Functional involvement of cathepsin W in the cytotoxic activity of NK-92 cells

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Abstract Human cathepsin W (lymphopain) is a papain-like cysteine protease of unknown function that is specifically expressed in natural killer (NK) cells and to a lesser extent in cytotoxic T cells (CTL). In order to analyze the functional importance of cathepsin W for the cytotoxic process, we investigated NK-92 cells that have an NK cell-like phenotype and express cathepsin W. NK-92 cells possess strong cytotoxic activity against Jurkat and K562 cells. The cytotoxic activity of NK-92 cells against K562 was decreased in the presence of antisense phosphorothioate oligonucleotides against the cathepsin W-cDNA. Western blot analysis showed that the impaired cytotoxic activity of NK-92 cells was accompanied by reduced amounts of cathepsin W in the antisense-treated cells. In addition, co-cultivation experiments between NK-92 and K562 cells revealed a time-dependent decrease of cathepsin W by Western blot and immunofluorescence analysis during the cytotoxic attack, whereas CD56 expression of NK-92 cells was not affected. During cytotoxic attack, cathepsin W was neither targeted to K562 cells or other subcellular compartments, as shown by immunofluorescence analysis. The decrease of cathepsin W protein was associated with stable cathepsin W transcript levels. Control experiments using HT-29 cells, which are resistant against NK-92-mediated cytotoxicity, showed no change of cathepsin W expression, implying that the decrease of cathepsin W in the NK-92/K562 assay is linked to the cytotoxic process. Although the exact function of cathepsin W with respect to its enzymatic activity and its site of action still needs to be elucidated, our data demonstrate for the first time that cathepsin W is important for cellular cytotoxicity mediated by NK cells.
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1. Introduction

Cathepsin W (also named lymphopain) represents a cysteine protease that belongs to the C1A-family of papain-like proteases [1] and was found to be expressed in cytotoxic lymphocytes [2,3]. In peripheral blood the majority of the cathepsin W-expressing cells represent natural killer (NK) cells which exhibit higher cathepsin W gene expression levels than cytotoxic T cells (CTL) [4]. As yet, the proteolytic activity of cathepsin W has not been characterized. Based on restricted gene expression pattern in cytotoxic cells, a functional role of cathepsin W in the cytotoxic process mediated by NK cells and CTL has been suggested [2,4]. Both NK cells and CTL play a major role in the immune response against tumor cells, viral infections and allografts [5,6] as well as in inflammatory diseases such as arthritis [7], chronic obstructive pulmonary disease [8] and inflammatory bowel disease (IBD) [9]. In the search for a pathophysiological role of cathepsin W, we and others showed that cathepsin W is differentially expressed in IBD and autoimmune gastritis [10] and that cathepsin W is up-regulated in large granular leukemia [11].

To investigate the functional role of cathepsin W in the cytotoxic process, NK-92 cells, which represent an NK cell phenotype, were used as a model. Here, we demonstrate that the antisense-mediated inhibition of cathepsin W leads to a decrease of cytotoxic activity, and provide further evidence for a functional involvement of cathepsin W in cell-mediated cytotoxicity.

2. Materials and methods

2.1. Cell culture and treatment with oligonucleotides

NK-92 cells were cultivated in MEM-α containing 12.5% horse serum, 12.5% fetal calf serum (FCS), 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, antibiotics, and 100 U/ml recombinant IL-2 (Pepro-Tech Inc., USA). K562 and HT-29 cells were cultivated in RPMI-1640 containing antibiotics and 10% FCS. All reagents were purchased from PAA (Colbe, Germany). For co-cultivation, NK-92 cells and K562 or HT-29 were mixed at different cell ratios and incubated in a 25 cm^2 flask in a cell incubator at 37°C and 5% CO_2 up to 2 h.

For antisense-mediated inhibition of cathepsin W, 1×10^6 cells were cultivated in the presence of 10 μM antisense phosphorothioate oligonucleotides (Table 1) for 3 days (8 ml medium) and expanded to 12 ml medium for another 2–3 days. Analyses were performed at day 5 or 6.

2.2. Fluorescence-activated cell sorter (FACS) analysis of NK cell-mediated cytotoxicity

Cytotoxic assay was performed by a FACS-based method using K562 as target cells as described by others [12,13]. Briefly, K562 cells were split 1 day before testing. Next day, cells were washed with complete medium and labeled 0.3 μM DiO (3,3′-dioctadecyloxacarbocyanine, a lipophilic membrane dye) in complete medium. Effector cells (NK-92, 2×10^6/ml) and labeled target cells (K562, 5×10^5/ml) were mixed in Falcon polystyrene 12×75 assay tubes to different effector:target ratios as well as effector and target cells only. Cells were centrifuged for 10 min at $200 \times g$ and without removing the supernatant incubated for 1 h at 37°C and 5% CO_2 in a cell culture

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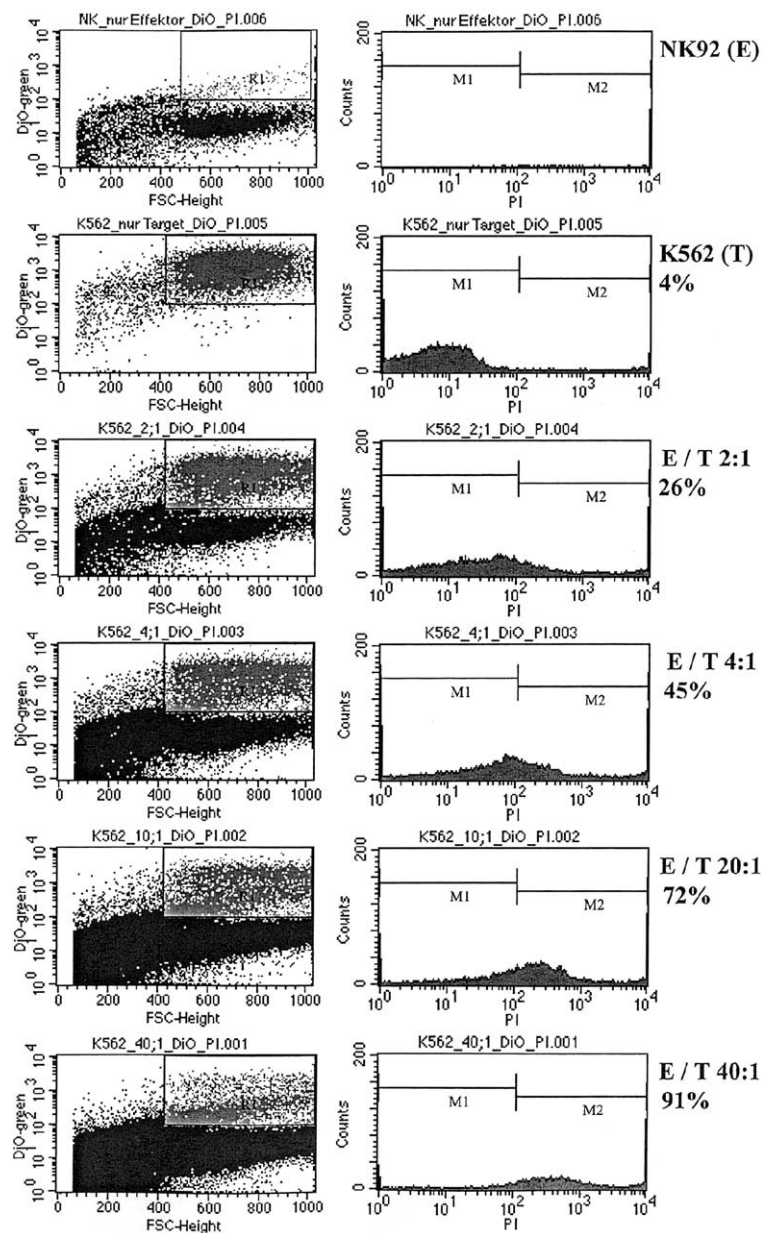*E-mail address:* thomas.wex@medizin.uni-magdeburg.de (T. Wex).

Fig. 1. FACS-based analysis of cell-mediated cytotoxicity. The assay was performed as described for 90 min using different ratios of effector (E) and target (T) cells as indicated in the right margin. The left panel shows dot plots of fluorescence intensity, NK-92 and K562 cells are represented as black and gray dots (R1), respectively. The right panel represents the corresponding histograms of DiO-positive target cells in R1 documenting viable cells (M1) and dead cells (M2). The relative proportion of killed K562 cells depends on the E/T ratio and time course.

incubator. After incubation, cells were centrifuged and the pellet was resuspended in 500 μl Cell wash (Becton Dickinson, Heidelberg, Germany). The viability of labeled and non-labeled cells was evaluated by propidiumiodid incorporation (10 μg/ml). Analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson). Cali-BRITE™ beads (Becton Dickinson) were used for calibration. In or-

der to exclude cell debris from analysis, the threshold was set to channel 52 of forward scatter on a linear scale. Data acquisition was performed by the Cellquest software (BDIS). Subsequently, dot blot combinations of FSC/FL1 (DiO) and FL1/FL3 (DiO/PI) were created and DiO-positive cells were gated from which at least 2000 cells per sample were examined. The percentage of dead target cells

Table 1
Sequence and localization of antisense oligonucleotides against cathepsin W-cDNA

Name	Sequence	Position in catW-cDNA
anti-catW 3	5′-ggg-agg-gca-gga-gac-tcg-ggg-ctt-c-3′	1170–1149
anti-catW 4	5′-ggc-cag-gta-ctg-cgc-aat-tc-3′	807–788
anti-catW 5	5′-gag-gct-gag-gct-gag-act-gcg-atg-ga-3′	979–1003
sense catW 3	5′-gaa-gcc-ccg-agt-ctc-ctg-ccc-tcc-g-3′	1149–1170
scrambled catW 3	5′-gag-cga-ggt-acg-gag-ggc-tcg-ct-3′	–

The positions of the oligonucleotides are based on the cDNA sequence (accession number NM_001335 [2].
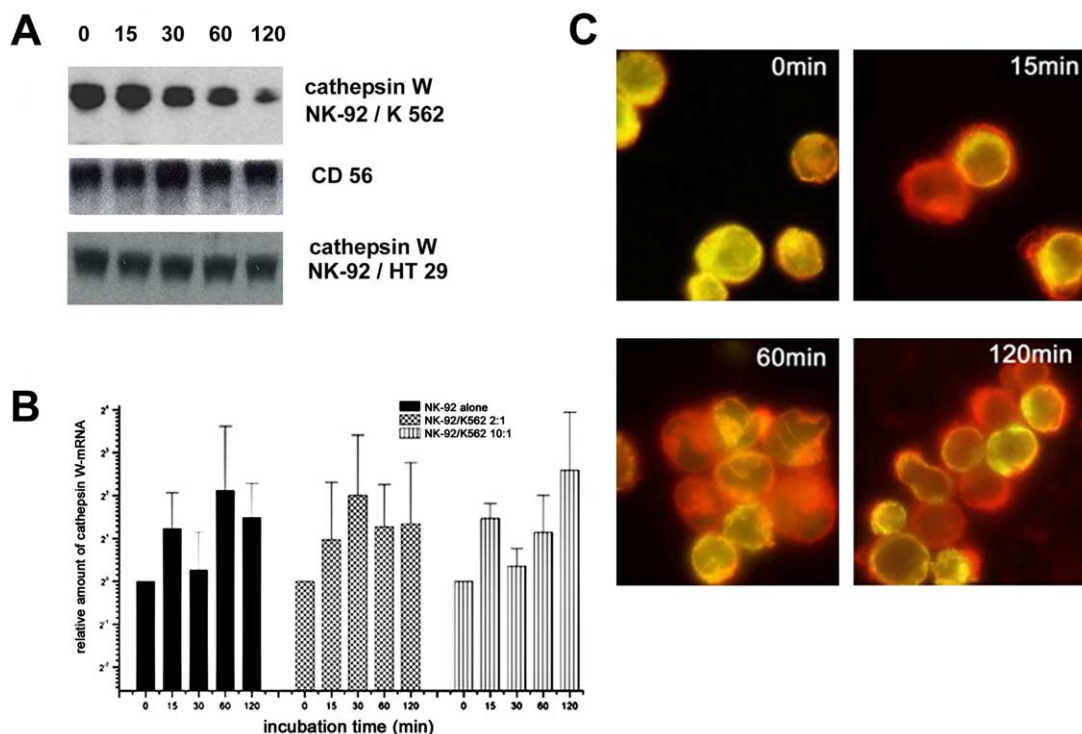
Fig. 2. Cathepsin W gene expression in NK-92 cells during the cytotoxic process. A: Aliquots were harvested between 0 and 120 min as indicated, and subjected to Western blot analysis. The upper blot demonstrates cathepsin W expression of the NK-92/K562 mixture at an E/T ratio of 2:1. The double band corresponds to the pro-form of cathepsin W at 52–55 kDa. The medium blot shows the corresponding expression of CD56. The lower panel documents cathepsin W expression of the NK-92/HT-29 mixture at an E/T ratio of 2:1. B: This demonstrates the cathepsin W-mRNA content of NK-92 cells during co-cultivation. Data represent relative values of cathepsin W transcript levels adjusted to time point 0. The results are shown as mean and standard deviations and represent three to five independent experiments. C: Indirect immunofluorescence analysis of cathepsin W was performed using the monoclonal anti-cathepsin W antibody CW40-1B1 detecting the propeptide and a secondary anti-mouse FITC-labeled antibody (green). Endoplasmic reticulum was stained by Rhodamine-succinylated ConA (Vector Laboratories). Areas of co-localization of both cathepsin W and endoplasmic reticulum are shown in yellow. Controls performed using corresponding isotype antibody did not show specific staining (data not shown).

[FL1: DiO- and FL3: PI-double-positive cells], which reflects active cell lysis, was documented in a histogram plot.

2.3. Western blot analysis and quantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis

Protein extraction, sodium dodecyl sulfate (SDS) electrophoresis and Western blot analysis were performed by standard methods as described previously [4]. Cathepsin W was detected using monoclonal antibody CW40-1B1 [10], the anti-CD56 antibody was obtained from Chemicon (Temecula, CA, USA). Total cellular RNA was isolated using the RNeasy kit (Qiagen, Hildesheim, Germany) as described by the manufacturer, and transcribed into cDNA using a standard protocol for AMV reverse transcriptase (Promega, Mannheim, Germany). Quantitative RT-PCR was performed using an iCycler (Bio-Rad, Munich, Germany) in 30 µl reaction mixture consisting of 15 µl HotStartTaq™ Master Mix (Qiagen), 1.2 µl of the RT reaction, SYBR-Green I (Molecular Probes, Eugene, OR, USA) and 0.5 µM of the specific primers for β-actin or cathepsin W using standard conditions for amplification. Initial template mRNA amounts were calculated using the iCycler software. β-Actin mRNA amounts were used to normalize the cDNA contents of the different samples. The following primers were designed for the RT-PCR analysis: Cathepsin W (Genbank™ accession no. NM001335) fw: 5′-gct-cac-cgc-ctg-gac-atc-ttg-c-, rev: 5′-tat-gtt-gcc-tgc-cgc-tgc-cat-g-; and β-actin (accession no. NM001101) fw: 5′-cat-gcc-atc-ctg-cgt-ctg-gac-c-, rev: 5′-aca-tgg-tgg-tgc-cgc-cag-aca-g-. The lengths of the resulting RT-PCR fragments were 308 bp (cathepsin W) and 400 bp (β-actin).

2.4. Immunofluorescence analysis

Cells were incubated on polylysine-covered slides at room temperature for 15 min and immediately fixed in phosphate-buffered saline (PBS) (pH 7.4) containing 3.7% PFA and 0.025% glutaraldehyde for 10 min. Cells were permeabilized in PBS containing 0.2% Triton X100

for 10 min, twice rinsed with PBS and blocked with 1% bovine serum albumin in PBS (pH 7.4) for 20–30 min. Cells were incubated with primary antibody for 1–2 h and subsequently blocked again as described above. Then, specimens were treated with both a secondary fluorescein isothiocyanate (FITC)-labeled anti-mouse antibody (Sigma) and Rhodamine-succinylated concanavalin A (Vector Laboratories, Burlingame, CA, USA) for 1 h, washed, and postfixed in PBS (pH 7.4) containing 3.7% PFA and 0.05% glutaraldehyde for 10 min. After washing with PBS (pH 7.4), specimens were washed once in PBS (pH 8.9), and subsequently embedded. The specimens were analyzed using the fluorescence microscope (Leica DMRE7, Leica Wetzlar, Germany) equipped with a CCD camera (Spot RT, Diagnostic Instruments, Burroughs, MI, USA) and a 40/1.25 objective. Separate images were taken in the corresponding channels, and later merged using the Photoshop™ software. Image acquisition of the controls and data processing was performed under the same conditions.

3. Results and discussion

3.1. Cell-mediated cytotoxicity of NK-92 cells and cathepsin W expression

To study the role of cathepsin W, the NK cell-related line NK-92 was used as a model. NK-92 cells represent large granular lymphocytic cells with strong cytotoxic activity and NK cell-like phenotype (CD3⁻, CD4⁻, CD8⁻, CD45⁺, CD56⁺). NK-92 cells possess a potent cytotoxic activity against the erythropoietic cell line K562 and the T-cell line Jurkat. The rate of cytotoxicity was dose- (Fig. 1) and time-dependent (data not shown). In addition, NK-92 cells showed a strong
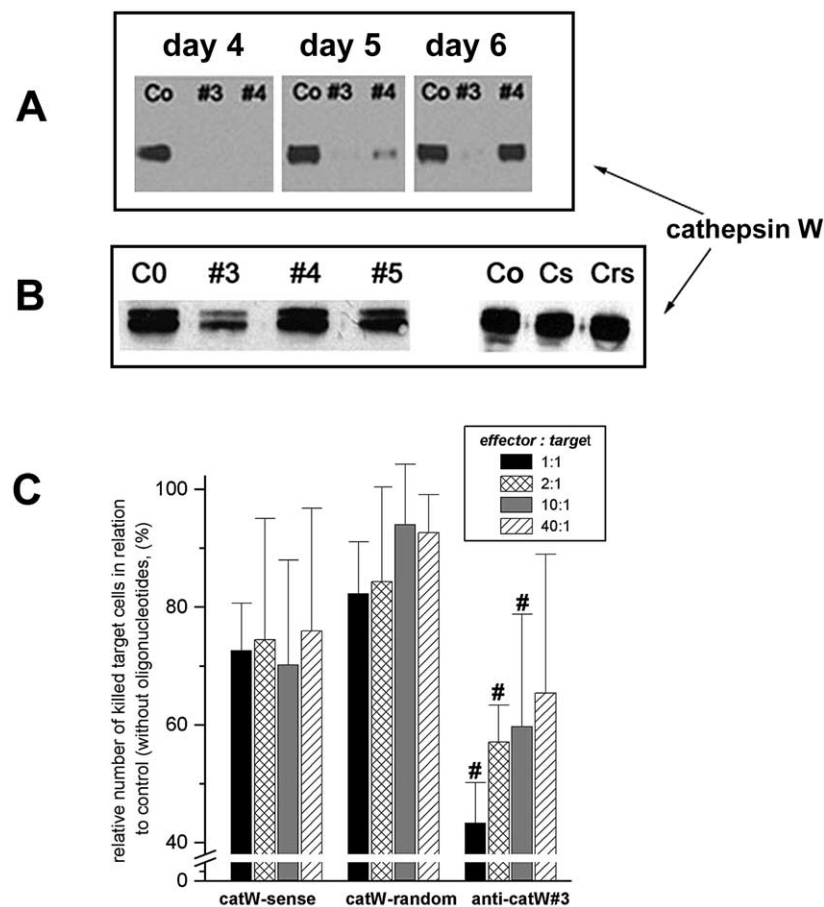
Fig. 3. Cell-mediated cytotoxicity of antisense-treated NK-92 cells. A: 5×10^3 cells were cultivated in 200 μl medium (96-well plate) in the presence of 10 μM anti-cathepsin W oligonucleotides 3, 4 and without oligonucleotides (Co) harvested at days 4–6, and subjected to Western blot analysis. B: Western blot analyses illustrate a partial and slight inhibition of endogenous cathepsin W expression by anti-cathepsin W 3 and anti-cathepsin W 4+5, respectively (left panel). Both control oligonucleotides (Cs: sense; Crs: randomly scrambled) as well as control without oligonucleotides (Co) did not affect endogenous cathepsin W expression of NK-92 cells (right panel). C: NK-92 cells were cultivated in the presence of oligonucleotides as described in Section 2 and their concentration was adjusted to 2×10^6 cells/ml before analyzing their cytotoxic activity. The cytotoxic activity of NK-92 cells treated with different oligonucleotides is shown as relative values (Y-axes) based on the control without oligonucleotides set to 100%. Data represent mean values and standard deviations of three to five independent experiments. Values marked by '*' were found to be significantly different from their corresponding controls as analyzed by independent Fisher's T-test ($P < 0.05$).

constitutive expression of cathepsin W [10]. In order to investigate the proposed functional involvement of cathepsin W in the cytotoxic process, cathepsin W expression was analyzed during 'cytotoxic attack'. The co-cultivation of NK-92 and K562 cells resulted in a reduction of cathepsin W as shown by Western blot (Fig. 2A) and immunofluorescence (Fig. 2C). The strong co-staining with concanavalin A, a lectin that preferentially binds mannose-glycosylated proteins in the endoplasmic reticulum, implies a localization of pro-cathepsin W in the vicinity of this compartment. The fact that we did not see any change in the co-localization during the 2 h would suggest that pro-cathepsin W is not targeted to another compartment in NK-92 cells. In order to prove the specificity of the observed down-regulation of pro-cathepsin W, the CD56 expression of NK-92 cells was analyzed by Western blot (Fig. 2A) and immuncytometry. As shown by Western blot (Fig. 2A), CD56 expression of NK-92 cells was unchanged during 'cytotoxic attack'. The corresponding cytofluorimetric analysis of CD56 expression revealed identical results; both the number of CD56-expressing cells as well as the CD56 expression per cell was found to be stable during the co-incubation of 2 h (data not shown). Furthermore, we investigated the cathepsin

W expression during the co-cultivation of NK-92 cells with cells that are resistant against NK cell-mediated cytotoxicity. Interestingly, several cell models (e.g. U937, Raji, Daudi) known to be resistant against NK cells were efficiently killed by NK-92 cells (data not shown). Only HT-29 cells, an epithelial cell line derived from colon adenocarcinoma, were found to be resistant against NK-92 cell-mediated cytotoxicity. During co-cultivation between NK-92 and HT-29 cells, cathepsin W expression did not change, as shown by Western blot analysis (Fig. 2A). Together, both control experiments show that: (I) the 'down-regulation' of cathepsin W documented for NK-92+K562 cells is linked to the cytotoxic action, and (II) that this effect is specific for cathepsin W since CD56 expression was not affected (Fig. 2A).

The analysis of the cathepsin W transcript during co-incubation with K562 cells revealed only a slight increase of cathepsin W-mRNA levels that were not significantly different from those observed in NK-92 cells alone (Fig. 2B). This finding suggests that there is no transcriptional up-regulation of the cathepsin W gene expression during the 'cytotoxic attack'. However, it is notable that NK-92 cells are IL-2-dependent, and therefore need to be cultivated in the presence of

IL-2. Since it has been shown that IL-2 induces cathepsin W gene expression of peripheral NK cells [4], additional effects on the transcriptional regulation after the 'cytotoxic attack' might be prevented in this model.

3.2. Antisense-mediated inhibition of cathepsin W

To investigate the functional importance of cathepsin W during the cytotoxic process, five antisense oligonucleotides were employed to find out their capability of inhibiting endogenous cathepsin W expression in NK-92 cells. These experiments were performed initially at a small scale (5×10^3 cells) and revealed three potential candidates that partially (anti-catW 4 and 5) or completely (anti-catW 3) inhibited cathepsin W expression in NK-92 cells as shown by Western blot analysis (partially shown in Fig. 3A). For studying cytotoxic activity, anti-catW 3, 4 and 5 were used to inhibit cathepsin W expression at a larger scale ($1–2 \times 10^6$ cells). Here, all three oligonucleotides were not as effective as in the small-scale experiments. Cells treated with anti-catW 3 demonstrated the strongest inhibition, whereas anti-catW 4 and 5 showed only slight effects on the cellular cathepsin W level (Fig. 3B). Furthermore, two controls (sense and randomly scrambled) of the most effective antisense oligonucleotide anti-catW3 did not significantly affect cellular cathepsin W expression (Fig. 3B). The Western blot analyses clearly demonstrated a down-regulation of cathepsin W, in particular by anti-catW3. To investigate the specificity of the antisense-mediated inhibition, the cathepsin F expression of NK-92 cells was analyzed. Cathepsin F represents the most closely related human cathepsin to cathepsin W [14] and could therefore be a potential target for the anti-cathepsin W antisense oligonucleotides. However, cathepsin F expression was neither detected on the protein or RNA level in these cells (data not shown). The anti-catW3-mediated inhibition of cathepsin W resulted in a decrease of cell-mediated cytotoxicity, as shown in Fig. 3B. Anti-catW 3 significantly inhibited the cytotoxic activity of NK-92 cells compared to control oligonucleotides (Fig. 3B), whereas the two other antisense primers (anti-catW 4+5) did not significantly differ from controls (data not shown). Altogether, the decrease of endogenous cathepsin W expression mediated by anti-catW3 corresponded with the impaired cytotoxic activity of NK-92 cells.

In summary, the results of our experiments support the hypothesis that cathepsin W plays an important role in cellular cytotoxic processes mediated by NK cells. Since the proteolytic activity of cathepsin W has not been identified, the underlying mechanism of its functional involvement in the cytotoxic process is currently unknown. Taking into account the primary structure of cathepsin W, which has all the features of an active cysteine protease, cathepsin W should possess proteolytic activity. The characterization of its enzymatic activity as well as the location of its action are key issues to understand the functional role of cathepsin W.

References

[1] Rawlings, N.D. and Barrett, A.J. (1994) Methods Enzymol. 244, 461–486.
[2] Linnevers, C., Smeekens, S.P. and Bromme, D. (1997) FEBS Lett. 405, 253–257.
[3] Brown, J., Matutes, E., Singleton, A., Price, C., Molgaard, H., Buttle, D. and Enver, T. (1998) Leukemia 12, 1771–1781.
[4] Wex, T., Buhling, F., Wex, H., Gunther, D., Malfertheiner, P., Weber, E. and Bromme, D. (2001) J. Immunol. 167, 2172–2178.
[5] Moretta, L., Bottino, C., Pende, D., Mingari, M.C., Biassoni, R. and Moretta, A. (2002) Eur. J. Immunol. 32, 1205–1211.
[6] Barry, M. and Bleackley, R.C. (2002) Nat. Rev. Immunol. 2, 401–409.
[7] Dulphy, N., Rabian, C., Douay, C., Flinois, O., Laoussadi, S., Kuipers, J., Tamouza, R., Charron, D. and Toubert, A. (2002) Int. Immunol. 14, 471–479.
[8] Barnes, P.J. (2003) Annu. Rev. Med. 54, 113–129.
[9] Kappeler, A. and Mueller, C. (2000) Histol. Histopathol. 15, 167–172.
[10] Buhling, F., Kellner, U., Guenther, D., Kahl, S., Bromme, D., Weber, E., Malfertheiner, P. and Wex, T. (2002) Biol. Chem. 383, 1285–1289.
[11] Kothapalli, R., Bailey, R.D., Kusmartseva, I., Mane, S., Epling-Burnette, P.K. and Loughran, T.P.J. (2003) Int. J. Oncol. 22, 33–39.
[12] Papa, S., Vitale, M., Mariani, A.R., Roda, P., Facchini, A. and Manzoli, F.A. (1988) J. Immunol. Methods 107, 73–78.
[13] Piriou, L., Chilmonczyk, S., Genetet, N. and Albina, E. (2000) Cytometry 41, 289–297.
[14] Wex, T., Levy, B., Wex, H. and Bromme, D. (1999) Biochem. Biophys. Res. Commun. 259, 401–407.