Cell surface-bound heat shock protein 70 (Hsp70) renders tumor cells more sensitive to the cytolitic attack mediated by natural killer (NK) cells. A 14-amino acid Hsp70 sequence, termed TKD (TKDNNLLGRFELSG, aa450–463), could be identified as the extracellular localized recognition site for NK cells. Here, we show by affinity chromatography that both, full-length Hsp70-protein and Hsp70-peptide TKD, specifically bind a 32-kDa protein derived from NK cell lysates. The serine protease granzyme B was uncovered as the 32-kDa Hsp70-interacting protein using matrix-assisted laser desorption ionization time-of-flight mass peptide fingerprinting. Incubation of tumor cells with increasing concentrations of perforin-free, isolated granzyme B shows specific binding and uptake in a dose-dependent manner and results in initiation of apoptosis selectively in tumor cells presenting Hsp70 on the cell surface. Remarkably, Hsp70 cation channel activity was also determined selectively in purified phospholipid membranes of Hsp70 membrane-positive but not in membrane-negative tumor cells. The physiological role of our findings was demonstrated in primary NK cells showing elevated cytoplasmic granzyme B levels following contact with TKD. Furthermore, an increased lytic activity of Hsp70 membrane-positive tumor cells could be associated with granzyme B release by NK cells. Taken together we propose a novel perforin-independent, granzyme B-mediated apoptosis pathway for Hsp70 membrane-positive tumor cells.

Elevated cytoplasmic Hsp701 levels have been found to protect tumor cells against programmed cell death (1). However, evidence has accumulated indicating that the presence of Hsp70 on the plasma membrane or in the extracellular milieu is highly immunogenic and exposes target cells to immunological attack (2). Following receptor-mediated uptake (3) and re-presentation by antigen presenting cells, HSP-chaperoned peptides elicit a cytotoxic, CD8+ T cell response (4). Recently, several receptors, including CD91, Toll-like receptors 2/4 (TLR2/4), and CD40 (5) have been identified to mediate the interaction of HSP90- (gp96), HSP70- (Hsp70, Hsc70), and HSP60-peptide complexes with antigen presenting cells (6–9).

The amount of membrane-bound Hsp70 on tumor cells positively correlates with their sensitivity to lysis mediated by natural killer (NK) cells. Physical (i.e., heat, irradiation) and chemical (i.e., cytostatic drugs, alkyllysophospholipids) stress has been found to increase Hsp70 surface expression on tumor cells and thereby renders them better targets for NK cells (16–18). Incubation of purified NK cells with recombinant Hsp70 increases the cytolitic activity against Hsp70 membrane-positive tumor cells (19). NK cells have been found to interact specifically with a 14-amino acid Hsp70 sequence, termed TKD (TKDNNLLGRFELSG, aa450–463), on the C-terminal of this protein. This region (TKD) is present in the ectoplasmic domain of viable tumor cells (20). Therefore, it was not surprising that similar to full-length Hsp70 (19), Hsp70-peptide TKD exhibits a comparable immunostimulatory capability to NK cells (20). Although the preceding observations indicate that Hsp70-peptide functions as a tumor-selective target recognition structure for NK cells (21), the mechanism by which NK cells lyse Hsp70 membrane-positive tumor target cells remained to be elucidated.

EXPERIMENTAL PROCEDURES

Cells

The NK cell line YT was cultured at low cell densities ranging between 0.1 and 0.5 × 106 cells/ml in RPMI 1640 medium (Invitrogen) containing 10% fetal calf serum (Invitrogen) supplemented with 6 mM l-glutamine and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin; Invitrogen). The tumor sublines CX+ /CX– and Colo+ /Colo– were derived by cell
sorted of CX-2 colon (Hsp70 positive: 60%) and Colo357 pancreas (Hsp70 positive: 60%) carcinoma cell lines using the Hsp70-specific monoclonal antibody C92F3B1 (Multimmune GmbH, Regensburg, Germany), according to a previously described protocol (21). Hsp70-stably high expressing CX- (+ Hsp70 positive: 80%) and Colo+ (Hsp70 positive: 85%) carcinoma sublines differ significantly from Hsp70-stably low expressing CX- (Hsp70 positive: 25%) and Colo- (Hsp70 positive: 35%) carcinoma sublines. Carcinoma sublines and the non-NK leukemia cell line K562 were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 6 mM l-glutamine and antibiotics. Exponentially growing tumor cells (day 1 after feeding of the cells) were used for granzyme B and camptothecin treatment.

All cell lines were screened regularly for mycoplasma contaminations by an enzymoassay detecting Mycoplasma arginini, Mycoplasma hyorhinis, Acholeplasma laidlawii, and Mycoplasma orale (Roche Diagnostics). Only mycoplasma-free cell lines were used.

Primary NK cells were generated by CD3-depletion of peripheral blood mononuclear cells using a standard Miltenyi bacterin separation method (Miltenyi, Bergisch Gladbach, Germany). Sorted cells were activated by incubation of the NK cells for 3 days in supplemented RPMI 1640 medium containing 100 IU/ml interleukin-2 (Chiron, Ratingen, Germany) and 2 μg/ml Hsp70-peptide TKD (TDKNLL-GRFELSG, aa450–463 Bachem Bubendorf, Switzerland).

Affinity Chromatography and Immunoprecipitation

Bovine serum albumin (BSA, 1 mg/ml, Sigma), lyophilized, recombinant human Hsp70-protein (1 mg/ml, StressGen, British Columbia, Canada), and Hsp70-peptide TKD (1 mg/ml, Bachem, Bubendorf, Switzerland) were incubated with equilibrated AminoLink-agaroose beads (2 ml, Pierce) for 6 h, together with the reductive NaCNBH4-followed by removal of uncoupled material by extensive washes with Tris buffer and quenching non-reactive groups. Binding capacity of BSA, Hsp70-protein, and Hsp70-peptide TKD was greater than 95%. Cell lysates of NK cells and the leukemic non-NK cell line K562 were loaded onto BSA, Hsp70-protein, and Hsp70-peptide TKD-conjugated columns for 1 h at room temperature.

Columns were washed with 10 column volumes of 20 mM Tris buffer, bound proteins were eluted with 3 M sodium chloride in 20 mM Tris buffer containing 5 mM CaCl2 and incubated with annexin V-FITC followed by removal of uncoupled material by extensive washes with Tris buffer and quenching non-reactive groups. Binding capacity of BSA, Hsp70-protein, and Hsp70-peptide TKD was greater than 95%. Cell lysates of NK cells and the leukemic non-NK cell line K562 were loaded onto BSA, Hsp70-protein, and Hsp70-peptide TKD-conjugated columns for 1 h at room temperature.

Columns were washed with 10 column volumes of 20 mM Tris buffer, bound proteins were eluted with 3 M sodium chloride in 20 mM Tris buffer, in 5 fractions (1 ml). Each fraction was subjected to a SDS-PAGE using a 10% polyacrylamide slab gel and transferred onto polyvinylidene difluoride membranes.

**Western Blot Analysis**

Blots were blocked with skim milk (0.1%) and incubation with monoclonal antibody directed against granzyme B (2C5, IgG2a, BD Biosciences, Germany), for 5 h at 4 °C. Blots were washed and incubated with a secondary mouse anti-IgG horseradish peroxidase antibody (Dianova, Hamburg, Germany), for 1 h at 4 °C. Proteins were detected using the ECL kit (Amersham Biosciences) for 5 s.

**Protein Identification by Peptide Mass Fingerprinting**

A 32-kDa protein band was isolated by affinity chromatography on immobilized Hsp70-protein or Hsp70-peptide TKD, which was excised from Coomassie Blue-stained gels, digested with trypsin, and desalted using reverse phase ZIF tips (Millipore, Eschborn, Germany). The samples were embedded in 4-hydroxy-cyanocinnamic acid and the peptide masses were determined with a Perseptive Voyager DePro matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer in reflective mode. A peaklist was compiled with the m/z software (ProteomeTools) and used for peak selection; the resulting peptide mass fingerprint was used to search the non-redundant NCBI protein data base using the Profound search engine (Proteometrics). Granzyme B was identified with 100% probability and >95% confidence.

**Membrane Preparation**

Membrane purification was performed by Deuche homogenization (100 x 10^6 tumor cells) in hypotonic, EDTA-free buffer containing the protease inhibitor phenylmethylsulfonyl fluoride followed by sequential centrifugation at 1,000 x g for 5 min and at 100,000 x g, at 4 °C, for 60 min. The pellet containing membranes was resuspended in 2 ml of 0.3 M NaCl in 50 mM Tris buffer, 0.5% Nonidet P-40, pH 7.6.

**Cation Channel Activity Measurements**

Large unilamellar liposomes were prepared from plasma membranes derived of Hsp70 membrane-positive and membrane-negative tumors and applied to an orifice of about 100–120 μm in diameter with a Teflon film separating two compartments. The ionic solutions containing ei-

**Flow Cytometry**

Cells (0.5 x 10^6) were fixed in paraformaldehyde (1% paraformaldehyde in PBS) for 10 min, and permeabilized in PBS containing BSA (0.5%, NaN3, (0.1%, and saponin (0.1%). Permeabilized cells were incubated either with the granzyme B-pyrocoerythrin (PE)-conjugated monoclonal antibody HC2-PE (IgG1; Holzel Diagnostika, Cologne, Germany) or with an isotype-matched IgG1 control antibody, at 4 °C for 1 h, in the dark. Following washing intracellular localized granzyme B was analyzed on a FACScalibur instrument (BD Biosciences).

**Treatment**

Stock solutions of camptothecin (4 mg/ml, Sigma, Munich, Germany) were diluted in dimethyl sulfoxide and stored at 4 °C in the dark. Granzyme B (Holzel Diagnostics, Cologne, Germany) solutions were freshly prepared directly before usage. Exponentially growing cells (0.5–1.5 x 10^6) were incubated either with camptothecin at a final concentration of 4 μg/ml or with purified, enzymatically active granzyme B (10 ng/ml, 1 μg/ml, 2 μg/ml, 4 μg/ml) for 10 min, and 30 min either at 4 or 37 °C. After washing in RPMI 1640 medium binding and uptake was determined in non-permeabilized and permeabilized tumor cells by flow cytometry and fluorescence microscopy on a Axiocap 25 scanning microscope (Zeiss, Jena, Germany) equipped with a x40 objective and standard filters. Images were treated by multiplicative shading correction using the software Axiovision (Zeiss Vison, Jena, Germany). Granzyme B was visualized in red by using the HC2-PE antibody.

Apoptotic cell death was measured after incubation of tumor cells with 10 ng/ml granzyme B for 4, 12, and 24 h by different apoptosis assays, as described below.

**Apoptosis Assays**

**Annexin V-FITC Staining**—Briefly, cells were washed twice in Hepes buffer containing 5 mM CaCl2 and incubated with annexin V-FITC (Roche Diagnostics) for 10 min at room temperature. Annexin V-FITC positively stained cells were measured on a FACScalibur flow cytometer (BD Biosciences).

**DAPI Staining**—Methanol/acetone fixed cells (0.1 x 10^6 cells/100 μl) were incubated with 0.5 μg/ml 4,6-diamino-2-phenylindole (DAPI) in PBS/glycerol (3:1) for 15 min in the dark. Following washing in PBS the cells were mounted with fluorescent mounting medium (Dako, Glostrup, Denmark) and analyzed for fluorescence using a Zeiss model AxioScope 2 scanning microscope (Zeiss, Jena, Germany) equipped with a x40 objective and standard filters. Apoptosis was visualized with DAPI staining in 50 cells, each. Images were treated by multiplicative shading correction using the software Axiovision (Zeiss).

**Cytochrome c**—Cytochrome c was determined using a quantitative immunosassay (DCDCO, R&D Systems, Wiesbaden, Germany). Briefly, untreated, camptothecin (4 μg/ml), or granzyme B (10 ng/ml)-treated cells (1.5 x 10^6) were washed in PBS and treated with lysis buffer for 1 h at room temperature. Following centrifugation at 1,000 x g for 15 min, supernatants were removed and 200 μl of a 1:100, 1:250, and 1:500 dilution was used for a sandwich enzyme-linked immunosorbent assay. Following incubation with substrate solution in the dark for 30 min the reaction was stopped. The optical density of each well was determined on an enzyme-linked immunosorbent assay reader at 450 nm. The amount of cytochrome c was determined according to a calibration curve.

**51Cr Release Assay and Inhibition Assay**

NK cell-mediated cytotoxicity was measured using a 12-h 51Cr radiolabeled cytotoxicity assay. As target cells the colon carcinoma sublines CX- and CX+ were used. For blocking studies the monoclonal antibody C92F3B1 and an isotype matched control antibody (IgG1) were used at a final concentration of 5 μg/ml x 10^6 cells. Following incubation of CX- and CX+ target cells with the antibodies for 30 min at 4 °C, the cells were labeled with 51Cr and the cytotoxicity assay was performed as described.
Bound proteins were eluted from the columns in 5 fractions (with cell lysates of the NK cell line YT or the non-NK cell line K562. (Hsp70), BSA, and Hsp70-peptide (TKD) columns were incubated either for Hsp70-protein and Hsp70-peptide TKD. A protein band in fractions two (derived from Hsp70 and TKD columns revealed a dominant 32-kDa resolved on SDS-PAGE. Following silver stain, eluates of YT cells band was detectable in YT eluates derived from BSA columns and in

**FIG. 1.** Identification of granzyme B by MALDI-TOF analysis. A, Coomassie Blue-stained 32-kDa band of fraction 3 (F3), derived from the TKD column, correspond to human granzyme B. The probability of identification was 100% and the estimated Z-score was 1.89 corresponding to >95% confidence. C, Corresponding Western blot analysis of YT and K562 cell eluates (F3) following incubation with Hsp70-protein (Hsp70) and Hsp70-peptide (TKD) columns. The blot was autoradiographed and the localization of granzyme B was visualized by immunostaining with the granzyme B specific monoclonal antibody 2C5. The identity of the 32-kDa protein

K562 eluates derived from TKD columns. The position of the 32-kDa band is indicated with an arrow. A, The tryptic peptides of the Coomassie Blue-stained 32-kDa band of fraction 3 (F3), derived from the TKD column, correspond to human granzyme B. The probability of identification was 100% and the estimated Z-score was 1.89 corresponding to >95% confidence. C, Corresponding Western blot analysis of YT and K562 cell eluates (F3) following incubation with Hsp70-protein (Hsp70) and Hsp70-peptide (TKD) columns. The blot was autoradiographed and the localization of granzyme B was visualized by immunostaining with the granzyme B specific monoclonal antibody 2C5. Eluates of YT cells (left), but not K562 cells (right) revealed a 32-kDa granzyme B protein band. D, Intracellular flow cytometry of permeabilized YT cells (left) and K562 cells (right) using the PE-conjugated granzyme B-specific monoclonal antibody H2C2-PE (solid line), as compared with an isotype-matched negative control antibody (dashed line). Only YT cells, but not K562 cells, contain cytoplasmic granzyme B.
band as granzyme B was further confirmed by Western blot analysis using a specific antibody (2C5) against granzyme B. Fractionation of YT cell lysates on Hsp70-protein (Hsp70) and Hsp70-peptide (TKD) columns revealed the presence of granzyme B protein band by Western blotting (Fig. 1C). However, no granzyme B was detected in the eluted fraction of Hsp70 and TKD affinity columns loaded with K562 cell lysates (Fig. 1C). Also flow cytometry using a phycoerythrin-conjugated granzyme B antibody HC2-PE showed positive staining for cytoplasmic granzyme B in YT cells, but not in K562 cells (Fig. 1D). In summary, these data indicate that granzyme B interacts with full-length Hsp70-protein as well as Hsp70-peptide TKD.

Specific Binding and Internalization of Granzyme B in Hsp70 Membrane-positive Tumor Cells—The preceding findings posed the question whether membrane-bound Hsp70 might enable specific binding and entry of granzyme B into the cytosol. Therefore, perforin-free, purified granzyme B was co-incubated with tumor cell sublines CX+/CX− and Colo+/Colo− that exhibit differential Hsp70 membrane expression. A light microscopic analysis of untreated CX+ and Colo+ cells (control) at 4 versus 37 °C is shown in the upper row of each panel (Fig. 2A). The corresponding immunofluorescence microscopy of the cells at 4 and 37 °C is illustrated below (control). Initially, none of the cells showed any granzyme B staining, neither on the cell surface nor in the cytoplasm. However, after a 15-min incubation period of the cells with purified granzyme B (grB) at 4 °C, a ring-shaped fluorescence, indicating a typical cytoplasmic staining pattern in CX+ and Colo+ tumor sublines (Fig. 2A, left panel). A temperature shift from 4 to 37 °C during the 30-min incubation period resulted in uptake of granzyme B, as determined by a cytoplasmic staining pattern in CX+ and Colo+ tumor sublines (Fig. 2A, right panel). In contrast, the Hsp70 membrane-negative counterparts CX− and Colo− neither exhibited any granzyme B cell surface binding at 4 °C nor uptake at 37 °C (data not shown). Flow cytometry analysis of permeabilized cells revealed a faint shift of the granzyme B peak to the right selectively in Hsp70 membrane-positive CX+ and Colo−, but not in CX− and Colo− tumor sublines, if the cells were co-incubated with 1 μg/ml granzyme B for 30 min at 37 °C (Fig. 2B, upper graph). A dose-dependent increase in granzyme B uptake, in Hsp70 membrane-positive tumor cells (CX+/Colo+), was detected after co-incubation with 2 and 4 μg/ml granzyme B (Fig. 2B, lower graph). However, even at the highest concentration of 4 μg/ml, granzyme B was internalized much more pronounced by Hsp70-negative tumor cells (CX−/Colo−).

Potential ion channels formed by Hsp70 may play a role in the mechanism of selective granzyme B uptake in Hsp70 membrane-positive tumor cells. Indeed, a particular ion conductance pathway was observed after incubation of vesicles derived from purified phospholipids of Hsp70 membrane-positive (CX+) tumor sublines. This was not seen in vesicles obtained from Hsp70 membrane-negative (CX−) tumor cells (data not shown). Based on these results one might speculate about an ion channel activity facilitating uptake of granzyme B selectively into Hsp70 membrane-positive tumor cells.

In Vitro Provided Granzyme B Induces Apoptosis Selectively in Hsp70 Membrane-positive Tumor Cells—Differences in the inducibility of apoptosis were studied by co-incubation of Hsp70-high (CX+/Colo+) and low (CX−/Colo−) expressing carcinoma cells (21) with 10 ng/ml enzymatically active granzyme B (22) for 4, 12, and 24 h. The topoisomerase inhibitor camptothecin at a final concentration of 4 μg/ml served as a positive control for apoptosis. Programmed cell death was determined by using three different apoptosis assays including annexin V-FITC, DAPI staining, and mitochondrial cytochrome c release. After a 4-h incubation period, neither camptothecin nor granzyme B initiated apoptosis in any of the tested tumor cells (data not shown), indicating that our tumor carcinoma cell lines are more resistant to apoptotic cell death, as compared with the acute T cell leukemia cell line Jurkat. After a 12- and 24-h incubation period with camptothecin significant apoptosis was observed in all tumor sublines (Fig. 3A). It appeared that the colon carcinoma sublines CX+/CX− are better protected toward a camptothecin-mediated cell death as compared with the pancreas carcinoma sublines Colo+/Colo−. However, no significant differences in the inducibility of apoptosis by using camptothecin was observed between Hsp70 membrane-positive and Hsp70 membrane-negative tumor cell lines. Interestingly, this was not the case if the tumor sublines were incubated with granzyme B at a concentration that is found in the serum under physiological conditions (10 ng/ml): 12 h post-treatment the amount of annexin V-FITC positive cells was equally up-regulated in Hsp70 membrane-positive CX+/Colo+ tumor cells (1.3-fold); after 24 h the increase in apoptotic cells was significant in Hsp70 membrane-positive CX+ (1.8-fold) and in Colo+ (2.4-fold) tumor cells (Fig. 3A). In line with these results the amount of Hsp70 membrane-positive leukemic K562 cells (25) was similarly up-regulated (1.8-fold) following contact with granzyme B (data not shown). In contrast, the amount of apoptotic CX− and Colo− tumor cells with stably low Hsp70 membrane expression levels remained unaltered before and after a 12- and 24-h co-incubation period with granzyme B (Fig. 3A).

To exclude apoptosis initiated by anoikis light microscopic analysis of untreated (control), camptothecin- (cam), and granzyme B (grB)-treated CX+/CX− and Colo+/Colo− tumor cells were performed. As shown in Fig. 3B, 24 h post-treatment with granzyme B, neither Hsp70 membrane-positive nor negative tumor cell lines exhibited any signs of loss in plastic adherence. Regarding these findings we ruled out the possibility that anoikis might be a possible mechanism for the induction of apoptotic cell death in Hsp70 membrane-positive tumor sublines. It is important to note that all apoptosis assays were determined within the adherent cell population following a short term (<1 min) trypsinization.

Consistent with the results derived by annexin V-FITC staining all cell types, CX+/CX−, Colo+/Colo−, exhibited a positive DAPI nuclear fragmentation staining, as a typical sign of apoptosis at a later stage, following treatment with camptothecin, as compared with untreated control cells (Fig. 3C). Again, DNA fragmentation was detected only in Hsp70 membrane-positive CX+ and Colo+ tumor cells, 24 h post-treatment with granzyme B (grB). In line with the annexin V-FITC staining results, no signs of DNA fragmentation were observed in Hsp70 membrane-negative CX− and Colo− cells (Fig. 3C). As an additional test for apoptotic cell death, cytochrome c release was measured following incubation of CX+ and CX− cells with granzyme B and camptothecin. As summarized in Table I, following incubation with granzyme B for 24 h, cytochrome c concentration was elevated 1.8-fold (0.382 mg/ml versus 0.690 mg/ml) in CX+ cells. However, no increase in cytoplasmic cytochrome c was observed in CX− cells following treatment with granzyme B (0.452 versus 0.425 mg/ml). An incubation with camptothecin (4 μg/ml) for 24 h resulted in a comparable, 1.5-fold increase in cytochrome c concentrations in CX+ and CX− tumor cells. In summary these results indicate that following binding and selective uptake, via membrane-bound Hsp70, granzyme B initiates apoptosis in a perforin-independent manner.
**FIG. 2.** Specific cell surface binding and uptake of granzyme B (grB) by Hsp70 membrane-positive tumor cells. A, comparative binding of granzyme B (2 μg/ml) to the cell surface of CX+/CX− and Colo+/Colo− tumor cells at 4 °C, and uptake into the cytosol after a temperature shift to 37 °C for 30 min, using the PE-conjugated granzyme B-specific monoclonal antibody HC2-PE. *First row,* light microscopy; *second row,* immunofluorescence of cells without granzyme B (control); *third row,* immunofluorescence of cells after addition of granzyme B, as specified (grB). One representative fluorescence microscopy of three showing identical results is illustrated; magnification ×40. B, intracellular flow cytometry of permeabilized CX+/CX− (n = 2) and Colo+/Colo− (n = 4) tumor cells using granzyme B-specific monoclonal antibody HC2-PE before (dashed line) and after (solid line) incubation of the tumor cells with 1, 2, and 4 μg/ml granzyme B at 37 °C for 30 min. Only CX+ and Colo+, but not CX− and Colo− cells showed a dose-dependent shift of the granzyme B peak to the right, indicating uptake of extracellular offered granzyme B. **ctrl**, control.
Isolated granzyme B (grB) induces apoptosis selectively in Hsp70 membrane-positive tumor cells. A, percentage of annexin V-FITC positive and propidium iodide-negative CX+/Colo+ (left) and CX−/Colo− (right) cells, either untreated (black bars), or following incubation either with camptothecin (4 μg/ml; light gray bars) or granzyme B (10 ng/ml; dark gray bars) for 12 and 24 h. The data represent the mean of three to four independent experiments ± S.D.; * marks granzyme B values significantly different from control (p < 0.05). B, light microscopical analysis.
Granzyme B Released by TKD-activated NK Cells Mediates Apoptosis in Hsp70 Membrane-positive Tumor Cells—The physiological role of our findings was tested in functional assays using naive and Hsp70-peptide (TKD)-stimulated human NK cells. Previously, we have shown that incubation of NK cells with Hsp70-protein at concentrations between 10 and 50 μg/ml or with equivalent TKD concentrations between 0.2 and 2.0 μg/ml resulted in increased cytolytic activity against Hsp70 membrane-positive tumor target cells. Concomitantly, the expression of CD94, the killer cell inhibitory/activatory C-type lectin receptor, was up-regulated (24). Although it was known that Hsp70 acts as a tumor-selective recognition structure for NK cells (21, 25), it remained unclear whether tumor cells die by necrosis or apoptosis. To elucidate this question, NK cells were incubated with Hsp70-peptide TKD (2 μg/ml) for 3 days. A significant increase (1.4-fold) in cytoplasmic granzyme B levels was observed within 3 days of stimulation in CD3-negative NK cells. In contrast, granzyme B expression was not up-regulated in CD3-positive T cells following identical treatment conditions.

Killing of CX+/CX− and Colo+/Colo− tumor cells following contact with freshly isolated, unstimulated (NK d0), or TKD-stimulated NK cells (NK d3) was compared in a standard 12-h 51Cr release assay (Fig. 4). Because of experimental limitations, the co-incubation period of NK and tumor cells in the cytotoxicity assay could not be extended to 24 h, like in our in vitro apoptosis assays.

Concomitant with the increase in cytoplasmic granzyme B, the cytolytic activity of TKD-stimulated NK cells (NK d3) against CX+ target cells was enhanced 1.5-fold and that of Colo+ target cells 2.0-fold at effector to target (E:T) ratio ranging between 20:1 and 2:1. In contrast, the cytolytic activity against Hsp70 membrane-negative CX− and Colo− cells was not elevated. The increased lysis of Hsp70 membrane-positive tumor cells was decreased in both cell systems down to the degree of lysis of Hsp70 membrane-negative tumor cells, by Hsp70-specific monoclonal antibody, that is known to recognize membrane-bound Hsp70-peptide TKD on viable tumor cells (25). In contrast, the lower lysis of Hsp70 membrane-negative tumor cells remained unaffected after incubation with Hsp70 antibody. Technically, it is not possible to quantify the absolute amount of granzyme B that is transferred from NK cells into tumor cells by cell-to-cell contact. However, relative values of granzyme B release could be determined by ELISPOT analysis. Therefore, a comparison of the cytolytic response of freshly isolated, unstimulated (NK d0) and TKD-stimulated NK cells (NK d3) against CX+/CX− and Colo+/Colo− tumor cells was performed concomitantly with the definition of the granzyme B release. Irrespective of the tumor cell line and the E:T cell ratio, co-incubation of tumor cells with unstimulated NK cells (NK d0) always results in very low granzyme B release; the number of spots was always less than 20. After a 3-day stimulation period with TKD (NK d3) followed by a 4-h co-incubation time with tumor cells, granzyme B release was significantly up-regulated. At an E:T ratio of 5:1, the number of granzyme B spots, as determined in three independent experiments, was as follows: CX+, 260 ± 20; CX−, 165 ± 6; Colo+, 137 ± 55; Colo−, 66 ± 8. Concomitantly, Hsp70 membrane-exhibited nuclear DNA fragmentation (lower panel, left). No signs of apoptosis were observed in CX− and Colo− tumor cells following incubation with granzyme B (lower panel, right). Scale bar represents 10 μm.
positive tumor target cells (CX+/Colo+) were lysed significantly better as compared with their negative counterparts (CX−/Colo−). These data strongly suggest that lysis of Hsp70 membrane-positive tumor cells by TKD-activated NK cells is associated with granzyme B release. Proteases, like granzyme B, initiate apoptosis by an intracellular mechanism (35). In Fig. 2, A and B, specific binding and a dose-dependent internalization of in vitro provided granzyme B was detected selectively in Hsp70 membrane-positive tumor cells. With respect to these findings, we assumed that differences in lysis of CX+/CX− and Colo+/Colo− tumor cells are because of a different capacity of the tumor cells to internalize granzyme B.

DISCUSSION

Previously we have demonstrated that tumor cells, but not normal cells, present Hsp70 on their plasma membrane (14). Antibody mapping revealed that part of the C-terminal substrate binding domain is exposed to the extracellular milieu (26). The epitope of this antibody corresponds to a 14-amino acid sequence, termed TKD (TKDNNLLGRFELS, aa450–463). Thus, exposure of TKD on the cell surface sensitizes tumor cells to the cytolytic activity of NK cells (20). Furthermore, Hsp70-peptide TKD exhibits activatory properties on NK cells, which is comparable with the activity observed by equivalent amounts of full-length Hsp70 (19). Although it is obvious that Hsp70-protein and Hsp70-peptide TKD efficiently trigger NK cell activity, the mechanism how NK cells kill Hsp70 membrane-positive tumor target cells remained unclear.

To identify molecules that are involved in the interaction of NK cells with Hsp70-positive tumor cells, Hsp70 conjugated to Sepharose was used as affinity bait to isolate Hsp70-binding proteins from lysates of the human NK cell line YT (27). In the cytoplasm, Hsp70 binds hydrophobic residues of denatured polypeptides and co-chaperones via the substrate binding pocket, localized in the C-terminal region of the protein, in an ATP-dependent manner (28–31). To characterize interacting partners specific for membrane-bound Hsp70, an affinity column with Hsp70-peptide TKD, was also used (20). A dominant 32-kDa protein band was eluted from Hsp70-protein and Hsp70-peptide columns with lysates of NK cells. This protein band was not detected with lysates of non-NK cells. The intensity of the 32-kDa protein band was more pronounced in eluates of TKD columns because at the molar level a 50–100-fold excess Hsp70-peptide was coupled to the column, as compared with full-length Hsp70-protein. By MALDI-TOF and Western blot analysis the Hsp70-interacting partner could be identified as granzyme B (M, expected 32,000). This observation is consistent with a prior report indicating binding of Hsp70 and Hsp27 to granzyme-immobilized Sepharose columns (32).

One mechanism of NK cell-mediated killing involves the exocytosis of cytotoxic granules containing perforin and serine proteases (33). After internalization the protease granzyme B cleaves procaspases into their activated form, and thereby induces programmed cell death by promoting DNA fragmentation, and cytochrome c release. Cell viability of Hsp70 membrane-negative tumor sublines was not affected by granzyme B. These differences in the induction of apoptosis were not observed with the topoisomerase inhibitor camptothecin. Therefore, we speculate that initiation of apoptosis by granzyme B and camptothecin involves different routes. Our results that binding of granzyme B to the extracellular exposed region of Hsp70, defined by the Hsp70-peptide TKD, may be critical for uptake and for the induction of apoptosis. This observation is supported by the finding that the enhanced cytolytic activity of NK cells against Hsp70 membrane-positive tumor cells could be blocked by an Hsp70-specific monoclonal antibody, which binds to TKD. It is conceivable that cell surface-bound Hsp70 antibody prevents binding and internalization of granzyme B to tumor cells and thus protects them from apoptosis.

Earlier observations have shown that only an Hsp70 antibody directed against the C-terminal part TKD was able to recognize cell surface-localized Hsp70 on viable tumor cells (26). This suggests that the protein may be incorporated into the lipid bilayer. This option has been supported by recent evidence indicating interaction of Hsp70 with phospholipids of the plasma membrane (42). Moreover, an ion conductance pathway was observed in artificial lipid bilayers after incorporation of Hsc70 (43). More recently, these proteins were found to interact with lipids in a liposome aggregation assay (44). Therefore, it is possible that Hsp70 is also embedded in the plasma membrane of tumor cells. Here we report on a selective cation channel activity in plasma membrane of Hsp70 positive tumor cells. Based on these results, one might speculate about channel formation, which facilitate binding and uptake of granzyme B into Hsp70 membrane-positive tumor cells. In the absence of Hsp70 in the plasma membrane, these channels cannot be created and thus uptake of granzyme B is prohibited. In the cytosol Hsp70 frequently cooperates with other proteins. Therefore, we were interested to identify molecules that may be tightly associated with Hsp70 in the plasma membrane. Unpublished data of our group suggest that the silencer of death domain, also termed Bag-4, is co-localized with Hsp70 on the cell surface. Ongoing studies investigate the role of Hsp70 in concert with Bag-4 in the formation of ion channels.

Keeping in mind that tumor sublines with differential Hsp70 membrane expression did not differ with respect to their intracellular Hsp70 levels, neither under physiological conditions nor following stress (21), differences in the sensitivity to granzyme B-mediated apoptosis cannot be explained by differential cleavage of Hsp70 inside the cell. Also modulations in the expression of major histocompatibility complex class I molecules, known to differentially induce killer cell inhibitory/activating receptors on NK cells, could be ruled out, because the major histocompatibility complex class I cell surface pattern was identical in Hsp70 membrane-positive and -negative tumor sublines (21).

Apart from the observation that Hsp70 serves as an entry port for granzyme B into Hsp70 membrane-positive tumor target cells, Hsp70-protein and TKD stimulate the production and release of granzyme B in primary NK cells. This finding can explain why high Hsp70 membrane expression not only predisposes tumor target cells to apoptotic cell death but also favors NK cell activation. In contrast to in vitro applied granzyme B, activated NK cells also kill Hsp70 membrane-negative tumor cells to a certain extent. However, this weak lysis was not

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2 M. Gehrmann, J. Marienhagen, H. Eichholtz-Wirth, E. Fritz, J. Ellwart, M. Jaäntellä, T. Zilch, and G. Multhoff, unpublished observations.
blockable with Hsp70 antibody. Together with the result that lysis of Hsp70 membrane-positive tumor cells was reduced to the degree of lysis observed with Hsp70 membrane-negative tumor cells by Hsp70 antibody, we assumed that Hsp70-mediated granzyme B internalization and apoptosis induction is only one mode how activated NK cells kill tumor cells. Furthermore, in comparison to in vitro provided granzyme B, killing mediated by NK cells engage a physical cell-to-cell contact that is likely to result in high local granzyme B concentrations at the tumor cells that cannot be quantified experimentally. Therefore, our in vitro apoptosis assays were conducted with granzyme B concentrations similar to those found in human serum (41). These concentrations are undoubtedly different to granzyme B concentrations similar to those found in human more, in comparison to only one mode how activated NK cells kill tumor cells. Further-
tumor cells by Hsp70 antibody, we assumed that Hsp70 medi-
elucidates one part of the puzzling role of membrane-bound Hsp70 in the natural defense mechanisms against tumor cells. We propose the Hsp70 epitope TKD is a of granzyme B by NK cells, on the other hand it facilitates cer. On the one hand it stimulates the production and delivery of granzyme B into the medium, Kinetical studies indicated with the relative release of granzyme B into the medium, cells could not be defined, the cytotoxic response of activated amount of granzyme B transferred from NK cells into tumor serum (41). These concentrations are undoubtedly different to granzyme B concentrations similar to those found in human.

Acknowledgments—We thank Marja Ja¨a¨ttela¨ and Christopher Froelich for constructive suggestions. We also thank Gerald Thonigs and Lydia Rossbacher for excellent technical assistance.

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