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Melanoma-associated antigen tyrosinase but not Melan-A/MART-1 expression and presentation dissociate during the heat shock response

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Abstract

Heat shock has been shown to have pleiotropic effects on tumor physiology besides a direct cytotoxic effect. In the present study, we address the question whether heat shock treatment has an impact on the antigenicity of human melanoma cells and their specific recognition by cytotoxic lymphocytes. The heat shock response was induced by treating the cells with two different thermal isoeffect doses, which resulted in equivalent clonogenic survival, mimicking doses achieved during clinical hyperthermia treatment of tumors. Antigen expression and immune recognition by cytotoxic T cells was studied using the human melanoma cell lines 624.38-MEL, SK-MEL23, WM115 and WM266-4, which naturally express, process and present tyrosinase and Melan-A/melanoma antigen recognized by T cells (MART)-1-derived peptides in the context of HLA-A2 molecules. We demonstrate that during the heat shock response following the two thermal doses, heat shock protein 70 (M_r 72 kDa) (HSP70) was induced with differential kinetics; tyrosinase protein and mRNA levels dissociated with a significant increase in tyrosinase protein and a decrease in transcript levels. A similar dissociation was not observed for Melan-A/MART-1. Furthermore, tyrosinase-specific T-cell recognition did not correlate with changes in HSP70 and antigen protein levels. These results suggest that caution has to be taken when considering protein levels as a marker for the antigenic status of a tumor. Moreover, these results document the maintenance of immunological homeostasis during recovery from heat treatment, thus challenging the view that tumor cells subjected to heat shock become resistant to CTL recognition.

Introduction

The clinical results of hyperthermia in the combined treatment of several human cancers have recently been updated (1). By acting as a chemo- and radiosensitizer and by increasing the blood flow, loco-regional hyperthermia was found to be effective as an adjuvant in clinical trials for melanoma (2) as well as for certain other solid tumors (3). The initial rationale for the use of hyperthermia in the treatment of cancer was based on the observation that temperatures >43°C are directly cytotoxic to tumor cells as a function of time. However, the heterogeneous tumor temperature distribution achieved at the tumor site ranges between 40 and 44°C and is frequently <43°C (4). Furthermore, fever-like whole-body hyperthermia which ranges between 39 and 40°C also shows efficacy in mouse tumor models and is currently being investigated in phase I clinical trials (5, 6). These studies indicate that the biological efficacy of hyperthermia is most likely not only the result of direct cytotoxicity but also might include other mechanisms (7, 8).

Several groups currently investigate the immunologically relevant changes in tumor cell physiology and the susceptibility to immune effector cells after heat shock treatment. Davies *et al.* published the first studies on the effect of hyperthermia on antigen expression, showing a heat-related, dose-dependent decrease of melanoma surface antigens by shedding and masking of surface antigens (9, 10). Further studies showed

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a decrease in the presentation of exogenous antigens by MHC class II (11) and an abrogation of co-stimulatory functions in antigen-presenting cells after heat shock (12). Some groups showed that heat shock conditions reduce the susceptibility of target cells to monocyte cytotoxicity (13), to CTL attack (14–16) and to the effect of cytokines, such as tumor necrosis factor-α (17–21).

Another aspect of heat-inducible heat shock protein 70 (M_r 72 kDa) (HSP70) is its role in protection against stress-induced apoptosis (22). Overall, these results suggest that heat shock induces a state of immunological resistance in tumor cells.

On the other hand, evidence has accumulated that hyperthermia and the associated heat shock response increase the immunogenicity of cancer cells (23, 24). These changes include induction of MHC class II-restricted presentation of endogenous antigens (25) and the enhancement of MHC class I antigen presentation via heat shock protein (hsp) expression (26). Expression of inducible HSP70 was also found to be associated with increased tumor immunogenicity (27–29) and with enhanced susceptibility of tumor cells to cytotoxic lymphocytes (30–32).

With regard to these heat-induced immunological effects, the results reported so far are inconsistent, claiming heat-induced immunoresistance as well as immune stimulation.

In the present study, we have evaluated more specifically the time–temperature-dependent effects of heat exposure. We employed an experimental system that allowed us to follow the time–temperature-dependent expression of specific antigens and their processing and presentation via MHC class I. We selected the melanoma system because it is the most widely characterized tumor with regard to tumor-associated antigens, like tyrosinase and Melan-A/MART-1, their epitopes and their restriction elements for MHC class I and II presentation (33, 34). Based on the dose-dependent effects of clinical hyperthermia treatment of melanoma (2), we selected two different thermal doses, one below and one above the breakpoint temperature that mimic the heterogenicity of the temperature distribution achieved within tumors under clinical conditions. We demonstrate that following heat exposure, HSP70 and antigen expression display distinct expression and kinetics that reflect the thermohistory of the cells. Immunologically, low thermal dose (41.8°C/120 min) did not alter immune recognition of the cells, whereas high thermal dose induced pleiotropic effects, altering antigen expression, surface presence of HLA-A2 and T-cell recognition, however, only in a transient manner. These results challenge previous observations proposing that cells subjected to heat shock lose antigenicity and the capacity to activate immune effector cells.

Methods

Tumor cell lines and lymphocyte effector cells

The human melanona cell lines 624.38-MEL and SK-MEL23 (tyrosinase and Melan-A/MART-1 positive; HLA-A*0201 positive) were a kind gift from M. C. Panelli (National Institutes of Health, Bethesda, MD, USA). 624.38-MEL is a clone of the bulk melanoma cell line 624 expressing high levels of HLA-A2 molecules on the cell surface (35). WM115 and WM266-4 were purchased from the ESTDAB (European Searchable Tumour Cell Bank and Database) co-ordinated by G. Pawelec (Tübingen, Germany). WM115 (ESTDAB-066) and WM266-4 (ESTDAB-076) are human melanoma cell lines derived from primary tumor and from metastatic tumor, respectively. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% humidity, unless otherwise stated, in medium supplemented with 1% streptomycin sulfate and sodium penicillin G and 1% MEM non-essential amino acids.

The HLA-A*0201-restricted tyrosinase peptide $tyr_{368–376}$ (YMNGTMSQV)-specific cytotoxic T-cell clone, TyrF8 (36), was kindly provided by P. Schrier (Department of Clinical Oncology, Leiden University Hospital, the Netherlands). The HLA-A2-restricted Melan-A/MART-1 peptide (AAGIGILTV)-specific cytotoxic T-cell clone, A42 (37, 38), was kindly provided by M. C. Panelli. TyrF8 and A42 cells were cultured in 24-well plates using RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 7.5% heat-inactivated FCS, 7.5% heat-inactivated pooled human serum and 50 U ml^{-1} of rIL-2 (Proleukin, Cetus Corp., Emeryville, CA, USA). TyrF8 and A42 CTLs were stimulated biweekly with irradiated [10 000 rad, using a ^{137}Cs-source Gammacell 40 (Atomic Energy of Canada, Ottowa, Canada)] IL-2-secreting HLA-A*0201-positive and tyrosinase- and Melan-A/MART-1-positive melanoma cells, irradiated (5000 rad) pooled allogeneic PBMC and irradiated (15 000 rad) EBV-transformed allogeneic B-lymphoblastoid cell lines. Fresh medium was given every third day and TyrF8 and A42 cells were split when cells were confluent. For functional assays measuring antigen-specific induction of cytokine secretion, TyrF8 and A42 were used between days 8 and 14 after the last stimulation.

JB4 is a T-cell clone recognizing HLA-A2-expressing cell lines independent of specific peptides and not discriminating among the HLA-A2 molecular subtypes (HLA-A*0201, A*0205 and A*0220). The clone was generated in our own facility (by E.N.) by limiting dilution cloning of an allogeneic mixed lymphocyte tumor cell culture. Briefly, peripheral blood lymphocytes (PBLs) from the healthy donor J.B. (HLA-A2 negative) were used as responder cells against an in-house renal cell carcinoma line (RCC-26, HLA-A2 positive) (39). After two rounds of stimulation, responding T lymphocytes were cloned at a density of 0.3 cells per well in a 96-well round-bottom plate, using irradiated (10 000 rad) RCC-26 cells and pooled allogeneic PBMC (5000 rad) as feeder cells. The specificity of JB4 was determined in cytotoxicity and cytokine stimulation assays, using various allogeneic HLA-typed cell lines and was found to be HLA-A2 specific. JB4 was propagated like TyrF8, with the exception that irradiated RCC-26 cells were used instead of melanoma cells.

Clonogenic assay for survival of heat-treated melanoma cells

The ability of tumor cells to form colonies after various hyperthermia exposures was assessed using the clonogenic assay as described previously (40, 41). Briefly, exponentially growing cultured melanoma cells were exposed to different temperatures between 41.8 and 45°C for different incubation times (10–150 min). At defined time points, viable, adherent cells were harvested by trypsinization and seeded in duplicate T25 tissue culture flasks at 100 and 500 cells per flask and allowed to form colonies in an undisturbed, humidified,

37°C/5% CO_2 air atmosphere. After 7 days, flasks were washed with 0.9% NaCl solution and cell colonies were stained with crystal violet solution (20% ethanol, 0.8% ammonium oxalate and 2% crystal violet). Only colonies containing at least 50 cells were considered to be viable survivors. The number of colonies was counted and colony efficiency of each treatment was scored as survival fraction (SF) according to the formula SF = number of colonies for a defined temperature and duration/(number of plated cells × plating efficiency). Plating efficiency (PE) is determined by dividing the number of colonies of untreated cells by the number of plated cells. The mean PE in three independent experiments was 89% (0.89 ± 0.01) for 624.38-MEL, 20% for SK-MEL23 and WM115 and 11% for WM266-4. The SFs were plotted on a logarithmic scale (*y*-axis) against the time of heat exposure (in min) on a linear scale (*x*-axis) (Fig. 1).

The 'breakpoint temperature' was defined as the critical temperature above which cells start to die exponentially. The breakpoint temperature for 624.38-MEL was 43.5°C (Fig. 1), for SK-MEL23 and WM115 was 43°C and for WM266-4 was 42°C (data not shown).

Thermal isoeffect doses and cell viability

Heat exposure as a function of time was performed using temperatures below and above the breakpoint temperature as determined for every individual cell line. The duration of heat exposure was adjusted to achieve equivalent SFs at the specified temperatures [thermal isoeffect dose (TID)], according to the classical methods (42). For 624.38-MEL and SK-MEL23 cells isosurvival fractions of 45% were achieved when cells were heated either at 41.8°C for 120 min or at 45°C for 22 min (Fig. 1). Isosurvival thermal doses for WM115 were 41.8°C for 120 min and 44°C for 25 min (data not shown). Isothermal doses for WM266-4 cells were 41.8°C/120 min and 43°C/10 min (data not shown). Heating was performed by directly immersing the cell culture flasks, sealed with parafilm in a temperature-controlled water bath. Control cells were sealed and incubated at 37°C for exactly the same time as the heat-exposed samples. After treatment, the cells were returned to 37°C/5% CO_2 and allowed to recover for different time periods (up to 7 days), as indicated in the figure legends. At the defined time points of recovery, non-viable cells in the supernatant were washed out and only adherent viable cells were trypsinized and harvested for analysis. Cell viability of adherent cells was evaluated by trypan blue dye exclusion and was always >90% (data not shown).

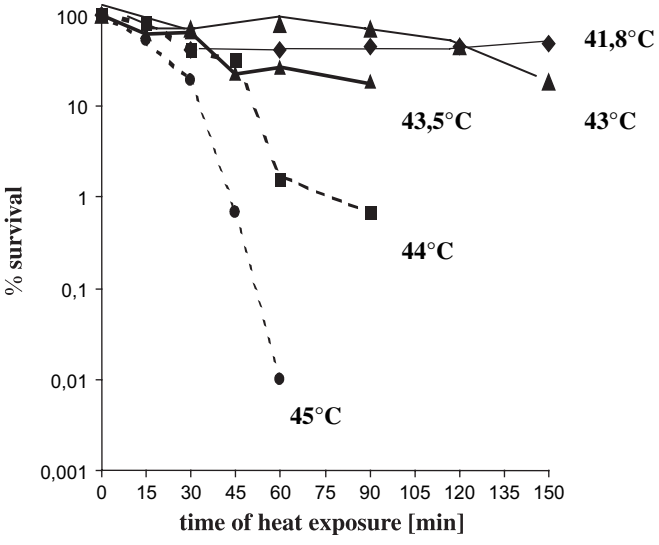

Fig. 1. Clonogenic cell survival of 624.38-MEL after heat exposure. 624.38-MEL cells were exposed to temperatures of 41.8/43/43.5/44/45°C for various time periods up to 150 min. At intervals of 15 min, 624.38-MEL were trypsinized and plated at 100 and 500 cells per T25 flask and allowed to grow and form colonies in a 37°C/5% CO_2 environment. After 7 days cells were fixed and stained and colonies were counted. The survival fraction was determined and plotted on a logarithmic scale on the *y*-axis versus the time of heat exposure on the *x*-axis in a linear scale. The temperature 43.5°C was determined as the 'breakpoint temperature', defined as the critical temperature at which cells above this level start to die exponentially. One experiment representative of three is shown. Similar clonogenic survival profiles were found for SK-MEL23, WM266-4 and WM115 with cell individual breakpoint temperatures (see Methods).

Flow cytometric analysis of surface expression of MHC class I and intracellular expression of HSP70 and HSC70

Heated and non-heated cultures were analyzed after 4, 15, 24, 48 and 72 h of recovery at 37°C after heat exposure. At these time points, cultures were washed with PBS to remove dead (non-adherent) cells and viable adherent cells were harvested by trypsinization. For surface immunostaining, cells were incubated with primary antibodies for 30 min at 4°C, washed with PBS containing 5% FCS and then incubated with phycoerythrin-conjugated goat anti-mouse IgG (DAKO, Hamburg, Germany) for 30 min at 4°C. To measure overall MHC class I molecule expression, the hybridoma cell supernatant W6/32 (43) was used at a 1 : 2 dilution. To detect the HLA-A2 allotype, the hybridoma supernatant HB54 (44) was used undiluted. Isotype control antibodies were purchased from Immunotech (Hamburg, Germany).

Intracellular staining for HSP70 and constitutively expressed heat shock protein cognate 70 (M_r 73 kDa) (HSC70) was performed using the permeabilization kit 'Fix and Perm' (Dianova, Hamburg, Germany), according to the manufacturer's instructions. Briefly, cells were washed with PBS plus 5% FCS and permeabilized with reagent A for 15 min at room temperature. After washing, cells were incubated with reagent B and the primary antibody for 30 min at 4°C in the dark. Cells were washed with PBS/5% FCS and then incubated with reagent B containing the secondary antibody for 30 min at 4°C in the dark. After washing, cells were fixed in 1% PFA and analyzed for fluorescence staining using a FACScan (Becton Dickinson, Mountain View, CA, USA) and standard Cellquest software. Primary antibodies included the monoclonal rat IgG1 6B3 (45) recognizing the human inducible HSP70, the monoclonal rat SPA-815 antibody (StressGen Biotechnologies, Victoria, Canada) specific for the HSC70. All rat-derived primary antibodies were detected using a rabbit anti-rat FITC-conjugated secondary antibody (DAKO).

Western blot analysis

Melanoma cells were lysed in 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS) buffer (2% CHAPS in 50 mM HEPES and 200 mM NaCl, pH 7.5) containing a mixture

of protease inhibitors (10 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.2 mM sodium vanadate, 10 μl ml^{-1} phenylmethylsulfonylfluoride and pepstatin A, leupeptin A, aprotinin, antipain and chymostatin, all at 1 μl ml^{-1}, freshly added just before cell lysis) for 30 min at 4°C. Whole-cell lysates containing 40 μg protein, as determined by the Lowry method (BioRad, Munich, Germany), were denatured by boiling for 5 min in SDS sample buffer, loaded onto one lane and separated by 10% SDSP. After electrophoresis, proteins were transferred to nitrocellulose membranes (Sartorius, Goettingen, Germany) and probed with appropriate antibodies. Detection of the protein–antibody complexes was achieved using the enhanced chemiluminescence system (Amersham Life Science, Karlsruhe, Germany). Tyrosinase was detected using the C-19 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), which was raised against the C-terminal peptide of human tyrosinase. Human Melan-A/MART-1 was detected using the A103 mouse mAb (DAKO, Glostrup, Denmark) (38). HSP70 and HSC70 were detected using the monoclonal rat IgG1 6B3 (45) and the rat monoclonal SPA-815 (StressGen Biotechnologies), respectively. Equal protein loading was verified by immunodetection of tubulin using the rabbit anti-human β-tubulin antibody (Santa Cruz Biotechnology).

Analysis of mRNA levels of tyrosinase, Melan-A/MART-1 and HLA-A2 by quantitative reverse transcription PCR

A two-step reverse transcription–PCR (RT-PCR) protocol was performed for the semi-quantitative detection of tyrosinase, Melan-A/MART-1 and HLA-A2 transcripts for all melanoma cell lines after heat exposure. Quantitative real-time RT-PCR was performed by LightCycler technology (Roche Diagnostics, Mannheim, Germany) using SYBR green fluorescence. In brief, 0.5×10^6 to 0.6×10^6 cells were harvested and lysed in 200 μl TriReagent (Biozol, Eching, Germany) at different time points (4, 15, 24 and 48 h), after being exposed to isoeffect thermal doses either below or above the breakpoint temperature. An equal number of cells cultured at 37°C were harvested and used as a control. After isolation of total cellular RNA according to manufacturer's instructions (TriReagent, Sigma, Deisenhofen, Germany), an aliquot of 5 μl of RNA was reverse transcribed using oligo$(dT)_{15}$ primer and AMV reverse transcriptase (first strand cDNA synthesis kit for RT-PCR, Roche Diagnostics). For the detection of tyrosinase transcripts, tyrosinase-specific primers HTYR1 (sense 5′-TTG GCA GAT TGT CTG TAG CC-3′) and HTYR2 (anti-sense 5′-AGG CAT TGT GCA TGC TGC TT-3′) were used, as previously described by Smith *et al.* (46), resulting in an amplicon of 284-bp length. Primers for Melan-A/MART-1 were 5′-Melan-A/MART-1 (sense 5′-ATG CCA AGA GAA GAT GCT CAC-3′) and 3′ Melan-A/MART-1 (anti-sense 5′-AGC ATG TCT CAG GTG TCT CG-3′), resulting in an amplicon of 384-bp length. HLA-A2-specific amplicons were generated using the primers 5′-HLA-A2 (sense 5′-CTT CAT CGC AGT GGG CTA CGT-3′) and 3′-HLA-A2 (anti-sense 5′-TCC CAC TTG TGC TTG GTG GT-3′).

As an internal control, the housekeeping gene α-enolase was used to confirm that comparable amounts of total RNA were used for all samples in RT-PCR. A 619-bp amplicon was generated with the primers α-Eno1 (sense 5′-GTT AGC AAG AAA CTG AAC GTC ACA-3′) and α-Eno2 (anti-sense 5′-TGA AGG ACT TGT ACA GGT CAG-3′). All primers were selected to span exon–intron boundaries, to prevent occurrence of co-amplification of genomic DNA. Amplification of α-enolase was performed in separate capillaries but in the same real-time PCR run as the tyrosinase, Melan-A/MART-1 and HLA-A2 samples.

For each LightCycler reaction, a master mix of 18 μl was prepared, containing 4 mM $MgCl_2$, 10 pmol of both primers and 2 μl of the ready-to-use LightCycler Fast Start DNA Master SYBR Green I (Roche Diagnostics). A total of 18 μl master mix and 2 μl of each cDNA were added to glass capillaries placed in adapters. Sealed capillaries were centrifuged briefly in a microcentrifuge and placed in the LightCycler rotor. For online detection of amplified products using SYBR Green I, the following protocol including a three-segment PCR amplification and melting curve program was used: initial denaturation at 95°C for 10 min, 35 cycles of amplification with 1 s at 95°C, 10 s at 55°C and 25 s at 72°C for tyrosinase; initial denaturation at 95°C for 10 min, 40 cycles of amplification with 1 s at 95°C, 10 s at 57°C and 20 s at 72°C for Melan-A/MART-1 and 10 s at 60°C and extension 30 s at 72°C for HLA-A2. Melting curve analysis and cooling was done according to the manufacturer's instructions. Specificity of reaction and fragment size were confirmed by running PCR products on a 2% agarose gel stained with ethidium bromide.

Evaluation of results was done by conversion of the crossing points, given by the LightCycler instrument, into relative concentration of transcripts. Transcript levels obtained from cell lines cultured at 37°C were set at 100% to determine an increase or decrease of transcripts in heat-treated cells. Experiments were repeated three times to confirm reproducibility.

T-cell stimulation assay

At different time points after heat shock viable, adherent melanoma cells were harvested, counted and plated in triplicate in 96-well flat-bottom plates, CD8+ T-cell clones were added at ratios of 5 : 1 or 2.5 : 1. Culture supernatants were harvested after 24 h and the content of IFN-γ, as a surrogate marker for T-cell activation, was determined using a commercially available ELISA (OptEIA, Becton Dickinson, Heidelberg, Germany). Background values arising from T cells or melanoma cells cultured alone were generally <5 pg ml^{-1} and were subtracted from the experimental values.

Statistical analysis

The statistical significance of experimental values was assessed by means of independent Student's *t*-test on two populations. *P*-values of $P < 0.05$ (*) are significant and $P < 0.01$ (**) is considered as highly significant.

Results

Selection of two TIDs in 624.38-MEL cells

The breakpoint temperature was defined for all four melanoma cell lines by exposing them to different temperatures from 41.8 to 45°C for various time periods ranging from 15 to 150 min. After heat exposure, cells were seeded at low density and allowed to grow at 37°C/5% CO_2. After 7 days, the number of colonies was counted and expressed as the SF, which is plotted against the duration of heat exposure. Figure 1 shows

the clonogenic cell survival of 624.38-MEL cells in response to different temperatures as a representative example. In 624.38-MEL and SK-MEL23 exposure to temperatures <43.5°C had a minimal impact on their survival, whereas temperatures >43.5°C exponentially reduced tumor cell survival, thus defining 43.5°C as the breakpoint temperature for these two cell lines (Fig. 1). The TIDs at the specified temperatures of 41.8 and 45°C were defined by adjusting exposure times (120 and 22 min, respectively). These thermal doses resulted in equivalent clonogenic survival rates (isosurvival rate: 45%). Breakpoint temperatures and isosurvival doses for the other cell lines are listed in Methods.

Thermal dose-related differential kinetics of HSP70 protein expression

Increased levels of misfolded or aggregated proteins induced by heat shock overwhelm the binding capacity of the basal pool of cytosolic molecular chaperones and induce the heat shock response. The heat shock response is the time period after heat exposure during which expression of hsps is transcriptionally and translationally upregulated to assist in the renaturation or degradation of misfolded proteins (47–49).

To assess the characteristics of the heat shock response for the different melanoma cell lines, the cells were treated with the determined TIDs and the expression levels of HSC70 (constitutive) and HSP70 (inducible) were investigated by western blot analysis and flow cytometry.

At a physiological growth temperature of 37°C, both the HSC70 and the inducible HSP70 were detectable in 624.38-MEL cells (Fig. 2A, 37°C) by western blot. Heat exposure did not change the expression of the constitutive form of the HSP70 family (HSC70) but stimulated the expression of inducible HSP70 protein (Fig. 2A). Quantitating HSP70 induction using FACS analysis revealed different kinetics after the two thermal doses (Fig. 2B). For cells heated at 41.8°C/120 min, a 4-fold increase HSP70 was already detected at 4 h after heat treatment and levels increased linearly to peak levels of a 9-fold increase (8.9 ± 0.4) at the 48-h time point (Fig. 2B, left panel). Cells exposed to 45°C/22 min showed no increase in HSP70 protein expression at 4 h of recovery. Induction started at the 15-h time point and reached peak levels of 15-fold induction at 48 h of recovery (14.9 ± 0.6 at 48 h) (Fig. 2B, right panel).

HSP70 protein expression decreased after 48 h but remained above the pre-heat shock level for several days after heat shock (day 7+) (data not shown). Comparable HSP70 and HSC70 kinetic profiles were detected in SK-MEL23. In WM115 and WM266-4 maximal expression level of HSP70 was detected earlier (between 3 and 24 h of recovery at 37°C) (data not shown).

Changes in expression levels of immunologically relevant proteins: MHC class I, HLA-A2, tyrosinase and Melan-A/MART-1 antigens during the heat shock response

All selected human melanoma cell lines express tyrosinase and Melan-A/MART-1 and are recognized by antigen-specific HLA-A2-restricted CD8+ T-cell clones TyrF8 and A42, respectively, indicating that tyrosinase and Melan-A/MART-1 are processed into peptides that bind to the class I allotype HLA-A2. The four cell lines were used to study the influence over

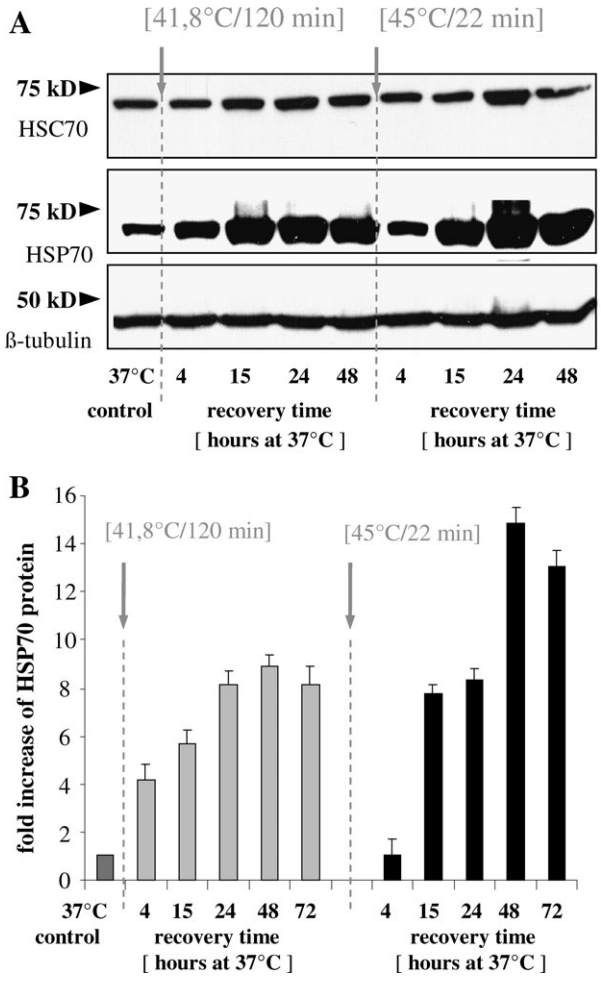

Fig. 2. (A) Western blot analysis for HSC70 and HSP70 expression after heat exposure for 624.38-MEL (as a representative example). 624.38-MEL cells were exposed to 41.8°C for 120 min or to 45°C for 22 min and then returned to 37°C for recovery. Cells were harvested 4, 15, 24 and 48 h after heat treatment and counted and an equal amount of viable cells as lysed and separated by 10% SDSP. After electrophoresis, proteins were blotted onto a nitrocellulose membrane and stained with antibodies against HSP70 (6B3) or HSC70 (SPA-815). To control for equal protein loading, staining for β-tubulin was performed. The blot is representative of five independent experiments. (B) Intracellular flow cytometry for inducible HSP70. 624.38-MEL cells were subjected to 41.8°C/120 min (light gray histograms) or 45°C/22 min (black histograms) and harvested at different time points after heat treatment. Viable cells were analyzed for intracellular protein levels of HSP70 by intracellular flow cytometry using the mAb 6B3 and isotype control antibody. Protein expression level was calculated as the difference in mean fluorescence intensity (MFI) of 6B3 and the isotype control. MFI of isotype control staining was always <20. Fold increase values were calculated using the value at 37°C as a reference value (1-fold, dark gray histogram; absolute value for ΔMFI of HSP70 at 37°C was 47 ± 17). Shown is the mean of five independent experiments (±standard deviations).

time of the two selected thermal doses on antigen expression, processing and presentation via MHC class I. The stimulation of IFN-γ secretion by immune effector cells was measured to assess the antigen presentation of tyrosinase. Expression levels of MHC class I molecules, HLA-A2, tyrosinase and Melan-A/MART-1 antigen were determined by FACS analysis, western blot and quantitative RT-PCR.

Thermal dose-related expression of human melanoma antigens tyrosinase and Melan-A/MART-1

First, we determined the effects of the two thermal doses on transcript levels of tyrosinase and Melan-A/MART-1 by quantitative RT-PCR. For all four cell lines, no significant changes in Melan-A/MART-1 transcripts were detected during the recovery period independent of the thermal doses. Figure 3(A) shows Melan-A/MART-1 transcript levels of 624.38-MEL as a representative example. Tyrosinase transcript level remained unchanged when exposing the cell lines to thermal doses below the breakpoint temperature (41.8°C/120 min). However, temperatures above the breakpoint resulted in a significant drop in transcript levels to 10% (0.1-fold increase, $P < 0.01$) and to 13% (0.13-fold increase, $P < 0.01$) of control levels (37°C = 1-fold) at 15 and 24 h of recovery. Figure 3(B) shows representative examples for 624.38-MEL and SK-MEL23.

To determine whether changes in transcript level translated to the protein level, western blot analysis was performed for Melan-A/MART-1 and tyrosinase. For tyrosinase, a significant increase in tyrosinase protein levels was observed for both thermal doses in all cell lines despite the reduction in transcript level. 624.38-MEL is shown as a representative example in Fig. 3(C). Protein and transcript levels of tyrosinase returned to starting levels 6–7 days after heat shock (data not shown). Melan-A/MART-1 protein levels remained unchanged during the heat shock response for all thermal doses in all four human melanoma cells studied (Fig. 3C shows 624.38-MEL as a representative example).

Thermal dose-related expression of MHC class I and HLA-A2 molecules

MHC class I molecules on tumor cells have an essential role in antigen presentation for T-cell recognition and are the prime determinators for the cellular immunogenicity. Therefore, the expression level was analyzed during the heat shock response. Since tyrosinase and Melan-A/MART-1 are processed into peptides presented by HLA-A2 (34, 37), the antibody HB54 was used to specifically detect the HLA-A2 allotype.

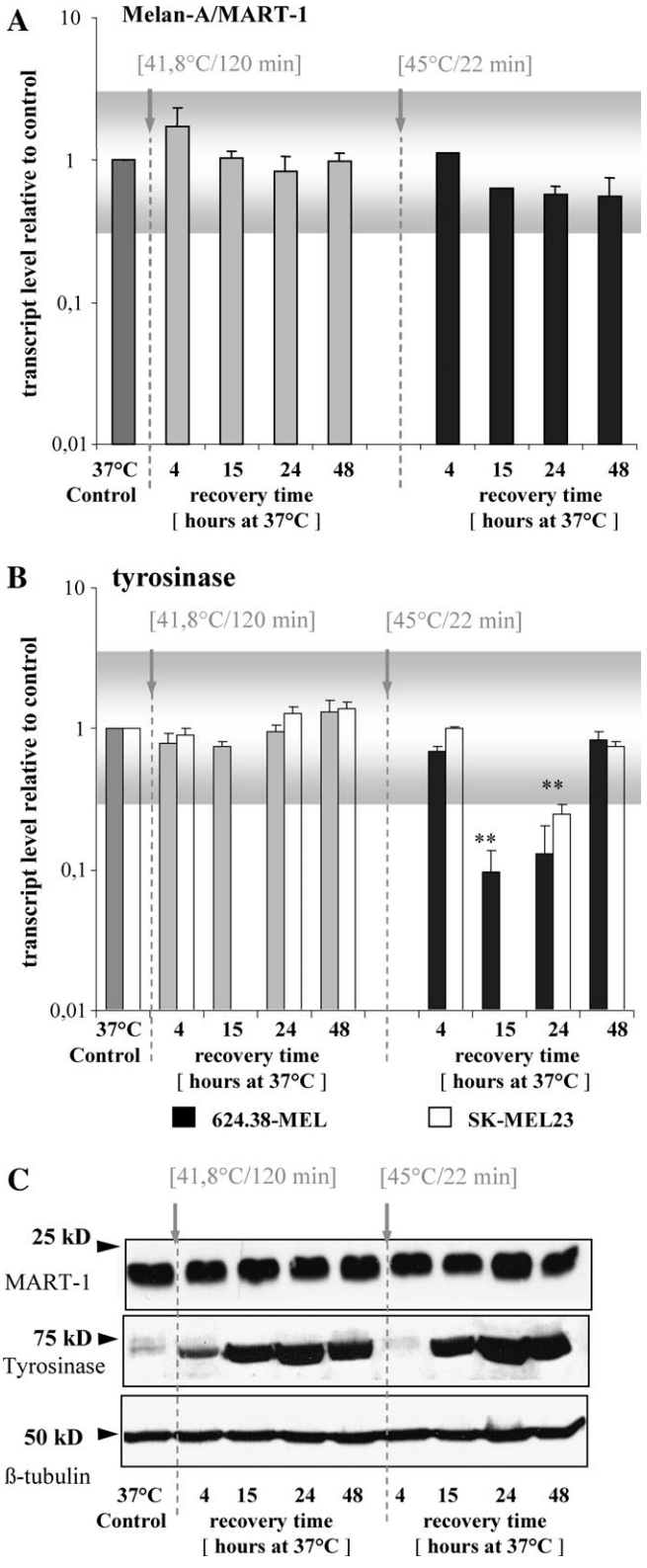

Fig. 3. Quantification of Melan-A/MART-1 transcripts in 624.38-MEL (A) and of tyrosinase transcripts (B) in 624.38-MEL cells (filled bars) and SK-MEL23 (empty bars). 624.38-MEL and SK-MEL23 cells were harvested 4, 15, 24 and 48 h after exposure to 41.8°C for 120 min or 45°C for 22 min. Equal amounts of total RNA were analyzed by quantitative real-time RT-PCR using the LightCycler system and SYBR green fluorescence. No signals were observed in the negative control (water in place of template). Amplification of the housekeeping gene α-enolase was performed in separate capillaries but in the same real-time PCR run as the tyrosinase and Melan-A/MART-1 samples to confirm that comparable amounts of total RNA were used for all samples. Blotted is the fold-change in transcript levels with the level at 37°C set to one (crossing points at 37°C were ~13 for Melan-A/MART-1 and 16 for tyrosinase). Values are the mean of three independent experiments (±standard deviation). The gray horizontal box crossing the bars defines the confidence interval in which all values show a difference of ±1.5 cycles compared with the reference value at 37°C. According to the manufacturer's instructions this cutoff interval was defined to discriminate between significant over-expression of transcripts from significant under-expression (0.35–2.8). (C) Representative example of western blot analysis of Melan-A/MART-1 and tyrosinase expression in 624.38-MEL cells. 624.38-MEL cells were exposed to 41.8°C for 120 min or to 45°C for 22 min and then returned to 37°C for recovery. Cells were harvested 4, 15, 24 and 48 h after heat treatment and counted and an equal amount of viable cells was lyzed and separated by 10% SDSP. After electrophoresis, proteins were blotted onto a nitrocellulose membrane and stained with antibodies against tyrosinase (C-19) and Melan-A/MART-1 (A103). To control for equal protein loading, staining for β-tubulin was performed. The blot is representative of five independent experiments.

No changes in HLA-A2 surface expression were observed for WM115 and WM266-4 cell lines, irrespective of the thermal dose (Tables 1 and 2). For SK-MEL23, HLA-A2 surface levels increased steadily over recovery time after exposure to both thermal doses. For 624.38-MEL, a decrease at 4 and 15 h of recovery from both thermal doses was observed. This decrease of HLA-A2 was transient and HLA-A2 reached levels higher than control levels at 72 h of recovery (Tables 1 and 2). Representative FACS histograms are shown in Fig. 4. HLA-A2 mRNA levels were determined by real-time PCR and were found unchanged after heat treatments (data not shown).

Similar results were obtained for pan-MHC class I as determined by staining with antibody W6/32.

To evaluate whether the changes in HLA-A2 surface expression observed for 624.38-MEL and SK-MEL23 were immunologically relevant, the immunostimulatory capacity of the heat-treated tumor cells for T cells was determined. JB4 recognizes the HLA-A2 protein itself, independently from its bound peptide repertoire and is stimulated to secrete IFN-γ. The relative amount of IFN-γ produced directly correlates with the expression level of HLA-A2 (data not shown). For the stimulation assays, low stimulator to effector ratios were selected (5 : 1, 2.5 : 1) to ensure that the response capacity of JB4 was not saturated. The increase in surface HLA-A2 resulted in better T-cell stimulation for SK-MEL23, but not for 624.38-MEL (Fig. 5). Possibly the high starting level of HLA-A2 observed for 624.38-MEL had already saturated the T-cell response, such that higher levels did not translate in more T-cell stimulation. In contrast, T cells produced less IFN-γ when cultured with 624.38-MEL that had reduced HLA-A2 surface expression (Fig. 5; Table 2, Fig. 4: time points 4 and 15 h of recovery from thermal dose above breakpoint temperature). This indicates that an increase in HLA-A2 expression can be immunologically relevant in situations of low basal level expression (i.e. SK-MEL23). On the other hand, JB4 T cells seem to be very sensitive to reduction in HLA-A2.

Thermal dose-related endogenous HLA-A2-restricted tyrosinase and Melan-A/MART-1 peptide presentation

The tyrosinase-specific CTL clone, TyrF8, and the Melan-A/MART-1-specific CTL, A42, were used to test the influence of heat shock on the capacity of the melanoma cell lines to process and present peptides derived from the antigen tyrosinase and Melan-A/MART-1 in the context of HLA-A*0201. This was of particular interest since a significant increase in tyrosinase protein expression was observed after heat shock that could lead to higher numbers of presented peptides. Melanoma cells were subjected to the pre-determined isoeffect thermal doses and were used to stimulate the TyrF8 and A42 T-cell clones at different time points of recovery. The amount of secreted IFN-γ was determined from supernatants because of its close correlation to the extent of antigen presentation and the relevance of IFN-γ to control tumor growth *in vivo* (50–52).

The IFN-γ secretion by the tyrosinase-specific TyrF8 incubated with melanoma cells treated at 41.8°C/120 min did

Table 1. Changes in HLA-A2 expression following exposure to isothermal doses below the breakpoint temperature[a]

Cell line	Mean (±SD) of ΔMFI[b] for HLA-A2 (HB54)					
	Control 37°C	Recovery at 37°C				
		4 h	15 h	24 h	48 h	72 h
624.38-MEL	458 ± 92	354 ± 131	437 ± 157	511 ± 165	584 ± 169	669 ± 59
SK-MEL23	72 ± 9	71 ± 22	nt	**171 ± 56**	**209 ± 34***	**188 ± 39**
WM266-4	90 ± 11	101 ± 5	nt	104 ± 16	108 ± 17	98 ± 8
WM115	100 ± 55	97 ± 41	nt	128 ± 63	136 ± 76	123 ± 76

Bold values are different from control values and relevant for T-cell stimulation. nt is not tested.
[a]At 41.8°C/120 min for all four cell lines. [b]ΔMean fluorescence intensity (ΔMFI) was calculated by subtracting the mean fluorescence values of the isotype control (MOPC21) from the mean fluorescence values obtained with the specific antibody HB54 directed against HLA-A2 molecules. MFI of isotype control ranged between 3 and 25. Results are the mean values of ΔMFI ± standard deviations from four and three independent experiments.
*$P < 0.001$ compared with control.

Table 2. Changes in HLA-A2 expression following exposure to isothermal doses above the breakpoint temperature[a]

Cell line	Mean (±SD) of ΔMFI[b] for HLA-A2 (HB54)					
	Control 37°C	Recovery at 37°C				
		4 h	15 h	24 h	48 h	72 h
624.38-MEL	458 ± 92	**367 ± 31**	**385 ± 10**	578 ± 109	622 ± 75	620 ± 220
SK-MEL23	72 ± 9	125 ± 82	nt	**152 ± 32**	**312 ± 87***	**264 ± 122**
WM266-4	90 ± 11	95 ± 7	nt	97 ± 23	97 ± 12	83 ± 27
WM115	100 ± 55	91 ± 41	nt	151 ± 66	118 ± 57	79 ± 40

Bold values are different from control values and relevant for T-cell stimulation. nt is not tested.
[a]At 45°C/22 min for 624.38-MEL and SK-MEL23, 43°C/10 min for WM266-4 and 44°C/25 min for WM115. [b]As footnote 'b' in Table 1.
*$P < 0.001$ compared with control.

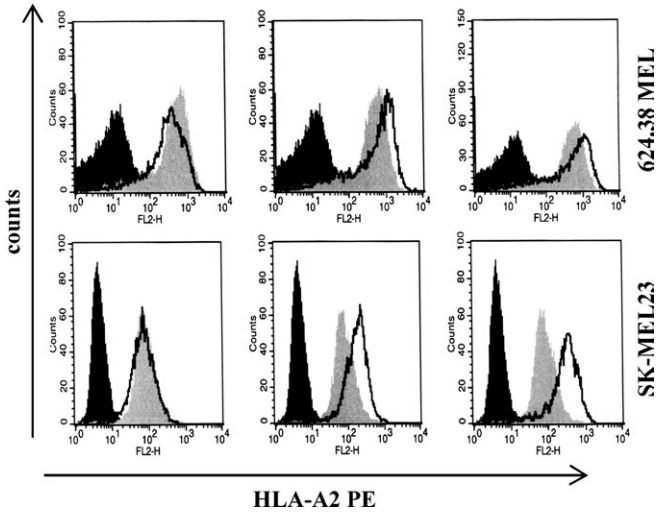

Fig. 4. Flow cytometric analysis for surface levels of HLA-A2 in 624.38-MEL and SK-MEL23. Melanoma cells were cultured at 37°C and exposed to 45°C for 22 min. Flow cytometry was performed on viable cells harvested at 4 h (left panel), at 24 h (middle panel) and 48 h (right panel) of recovery after treatment. Isotype control and HLA-A2 at 37°C are the black and gray histograms, respectively, shown in all panels. Histograms for HLA-A2 are shown as dark gray continuous lines. One experiment representative of four is shown.

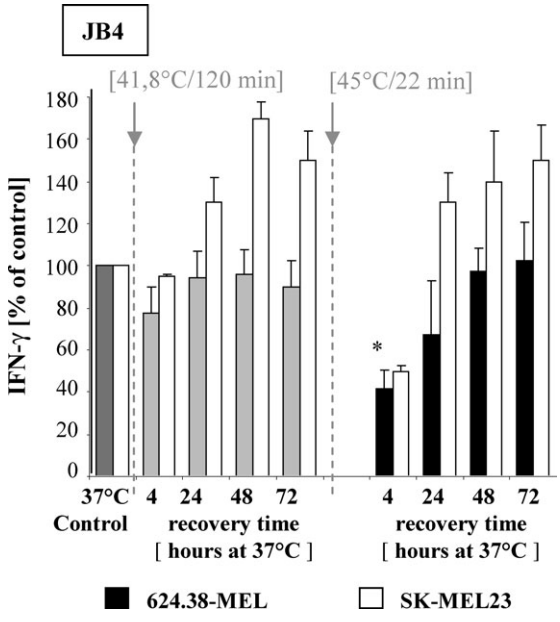

Fig. 5. IFN-γ secretion by JB4 induced by heat-treated 624.38-MEL (filled bars) and SK-MEL23 (empty bars). Melanoma cells were exposed to 41.8°C/120 min or 45°C/22 min and harvested after 4, 24, 48 and 72 h of recovery at 37°C. Viable cells were co-cultured with the CTL clone JB4. Results of a stimulator to effector cell ratio of 5 : 1 are shown. Similar results were obtained at a ratio of 2.5 : 1 (data not shown). IFN-γ secretion by JB4 is expressed as percent of IFN-γ secretion relative to control cells grown at 37°C (absolute values for IFN-γ at 37°C at a ratio 5 : 1 were 987 ± 98 pg ml^{-1} for 624.38-MEL and 458 ± 34 pg ml^{-1} for SK-MEL23); values represent the mean and standard deviations of six independent experiments for the time points 24, 48 and 72 h and of two independent experiments for the time point 4 h of recovery at 37°C. *$P < 0.05$.

not change significantly over the recovery up to 72 h, except for SK-MEL23. This cell line showed an increased stimulatory capacity possibly due to the increased surface HLA-A2 level (Fig. 6A, Table 1). All melanoma cell lines when exposed to thermal doses above the breakpoint temperature showed a decreased stimulatory capacity for TyrF8 at 4 and 24 h ($P < 0.01$, $N = 6$) even in the case of SK-MEL-23, where HLA-A2 surface expression was enhanced (Fig. 6A, Table 2). In all cases, the stimulatory capacity was restored or even enhanced (SK-MEL23) at 72 h of recovery (Fig. 6A).

IFN-γ secretion by the Melan-A/MART-1-specific A42 T cells incubated with heat-treated melanoma cells showed a kinetic similar to that of JB4 with a moderate and transient decrease of IFN-γ secretion until 24 h of recovery from temperatures above the breakpoint ($P < 0.05$, $N = 6$) (Fig. 6B).

Comparing the results obtained for T-cell stimulation with levels of HSP70, melanoma-associated antigen transcripts and proteins, and surface HLA-A2 after heat exposure, the following conclusion can be drawn. The capacity to stimulate IFN-γ secretion did not correlate with HSP70 protein (Fig. 2) or tyrosinase or Melan-A/MART-1 protein levels (Fig. 3C). Indeed, antigen protein levels increased (tyrosinase) or stayed constant (Melan-A/MART-1) during recovery from thermal exposure (Fig. 3C).

The IFN-γ secretion of tyrosinase-specific TyrF8 was decreased when stimulated with 624.38-MEL and SK-MEL23 that had recovered from a thermal dose above the breakpoint temperature for 4 or 24 h. At 24 h of recovery, 624.38-MEL had normal and SK-MEL23-enhanced HLA-A2 surface level (Table 2). This 24-h time point of diminished stimulatory capacity was found to correspond to the time point of lowest transcript levels for tyrosinase (Fig. 3B). For the second time point of low stimulatory capacity (4 h), tyrosinase transcript levels were normal. At this time point, the reduced HLA-A2 surface expression observed for 624.38-MEL (Table 2) might limit presentation of tyrosinase epitopes. Thus, temperature exposure appears to influence tyrosinase presentation through mechanisms involving modulations on both HLA-A2 surface and tyrosinase transcript levels. The reduced HLA-A2 surface expression of 624.38-MEL is reflected also by a lower IFN-γ production of JB4 (Fig. 5).

However, it is noted, that the lower stimulatory capacity of SK-MEL23 at the 4-h time point after recovery from thermal dose above the breakpoint temperature cannot solely be explained on the basis of either HLA-A2 level or antigenic protein or transcript levels. SK-MEL23 had HLA-A2 levels above or at control level, and control levels for tyrosinase or Melan-A/MART-1 transcript and protein. Still, they were unable to fully stimulate antigen-specific (TyrF8: Fig. 6A; A42: Fig. 6B) or allorestricted T cells (JB4: Fig. 5). Thus, factors not determined by our analyses appear to influence T-cell stimulation at this early time point of recovery from temperatures above the breakpoint.

Discussion

In this study we present a comprehensive analysis of effects induced by heat shock of two different TIDs (below and above their breakpoint temperatures) on four melanoma cell lines. These doses resulted in equivalent clonogenic survival rates

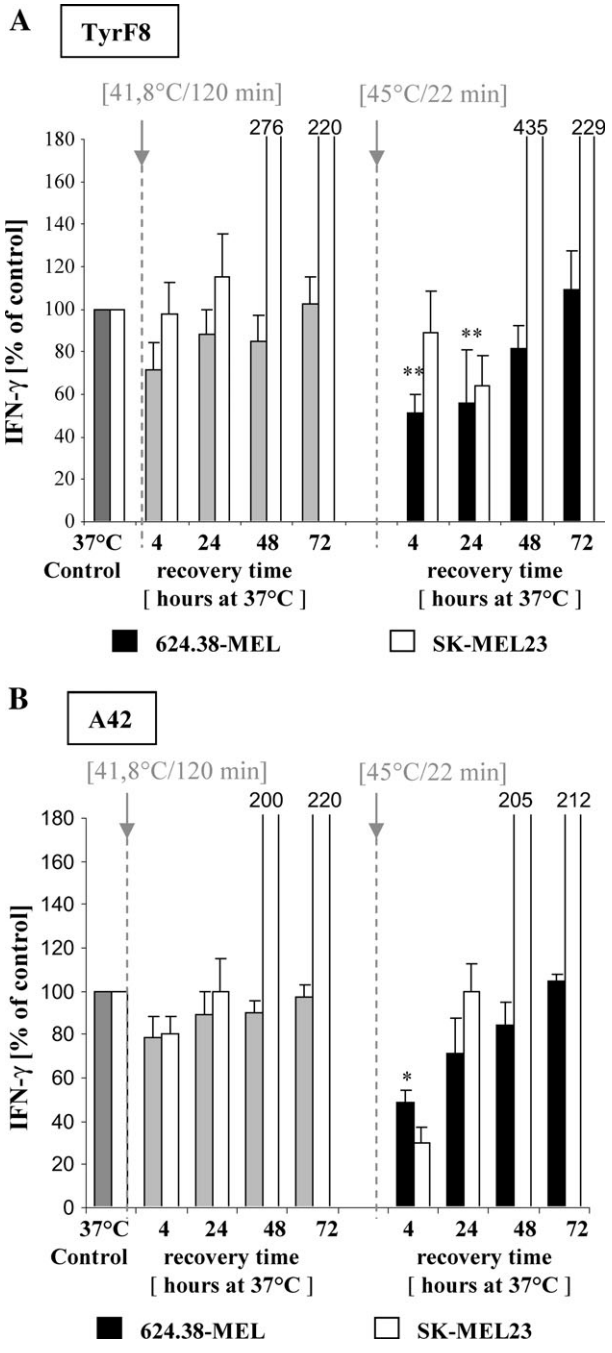

Fig. 6. IFN-γ secretion by TyrF8 (A) and A42 (B) induced by heat-treated 624.38-MEL (filled bars) and SK-MEL23 (empty bars). Melanoma cells were exposed to 41.8°C/120 min or 45°C/22 min and harvested after 4, 24, 48 and 72 h of recovery at 37°C. Viable cells were co-cultured with the CTL clones TyrF8 (A) and A42 (B). Results of a stimulator to effector cell ratio of 5 : 1 are shown. Similar results were obtained at a ratio of 2.5 : 1 (data not shown). IFN-γ secretion by TyrF8 and A42 are expressed as percentage of IFN-γ secretion relative to control cells grown at 37°C (absolute values of IFN-γ at 37°C were 477 ± 50 pg ml^{-1} for TyrF8 and 1606 ± 100 pg ml^{-1} for A42 stimulated with 624.38-MEL; 768 ± 168 pg ml^{-1} for TyrF8 and 1496 ± 209 pg ml^{-1} for A42 stimulated with SK-MEL23). Values represent the mean ± SD of six independent experiments for the time points 24, 48 and 72 h and of two independent experiments for the time point 4 h of recovery at 37°C. *$P < 0.05$, **$P < 0.01$. Similar IFN-γ secretion profiles were found for WM266-4 and WM115 (data not shown).

and mimic those achieved during clinical hyperthermia treatment of tumors. We specifically evaluated the time–temperature-dependent effects on tumor antigen expression, processing and presentation by MHC class I and on the recognition of heat-treated cells by antigen-specific cytotoxic T cells. This study thereby addresses the concern that heat shock treatment of tumor cells may reduce the presence of tumor antigens, due to heat shock-related reduced transcription, translation or protein degradation (47, 48), enabling tumor cells to escape immune recognition and immune surveillance. It is already known that during the progression of malignant melanoma, changes in antigenic profile occur, with concordant loss of multiple melanocyte differentiation proteins (53).

Our study demonstrates for the first time that during the heat shock response, tyrosinase protein and mRNA levels dissociate with a significant increase in tyrosinase protein and a parallel decrease in its transcripts. This was not observed for Melan-A/MART-1, indicating that the response pattern can differ for individual proteins. Interestingly, tyrosinase-specific T-cell recognition did not correlate with changes in antigen protein level. Furthermore, the kinetics and the degree of induction of HSP70, as well as the degree of dissociation between tyrosinase protein and tyrosinase mRNA levels, correlated with the severity of the initial stress, demonstrating that the two different thermal doses have differential long-term biological effects, even though they result in the same clonogenic survival ability.

Since tyrosinase protein levels did not correlate with its transcript levels, we reasoned that the mechanism by which tyrosinase protein could increase was a heat-dependent decrease in the degradation rate. Tyrosinase resides in the endoplasmic reticulum (ER) for extended time to complete the extensive glycosylation process. Through retrograde translocation from the ER into the cytoplasm it becomes a target for proteasome-dependent proteolytic degradation (54, 55). It is known that heat shock blocks the translocation of proteins from the ER into the cytosol. This will result in ER retention and reduced degradation (56), providing an explanation for the increased tyrosinase level that we observed. Our results also suggest that a thermal dose above the breakpoint temperature increases the magnitude of ER retention and the levels of tyrosinase protein. Melan-A/MART-1 is a small protein without glycosylation and thus has a short retention time in the ER. It is therefore conceivable that it is less sensitive to heat-induced inhibition of retrograde ER/cytosol translocation. This could be an explanation why we did not observe protein accumulation following heat treatment.

At the transcriptional level, it is known that severe heat shock blocks RNA splicing and represses normal transcription (57), correlating with our observed reduction in transcript levels after 15 and 24 h of recovery after heating melanoma cells to 45°C for 22 min. After a mild heat shock, RNA splicing is protected and mature mRNA accumulates (57), which was reflected by the unaltered level of tyrosinase transcripts after a heat shock of 41.8°C/120 min (Fig. 3B). Surprisingly, we did not find changes in mRNA and protein levels for Melan-A/MART-1 (Fig. 3A) and HLA-A2. This indicates that not all genes are affected equally. The differences may reflect a selection of those housekeeping genes and constitutively expressed genes that are essential for cell survival and may impact on antigen dominance within the cell.

The heat-induced quantitative changes of surface expression of MHC class I and the HLA-A2 allotype were also a function of the initial thermal dose and the time of recovery. Previous studies demonstrated an increase in MHC class I surface expression 24 and 48 h after a heat shock of 43°C/60 min (cytostatic, sub-lethal) (26) and after 5 days at 39°C (58). We found heat-induced increase in surface expression of MHC class I and HLA-A2 in two (624.38-MEL and SK-MEL23) of four melanoma cell lines. The increase was immunologically relevant for antigen presentation and T-cell stimulation only in the cell line that had low starting levels (SK-MEL23) of HLA-A2. Reduction in HLA-A2 observed for 624.38-MEL translated into a reduced capacity to stimulate T cells, as was observed previously by others (31, 59). The transient nature of the decrease, however, has not been described to date.

In the B16 mouse model, Wells *et al.* (60) correlated the augmentation of MHC class I with the level of HSP70 and they observed better antigen presentation. Our results did not show a correlation between heat-induced HSP70 over-expression and increase in MHC class I or HLA-A2 surface expression or antigen presentation. One explanation could be the different basal level of HSP70 in human and mouse cells. Indeed, human tumor cells such as our melanoma cell lines express high levels of HSP70 already at 37°C (61). Thus, HSP70, if required for antigen presentation, might not be a limiting factor in human tumor cells. In the murine system, induced over-expression of HSP70 after transfection may generate B16 cell clones with higher class I expression and better CTL susceptibility.

In studying the capability of tumor cells to stimulate the tyrosinase-specific CTL clone, it was observed that the response pattern was determined by the combination of HLA-A2 and tyrosinase mRNA, supporting the notion that the expression level of melanoma-associated antigens is a significant co-factor in addition to MHC class I in determining antigen presentation (62). For tyrosinase, mRNA levels, and not protein levels, impacted on antigen presentation. The observation of tyrosinase antigen presentation and the recently published studies on MUC-1 (63) support the defective ribosomal products (DRiPs) hypothesis (64, 65). According to Yewdell *et al.* (64), antigenic peptides may not derive from native proteins and thereby they are not proportional to the relative abundance of the corresponding protein. The DRiP hypothesis and the results from tyrosinase and MUC-1 (63) suggest that caution should be taken when evaluating the protein level of a given antigen as a marker to predict immune recognition. In the case of Melan-A/MART-1, our data are indecisive regarding the DRiP postulate, since changes were observed neither at the protein nor at the transcript level and the marginal changes in Melan-A/MART-1-specific T-cell stimulation were best correlated to changes in HLA-A2 surface expression.

Finally, addressing the time and temperature dependency of antigen expression on immune recognition, our study may have importance for the clinical application of hyperthermia. Our observations demonstrate that elevated temperatures induce a pleiotropy of changes, including changes on antigen expression and HLA surface expression that are relevant for immune effector mechanisms. Nevertheless, over time tumor cells maintain immunological homeostasis and remain susceptible to immune effector cells.

Acknowledgements

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Abbreviations

CHAPS	3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate
DRiP	defective ribosomal product
ER	endoplasmic reticulum
HSC70	constitutively expressed heat shock protein cognate 70 (M_r 73 kDa)
hsp	heat shock protein
HSP70	heat shock protein 70 (M_r 72 kDa)
MART-1	melanoma antigen recognized by T cells-1
PBL	peripheral blood lymphocyte
PE	plating efficiency
RT-PCR	reverse transcription PCR
SF	survival fraction
TID	thermal isoeffect dose

References

1 Wust, P., Hildebrandt, B., Sreenivasa, G. *et al.* 2002. Hyperthermia in combined treatment of cancer. *Lancet Oncol.* 3:487.
2 Overgaard, J., Gonzalez Gonzalez, D., Hulshof, M. C. *et al.* 1995. Randomised trial of hyperthermia as adjuvant to radiotherapy for recurrent or metastatic malignant melanoma. *Lancet* 345:540.
3 Falk, M. H. and Issels, R. D. 2001. Hyperthermia in oncology. *Int. J. Hyperthermia* 17:1.
4 Hildebrandt, B., Wust, P., Ahlers, O. *et al.* 2002. The cellular and molecular basis of hyperthermia. *Crit. Rev. Oncol. Hematol.* 43:33.
5 Kraybill, W. G., Olenki, T., Evans, S. S. *et al.* 2002. A phase I study of fever-range whole body hyperthermia (FI WBH) in patients with advanced solid tumours: correlation with mouse models. *Int. J. Hyperthermia* 18:253.
6 Basu, S. and Srivastava, P. 2003. Fever-like temperature induces maturation of dendritic cells through induction of HSP90. *Int. Immunol.* 15:1053
7 Wang, X. Y., Kazim, L., Repasky, E. A. and Subjeck, J. R. 2001. Characterization of heat shock protein 110 and glucose-regulated 170 as cancer vaccines and the effect of fever-range hyperthermia on vaccine activity. *J. Immunol.* 166:490.
8 Atanackovic, D., Nierhaus, A., Neumeier, M., Hossfeld, D. K. and Hegewisch-Becker, S. 2002. 41.8°C whole body hyperthermia as an adjunct to chemotherapy induces prolonged T-cell activation in patients with various malignant diseases. *Cancer Immunol. Immunother.* 51:603.
9 Davies, C. de L., Rofstad, E. K. and Lindmo, T. 1985. Hyperthermia-induced changes in antigen expression on human FME melanoma cells. *Cancer Res.* 45:4109.
10 Davies, C. L. and Lindmo, T. 1990. Hyperthermia-induced shedding and masking of melanoma-associated antigen. *Int. J. Hyperthermia* 6:1053.
11 Pepin, E., Villiers, C. L., Gabert, F. M., Serra, V. A., Marche, P. N. and Colomb, M. G. 1996. Heat shock increases antigenic peptide generation but decreases antigen presentation. *Eur. J. Immunol.* 26:2939.
12 Kuperberg, G., Ellis, J., Marcinkiewicz, J. and Chain, B. M. 1991. Temperature-induced stress abrogates co-stimulatory function in antigen-presenting cells. *Eur. J. Immunol.* 21:2791.
13 Jaattela, M. and Wissing, D. 1993. Heat-shock proteins protect cells from monocyte cytotoxicity: possible mechanism of self-protection. *J. Exp. Med.* 177:231.
14 Geginat, G., Heine, L. and Günther, E. 1993. Effect of heat shock on susceptibility of normal lymphoblasts and of a heat shock protein 70-defective tumor cell line to cytotoxic T lymphocytes *in vitro*. *Scand. J. Immunol.* 37:314.

15 Sugarawa, S., Nowicki, M., Xie, S., Song, H.-J. and Dennart, G. 1990. Effects of stress on lysability of tumor targets by cytotoxic T cells and tumor necrosis factor. *J. Immunol.* 145:1991.
16 Jackson, K. M., DeLeon, M., Sistonen, L. and Verret, C. N. 2000. Heat-shocked A20 lymphoma cells fail to induce degranulation of cytotoxic T lymphocytes: possible mechanism of resistance. *Cell. Immunol.* 203:12.
17 Gromkowski, S. H., Yagi, J. and Janeway, C. A. 1989. Elevated temperature regulates tumor necrosis factor-mediated immune killing. *Eur. J. Immunol.* 19:1709.
18 Kusher, D. I., Ware, C. F. and Gooding, L. R. 1990. Induction of the heat shock response protects cells from lysis by tumor necrosis factor. *J. Immunol.* 145:2925.
19 Jaattela, M. 1990. Effects of heat shock on cytolysis mediated by NK cells, LAK cells, activated monocytes and TNFs alpha and beta. *Scand. J. Immunol.* 31:175.
20 Jaattela, M. 1995. Over-expression of hsp70 confers tumorigenicity to mouse fibrosarcoma cells. *Int. J. Cancer* 60:689.
21 Van Molle, W., Wielockx, B., Mahieu, T. *et al.* 2002. HSP70 protects against TNF-induced lethal inflammatory shock. *Immunity* 16:685.
22 Mosser, D. D., Caron, A. W., Bourget, L. *et al.* 2000. The chaperone function of hsp70 is required for protection against stress-induced apoptosis. *Mol. Cell. Biol.* 20:7146.
23 Mise, K., Kann, N., Okino, T. *et al.* 1990. Effect of heat treatment on tumor cells and antitumor effector cells. *Cancer Res.* 50:6199.
24 Wells, A. D. and Malkovsky, M. 2000. Heat shock proteins, tumor immunogenicity and antigen presentation: an integrated view. *Immunol. Today* 21:129.
25 Michalek, M. T., Benacerraf, B. and Rock, K. L. 1992. The class II MHC-restricted presentation of endogenously synthesized ovalbumin displays clonal variation, requires endosomal/lysosomal processing, and is upregulated by heat shock. *J. Immunol.* 148: 1016.
26 Ito, A., Shinkai, M., Honda, H., Wakabayashi, T., Yoshida, J. and Kobayashi, T. 2001. Augmentation of MHC class I antigen presentation via heat shock protein expression by hyperthermia. *Cancer Immunol. Immunother.* 50:515.
27 Menoret, A., Patry, Y., Burg, C. and Le Pendu, L. 1995. Co-segregation of tumor immunogenicity with expression of inducible but not constitutive HSP70 in rat colon carcinomas. *J. Immunol.* 10:609.
28 Melcher, A., Todryk, S., Hardwick, N., Ford, M., Jacobson, M. and Vile, R. G. 1998. Tumor immunogenicity is determined by the mechanism of cell death via induction of heat shock protein expression. *Nat. Med.* 4:581.
29 Clark, P. R. and Menoret, A. 2001. The inducible HSP70 as a marker of tumor immunogenicity. *Cell Stress Chaperones* 6:121.
30 Dressel, R., Lubbers, M., Walter, L., Herr, W. and Gühnter, E. 1999. Enhanced susceptibility to cytotoxic T lymphocytes without increase of MHC class I antigen expression after conditional overexpression of heat shock protein 70 in target cells. *Eur. J. Immunol.* 29:3925.
31 Dressel, R., Elsner, L., Quentin, T., Walter, L. and Günther, E. 2000. Heat shock protein 70 is able to prevent heat shock-induced resistance of target cells to CTL. *J. Immunol.* 164:2362.
32 Multhoff, G., Botzler, C., Jennen, L., Schmidt, J., Ellwart, J. and Issels, R. 1997. Heat shock protein 72 on tumour cells: a recognition structure for natural killer cells. *J. Immunol.* 158:4341.
33 Woelfel, T., Van Pel, A., Brichard, V. *et al.* 1994. Two tyrosinase nanopeptides recognized on HLAA2 melanoma by autologous cytolytic T lymphocytes. *Eur. J. Immunol.* 24:759.
34 Renkvist, N., Castelli, C., Robbins, P. and Parmiani, G. 2001. A listing of human tumor antigens recognized by T cells. *Cancer Immunol. Immunother.* 50:3.
35 Rivoltini, L., Barracchini, K. C., Viggiano, V. *et al.* 1995. Quantitative correlation between HLA class I allele expression and recognition of melanoma cells by antigen-specific cytotoxic T lymphocytes. *Cancer Res.* 55:3149.
36 Visseren, M. J., van Elsas, A., van der Voort, E. I. *et al.* 1995. CTL specific for tyrosinase autoantigen can be induced from healthy donor blood to lyse melanoma cells. *J. Immunol.* 154:3991.
37 Kawakami, Y., Eliyahu, S., Sakaguchi, K. *et al.* 1994. Identification of the immunodominant peptides of the Melan-A/MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. *J. Exp. Med.* 180: 347.
38 Kawakami, Y., Eliyahu, S., Delgado, C. *et al.* 1994. Cloning of the gene coding for a shared melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proc. Natl Acad. Sci. USA* 91:3515.
39 Schendel, D. J., Gansbacher, B., Oberneder, R. *et al.* 1993. Tumor-specific lysis of human renal cell carcinomas by tumor-infiltrating lymphocytes. I. HLA-A2 restricted lysis of autologous and allogeneic tumor lines. *J. Immunol.* 151:4209.
40 Overgaard, J. and Suit, H. D. 1979. Time-temperature relationship in the hyperthermic treatment of malignant and normal tissue *in vivo*. *Cancer Res.* 39:3248.
41 Issels, R. D. and Nagele, A. 1990. Influence of thiols on thermosensitvity of mammalian cells *in vitro*. *Methods Enzymol.* 186:696.
42 Dewey, W. C. 1994. Arrhenius relationships from the molecule and cell to the clinic. *Int. J. Hyperthermia* 10:457.
43 Barnstable, C. J., Jones, E. A. and Crumpton, M. J. 1978. Isolation, structure and genetics of HLA-A,-B and DRw (Ia) antigens. *Br. Med. Bull.* 34:241.
44 McMichael, A. J., Parham, P., Rust, N. and Brodsky, F. 1980. A monoclonal antibody that recognizes an antigenic determinant shared by HLA A2 and B17. *Hum. Immunol.* 1:121.
45 Noessner, E., Gastpar, R., Milani, V. *et al.* 2002. Tumor-derived heat shock protein 70 peptide complexes are cross-presented by human dendritic cells. *J. Immunol.* 169:5424.
46 Smith, B., Selby, P., Southgate, J., Pittman, K., Bradley, C. and Blair, G. E. 1991. Detection of melanoma cells in peripheral blood by means of reverse transcriptase and polymerase chain reaction. *Lancet* 338:1227.
47 Lindquist, S. 1986. The heat-shock response. *Annu. Rev. Biochem.* 55:1151.
48 Welch, W. J. and Suhan, J. P. 1986. Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. *J. Cell Biol.* 103:2035.
49 Georgopoulos, C. and Welch, W. J. 1993. Role of the major heat shock proteins as molecular chaperones. *Annu. Rev. Cell Biol.* 9:601.
50 Ghanekar, S. A., Nomura, L. E., Suni, M. A., Picker, L. J., Maecker, H. T. and Maino, V. C. 2001. Gamma interferon expression in CD8+ T cells is a marker for circulating cytotoxic T lymphocytes that recognize an HLA-A2-restricted epitope of human cytomegalovirus phosphoprotein pp65. *Clin. Diagn. Lab. Immunol.* 8:628.
51 Pittet, M. J., Zippelius, A., Speiser, D. E. *et al.* 2001. *Ex vivo* IFN-gamma secretion by circulating CD8 T lymphocytes: implications of a novel approach for T cell monitoring in infectious and malignant diseases. *J. Immunol.* 166:7634.
52 Street, S. E., Cretney, E. and Smyth, M. J. 2001. Perforin and interferon-gamma activities independently control tumor initiation, growth, and metastasis. *Blood* 97:192.
53 Slingluff, C., Colella, T., Thompson, L. *et al.* 2000. Melanomas with concordant loss of multiple melanocytic differentiation proteins: immune escape that may be overcome by targeting unique or undefined antigens. *Cancer Immunol. Immunother.* 48: 661.
54 Halaban, R., Cheng, E., Zhang, Y. *et al.* 1997. Aberrant retention of tyrosinase in the endoplasmic reticulum mediates accelerated degradation of the enzyme and contributes to the dedifferentiated phenotype of amelanotic melanoma cells. *Proc. Natl Acad. Sci. USA* 94:6210.
55 Mosse, C. A., Hsu, W. and Engelhard, V. H. 2001. Tyrosinase degradation via two pathways during reverse translocation to the cytosol. *Biochem. Biophys. Res. Commun.* 285:313.
56 VanSlyke, J. K. and Musil, L. S. 2002. Dislocation and degradation from ER are regulated by cytosolic stress. *J. Cell Biol.* 157:381.
57 Yost, H. J., Petersen, R. B. and Lindquist, S. 1990. RNA metabolism: strategies for regulation in the heat shock response. *Trends Genet.* 6:223.

58 Aboud, M., Segal, S., Priel, E., Blair, D. G. and O'Hara, B. 1992. Effect of temperature on the expression of major histocompatibility complex class-I antigens. *Immunol. Invest.* 21:219.
59 Blom, D. J., De Waarg-Siebinga, I., Apte, R. S., Luyten, G. P., Niederkorn, J. Y. and Jager, M. J. 1997. Effect of hyperthermia on expression of histocompatibility antigens and heat-shock protein molecules on three human ocular melanoma cell lines. *Melanoma Res.* 7:103.
60 Wells, A. D., Rai, S. K., Salvato, M. S., Band, H. and Malkovsky, M. 1998. Hsp72-mediated augmentation of MHC class I surface expression and endogenous antigen presentation. *Int. Immunol.* 10:609.
61 Protti, M. P., Heltai, S., Bellone, M., Ferrarini, M., Manfredini, A. A. and Rugarli, C. 1994. Constitutive expression of the heat shock protein 72 kDa in human melanoma cells. *Cancer Lett.* 85:211.
62 Cormier, J. N., Panelli, M. C., Hackett, J. A. *et al.* 1999. Natural variation of the expression of HLA and endogenous antigen modulates CTL recognition in an *in vitro* melanoma model. *Int. J. Cancer* 80:781.
63 Kayser, S., Watermann, I., Rentzsch, C., Weinschenk, T., Wallwiener, D. and Guckel, B. 2003. Tumor-associated antigen profiling in breast and ovarian cancer: mRNA, protein or T cell recognition? *J. Cancer Res. Clin. Oncol.* 129:397.
64 Yewdell, J. W., Anton, L. C. and Bennick, J. R. 1996. Defective ribosomal products (DriPs). A major source of antigenic peptides for MHC class I molecules? *J. Immunol.* 157:1823.
65 Schubert, U., Anton, L., Gibbs, J. *et al.* 2000. Rapid degradation of a large fraction of newly synthesised proteins by proteasomes. *Nature* 404:770.