Influence of Hsp70 and HLA-E on the killing of leukemic blasts by cytokine/Hsp70 peptide-activated human natural killer (NK) cells

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Abstract This study compared the effects of the human 70-kDa stress protein (Hsp70) peptide, TKDNNLLGRFELSG (TKD), proinflammatory cytokines, or a combination of both on the repertoire of receptors expressed by human natural killer (NK) cells and their capacity to kill human CX colon carcinoma cells, K562 erythroleukemic cells, and leukemic blasts from two patients with acute myelogenous leukemia. Low-dose interleukin (IL) 2/IL-15 and TKD increase the expression density of activatory (NKG2D, NKp30, NKp44, NKp46, CD94/NKG2C) and inhibitory (CD94/NKG2A) receptors on NK cells. Con-

comitantly, IL-2/TKD treatment enhances the cytotoxicity of NK cells (as reflected by their secretion of granzyme B) against Hsp70 membrane-positive and human leukocyte antigen (HLA)-E membrane-negative (Hsp70⁺/HLA-E⁻) CX⁺ and K562 cells. However, it had no effect on the responsiveness to Hsp70⁻/HLA-E⁻ CX⁻ cells over that induced by IL-2 alone. The cytotoxicity of IL-2/TKD-activated, purified NK cells and peripheral blood mononuclear cells against Hsp70⁺/HLA-E⁺ leukemic blasts was weaker than that against Hsp70⁺/HLA-E⁻ K562 cells. Hsp70-blocking and HLA-E transfection experiments confirmed membrane-bound Hsp70 as being a recognition/activatory ligand for NK cells, as cytotoxicity was reduced by the presence of the anti-Hsp70 monoclonal antibody cmHsp70.2 and by inhibiting Hsp70 synthesis using short interference ribonucleic acid. HLA-E was confirmed as an inhibitory ligand, as the extent of NK cell-mediated lysis of K562 cell populations that had been transfected with HLA-E^R or HLA-E^G alleles was dependent on the proportion of HLA-E-expressing cells. These findings indicate that Hsp70 (as an activatory molecule) and HLA-E (as an inhibitory ligand) expression influence the susceptibility of leukemic cells to the cytolytic activities of cytokine/TKD-activated NK cells.

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Introduction

Although for many years natural killer (NK) cells have been considered to be nonspecific killers, it is now apparent that these cells are highly sophisticated in their capacity to distinguish between normal and malignant cells. The

effector functions of NK cells are regulated by killer cell inhibitory and activating receptors belonging to the killer cell immunoglobulin-like, the immunoglobulin-like transcript, C-type lectin receptor (Lanier et al. 1998), or the natural cytotoxicity receptor families (Moretta et al. 2001). The activating and inhibitory functions of these receptors depend on the intracellular immunoreceptor tyrosine-based inhibitory or activation motifs, respectively (Long 1999; Moretta et al. 2001).

The “missing self” hypothesis proposed that tumor cells that display an altered pattern of major histocompatibility complex (MHC) expression or did not express MHC antigens were targets for the cytolytic activity of NK cells (Ljunggren and Karre 1990). However, it is also known that NK function is determined by a fine balance of inhibitory and activatory receptors that recognize a variety of different ligands (Moretta et al. 2001), including nonclassical stress-inducible MHC class I-related chain (MIC) A and MICB glycoproteins, the glycosylphosphatidylinositol-linked UL-16 binding proteins (ULBP), the retinoic acid early inducible-1 protein, and HA60, a minor histocompatibility antigen (Lanier et al. 1998; Bauer et al. 1999; Cosman et al. 2000). The C-type lectin receptor CD94 expressed on NK cells forms heterodimers with members of the NKG2 family. The CD94/NKG2A inhibitory heterodimeric receptor complex binds to HLA-E molecules presenting leader peptides of human leukocyte antigen (HLA)-A, HLA-B, and HLA-C alleles and mediates inhibitory signals via the tyrosine phosphatase SHP-1, whereas binding of HLA-E to the CD94/NKG2C heterodimeric complex elicits activatory signals via the activation of Syk family tyrosine kinases (Radons et al. 2006). NKG2D forms homodimeric complexes to the exclusion of CD94 and binds MHC-like ligands MICA and MICB and ULBP family members (Farag and Caligiuri 2006; Radons et al. 2006).

In addition to nonclassical HLA-E, we have reported that the major stress-inducible form of the human 70-kDa stress protein family, Hsp70, is frequently expressed on the plasma membranes of colon, lung, pancreas, mammary, head, and neck cancers and metastases derived there from (Multhoff et al. 1995a, b). Mapping has revealed that the Hsp70 sequence, which is recognized by NK cells, is a 14-mer peptide (TKDNNLLGRFELSG, amino acids 450–463, termed TKD), which represents a part of the C-terminal substrate-binding domain of Hsp70 (Botzler et al. 1998; Multhoff et al. 2001). TKD peptide plus low-dose interleukin (IL)-2 stimulates NK cells toward Hsp70-expressing tumors in vitro (Botzler et al. 1998; Multhoff et al. 1999) and in tumor mouse models in vivo (Multhoff et al. 2000; Moser et al. 2002; Stangl et al. 2006).

The expression density of CD94 is essential for the cross-talk of NK cells with Hsp70 membrane-positive tumor cells (Gross et al. 2003c), and the enhanced cytolytic

activity of NK cells against such tumor cells can be blocked by a CD94-positive monoclonal antibody (mAb) on the effector side and by an Hsp70-specific mAb recognizing the Hsp70 epitope TKD on the target side (Gross et al. 2003c). These findings suggest that in the absence of HLA-E, membrane-bound Hsp70 acts as a dominant tumor-specific recognition structure for NK cells and that the administration of cytokine/TKD-activated NK cells might therefore offer an immunotherapeutic approach for the treatment of Hsp70 membrane-positive tumors. This study further investigates the interplay of HLA-E and Hsp70 on leukemic blasts and their role on NK cell-mediated tumor killing. It appeared that HLA-E as an inhibitory and Hsp70 as an activatory ligand both affect the susceptibility of leukemic blasts to the cytolytic activities of cytokine/TKD-activated NK cells expressing CD94/NKG2A and CD94/NKG2C.

Materials and methods

Hsp70 peptide, TKD

Good manufacturing practice (GMP)-grade Hsp70 peptide TKD, which exhibits a stimulatory activity on NK cells, which is comparable to that induced by the full-length Hsp70 protein (Multhoff et al. 2001), was produced by Bachem AG (Bubendorf, Switzerland). This was diluted in sterile water for injection (1 mg/mL, Ecotainer®, B. Braun AG, Melsungen, Germany) and aliquoted into 1-mL syringes according to GMP guidelines by the Pharmaceutical Department at the University Hospital Regensburg, Regensburg, Germany.

Cells and cell culture

The human erythroleukemic cell line K562 (Hsp70⁺/HLA-E⁻) was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% v/v fetal calf serum (FCS, Life Technologies, Eggenstein, Germany), 6 mM L-glutamine, and antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin; Life Technologies).

The HLA-E-negative, Hsp70 membrane-positive (HSP70.1; Expasy accession number: HSP71_Human) and Hsp70 membrane-negative human colon carcinoma sublines (CX⁺ and CX⁻, respectively) that were generated from the parental cell line CX-2 (Tumorzellbank, Deutsches Krebsforschungszentrum, Heidelberg, Germany) by cell sorting using the Hsp70-specific mAb cmHsp70.1 (multi-mune GmbH, Munich, Germany; Multhoff et al. 1997) were cultured in RPMI 1640 medium supplemented with 10% v/v FCS, antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin), and 2 mM L-glutamine. Expo-

nential cell growth was maintained by regular cell passages. Plating efficiency, doubling time (20 h), and protein content of the CX⁺ and CX⁻ sublines are comparable.

Adherent tumor cells were trypsinized for less than 1 min with trypsin/ethylenediamine tetraacetic acid (Life Technologies), and single-cell suspensions were seeded at constant cell densities of 0.5–10⁶ cells in 5 mL fresh medium in T-25-ventilated small culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany). Cell lines were regularly screened and confirmed as being negative for mycoplasma contamination using a Gen-Probe[®] Rapid Detection System (Gen-Probe; H. Biermann GmbH, Bad Nauheim, Germany).

Peripheral blood was obtained from healthy volunteers, and peripheral blood mononuclear cells (PBLs) were isolated by density gradient centrifugation using Ficoll-Paque[®] (GE Healthcare Europe GmbH, Munich, Germany). The study was approved by the institutional Ethics Committee, and all patients gave written informed consent. All procedures were performed in accordance with the Helsinki Declaration of 1975, as revised in 2000.

Inhibition of membrane Hsp70 expression on K562 cells using siRNA

The influence of membrane Hsp70 on NK cell-mediated cytotoxicity was also explored using K562 cells on which Hsp70 expression had been inhibited using short interference (si) ribonucleic acid (RNA) technology. These membrane Hsp70-negative K562 cells were generated in the laboratory of Prof. Alexander Asea (Scott and White Clinic, Temple, TX).

Briefly, cells were harvested by trypsinization, washed in ice-cold phosphate-buffered saline, and seeded into six-well plates. Into one tube (tube A1), 50 μ L OPTI-MEM[®]-reduced serum medium (Invitrogen, Carlsbad, USA) plus Hsp70 siRNA was added, and into a second tube (Tube A2), 50 μ L OPTI-MEM[®] plus scrambled-siRNA was added to act as a control. Into a third tube (Tube B), 96 μ L OPTI-MEM[®] plus 0.4 μ L Lipofectamine[™] 2000 transfection reagent (Invitrogen) was added. Tubes were incubated for 5 min at room temperature, at which time, 50 μ L of the contents of Tube B were added to 50 μ L of the contents of tube A1 (experimental, tube C), and 50 μ L of the contents of tube B were added to 50 μ L of the contents of tube A2 (control, tube D). Tubes were incubated for 20 min at room temperature, K562 cells were added, and the tubes were incubated for 2 min at room temperature. Complete medium supplemented with penicillin/streptomycin and 10% v/v FBS (1 mL) was added, and cells were transferred to six-well plates and incubated for approximately 72 h at 37°C, 5% v/v CO₂ atmosphere. Gene silencing was confirmed by Western blot analysis. Flow cytometric analysis revealed that the proportion of cells

expressing membrane Hsp70 reduced from 45 to 5% (data not shown).

Transfection of K562 cells with HLA-E

The influence of membrane HLA-E expression on NK cell-mediated cytotoxicity was evaluated using K562 cells (membrane HLA-E⁻) on which the membrane expression of HLA-E had been induced by transfection. Transfectants, which were generated by electroporation and selected by adding 0.4 mg/mL G418 and/or 0.4 mg/mL hygromycin B, as described previously (Ulbrecht et al. 2000), were kindly provided by Prof. Elisabeth Weiss (Institut für Anthropologie und Humangenetik, Munich, Germany). The transfection of the HLA-E alleles such as HLA-E^GB5, HLA-E^GC6, HLA-E^RA2, and HLA-E^RA4 had no influence on the proportion of membrane Hsp70-positive K562 cells (47 $\pm$ 19, 38 $\pm$ 11, 47 $\pm$ 18, and 36 $\pm$ 17 vs 40 $\pm$ 9% in untransfected cells, respectively) or the intensity of membrane Hsp70 expression (MFI: 11, 10, 10, and 11 vs 11 in untransfected cells, respectively).

Flow cytometric analysis

The phenotype of PBLs derived from healthy donors, blasts derived from two patients with acute myelogenous leukemia (AML), and the expressions of membrane Hsp70, MHC class I, HLA-E, ULBP1, ULBP2, ULBP3, and MICA/B by the K562 and CX⁺ and CX⁻ cell lines were determined by multicolor flow cytometry. The phenotype of PBLs from healthy donors was determined using fluorochrome-labeled mAb cocktails CD3/56, CD45/CD14 (Simultest[™], Becton Dickinson GmbH, Heidelberg, Germany), and mAbs directed against CD56 (IgG_{2a}, IgG₁, Becton Dickinson GmbH) and CD94 (IgG₁, Ancell via Alexis Deutschland, Grünberg, Germany and IgG_{2a}, Immunotech S.A.S., Marseille, France).

Blasts from two patients with leukemia, K562 cells, and the CX⁺ and CX⁻ sublines were immunophenotyped using CD45-PE (H130-PE, IgG₁, Caltag GmbH, Hamburg, Germany) in combination with fluorochrome-conjugated mAbs directed against MHC class I (W6/32-FITC, IgG_{2a}, Cymbus Biotechnology, Eastleigh, UK), HLA-E (MEM-E/06-PE, IgG₁, BIOZOL Diagnostica Vertrieb GmbH, Eching, Germany), and Hsp70 (cmHsp70.1-FITC, IgG₁, multimune GmbH, Munich, Germany). The expression of ULBP1, ULBP2, ULBP3, and MICA/B was determined using mAbs that were obtained from R&D Systems GmbH (Wiesbaden-Nordenstadt, Germany). Prof. Alexander Steinle (Inst. Cell Biology, Tübingen, Germany).

In all instances, flow cytometric analysis was performed using a FACSCalibur[™] flow cytometer and CELLQuest Pro[™] data acquisition and analysis software (Becton

Dickinson GmbH). Both the proportion of cells expressing a given antigen and the intensity of antigen expression (mean fluorescence intensity, MFI) were determined. All analyses included relevant isotype-matched control antibodies (Becton Dickinson GmbH).

Separation of NK effector cells

CD3⁻ NK cells were isolated from leukapheresis products obtained from human donors following a standard CD3/CD19 depletion protocol (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). CD3⁺ T cells were separated from the same donors by positive selection using a CD3⁺ cell isolation kit (Miltenyi Biotech GmbH), also according to the standard protocol. The purity of the NK and T cell populations, as determined by flow cytometry using CD3-FITC/56-PE (Becton Dickinson GmbH) and CD94-FITC (IgG₁, Ancell Corporation) mAbs on day 2 after separation, is indicated.

Determination of cytotoxicity using the granzyme B ELISPOT assay

We have previously characterized the lysis of Hsp70 membrane-positive tumor cells by TKD-activated cytolytic effector cells as being perforin-independent, granzyme B-mediated apoptosis (Gross et al. 2003b). In this study, cytotoxicity was determined on the basis of a 4-h ⁵¹Cr-release assay, which was performed as described previously (Multhoff et al. 1997), and also on the basis of granzyme B release, which was determined according to the manufacturer's recommended protocol using a commercially available enzyme-linked immunosorbent spot (ELISPOT) assay kit (Becton Dickinson GmbH). The high degree of correlation between these two methodological approaches is illustrated in Fig. 1. Because of the fact that labeling of primary tumor material with ⁵¹Cr is frequently not very efficient, the granzyme B ELISPOT assay provides a good alternative for the ⁵¹Cr-release assay.

For the granzyme B release assay, 96-well ELISPOT plates (Millipore GmbH, Schwalbach, Germany) were coated overnight at 4°C with capture antibody and blocked with RPMI 1640 culture medium containing 10% v/v FCS. Effector and target cells were added and plates were incubated for 4 h at 37°C. After washing in deionized water and wash buffers A and B, a biotinylated detecting antibody was added (2 µg/mL, 2 h). After an additional two washes, granzyme B was visualized by adding freshly prepared avidin-horseradish peroxidase (1 h) followed by 3-amino-9-ethyl-carbazole substrate solution (25 min). Spots were counted and data analyzed using an Immuno Spot Series 3A Analyzer (CTL-Europe GmbH, Aalen, Germany).

Fig. 1 Correlation of the granzyme B ELISPOT and ⁵¹Cr-release assays for the determination of NK cell-mediated cytotoxicity at effector to target cell ratios of 5:1, 2:1, and 1:1. Effector cells were human CD3⁻ NK cells that had been stimulated with low-dose IL-2 (100 IU/mL) plus Hsp70 peptide TKD (2 µg/mL). Hsp70 membrane-positive K562 erythroleukemic cells were used as targets. The experiment summarizes the results of three independent experiments. The correlation between ELISPOT dots and percentage lysis is highly significant ($P < 0.001$)

Effect of TKD peptide, IL-2, and IL-15 on NK cell-mediated cytotoxicity

PBLs or purified NK cells from healthy donors were incubated for 5 days at 37°C with TKD peptide alone (2 µg/mL), low-dose IL-2 alone (100 IU/mL), TKD peptide plus low-dose IL-2, or TKD peptide plus low-dose IL-15 (10 IU/mL, equivalent to 5 ng/mL) at constant cell densities of 5×10^6 cells in RPMI 1640 medium supplemented with 6 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin (Life Technologies), and 5% v/v heat-inactivated FCS. These unstimulated and stimulated PBL and NK cell populations were used as effector cells (effector to target, E/T ratios ranging from 1:1 to 10:1) against K562 cells and bone marrow-derived autologous and allogeneic leukemic blasts. Leukemic blasts were isolated from patients at first diagnosis using Ficoll-Paque® separation and were used after a recovery period of 5 to 8 h in a conditioned growth medium.

Antibody-mediated blocking of cytotoxicity

The involvement of membrane-bound Hsp70 in the observed cytotoxic responses was evaluated using an inhibition assay. For this, 1×10^6 ⁵¹Cr-labeled target cells were preincubated with the cmHsp70.2 mAb (10 µg/mL final concentration, multimmune GmbH). After 20 min at room temperature, the target cells were washed in RPMI 1640 medium and the 4 h ⁵¹Cr-release assay was performed as described (Multhoff et al. 1997).

Statistical analysis

Data from experimental groups were compared using the Student's *t* test. Two groups were compared by using the Cox regression; *P* values less than or equal to 0.05 were considered to reflect statistically significant differences.

Results

Effect of the treatment of PBL with either TKD, IL-2, alone, or IL-2/TKD and IL-15/TKD on the NK and T cell phenotype

Treatment of PBLs from healthy human donors with low-dose IL-2 (100 IU/mL) or low-dose IL-15 (10 IU/mL) plus the Hsp70 peptide TKD induced a significant upregulation in the cell surface density of NK cell-specific activating molecules CD94/NKG2C, NKG2D, CD56/NKp30, CD56/NKp44, CD56/NKp46, and CD69. However, the density of expression of the inhibitory receptor complex CD94/NKG2A was concomitantly enhanced (Table 1). In contrast, neither of the two treatments alone had any significant effect on the expression density of the tested markers (Table 1). The MFI of CD16 was downregulated by IL-2 or IL-15 (data not shown). Concentrations above 100 IU/mL of IL-15 and above 1,000 IU/mL of IL-2 resulted in apoptotic cell death of the effector cells within 2 to 3 weeks after stimulation (data not shown).

Cytolytic activity of IL-2/TKD, IL-2, or TKD activated PBLs, NK, and T cells against CX⁺/CX⁻ and K562 tumor cell lines

Activation of PBL effector cells with IL-2 alone increased their secretion of granzyme B in response to incubation with CX⁺, CX⁻, and K562 target cells compared to that of PBL effectors that had been treated with TKD alone (Fig. 2). Concomitant with the upregulated expression of activatory and inhibitory cell NK cell receptors, incubation of PBLs with IL-2/TKD significantly increased their secretion of granzyme B (a correlate of cytolytic activity) in response to CX⁺ and K562 tumor cell lines (*P*<0.05), both of which are membrane-positive for Hsp70 but membrane-negative for HLA-E (Hsp70⁺/HLA-E⁻). These cell lines differ with respect to their MHC class I, ULBP1, ULBP2, ULBP3, and MICA/B expression pattern (Table 2). The responsiveness of IL-2/TKD-stimulated PBLs to the Hsp70⁻/HLA-E⁻ tumor cell line CX⁻, which shows similarities in the expression of MICA, MICB, and ULBP molecules to its corresponding Hsp70⁺/HLA-E⁻ counterpart (CX⁺, Table 2), was not increased in comparison to PBL effector cells that had been stimulated with IL-2 alone (Fig. 2).

As an internal control, PBLs derived from a healthy donor, lacking Hsp70 and HLA-E on their cell surface (Hsp70⁻/HLA-E⁻), were also used as targets for different IL-2- and IL-2/TKD-activated effector cell populations. As expected, no significant release of granzyme B was observed when unstimulated, IL-2 (100 IU/mL) or IL-2/

Table 1 Phenotype of untreated (control) PBLs derived from healthy donors (*n*=8) or PBLs incubated with TKD (2 µg/mL), IL-2 (100 IU/mL), IL-2/TKD (100 IU/mL), or IL-15/TKD (10 IU/mL/2 µg/mL) for 5 days

NK cell markers	Control Positive cells, % (MFI)	TKD	IL-2	IL-2/TKD	IL-15/TKD
CD3 ⁻ /56 ⁺	12±1 (698)	7±2 (226)	8±3 (370)	9±3 (1041 ^a)	9±1 (1399 ^a)
CD94 ⁺	7±2 (177)	4±2 (109)	7±1 (138)	11±2 (707 ^a)	12±3 (782 ^a)
CD94 ⁺ /NKG2A ⁺	5±2 (129)	5±2 (171)	7±2 (215)	8±2 (318 ^a)	11±2 (309 ^a)
CD94 ⁺ /NKG2C ⁺	4±4 (125)	1±0 (161)	2±1 (130)	2±1 (208 ^a)	3±1 (191 ^a)
NKG2D ⁺	9±3 (72)	7±1 (52)	8±2 (86)	14±4 (460 ^a)	16±6 (533 ^a)
CD56 ⁺	7±2 (74)	9±5 (59)	8±3 (107)	11±2 (435 ^a)	12±3 (479 ^a)
CD56 ⁺ /NKp30 ⁺	3±1 (37)	2±1 (33)	3±2 (47)	7±3 (199 ^a)	9±4 (242 ^a)
CD56 ⁺ /NKp44 ⁺	0±0 (1)	1±0 (80)	2±1 (132)	6±3 (399 ^a)	7±3 (548 ^a)
CD56 ⁺ /NKp46 ⁺	9±2 (233)	7±3 (120)	8±2 (130)	11±2 (393 ^a)	10±2 (389 ^a)
CD56 ⁺ /CD69 ⁺	1±0	1±1 (52)	9±2 (128 ^a)	8±4 (242 ^a)	10±4 (367 ^a)
T cell markers CD3 ⁺ /56 ⁻	68±4	74±4	74±8	75±3	74±7
NK-like T cells CD3 ⁺ /56 ⁺	3±2	3±3	3±0	3±1	5±1

MFI Mean fluorescence intensity

^a Indicates MFI values that are significantly different from the control (*P*<0.01).

Fig. 2 Granzyme B release by IL-2/TKD- (100 IU/mL/2 μ g/mL), IL-2- (100 IU/mL), and TKD- (2 μ g/mL) stimulated PBLs in response to CX⁺ (Hsp70⁺/HLA-E⁻) and CX⁻ (Hsp70⁻/HLA-E⁻) colon carcinoma sublines and erythroleukemic K562 (Hsp70⁺/HLA-E⁻) cells. Specific lysis (as reflected by granzyme B release) was determined using the ELISPOT assay at E/T ratios ranging from 10:1 to 5:1. Data are means $\pm$ SD from four independent experiments. Asterisk, Compared to IL-2 only, granzyme B release by PBLs in response to CX⁺ and K562 cells was significantly greater following stimulation with IL-2/TKD ($P < 0.05$)

TKD (100 IU/mL/2 μ g/mL) PBLs, CD3⁻ NK cells (purity >90%), and CD3⁺ T cells (purity >97%) were used as effector cells against Hsp70⁻/HLA-E⁻ target PBLs in an allogeneic setting (data not shown). The phenotypic characteristics of the target cells are summarized in reply to Table 2.

Effect of the stimulation of PBL, NK, and T cells with either IL-2/TKD, IL-15/TKD, or IL-2 on the susceptibility of leukemic blasts to lysis

As indicated above, K562 cells are Hsp70 membrane positive but HLA-E and MHC class I negative and thus act as a classical target cell line for NK cell-mediated immune responses (Table 2). However, the release of granzyme B by PBLs stimulated with IL-2 alone in response to K562 cells was significantly lower ($P < 0.05$) than that by cells that had

been stimulated with IL-2/TKD or IL-15/TKD (Fig. 3a). Unstimulated, resting PBLs secreted no granzyme B in response to K562 cells at an E/T ratio of 10:1.

We next investigated whether allogeneic leukemic blasts derived from two separate patients (no. 19 and no. 6) with AML could also serve as targets for IL-2/TKD- and IL-15/TKD-activated PBLs. The two blast populations were selected as target cells because of differences in the expression pattern of NK cell receptor ligands such as ULBP1–3 and MICA/B. In addition to an Hsp70 membrane-positive phenotype, they both expressed HLA-E on their cell surface (Hsp70⁺/HLA-E⁺) and thus differed profoundly from the Hsp70 membrane-positive tumor cell lines K562 and CX⁺ (Table 2). Compared to the induction of granzyme B secretion by PBLs in response to Hsp70⁺/HLA-E⁻ K562 cells, the granzyme B secretion by IL-2- and IL-2/TKD-stimulated PBLs induced by leukemic blasts of

Table 2 Proportion of K562 erythroleukemic cells, CX⁺ and CX⁻ colon carcinoma cells, and leukemic blasts of AML patients that are Hsp70, MHC class I, HLA-E, ULBP1, ULBP2, ULBP3, and MICA/B membrane-positive and membrane-negative

Identity		CD45/Hsp70	MHC class I	CD45/HLA-E	ULBP1	ULBP2	ULBP3	MICA/B
		Proportion of positive cells, %						
K562	Erythroleukemic	45	0	0	1	2	1	94
CX ⁺	Colon carcinoma	95	99	0.3	14	63	99	59
CX ⁻	Colon carcinoma	19	97	0.3	13	68	99	81
Blast no. 6	AML (85% blasts)	50	96	96	12	17	20	18
Blast no. 19	AML (88% blasts)	76	99	90	27	24	50	0

Data present mean values of at least three independent experiments.

AML Acute myelogenous leukemia, MHC major histocompatibility complex, PBL peripheral blood lymphocytes.

Fig. 3 Granzyme B release (as an indicator of cytotoxic activity) by PBLs from healthy donors (**a**), CD3⁺ NK cells (purity >95%, **b**), and CD3⁺ T cells (purity >98%, **c**) in response to K562 cells and leukemic blasts from two patients (patient no. 19, *center panel*; patient no. 6, *right panel*) as target cells. Blasts from patient no. 19 comprised 76% Hsp70 membrane-positive cells and those from patient no. 6 comprised 50% Hsp70 membrane-positive cells (*right panel*). Effector cells were either unstimulated or stimulated either with IL-2/TKD (100 IU/mL/2 μ g/mL), IL-15/TKD-treated (10 IU/mL/2 μ g/mL) or IL-2 (100 IU/mL). Effector to target cell ratios of PBL and T cells ranged between 2:1 and 10:1; that of NK cells between 5:1 and 1:1. The phenotypic characteristics of the target cells are provided in Table 2. *Asterisk*, Compared to IL-2/TKD and IL-15/TKD, granzyme B release by PBLs and NK cells in response to K562 cells and leukemic blasts (no. 19) was significantly lower following stimulation with IL-2 only ($P<0.05$)

patients no. 19, containing 76% Hsp70 membrane-positive cells, and no. 6, containing 50% Hsp70 membrane-positive cells, was weaker than that induced by Hsp70⁺/HLA-E⁻ K562 cells (Fig. 3a). For reasons that are currently unclear, no such difference was observed when effector cells were stimulated with IL-15/TKD (Fig. 3a). Although positive for MICA/B, lysis of the blasts of patient no. 6 was lower compared than that of no. 19, which was MICA/B negative. The effector PBLs were significantly less efficient at lysing the leukemic blasts ($P < 0.05$) when they were stimulated with IL-2 in the absence of TKD. Sorting of the effector cell populations clearly demonstrated that the killing of K562 cells and the leukemic blasts was mediated by CD3⁻ and CD56⁺/CD94^{high} NK cells (purity >95%; Fig. 3b) and not by CD3⁺/56⁻ T cells (purity >98%; Fig. 3c). Again, lysis of K562 cells by purified cytokine/TKD-activated NK cells was significantly higher than that of leukemic blasts. It is important to note that the highest E/T ratio of NK cells was 5:1 compared to a ratio of 10:1, which was used for the cytotoxicity assays that involved PBLs and purified T cells. A significant lysis of K562 cells by resting NK cells was observed only at an E/T ratio above 10:1 and of leukemic blasts at an E/T ratio above 20:1 (data not shown). The lysis of K562 and leukemic blasts mediated by either IL-2- vs cytokine/TKD-activated NK cells was found to be significantly different ($P < 0.05$).

Influence of Hsp70 as an activatory ligand for IL-2/TKD-activated PBLs against K562 tumor cells

To prove that membrane-bound Hsp70 acts as an activatory ligand for IL-2/TKD-activated NK effector cells, membrane Hsp70 expression was masked using an Hsp70 mAb cmHsp70.2, and de novo Hsp70 synthesis was inhibited using (si)RNA technology. As indicated in Fig. 4, lysis of K562 cells by IL-2/TKD-activated NK cells was inhibited by the cmHsp70.2 Hsp70 mAb but not by other mAbs that are specific for ULBP or MICA/B (data not shown). The proportion of Hsp70 membrane-positive K562 cells was reduced from 45 to 5% by siRNA (as determined by flow cytometry), and these cells were much less susceptible to lysis by IL-2/TKD activated NK cells (Fig. 4). Killing activity was not completely abrogated by anti-Hsp70 (si) RNA because of the fact that K562 cells are lysed by two mechanisms, on the one hand via membrane-bound Hsp70 as a ligand and on the other hand by the missing MHC class I expression.

HLA-E as an inhibitory ligand for IL-2/TKD activated PBLs against untransfected and HLA-E transfected K562 cells

The expression of HLA-E on the membrane of K562 cells influenced their sensitivity to cytotoxicity mediated by IL-

Fig. 4 Effect of the anti-Hsp70 mAb (cmHsp70.2, 10 μ g/mL) and the inhibition of Hsp70 expression using siRNA (mut3) on the cytolysis of K562 target cells by IL-2/TKD-treated (100 IU/mL/2 μ g/mL) PBL effector cells. Specific lysis was determined using the 4-h 51 Cr-release assay at E/T ratios ranging from 5:1 to 0.5:1. Data show one representative experiment out of three independent assays

2/TKD-activated NK cells, in that the killing of HLA-E membrane-negative cells was superior to that of the transfected populations of K562 cells that were membrane positive for HLA-E (Fig. 5). The two common HLA-E alleles HLA-E^G and HLA-E^R, which are associated with β 2-microglobulin and peptides of the signal sequences of HLA-A, HLA-B, and HLA-C alleles such as HLA-E^RA4 (11.2%), HLA-E^RA2 (20.7%), HLA-E^GC6 (32.5%), and HLA-E^GB5 (73.2%) were chosen because of the differences

Fig. 5 Cytolytic activity of CD3⁻ NK effector cells against K562 cells (Hsp70⁺/HLA-E⁻) into which HLA-E^GB5 (73.2%), HLA-E^GC6 (32.5%), HLA-E^RA2 (20.7%), and HLA-E^RA4 (11.2%) alleles had been transfected. The Hsp70 positivity was comparably high in all transfectants (47 ± 19 , 38 ± 11 , 47 ± 18 , and 36 ± 17 vs $40 \pm 9\%$ in untransfected K562 cells, respectively). Specific lysis was determined using the 4-h 51 Cr-release assay at E/T ratios ranging from 20:1 to 0.5:1. Data show one representative experiment out of three

in the intensity of HLA-E expression after transfection of K562 cells; the proportion of cells that were membrane Hsp70 positive was comparably high in all transfectants (47 ± 19 , 38 ± 11 , 47 ± 18 , and 36 ± 17 vs $40 \pm 9\%$ in untransfected K562 cells, respectively). As shown in Fig. 5, the susceptibility of HLA-E membrane-positive cell populations to NK cell-mediated lysis was related to the proportion of cells in that population that expressed membrane HLA-E. The progressive reduction in the lysis of K562 cell populations containing 73.2 (HLA-E^GB5) and 32.5% (HLA-E^GC6) HLA-E⁺ cells was more than 50% at the E/T ratios of 20:1, 10:1, and 5:1. It therefore appears that membrane-bound Hsp70 can only partially compensate for the negative regulatory signals that are mediated HLA-E, which is recognized by the receptor complex CD94/NKG2A. As shown in Table 1, the expression density of both the inhibitory (CD94/NKG2A) and the activatory (CD94/NKG2C) receptor complexes are enhanced as a response to a cytokine/TKD treatment, and thus we hypothesize that Hsp70 might compete with HLA-E⁺ cells for binding to the heterodimeric CD94 receptor complexes.

Discussion

NK cells, which comprise 5–20% of PBLs, provide the first line of defense against bacteria, parasites, and viruses. These cells also play a key role in the protection against cancer (Multhoff et al. 1995a; Rosenberg et al. 1998; Whiteside et al. 1998; Farag et al. 2002). For some time, the low-affinity Fcγ receptor CD16, which mediates antibody-dependent cellular cytotoxicity (Lanier et al. 1988), and the homophilic adhesion molecule CD56 were the only known human NK cell receptors. However, it is now known that the cytotoxicity of NK cells is mediated and regulated by a number of killer cell inhibitory and activating receptors, the best characterized of which recognize classical or nonclassical MHC molecules such as nonclassical HLA-E, nonclassical stress-inducible MICA and MICB glycoproteins, and the glycosylphosphatidylinositol-linked ULBPs (Moretta et al. 2001; Kirwan and Burshtyn 2007). The recognition of these molecules involves C-type lectin receptor CD94/NKG2A and CD94/NKG2C heterodimers on NK cells binding HLA-E and NKG2D homodimers binding MICA/B and ULBPs (Farag and Caligiuri 2006; Radons et al. 2006).

We were the first to report that membrane-bound Hsp70 acts as a tumor-specific recognition structure for and activator of NK cells expressing high amounts of CD94 (Botzler et al. 1996, 1998; Gross et al. 2003a, c). We subsequently demonstrated that a Hsp70-derived 14-mer peptide (TKD) in combination with IL-2 enhances the cytolytic activity of resting NK cells against tumor cells

presenting Hsp70 on their cell membrane (Multhoff et al. 1999, 2001) and that it increases the density of CD56 and CD94 expression on these NK cells (Gross et al. 2003a, c). We have also reported that CD94 antibody molecule blocks Hsp70 binding to NK cells and also the lysis of Hsp70 membrane-positive cells (Gross et al. 2003a, c). From these findings, we have concluded that CD94, in combination with NKG2C, is involved in the cytolytic activity of IL-2/TKD-activated NK cells.

Similar to tumor cell lines, leukemic blasts frequently present Hsp70 on their cell surface; however, in contrast, leukemic blasts also express HLA-E, which is an inhibitory ligand for CD94⁺/NKG2A⁺ NK cells. This study used the NK-sensitive tumor cell line K562 (wild type, Hsp70⁺/HLA-E⁻) and HLA-E transfected (Hsp70⁺/HLA-E⁺) and allogeneic leukemic blasts (Hsp70⁺/HLA-E⁺) to further explore the influence of HLA-E and Hsp70 on NK cell-mediated killing. As controls, responses to the HLA-E membrane-negative colon carcinoma cell lines CX⁺ and CX⁻ having differential Hsp70 membrane expression pattern were also evaluated. In vitro, cytokine/TKD-activated NK cells acquired the capacity to kill wild-type HLA-E⁻/Hsp70⁺ K562 cells. Hsp70⁺/HLA-E⁺ double-positive allogeneic leukemic blasts are also lysed by these preactivated NK cells, albeit to a slightly lower degree. The pattern of ULBP and MICA/B expression appears to be of minor importance for this Hsp70-mediated reactivity, as the target cells show a very diverse expression pattern with respect to these markers. The observation that cytokine stimulation alone is a significantly weaker inducer of cytotoxic responses against Hsp70 membrane-positive targets indicates that signals delivered via the Hsp70 peptide TKD are a prerequisite for Hsp70-mediated cytotoxic responses.

In addition to inducing the expression of the activatory receptor CD94/NKG2C, cytokine/TKD treatment also upregulated the expression of the inhibitory receptor complex CD94/NKG2A (Houchins et al. 1997). This finding might explain why the lysis of Hsp70 membrane-positive but HLA-E membrane-negative wild-type K562 cells was superior to that of membrane Hsp70⁺/HLA-E⁺ leukemic blasts and also to that of HLA-E-transfected K562 cells.

In summary, we present further evidence to confirm that NK cells activated with cytokines and the Hsp70-derived TKD peptide can recognize and kill Hsp70 membrane-positive tumor cells. We also show that HLA-E exhibits inhibitory effects toward the CD94/NKG2A NK cell receptor, which is coexpressed together with activatory receptors. Low-dose IL-15, which is a differentiation cytokine for NK cells (Fehniger and Caligiuri 2001; Farag and Caligiuri 2006), in combination with TKD, is similarly effective in stimulating Hsp70 reactivity in NK cells as IL-2.

Overall, these data further support the proposition that the transfusion of in vitro cytokine/TKD-stimulated NK cells might provide an immunotherapeutic option for treating patients with Hsp70 membrane-positive, HLA E membrane-negative tumors.

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