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The stress-inducible heat shock protein (HSP) 70 is a molecular chaperone which is well known to protect cells against apoptosis (1). Overexpression of HSP70 has been described in various tumors and has been found to be associated with enhanced tumorigenicity and resistance to therapy (2). In accordance with these findings, experimental down-regulation of HSP70 in tumor cells has been reported to enhance tumor regression in animal models (3–5).

However, in several other animal models contrary observations have been made, in that HSP70 expression has been found to be associated with tumor regression (6–9). In these cases, HSP70 appeared to augment the immunogenicity of tumors. In numerous studies, HSP70 has been shown to activate innate and adaptive immune reactions (10, 11). HSP70 chaperones antigenic peptides and channels them in, a receptor-mediated manner, into the MHC class I presentation pathway of professional APCs, which then prime peptide-specific CTL (12). Therefore, HSP70 derived from tumors can be used as tumor-specific vaccines (13). HSP70 also elicits the release of proinflammatory cytokines from innate immune cells and augments the expression of costimulatory molecules (14, 15). Furthermore, HSP70 has been shown to activate NK cells to specifically kill tumor cells that express HSP70 at the cell surface (16). These features have led to HSP70 being viewed as an endogenous adjuvant and immunological danger signal (10, 17, 18).

Given that HSP70 is known to be anti-apoptotic but that it can also elicit a CTL response, we were interested to determine whether HSP70 protects tumor cells against apoptosis mediated by CTL. In the model of the human melanoma cell line Ge, we have shown previously that constitutive overexpression of the MHC-linked stress-inducible HSP70 does not protect against apoptosis mediated by CTL in the granule exocytosis pathway (19). Acute HSP70 overexpression can even increase the susceptibility against CTL in vitro (19, 20). The immune system appears to be able to kill target cells undergoing an otherwise protective stress response.

Our goal was now to determine the effect of HSP70 expression on the susceptibility of Ge melanoma cells to the cytotoxic effects of adoptively transferred CTL in vivo. However, in SCID mice that lack B and T lymphocytes, the growth of HSP70-overexpressing tumors was reduced compared with control tumors, even before any adoptive immunotherapy. More impressively, invasive growth and regional metastases were only observed in animals associated with tumor regression (6–9). In these cases, HSP70 appeared to augment the immunogenicity of tumors. In numerous studies, HSP70 has been shown to activate innate and adaptive immune reactions (10, 11). HSP70 chaperones antigenic peptides and channels them in, a receptor-mediated manner, into the MHC class I presentation pathway of professional APCs, which then prime peptide-specific CTL (12). Therefore, HSP70 derived from tumors can be used as tumor-specific vaccines (13). HSP70 also elicits the release of proinflammatory cytokines from innate immune cells and augments the expression of costimulatory molecules (14, 15). Furthermore, HSP70 has been shown to activate NK cells to specifically kill tumor cells that express HSP70 at the cell surface (16). These features have led to HSP70 being viewed as an endogenous adjuvant and immunological danger signal (10, 17, 18).

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bearing non-HSP70-overexpressing control tumors. We show in this study that the stress-inducible danger signal HSP70 activates mouse NK cells in SCID mice which recognize inducible NKG2D ligands on tumor cells. NKG2D (NK group 2, member D) is an activating NK receptor (21). The MHC class I chain-related (MIC) A and B molecules are NKG2D ligands in humans (21) and their expression was induced in the human tumors in SCID mice. MICA and MICB genes are encoded within the MHC, are stress inducible, and are expressed in a restricted manner in intestinal epithelial cells and in tumors (22). Although the MICA and MICB genes are not present in the mouse genome, the human molecules functionally interact with mouse NKG2D (23–25). In mice, the retinoic acid early inducible-1 (Rae-1), and UL16-binding protein-like transcript 1 (Mult1) gene products and a minor histocompatibility Ag (H60) have been reported as NKG2D ligands (21). The inducible HSP70 and inducible NKG2D ligands, which both appear to function as immunological danger signals, synergistically elicited a NK cell-mediated immune response against tumor cells. This two danger signals-driven innate immune response was able to reduce the growth of primary tumors and suppress metastatic disease.

Materials and Methods

Animal experiments

SCID (C-B-17Lzm-scid) and SCID/beige (C-B-17/IcrHsd-scid-bg) mice were bred in our own colony under pathogen-free conditions in individually ventilated cages. 129Sv mice serving as spleen donors in some experiments were bred under conventional conditions. The SCID mice were originally obtained from Dr. H. J. Hedrich (Medizinische Hochschule Hannover, Hannover, Germany) the SCID/beige mice were from Harlan Winkelmann. Female and male mice between 12 and 20 wk of age were used for experiments after excluding leaky mice by measuring serum immunoglobulins using an ELISA. All animal experiments had been approved by the local government and were in accordance with institutional guidelines for the welfare of animals. Tumor cells (1 × 106 in 100 μl PBS) were injected subcutaneously into the flank of mice. Tumor growth was monitored every second day by palpation and size was recorded using linear calipers. Tumor volume was calculated by the formula V = rabc/2, where a, b, c are the orthogonal diameters. Animals were sacrificed before a tumor volume of 1 cm3 was reached, when a weight loss of >10% occurred, or when any behavioral signs of pain or suffering were observable. Autopsies were performed and the abdomen and thoracic cavity were examined systematically for the presence of metastases. The spleens were removed for immunological analyses. Parts of primary tumors were immediately frozen in liquid nitrogen for gene expression analyses. Further tumor and metastatic tissue was placed in phosphate-buffered 4% formalin for 16 h and then embedded in paraffin. Tissue sections (2.5 μm) were stained with H&E for routine histological examinations. Immunohistochemical staining of the proliferation marker Ki67 was performed as described (26). For flow cytometric analyses of tumor cells, fresh tumor tissue was cut into small pieces and incubated in a 5 mg/ml collagenase solution (Sigma-Aldrich) at 37°C for 90 min. Isolated cells were collected by centrifugation and resuspended in PBS before staining.

Gene expression analysis

RNA was prepared and Northern blots were hybridized and analyzed by densitometry as described (27). The rat and human MHC-linked inducible HSP70 proteins are 96.3% identical and 98.4% similar, but they can be distinguished by size. The human HSP70-1 (positions 2875 to 3070; accession no. X77207) (28) and the rat MHC-linked rat Hsp70-1 (positions 2225 to 2407, accession no. M59830) (29) were derived from the 3′ untranslated region of the respective genes by genomic PCR amplification. A MICA gene probe encompassing exons 2 to 5 (30) was used to detect MICA and MICB transcripts, which can be distinguished by size. The human β-actin cDNA was purchased from Clontech.

Target cell lines and culture

The human melanoma cell line Ge, the Ge-Hsp70 and Ge-con sublines derived therefrom (clones Ge-Hsp70-A, Ge-Hsp70-C, Ge-TCR-C, and Ge-GFP-B) (19), human erythroleukemia K562 cells, and mouse lymphoma YAC-1 cells were maintained at 37°C in NaHCO3-buffered DMEM supplemented with 10% FCS (Biochrom), 2 mM t-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich). To induce MICA/B expression, Ge cells were cultured in DMEM with 10 μM of the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) (Qbiogene-Alexis) 20 h before the tests (31). The heat shock protein Hsp70 or Ge-con cells is shown. SCID mice in which tumor growth was observed after injection of Ge-con cells are shown. The mean of tumor size ± SD for SCID mice in which tumor growth was observed after injection of Ge-Hsp70 or Ge-con cells is shown.

FIGURE 1. Tumors derived from HSP70-overexpressing melanomas grow slower and do not give rise to metastases. a, Ge-Hsp70 or Ge-con cells were injected s.c. into the flank of SCID mice (1 × 106 cells in PBS/animal). Representative photographs are shown. The black arrow (b) points toward a noninvasively grown s.c. tumor (tumor). Invasive growth (I) was only observed for control tumors. Metastases in the mesentery (VIV), in an axillary lymph node (V, black arrow), and in the diaphragm (VI) after injection of Ge-con cells are shown. b, The mean of tumor size ± SD for SCID mice in which tumor growth was observed after injection of Ge-Hsp70 or Ge-con cells is shown.

To analyze in vitro proliferation 2 × 104 cells per well were seeded in 200 μl DMEM in micro titer plates for cell culture (Sarstedt) in triplicates for each time point of measurement (12, 24, 48, and 72 h). Twelve hours before harvesting 1 μCi [methyl-3H]thymidine (specific activity 5 Ci/mmol, Amersham) was added to the respective wells. Triplicates were harvested using a Titertek cell harvester 550 (Flow Laboratories). Incorporated radioactivity was determined by liquid scintillation counting using a Wallac MicroBeta TriLux counter (PerkinElmer Life Sciences). Apoptosis was induced by hypoxia and glucose starvation. To expose cells to hypoxic conditions, petri dishes were placed in a GasPak 100 system (BD Biosciences). After 2 h the O2 concentration in the system is <1% and the CO2 concentration reaches 10% (32). The GasPak system was placed for 24 h
in an incubator at 37°C. For glucose starvation the melanoma cells were cultured for 24 h in glucose-free DMEM (Sigma), which was supplemented as standard DMEM. Propidium iodide positive dead cells and apoptotic cells appearing in the sub G1 peak of DNA histograms were determined as described previously (27).

Preparation of exosomes and immunoblot analysis

The Ge-Hsp70 and Ge-con cell lines were grown to ~80% confluence before being cultured in fresh DMEM for 72 h. Cell viability was >95%, as determined by trypan blue exclusion. The supernatant was harvested and exosomes were prepared as described (33) and analyzed by SDS-PAGE. Immunoblotting was performed (33) using Abs specific for the inducible form of HSP70 (mAb C92, clone C92F3A-5, mouse IgG1, SPA-810; StressGen; Biomol) and against Rab4 (sc-312, rabbit Ab; Santa Cruz Biotechnology).

Effectors, effector cell culture and 51Cr Release assays

Splenocytes from mice were obtained using a Tenbroek homogenizer and erythrocytes were removed by incubation for 5 min in lysing buffer (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA (pH 7.4–7.8)). The cells obtained from tumor bearing mice were used either directly as cytotoxic effector cells or cultured for 24 h in DMEM with 10% FCS, 20% supernatant from Con A-stimulated lymphocytes before being used in 51Cr Release assays. A signifi-cant reduction in 51Cr release was observed in the presence of anti-CD49b Ab and Abs reactive against CD3 (clone 145-2C11, hamster IgM, FITC-conjugated, Immunotools), CD19 (clone 6D5, rat IgG2a, tricolor-conjugated, Immunotools), and Abs reactive against CD3 (clone 145-2C11, hamster IgM, FITC-conjugated, Immunotools), CD4 (clone CT-CD4, rat IgG2a, PE-conjugated, Immunotools), and CD8 (clone CT-CD8a, rat IgG2a, PE-conjugated, Immunotools), and CD19 (clone 6D5, rat IgG2a, FITC-conjugated, Immunotools). Isotype controls (rat IgG2a, rat IgM, and hamster IgG) were purchased from Caltag Laboratories.

Statistics

All data were analyzed using the SAS version 9.1 software. ANOVA was used to analyze designs involving two or more factors. The different factors were incorporated into a two-way or three-way ANOVA involving inter-actions. The t test was used for the analysis of paired and unpaired two samples. A repeated measures ANOVA was conducted in all experimental designs having replicates, such as 51Cr Release assays. A signifi-cance level of α = 0.05 was used. Adjustments for multiple comparisons were performed where appropriate.

Results

Reduced tumor growth of HSP70-overexpressing melanoma cells

The human melanoma cell line Ge was retrovirally transduced to constitutively overexpress the normally stress-inducible MHC-linked rat Hsp70-1 (Hspalb) gene. Control cell clones (Ge-con) were obtained by transduction with a rat TCRβ-chain or GFP expression construct derived from the same vector. Both the Ge-Hsp70 and the Ge-con clones have been previously described and were used to analyze designs involving two or more factors. The different factors were incorporated into a two-way or three-way ANOVA involving inter-actions. The t test was used for the analysis of paired and unpaired two samples. A repeated measures ANOVA was conducted in all experimental designs having replicates, such as 51Chromium release assays. A signifi-cance level of α = 0.05 was used. Adjustments for multiple comparisons were performed where appropriate.

Table 1. Tumor frequency and formation of metastases after subcutaneous inoculation of Ge-Hsp70 or Ge-con cells into SCID and SCID/beige mice

<table>
<thead>
<tr>
<th>Inoculated cells</th>
<th>Tumor frequency (day 24)</th>
<th>Tumor frequency (at autopsy)</th>
<th>Metastases (at autopsy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCID Mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ge-Hsp70</td>
<td>73% (30/41)</td>
<td>73% (30/41)</td>
<td>0% (0/30)</td>
</tr>
<tr>
<td>Ge-Hsp70-A</td>
<td>73% (16/22)</td>
<td>73% (16/22)</td>
<td>0% (0/16)</td>
</tr>
<tr>
<td>Ge-Hsp70-C</td>
<td>74% (14/19)</td>
<td>74% (14/19)</td>
<td>0% (0/14)</td>
</tr>
<tr>
<td>Ge-con</td>
<td>86% (38/44)</td>
<td>86% (38/44)</td>
<td>21% (8/38)</td>
</tr>
<tr>
<td>Ge-TCR-C</td>
<td>87% (20/23)</td>
<td>87% (20/23)</td>
<td>25% (5/20)</td>
</tr>
<tr>
<td>Ge-GFP-B</td>
<td>86% (18/21)</td>
<td>86% (18/21)</td>
<td>17% (3/18)</td>
</tr>
<tr>
<td>Ge</td>
<td>76% (35/46)</td>
<td>85% (39/46)</td>
<td>18% (7/39)</td>
</tr>
<tr>
<td>SCID/beige mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ge-Hsp70</td>
<td>88% (22/25)</td>
<td>88% (22/25)</td>
<td>23% (5/22)</td>
</tr>
<tr>
<td>Ge-Hsp70-A</td>
<td>92% (11/12)</td>
<td>92% (11/12)</td>
<td>18% (2/11)</td>
</tr>
<tr>
<td>Ge-Hsp70-C</td>
<td>85% (11/13)</td>
<td>85% (11/13)</td>
<td>22% (3/11)</td>
</tr>
<tr>
<td>Ge-con</td>
<td>92% (22/24)</td>
<td>96% (23/24)</td>
<td>22% (5/23)</td>
</tr>
<tr>
<td>Ge-TCR-C</td>
<td>84% (10/12)</td>
<td>92% (11/12)</td>
<td>27% (3/11)</td>
</tr>
<tr>
<td>Ge-GFP-B</td>
<td>100% (12/12)</td>
<td>100% (12/12)</td>
<td>17% (2/12)</td>
</tr>
</tbody>
</table>

*Ge-Hsp70 cells (clones Ge-Hsp70-A and Ge-Hsp70-C), Ge-con cells (clones Ge-TCR-C and Ge-GFP-B), as well as parental Ge melanoma cells were injected subcutaneously into the flank of SCID or SCID/beige mice (1 × 10^6 cells in PBS/animal). 

*Percentage and number of animals in which primary tumors were palpable at the time point of section (day 26 to day 36).

*Tumor-bearing animals were carefully inspected for metastases during autopsy and the percentage and number of animals with metastases is given.
characterized in detail by in vitro analyses (19). Two clones of the Ge-Hsp70 cells (Ge-Hsp70-A and Ge-Hsp70-C) and two clones of the control cells (Ge-TCR-C and Ge-GFP-B) were selected for this study. The expression of HSP70 and control proteins in these clones was controlled regularly (data not shown) and found to be stable and in the range which we have previously described (19). Ge-Hsp70 and Ge-con cells were injected subcutaneously into the flank of SCID mice, which lack T and B lymphocytes. The primary tumors grew progressively and at day 26 the first animals had to be sacrificed (Fig. 1a). Surprisingly, the growth of HSP70-overexpressing tumors was reduced compared with control tumors. The frequency of tumors (Table I) on day 24 was slightly decreased in animals injected with Ge-Hsp70 cells (73%) compared with animals injected with Ge-con cells (86%). Most tumors grew locally (Fig. 1a, I and II) but in several cases invasive growth of the primary tumors (III) and regional metastases in the mesentery (IV) were observed. Some metastases were additionally found in regional lymph nodes (V) and in the diaphragm (VI). Intriguingly, the frequency of metastases was 21% for the Ge-con and 18% for the parental Ge tumors, whereas none of the Ge-Hsp70 tumors gave rise to metastases (Table I). Thus, overexpression of HSP70 appears to reduce the malignancy of the Ge melanoma cells. Furthermore, even when tumors developed from Ge-Hsp70 cells, their growth rate was significantly reduced (p = 0.0039, ANOVA) compared with tumors derived from Ge-con cells (Fig. 1b).

No effect of constitutive overexpression of HSP70 on proliferation and apoptosis
Staining of tumors with the proliferation marker Ki67 did not reveal major differences between Ge-Hsp70 and Ge-con-derived tumors (data not shown). The in vitro proliferation of HSP70-overexpressing and control clones, as determined by [3H]thymidine incorporation and cell counting was similar (data not shown). Thus, the reduced growth of HSP70-overexpressing tumors could not be explained by differences in the proliferation rate. Therefore, we analyzed cell death and apoptosis after exposure of the cells to conditions that occur in tumors, such as hypoxia and glucose starvation. Again, no difference between the Ge-Hsp70 and the Ge-con cells was observed when apoptosis was assessed by sub G1 peak measurement or cell death by propidium iodide staining after exposure to hypoxia or glucose-free medium for 24 h (data not shown).

Augmented cellular cytotoxicity in SCID mice bearing HSP70-overexpressing tumors
We then speculated that the innate immune system, which is still present in SCID mice, might contribute to the partial control of the growth of HSP70-overexpressing melanomas. To address this question, the number and the activity of cytotoxic cells from mice bearing HSP70-overexpressing or control tumors was analyzed. The percentage of splenic NK cells of mice which rejected tumors and mice in which Ge-Hsp70 or Ge-con tumors grew did not differ significantly (Fig. 2a). Moderate numbers of NK cells were found in the established tumors by flow cytometric analysis of freshly prepared cell suspensions. They were present in a slightly higher frequency (p = 0.0081, t test) in Ge-Hsp70 than in Ge-con tumors at the time point of autopsy (Fig. 2b). More interestingly, the cytotoxic activity of splenocytes from mice with HSP70-overexpressing tumors against the NK cell sensitive target cell line experiment is representative of five independent assays. d. Ge-Hsp70 or Ge-con cells were injected subcutaneously into the flank of SCID/beige mice (1 × 106 cells in PBS/animal). The mean of tumor size ± SD for SCID mice in which tumor growth was observed is shown.
YAC-1 was augmented when compared with mice with control tumors or tumor-free mice (Fig. 2c). This result suggests an increased cellular cytotoxic activity in SCID mice bearing HSP70-overexpressing tumors. Although the in vitro cytotoxic activity of splenocytes derived from SCID mice against the Ge-Hsp70 and Ge-con cells was generally low (data not shown), these results pointed toward a role of cytotoxic cells present in SCID mice, e.g., NK cells, in controlling the growth of Ge-Hsp70 tumors.

Tumor growth of HSP70-overexpressing melanoma cells is not reduced in SCID/beige mice

To verify the suspected role of NK cells in vivo, we injected the same cell clones as before into SCID/beige mice which lack functional NK cells in addition to T and B lymphocytes. The comparative analysis of tumor growth in SCID and SCID/beige mice has been widely used to determine the role of NK cells in tumor regression (36–40). The frequency (Table I) and the growth of tumors (Fig. 2d) after injection of Ge-Hsp70 and Ge-con cells were similar in these animals. Tumors derived from Ge-Hsp70 cells resulted in the same metastatic frequency as tumors from Ge-con cells (Table I). Furthermore, splenocytes from tumor-bearing SCID/beige mice did not kill YAC-1 cells efficiently in vitro irrespective of the tumor type that was present in the mice (data not shown). These results clearly indicate that the differences observed in the SCID mice were due to the activity of NK cells, that NK cells partially control the growth of Ge-Hsp70-derived tumors and that they completely suppress metastases.

No HSP70 but MICA/B cell surface expression on tumors

It has been shown previously that HSP70 which is expressed at the cell surface of some tumor cells can function as a target structure for NK cells (16). Thus, we analyzed the expression of HSP70 on the melanoma cells using an Ab suitable for HSP70 cell surface staining (35). Cultured Ge-Hsp70 and Ge-con cells were negative for plasma membrane-associated HSP70 (data not shown). Cells obtained from freshly prepared tumors from SCID or SCID/beige mice also did not express cell surface HSP70 (Fig. 3a), although the transgenic rat Hsp70-1 mRNA was strongly expressed in the Ge-Hsp70-derived tumors (Fig. 3b) and some stress-inducible endogenous human HSP70-2 mRNA was detectable in all tumors (Fig. 3c). Therefore, we next asked whether other ligands for activating NK receptors might be present on the tumors. MICA and MICB molecules were among the candidates, because these human ligands have also been shown to interact structurally and functionally with the mouse activating NK receptor NKG2D (23–25). Expression of MICA and MICB mRNA in Ge-Hsp70, Ge-con and parental Ge cells was very low in vitro (Fig. 4, a and b). In tumors, however, the expression of both MICA genes was clearly induced (Fig. 4, a and b). Interestingly, MICA and MICB mRNA expression in tumors grown in SCID/beige was higher than that in SCID mice (MICA: p = 0.0146 and MICB: p < 0.0001, ANOVA). Furthermore, we observed a differential expression of MICB mRNA in Ge-Hsp70 vs Ge-con tumors that was clearly modified by the host. MICB mRNA expression was lower in Ge-Hsp70 than Ge-con tumors grown in SCID mice (interaction: p = 0.0437, ANOVA), whereas such a difference was not found in SCID/beige mice. A similar tendency for MICA mRNA expression was observed, although not on a statistically significant level. In subsequent experiments, MICA/B molecules were confirmed to be expressed at the cell surface of tumor cells in vivo by flow cytometry after staining single cell suspensions derived from fresh tumors with an anti-MICA/B mAb or recombinant mouse NKG2D (Fig. 4, a and b).
In accordance with the mRNA expression data, a reduced MICA/B cell surface expression was observed on Ge-Hsp70 tumors grown in SCID mice \((p < 0.002, t\text{-test})\) compared with Ge-Hsp70 tumors grown in SCID/beige hosts (Fig. 4d). Furthermore, the MICA/B expression was different in Ge-Hsp70 and Ge-con tumors grown in SCID \((p < 0.0048, t\text{-test})\) but not in those grown in SCID/beige mice. These data suggest a functional role of MICA/B expression in the Ge-Hsp70 tumors. We assume that in SCID mice tumor cells which express MICA/B become targets for NK cells. Therefore, a selection against MICA/B expressing tumor cells in SCID but not in NK cell deficient SCID/beige mice might have occurred. This selection pressure was more apparent for HSP70-overexpressing tumors than for control tumors, perhaps due to a higher cytotoxic activity of NK cells in mice with HSP70-overexpressing tumors.

Augmented cellular cytotoxicity in SCID mice with HSP70-overexpressing tumors depends on the expression of NKG2D ligands on target cells

Splenocytes from SCID mice with HSP70-overexpressing tumors were able to lyse YAC-1 target cells (Fig. 2c). However, in vitro they did not lyse the human Ge melanoma cells (data not shown). This low cytotoxic activity against the Ge target cells might be explained by the fact that MICA/B molecules were not expressed in these cells under normal cell culture conditions. YAC-1 cells are a typical target cell line for murine NK cells and they express constitutively high amounts of NKG2D ligands in contrast to the human Ge melanoma cells (Fig. 5a). However, NKG2D ligands were inducible on the melanoma cells by treatment with the histone deacetylase inhibitor SAHA (Fig. 5a) that is known to induce MICA/B (31). Splenocytes from naive, tumor-free SCID mice did not kill YAC-1 or Ge melanoma cells irrespective of the expression of NKG2D ligands (Fig. 5b). Splenocytes from SCID mice with HSP70-overexpressing tumors (Ge-Hsp70) were able to lyse YAC-1 cells and SAHA-treated Ge-con or Ge-Hsp70 cells, which expressed NKG2D ligands but not untreated melanoma cells which were negative for NKG2D ligands (Fig. 5, c and d). Splenocytes from SCID mice with control tumors (Ge-con) had a much lower cytotoxic activity and killed even YAC-1 cells only inefficiently (Fig. 5, c and d). These findings suggest a role of tumor-derived HSP70 in the activation of NK cells which subsequently recognize NKG2D ligands on target cells.

No regulation of MICA/B expression by HSP70 overexpression

To explain the role of HSP70 in the tumors, one might alternatively speculate that overexpression of HSP70 as a molecular chaperone could allow for an increased cell surface expression of MICA/B molecules which subsequently augment the sensitivity of HSP70-overexpressing tumor cells to NK cells. However, in vivo the expression of MICA/B was not different in Ge-con and Ge-Hsp70 tumors grown in SCID/beige mice (Fig. 4). Also in vitro the expression of MICA/B on Ge-con and Ge-Hsp70 cells was similar.
(Fig. 6a). MICA/B expression was also inducible to the same extent on Ge-con and Ge-Hsp70 cells (Fig. 6a) by the histone deacetylase inhibitor SAHA. Induction of intracellular HSP70 in Ge-con and Ge-Hsp70 cells by heat shock (Fig. 6b) was also not accompanied by an induction of MICA/B molecules (Fig. 6a).

Moreover, no synergistic effect of heat shock and SAHA treatment on MICA/B cell surface expression was observed (Fig. 6a). Thus, neither constitutive nor induced HSP70 overexpression did affect the expression of MICA/B on the melanoma cells, arguing strongly against an epistatic regulation of MICA/B expression by HSP70.

HSP70 containing exosomes are released from HSP70-overexpressing melanoma cells

The function as target structure for NK cells (16) is not the only role that has been assigned to HSP70 in NK cell biology. Extracellular HSP70 has been shown to activate NK cells (41). Therefore, we determined whether HSP70 is released from the melanoma cells. It is known that cells, including tumor cells, can release exosomes which contain heat shock proteins (33, 42). In contrast to Ge-con cells, viable Ge-Hsp70 cells did release exosomes containing the inducible HSP70 (Fig. 7a). These findings indicate that the HSP70-overexpressing melanoma cells are a source for extracellular HSP70 that might activate NK cells.

HSP70-positive exosomes activate mouse NK cells to kill NKG2D ligands expressing target cells

Taken together, our findings suggest that HSP70 is released via exosomes from the HSP70-overexpressing tumor cells and activates NK cells which then kill preferentially the MICA/B expressing human melanoma cells. To further test this hypothesis, splenocytes from SCID mice were cultured in the presence of IL-2 (20 ng/ml) or IL-2 plus exosomes (10 μg/ml) derived from Ge-con or Ge-Hsp70 cells. After two days NK cells were negatively isolated by MACS and used as effector cells in a 51Chromium release assay against YAC-1, Ge-con, and SAHA-treated Ge-con target cells (Fig. 7b). The HSP70-positive exosomes of Ge-Hsp70 cells in contrast to exosomes of Ge-con cells were able to stimulate the cytotoxic activity of NK cells against YAC-1 and SAHA-treated Ge-con cells. Untreated Ge-con cells, which did not express NKG2D ligands, remained resistant to lysis (Fig. 7b). The NK cell depleted fractions of the splenocyte cultures were not able to kill any of the targets even at higher E:T ratios (data not shown). A summary of
MICA/B expression is not regulated by HSP70. \(a\), Ge-con and Ge-Hsp70 cells were cultured under standard conditions (co), treated with the histone deacetylase inhibitor SAHA (10 \(\mu\)M) for 24 h, heat shocked (HS, 1 h at 42°C plus 23 h at 37°C), or treated with SAHA and heat shock before flow cytometric analysis of MICA/B cell surface expression (mAb BAMO-1). The mean + SD of the specific MFI (MFI for the specific staining minus MFI for the staining with the secondary Ab alone) of six experiments with Ge-con and seven with Ge-Hsp70 cells is shown. \(b\), The same cells were analyzed in parallel for intracellular HSP70 expression (mAb C92) by flow cytometry. The mean + SD of the specific MFI is shown.

Discussion

The stress response is destined to maintain survival of cells that have been exposed to adverse environmental conditions. To be successful the immune system must have the capacity to destroy target cells, even during an ongoing stress response which has been initiated for cellular protection. Moreover, stressed cells, e.g., virus-infected cells or tumor cells, appear to be usually a more appropriate target for cytotoxic effector cells of the immune system than unstressed cells (43). We have previously shown that HSP70, which is known to protect cells efficiently against various adverse conditions, fails to protect against specific cytotoxic effector mechanisms of CTL mediated in the granule exocytosis pathway (19, 20, 27). Thus, cytotoxic effector mechanisms of the cellular immune system seem to dominate over the protective stress response. In accordance with this assumption, it has been suggested that components of the stress response system function as endogenous danger signals which trigger the initiation of an immune response (17, 18). HSP70, e.g., appears capable of activating and connecting innate and adaptive immune reactions (10, 18), and fulfills the criteria of endogenous danger signals. Further examples of potential endogenous danger signals are the ligands of the NKG2D receptor (44). NKG2D has been shown to serve as an activating receptor triggering NK cell responses against tumors and expression of NKG2D ligands in tumors has been reported to induce tumor rejection (22, 45, 46). NKG2D ligands include in humans MICA and MICB. These NKG2D ligands appear to be up-regulated in response to stress (21, 22, 47, 48), and they signal the
presence of potentially dangerous cells to the immune system (43, 49).

Herein we show that HSP70-positive exosomes derived from HSP70-overexpressing tumor cells activate in vitro mouse NK cells to kill tumor cells which express NKG2D ligands either constitutively, such as mouse YAC-1 cells, or in an inducible manner, such as the human Ge melanoma cells. The stimulation of splenocyte cultures by HSP70-positive exosomes increased the capability of the NK cells to kill targets. This stimulatory effect on NK cells seems to be a specific property of the stress-inducible HSP70. Exosomes which are released from the HSP70-overexpressing melanoma cells contained the stress-inducible HSP70 in contrast to exosomes from the HSP70-negative control cells. The cytotoxic activity of NK cells could also be stimulated by soluble HSP70 (own unpublished results). Thus, HSP70 released from exosomes might directly contribute to the observed effects.

The cytotoxic activity of NK cells could be inhibited by soluble NKG2D, confirming the requirement of NKG2D ligands for target cell recognition by the HSP70-stimulated NK cells. It might be important that the naive NK cells were stimulated first by the HSP70-positive exosomes. These preactivated NK cells were subsequently in the effector phase able to kill target cells which expressed NKG2D ligands. Strong and prolonged ectopic expression of NKG2D ligands such as MICA/B has been reported to result in an over-stimulation of NKG2D expressing cells, and in a consecutive down-regulation of NKG2D and inactivity of NK cells (50–52). Thus, the activation of NK cells might depend on the correct order and strength of the HSP70 and MICA/B signals.

In the melanoma cell lines used in this study, we observed an induction of the NKG2D ligands MICA/B in vivo in tumors. In vitro MICA/B was induced only in response to genotoxic stress, i.e., the chromatin-modifying treatment with the histone deacetylase inhibitor SAHA (31), but not in response to the proteotoxic stress of a heat shock, as it has been described for other cell lines (21, 22, 47). Our findings are in agreement with a recent report showing that mouse and human NKG2D ligands were up-regulated in several cell lines by various genotoxic stresses but not by heat shock (48). Therefore, the regulation of NKG2D ligands might vary between cell lines. It might be interesting that in our melanoma cells HSP70, which was inducible by proteotoxic stress, and NKG2D ligands, which were inducible by genotoxic stress, concordantly promoted the cytotoxic activity of NK cells against these tumors. Thus, two signals which appear to be independently

129Sv mice at different E:T ratios is shown as determined in three independent experiments. In the individual experiments triplicates of the target cells were analyzed. YAC-1, Ge-con, and Ge-con cells exposed to 10 μM SAHA for 20 h before the tests served as targets. The MICA/B induction on the Ge-con cells by SAHA treatment was in the same range as shown in Fig. 6a. NK cells were isolated by MACS from splenocytes which were stimulated before in vitro for 2 days with IL-2 (20 ng/ml), IL-2 plus exosomes of Ge-con cells (10 μg/ml), or IL-2 plus exosomes of Ge-Hsp70 cells (10 μg/ml). The mean of CD49b (DX5)-positive NK cells + SD among the effector cells used in the three experiments is shown as determined by flow cytometry. c. The inhibition of specific lysis of target cells which express NKG2D ligands by soluble mouse NKG2D is shown as determined in an experiment representative for three tests. The means of specific lysis + SD of target cells by NK cells at an E:T ratio of 10:1 are shown. YAC-1, Ge-con, and Ge-con cells exposed to 10 μM SAHA for 20 h before the tests served as targets. NK cells were isolated by MACS from splenocytes which were stimulated before in vitro for 2 days with IL-2 (20 ng/ml), IL-2 plus exosomes of Ge-con cells (10 μg/ml), or IL-2 plus exosomes of Ge-Hsp70 cells (10 μg/ml). For inhibition of lysis soluble mouse NKG2D-Fc was added to the test at a concentration of 3 μg/ml.

**FIGURE 8.** NK cells from immunocompetent mice are stimulated by exosomes of Ge-Hsp70 cells to kill target cells which express NKG2D ligands. a. The mean of specific lysis + SD of target cells by NK cells from presence of potentially dangerous cells to the immune system (43, 49).

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regulated by different kinds of stress can activate synergistically the NK cell cytotoxicity. The activation of NK cells by HSP70 against target cells which express NKG2D ligands appears to be relevant for immune surveillance of tumors in vivo, in that the growth of HSP70-overexpressing Ge melanoma cells was significantly reduced in SCID mice. Moreover, in contrast to control tumors the HSP70-overexpressing tumors did not grow invasively and did not give rise to regional metastases. These effects in SCID mice could unambiguously be attributed to NK cells, because tumor growth and the rate of metastases of HSP70-overexpressing tumors was the same as observed for control tumors in SCID/beige mice, which lack functional NK cells in addition to B and T lymphocytes (36–40).

Human MICA/B molecules are known to interact structurally and functionally with mouse NKG2D (23–25). Therefore, we determined the MICA/B expression in the tumors. The Ge melanoma cells express MICA/B in vivo, but hardly in vitro. It might be important that the expression of the NK2D ligands MICA/B was endogenously regulated in the tumors in our model so that an overstimulation and consequent down-regulation of NK cell activity was avoided (50–52). The MICA/B expression in tumors appears to be functionally relevant in SCID mice, because the expression level of both genes, MICA and MICB, was reduced in HSP70-overexpressing tumors grown in SCID compared with SCID/beige mice. The expression of MICA/B was not regulated epistatically by HSP70. Therefore, we interpret this reduced MICA/B expression as being an example of "cancer immunoeediting" (53). MICA/B-expressing tumor cells likely become a preferential target for HSP70-activated NK cells present in SCID mice thereby leading to a loss of those cells. Splenocytes from SCID mice with HSP70-overexpressing tumors were ex vivo able to lyse only those melanoma cells in which NKG2D ligands including MICA/B were induced by pharmacological means. Although the reduced expression of MICA/B in vivo in SCID mice with HSP70-overexpressing tumors also indicates a potential immune escape mechanism, HSP70 overexpression in primary tumors and the subsequent activation of NK cells, which can kill MICA/B-expressing tumor cells, was apparently sufficient to completely suppress invasive growth and regional metastases. In accordance with others studies (54) we assume that the activity of NK cells was efficient in our model at an early time point in progression of the disease because massive tumors were established. This activity led at least to a delay of tumor growth. In addition, metastasizing cells were apparently completely eradicated by HSP70-activated NK cells.

The outcome of HSP70 overexpression might depend on the tumor model that we have analyzed. In our model the extracellular stimulatory effects of HSP70 on the immune system were apparently more important than intracellular effects of HSP70 which might increase the tumorigenicity of HSP70-overexpressing cells. We have shown before that HSP70 overexpression led to reduced HSC70 expression in the Ge melanoma cells and that HSP70 can replace functions of HSC70 as a chaperone (19). Importantly, the effects of HSP70 and HSC70 on the stimulation NK cells appear to be different (41). The strong overexpression of HSP70 in the Ge-Hsp70 cells might have allowed detecting the NK cell stimulatory effects of HSP70 in our tumor model. However, the endogenous HSP70 was also induced in vivo in the tumors as shown for HSP70-2 at the mRNA level. Therefore, HSP70 and NKG2D ligands such as MICA/B might become expressed also in other tumors and contribute together to the activation of NK cells.

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Disclosures
L. Elsner and R. Dressel declare that a patent application has been filled in describing the use of HSP70 and the HSP70-derived peptide TKD for activation of NK cells against tumors expressing NKG2D ligands. G. Mulfetoff is in addition to her academic position CEO of the small company multimune.

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