently Hsp70-overexpressing cells (Fig. 6C). Thus, acute Hsp70 induction appears to be able to augment the susceptibility of target cells to CTL irrespective of constitutive Hsp70 expression levels.

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Fig. 4. Heat shock can enhance lysability of target cells by CTL. Ge cells were heat shocked (hs) at 42°C for 1 h and allowed to recover at 37°C for 4 h or kept at 37°C (co) before being labeled with 35S and used as targets for alloreactive CTL in a standard 51Cr release assay. Mean of triplicates each of two individual experiments of eight are shown; bars, ±SD. Hsp70 induction by heat shock was tested in each experiment and was in the range shown in Fig. 6B.

Fig. 5. Acute as well as permanent Hsp70 overexpression lead to noncytosolic Hsp70 localization. A, the presence of Hsp70 in cytosolic and noncytosolic protein preparations was analyzed by immunoblot with the specific mAb C92 in parental Ge, Ge-Hsp70, and Ge-TCR cells. Ge cells were tested under control conditions (co) or after heat shock (hs, 1 h at 42°C without recovery time). B, cytosolic and noncytosolic protein preparations of Ge-tra and Ge-tet-1 cells were tested for Hsp70 by immunoblot after doxycycline treatment (5 μg/ml) for 0, 1, 5, and 8 days, the latter to mimic permanent Hsp70 overexpression in the Tet-on system. The blots containing cytosolic protein preparations was probed with a β-actin-specific mAb (Ac-15) as loading control.
pression level of Hsc70 was quantified by flow cytometry using a specific mAb (Fig. 7A). Hsc70 expression was indeed found to be significantly reduced (P < 0.001, unpaired t test) in Hsp70-overexpressing Ge-Hsp70 compared with Ge-TCR cell clones (Fig. 7, A and B). The same reduction of Hsc70 expression levels could be demonstrated by immunoblot analysis using the Hsc70-specific mAb 1B5. Ratios of Hsc70:β-actin in Ge-Hsp70 cell clones was significantly reduced (P < 0.001, unpaired t test) by 50% compared with Ge-TCR and Ge-GFP clones as demonstrated by densitometry of the immunoblots.

To analyze whether Hsc70 is down-regulated at the transcriptional level, expression of the endogenous human Hsc70 and Hsp70–1 mRNA, as well as the transgenic rat Hsp70–1 mRNA were determined with specific primers in Ge-Hsp70 cells by real-time PCR and evaluated as ct, i.e., the PCR cycle in which the amplification products become detectable against the background. Rat Hsp70–1 and human Hsp70–2 are orthologous genes encoded in the MHC (36). As expected, transcripts of the rat Hsp70–1 gene were found only in the Ge-Hsp70 (mean ct value 19.1) but not in the Ge-TCR cell clones (mean ct value 48.6; ct values >40 are usually interpreted to indicate that a gene is not expressed). The expression of the endogenous human Hsp70–2 and Hsc70 mRNA was not different between Ge-Hsp70 and Ge-TCR clones (Fig. 7C). Thus, the decrease of Hsc70 expression shown above at the protein level is not seen at the RNA level and, thus, appears to be due to post-transcriptional regulation.

Long-term but not short-term Hsp70 induction in the Tet-On system leads to a down-regulation of Hsc70. We then tested whether also in Ge-tet cells Hsp70 overexpression would affect the Hsc70 level. No effect of Hsp70 overexpression on Hsc70 expression was observed after short-term induction (24 h) of the transgene (Fig. 7). However, long-term induction of Hsp70 for 8 days in doxycycline-containing medium resulted in a significant reduction (P < 0.05, paired t test) of Hsc70 expression also in the
containing medium was changed each day.

To elucidate whether Hsp70 could replace Hsc70 in

nonsignificant effect of doxycycline alone was weak compared

with the effect observed in Ge-tet cells after Hsp70 overexpression

of Hsc70 expression after long-term culture with doxycycline was

Ge-tet cells compared with control cells (Fig. 8). A slight reduction

Hsp70 and Hsc70 expression by flow cytometry (mAbs C92 and 1B5, respectively) in Ge,

Ge-tra, and Ge-tet cells after doxycycline treatment (5 μg/ml, 24 h or 8 days) and in

untreated control cells (co); bars, ±SD. For long-term induction of Hsp70 the doxycycline

containing medium was changed each day.

Hsp70 Binds to Clathrin in Cells Overexpressing Hsp70 Permanently. To elucidate whether Hsp70 could replace Hsc70 in

cells that overexpress Hsp70 permanently we immunoprecipitated Hsp70 in Ge-Hsp70 and Ge-TCR cell clones, and analyzed coimmunoprecipitated molecules by SDS-PAGE (Fig. 9). One prominent band of ~180 kDa was reproducibly present in precipitates from Ge-Hsp70 but not Ge-TCR cells. This band was analyzed by MALDI-TOF mass spectrometry and identified as clathrin. Hsc70 is well known to function as clathrin-uncoating ATPase (37). Thus, binding of Hsp70 to clathrin suggests that Hsp70 is able to replace Hsc70 in cells that are forced to overexpress Hsp70 permanently.

DISCUSSION

We have previously transfected the human melanoma cell line Ge with a rat Hsp70 gene under the control of a tetracycline-inducible promoter (21). This system allowed for the study of the effect of acute Hsp70 overexpression without applying stress such as heat shock that is known to interfere with many cellular processes. Now, we have established clones that are derived from the same parental cell line Ge and overexpress Hsp70 permanently. Thus, we are able to compare effects of stress-free acute Hsp70 overexpression with stress-free permanent Hsp70 overexpression and, furthermore, with stress-induced Hsp70 expression. We have used these tools to determine the role of Hsp70 in CTL-induced apoptosis of tumor cells.

Induction of Hsp70 in Ge-tet cells had been shown by us to enhance susceptibility to CTL-mediated lysis independent of antigen processing and without affecting MHC class I cell surface expression (21). These findings were at variance with a report that permanent Hsp70 overexpression augments MHC class I cell surface expression (18, 38), and thereby increased lysability by CTL (18).

Thus, two questions were raised in this study. Firstly, does permanent unlike acute Hsp70 overexpression increase MHC class I expression at the cell surface in Ge cells? Secondly, does permanent like acute Hsp70 overexpression increase susceptibility of Ge cells to lysis by CTL?

The concept that Hsp70 is involved in antigen presentation was proposed in 1994 by Srivastava et al. (15), who suggested that antigenic peptides generated at the proteasome are chaperoned in the cytoplasm by Hsc70, Hsp70, or Hsp90 and protected from additional degradation before being delivered to the ER by the transporter associated with antigen processing for association with MHC class I molecules. An augmentation of MHC class I expression due to Hsp70 overexpression as described by Wells et al. (18) would support this concept (39). However, as shown here, MHC class I cell surface expression was not increased in permanently Hsp70 overexpressing Ge melanoma cell clones compared with parental Ge cells, or clones overexpressing a TCR-β chain or GFP as a control. Thus, neither acute (21) nor permanent Hsp70 overexpression affects MHC class I expression in Ge cells. In view of the report by Wells et al. (18) and our results, increased MHC class I cell surface expression due to Hsp70 overexpression cannot be a general phenomenon, even when overexpression of Hsp70 is permanent, but might depend on the cell line that is analyzed. A relative lack of peptides necessary for class I expression on the cell surface could be excluded as reason for our finding by the stimulating effect of IFN-α on class I expression.

In contrast with our results about acute overexpression of Hsp70 using the Tet-on system (21) permanent overexpression of Hsp70 did not increase lysability of Ge cells by CTL. Importantly, also no resistance against lysis was observed. Both findings are remarkable, because the antiapoptotic functions of Hsp70 are well established (1–6). Our results obtained with transfectants derived from the same parental cell line Ge indicate that the outcome of acute versus permanent Hsp70 overexpression is remarkably different and might be biologically highly relevant. It is important to note that Hsp70 overexpression in both systems is not achieved by treatments such as heat shock that lead to protein denaturation and subsequent activation of the cellular stress response system (40). Nevertheless, we also analyzed the effect of heat shock on lysability of Ge cells by CTL. Lysability was increased in most experiments, whereas resistance to CTL was never observed. Also in Ge-Hsp70 cells that overexpress Hsp70 permanently heat shock could augment CTL-mediated lysis. Thus, acute Hsp70 overex-
pression either due to stress-mediated induction by heat shock or to stress-free induction in the artificial Tet-on system can increase the susceptibility of target cells to CTL. Variations in the magnitude of Hsp70 induction due to stress in relation to the actual demand of the cell for additional chaperones could be associated with differences in the susceptibility of cells to CTL-mediated cytotoxicity and explain the more variable results of the heat shock experiments compared with the induction of Hsp70 in the Tet-on system.

The magnitude of Hsp70 induction due to heat shock is not diminished in the permanently Hsp70 overexpressing Ge cells compared with control Ge cells. Thus, the endogenous Hsp70 becomes strongly induced after heat shock when the demand of chaperones is increasing independent of constitutive Hsp70 expression levels. The Hsp70 that is permanently overexpressed in Ge-Hsp70 cells is obviously integrated functionally in the chaperone system at steady state conditions and, therefore, not available to meet the requirements for Hsp70 after stress. We reasoned that the permanent strong overexpression of Hsp70 in the retrovirally transduced Ge cells might affect the expression levels of other chaperones in the cell. The most likely candidate to be affected appeared to be the constitutively expressed cytosolic Hsp70 family member Hsc70. When we compared Hsp70 and Hsc70 protein levels in Ge-Hsp70 and Ge-TCR cells, we found that permanent Hsp70 overexpression had indeed resulted in reduced Hsc70 expression levels. The same observation was made in K562 cells that were transduced to overexpress Hsp70 permanently, indicating that the Hsc70 counter-regulation is not a particularity of Ge cells. We speculate that in the transductants Hsp70 takes over some physiological functions of Hsc70, because it might be more difficult for the cell to control the forced expression of the Hsp70 transgene than the expression of the endogenous Hsc70. This hypothesis is supported by our finding that Hsp70 is bound to clathrin in Ge-Hsp70 cells but not in control cells and might, therefore, partly replace Hsc70 as clathrin-uncoupling ATPase.

We also tested whether the differential effects of acute versus permanent Hsp70 overexpression on Hsc70 levels can be reproduced in the Ge-tet system. Indeed after 8 days of stimulating Hsp70 expression by doxycycline Hsc70 expression was reduced in Ge-tet cells. In contrast no effect was observed after 24 h indicating that the adaptation of the cells to higher Hsp70 levels takes some time and can be followed in vitro. Notably, this regulation occurs at the protein and not at the mRNA level.

A similar feedback regulation of chaperone expression levels has been described recently for the ER chaperone Grp78 (BiP; Ref. 41). Using a tetracycline-dependent system to overexpress mouse BiP in human HeLa cells these authors reported a replacement of human BiP by mouse BiP on activation of mouse BiP expression without increase of the total amount of BiP. This control of BiP expression level was found to occur at the post-transcriptional level (41). Our results obtained with Hsp70/Hsc70 are in accord with these data. In addition we show a compensatory replacement between different chaperones.

The Hsp70/Hsc70 self-regulation has functional consequences. Enhanced lysability by CTL due to Hsp70 overexpression in the Ge-tet system was only observed after acute Hsp70 induction that does not reduce Hsc70 expression. This would indicate that it depends on the balance between Hsp70 induction and the Hsp70/Hsc70 demand in an individual cell whether Hsp70 chaperones are available for an interaction with proteins that in turn would lead to enhanced CTL-mediated apoptosis. We speculate that the lysis-promoting effect after acute Hsp70 overexpression in the Ge-tet system is mediated by “free” Hsp70 that has not yet bound proteins or peptides and becomes available to interact with proteins that are involved in the execution of CTL-mediated apoptosis in the granule pathway (42). Granzyme A itself has been shown to bind to Hsp27 and Hsp70 (43), and is, therefore, an obvious candidate. Furthermore, Hsp70 has been shown recently to stabilize the function of the caspase-activated DNase (44).

Our results have several implications for the interpretation and understanding of Hsp70 in stress response and tumor biology. Firstly, an interdependence of Hsp70 and Hsc70 expression exists at the post-transcriptional level that points to a self-regulating chaperone network. Therefore, Hsp70 overexpression has to be interpreted carefully, because, as in our example, secondary effects on other chaperones, such as Hsc70, could occur. Secondly, many human tumor cells express the otherwise inducible Hsp70 permanently in the course of malignant transformation. In addition, acute overexpression of Hsp70 may occur in tumors due to hypoxia, starvation, or oxidative stress. The functional consequences of these two Hsp70 expression modes, acute versus permanent, might be different also in vivo. According to our in vitro model acute Hsp70 overexpression is able to augment susceptibility to CTL whereas permanent overexpression of Hsp70 has no such effect.

Thirdly, acute Hsp70 induction is possible irrespective of the constitutive Hsp70 expression level as shown here by the Ge and Ge-Hsp70 cells. Furthermore, tumor cells that, due to Hsp70 overexpression, are protected against apoptosis mediated by tumor necrosis factor α (45, 46) or chemotherapeutic drugs (47) might, according to our results, nevertheless be rendered more susceptible to CTL-mediated cytotoxicity by acute Hsp70 induction. Thus, immunotherapeutic approaches to elicit a CTL response against tumors might benefit from acute induction of Hsp70, e.g., by hyperthermia (48).

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