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Granulysin Delivered by Cytotoxic Cells Damages Endoplasmic Reticulum and Activates Caspase-7 in Target Cells

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Granulysin is a human cytolytic molecule present in cytotoxic granules with perforin and granzymes. Recombinant 9-kDa granulysin kills a variety of microbes, including bacteria, yeast, fungi, and parasites, and induces apoptosis in tumor cells by causing intracellular calcium overload, mitochondrial damage, and activation of downstream caspases. Reasoning that granulysin delivered by cytotoxic cells may work in concert with other molecules, we crossed granulysin transgenic (<i>GNLY<sup>+/−</sup></i>) mice onto perforin (<i>perf<sup>−/−</sup></i>) or granzyme B (<i>gzmb<sup>−/−</sup></i>)-deficient mice to examine granulysin-mediated killing in a more physiologic whole-cell system. Splenocytes from these animals were activated in vitro with IL-15 to generate cytolytic T cells and NK cells. Cytotoxic cells expressing granulysin require perforin, but not granzyme B, to cause apoptosis of targets. Whereas granzyme B induces mitochondrial damage and activates caspases-3 and -9 in targets, cytotoxic cell-delivered granulysin induces endoplasmic reticulum stress and activates caspase-7 with no effect on mitochondria or caspases-3 and -9. In addition, recombinant granulysin and cell-delivered granulysin activate distinct apoptotic pathways in target cells. These findings suggest that cytotoxic cells have evolved multiple nonredundant cell death pathways, enabling host defense to counteract escape mechanisms employed by pathogens or tumor cells. The Journal of Immunology, 2011, 186: 3497–3504.

Granulysin, another component of cytotoxic cell granules, is expressed constitutively by NK cells and by CTLs 3–5 d after activation (15). Granulysin is synthesized as a 15-kDa molecule that is cleaved at both the amino and the carboxyl termini to a 9-kDa form (16). Recombinant 9-kDa granulysin is broadly cytolytic against tumors and microbes, including Gram-positive and Gram-negative bacteria, yeast, fungi, and parasites (17). Granulysin has been implicated in a variety of human diseases, and expression of granulysin correlates with good clinical outcomes in cancer and infections (18–20). Granulysin homologs are found in humans, cows, pigs, and horses, but not in mice or rats (21, 22). To test the physiologic role of granulysin, we used a bacterial artificial chromosome to generate <i>GNLY</i> transgenic mice (23). These animals have normal development and phenotype, and their lymphocytes express granulysin in a manner similar to human T and NK cells.

In a series of studies, we described the mechanism by which recombinant 9-kDa granulysin causes apoptosis in Jurkat tumor cells (24–28). Positively charged granulysin interacts with the Jurkat cell surface on the basis of electrostatic charge (25) and then rapidly enters the cell, presumably by a scissoring action, as implicated by the crystal structure (26). There is a rapid increase in intracellular calcium and a decrease in intracellular potassium (27), resulting in mitochondrial depolarization and release of both cytochrome c and apoptosis-inducing factor (AIF) (27). Cytochrome c release activates caspase-3, which, together with AIF (28), induces endonuclease activation and apoptosis (27). Of note, experiments using recombinant 9-kDa granulysin may not accurately mimic the conditions of cytotoxic granule delivery by NK cells or CTLs in that 1) a relatively high (micromolar) concentration of recombinant granulysin is required to induce lysis, and 2) other molecules such as perforin, calreticulin, and granzymes are not present. To study the mechanism of granulysin-induced cell death under more physiologic conditions; we crossed <i>GNLY<sup>+/−</sup></i> mice onto <i>perf<sup>−/−</sup></i> and <i>gzmb<sup>−/−</sup></i> mice and used in vitro propagated cytotoxic NK cells from these strains to lyse target cells. In
accompanying previous reports (3, 29), NK cells lacking either granzyme B or perforin are defective in their ability to kill target cells. We found that NK cells expressing granulysin but lacking perforin are unable to kill target cells. Lysis by NK cells expressing granulysin but lacking granzyme B is similar to that of wild-type (WT) NK cells. Surprisingly, granulysin delivered by NK cells does not cause mitochondrial damage or activate either caspase-3 or caspase-9 in target cells, whereas recombinant 9-kDa granulysin activates these pathways. NK cells expressing granulysin cause both endoplasmic reticulum (ER) stress and caspase-7 activation in target cells, whereas 9-kDa granulysin does not. Thus, NK cell-delivered granulysin and recombinant granulysin induce target cell death through distinct pathways.

Materials and Methods

Mouse strains and cell lines

C57BL/6 perforin knockout mice (perf−/−) and C57BL/6 granocyte B knockout mice (gzmb−/−) were purchased from The Jackson Laboratory, Bar Harbor, ME. C57BL/6 granulysin transgenic mice (GNLY−/−) (23) were mated with perf−/− or gzmb−/− mice to obtain gzmb−/−GNLY−/−, perf−/− GNLY−/−, gzmb−/− perf−/−, and gzmb−/− perf−/− GNLY−/− strains. Mice were bred at the National Cancer Institute and mice >6 wk of age were used in all experiments. The Animal Care and Use Committee of the National Cancer Institute approved all animal experiments. YAC-1 (mouse lymphoma), K562 (human erythroleukemia), and THP-1 (human monocytic cells) were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS (Hyclone, Ogden, UT), 2 mM l-glutamine, and 100 U/ml penicillin-streptomycin (complete media). EL4 and EL4.F15 cells (30) were maintained in MEM with 10% FBS and 10 μM 2-mercaptoethanol (2-ME).

Activation of splenocytes and granulysin expression

Single-cell suspensions of splenocytes were cultured at 2.5 × 10^6 cells/ml in 24-well plates in complete medium supplemented with 25 ng/ml human rIL-15 (eBioscience, San Diego, CA) and 0.05 mM 2-ME. Fresh IL-15 was added on 4 and 8 d of culture. Granulysin expression was analyzed by intracellular staining and flow cytometry. The cells were labeled with fluorochrome-conjugated Abs specific for CD8 (clone 53-6.7) or NK1.1 (clone PK136) and CD3 (clone 145-2C11) (BD Biosciences, San Jose, CA), fixed and permeabilized and then stained with rabbit anti-granulysin antiserum, as described earlier (23). The flow cytometry data were analyzed with FlowJo analysis software (TreeStar, Ashland, OR).

Confocal microscopy

NK cells were isolated from IL-15–activated splenocytes by negative selection using MACS isolation kits (Miltenyi Biotec, Auburn, CA). Cells were immobilized on poly (l-lysine)–coated slides, fixed in 2% paraformaldehyde, and permeabilized (0.01% saponin and 0.1% Triton X-100). Cells were stained with rabbit anti-granulysin antisera (1:10,000) and counterstained with anti-rabbit IgG conjugated to Alexa Fluor 488 (Molecular Probes, Carlsbad, CA) (1:1000), and fluorescent images were acquired on an LSM 510 confocal system (Carl Zeiss) using a 63× Plan-Apochromat objective.

Flow-based killing assay

Target cells (YAC-1, EL4, EL4.F15, K562, or THP-1) were washed twice with PBS and labeled with 100 nM CFSE (Molecular Probes) for 15 min at 37°C. The reaction was stopped by addition of RPMI 1640 supplemented with 10% FBS. Cells were washed twice with PBS supplemented with 2% FBS, then resuspended in complete medium supplemented with 0.05 mM 2-ME and 25 ng/ml IL-15. Effector cells were mixed with 10^5 labeled target cells in 50 μl medium in 96-well plates and incubated at 37°C for 3–4 h at different E:T ratios. A total of 1 μg/ml 7-aminoactinomycin D (7-AAD) (Molecular Probes) was added to each well immediately before flow cytometry. Cell death (7-AAD incorporation and cell shrinkage) was analyzed by flow cytometry after gating on CFSE-positive target cells, as described earlier (23). Percentage of specific lysis was calculated by the following formula: % specific lysis = (% experimental lysis − % spontaneous lysis)/100 × 100.

In vitro assays using recombinant granulysin

Recombinant granulysin was expressed and purified as described previously (16). Cells were seeded in 96-well plates at a density of 4.5 × 10^4/ml (100 μl/well) in medium containing 0.1% FCS. Cells were cultured for 2 h in the presence of granulysin (25 μM). Mitochondrial membrane depolarization, reactive oxygen species (ROS) generation, and ER membrane integrity were measured as described below.

Mitochondrial membrane perturbation and ROS generation

Target cells were labeled with 100 nM DDAO-SE [7-hydroxy-9H-(1,3-dichloro-9-9-dimethylacridin-2-one)-succinimidyl ester] (Molecular Probes) for 15 min at 37°C, washed, and then mixed with effector cells at an E:T ratio of 10:1 for 2 h. For ROS detection, cells were incubated with 2 μM hydroxyethidine (2-HE) (Molecular Probes); for mitochondrial membrane depolarization, cells were incubated with 10 nM DIOC(3) (3,3-dihexyloxadecyl acryloacridoin) (Molecular Probes) for 15 min. Fluorescence was measured using two-color flow cytometry after gating on DDAO-SE+ target cells. The gates were set using target cells alone.

Detection of activated caspases

Effector and target cells were incubated for 2–3 h at a 10:1 E:T ratio, washed with PBS/1% BSA at room temperature, fixed, and permeabilized with the BD Cytofix/Cytoperm Kit (BD Biosciences) and then stained with either FITC-labeled mAb against the active form of caspase-3 (clone C296205; BD Pharmingen), caspase-7 (Cell Signaling Technology, Danvers, MA), caspase-9 (Cell Signaling Technology), or anti-m12D341 (rabbit serum specific for the active form of caspase-12, a kind gift from Dr. T. Momoi, National Institute of Neuroscience, Tokyo, Japan). In the case of anti-caspase-7, -9, or -12 Abs, cells were washed with perm/wash buffer and further stained with PE-conjugated anti-rabbit IgG (Invitrogen). After two washes with perm/wash buffer, cells were resuspended in 1% paraformaldehyde/PBS and analyzed by two-color flow cytometry.

Cytochrome c release

Cytochrome c release was quantified by flow cytometry as described (31). Briefly, effector and target cells were incubated at an E:T ratio of 10:1 for 2 h and then were mildly permeabilized with 25 μg/ml digitonin in 100 mM KCl on ice for 5 min. Cells were washed once with cold PBS, fixed in 4% paraformaldehyde, permeabilized with 0.05% saponin plus 3% BSA, and then incubated with the FITC-conjugated anti-cytochrome c mAb 6H2.B4 (BD Pharmingen). Cells were resuspended in 100 μl 4% paraformaldehyde in PBS and analyzed by FACS.

TUNEL assay

Effector cells were incubated at 37°C with 10^5 DDAO-SE–labeled target cells in 50 μl medium in 96-well plates. The cell mixtures were fixed in 2% paraformaldehyde and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice before staining with the In Situ Cell Death Detection Kit, Fluorescin (Roche Applied Sciences, Indianapolis, IN), following the manufacturer’s protocol. Stained cells were analyzed by flow cytometry. Target cells were reincubated with 40 μM pan-caspase inhibitor Quinoline-Val-Asp-CH2-OPh (Q-VD-OPh) (MP Biomedicals, Saloon, OH) for 1 h before the addition of effector cells.

Disruption of the ER

Effectors and targets were incubated for 1 h at various E:T ratios followed by incubation with 0.2 μg/ml brevetoxin A conjugated to BODIPY 558/568 (Bodipy, Molecular Probes) for another 30 min (32). Cells were washed twice with PBS and analyzed by flow cytometry.

Results

Granulysin delivered by cytotoxic cells requires perforin, but not granzyme B, for cytolysis of targets

To examine the mechanism of action of granulysin in cytolysis by cytotoxic cells, we used GNLY transgenic mice previously generated in our laboratory (23). Because granzyme B and perforin are both present in cytolytic granules with granulysin, the interaction of these three effector molecules was evaluated by crossing GNLY−/− mice with gzmb−/−, perf−/−, and gzmb−/− perf−/− mice. Splenocytes from these mice were activated in vitro using IL-15, and expression of granulysin was assessed by intracellular staining and
flow cytometry (Fig. 1A) (23). As shown earlier (23), IL-15 induces granulysin expression in NK1.1+ NK cells. By day 7, ~50% of NK1.1+ CD3+ cells express granulysin, but only 2% of CD8+ cells express granulysin (Fig. 1B). Splenocytes from GNLY+/+ animals lacking either perforin or granzyme B showed similar expression of granulysin (data not shown). Using confocal microscopy, we observed that granulysin is localized in the granules of NK cells (Fig. 1C) and colocalizes with granzyme B in these cytolytic granules (Supplemental Fig. 1). The presence or absence of granzyme B and/or perforin did not affect localization of granulysin in NK cells (Fig. 1C).

In vitro cytotoxicity assays were used to interrogate apoptotic pathways activated by granulysin. Splenocytes from WT, GNLY+/-, gzmB−/−, gzmB−/−GNLY+/+, perf−/−, perf−/−GNLY+/+, gzmB−/−perf−/−, and gzmB−/−perf−/−GNLY+/+ cultured with IL-15 for 7–10 d were used as cytotoxic cells. CFSE-labeled YAC-1 cells were used as target cells and assayed for incorporation of 7-AAD, which measures apoptosis/late cell death. Cytotoxicity was assayed over a range of E:T ratios and at different time points. As shown previously (23), there is no difference between GNLY+/+ and WT NK cells in apoptosis of YAC-1 targets (Fig. 2A–C). We also used EL4 and Fas-resistant EL4.F15 cells (30) to determine the role of Fas-mediated signaling on apoptotic pathways activated through granulysin. We found that cytotoxic cells from GNLY+/+ mice showed better killing of both EL4 and EL4.F15 cells, compared with cytotoxic cells from WT mice, but there was no effect of defective Fas signaling on cell death caused by either WT or GNLY+/+ NK cells (Supplemental Fig. 2A, 2B). In the absence of perforin, cell death was not observed over a range of E:T ratios (Fig. 2A). Although NK cells lacking granzyme B had reduced cytolytic capacity, compared with WT and GNLY+/+ effectors (Fig. 2B, 2C), NK cells from gzmB−/−GNLY+/+ mice showed cytolytic activity equivalent to that of WT and GNLY+/+ NK cells. (Fig. 2B, 2C). These findings suggest that target cell death by NK cells requires perforin plus either granzyme B or granulysin.

Cytotoxic cell-delivered granulysin does not mediate apoptosis through mitochondrial damage

We previously delineated the mechanisms by which recombinant 9-kDa granulysin causes cell death of Jurkat, a human tumor cell line: There is a rapid calcium influx and potassium efflux followed by mitochondrial perturbation, generation of ROS, release of cytchrome c and AIF, caspase activation, and apoptotic cell death (24, 25, 27, 28). To address the question of whether granulysin delivered by NK cells activates similar pathways in target cells, we monitored mitochondrial membrane depolarization and ROS generation in YAC-1 cells. Surprisingly, granulysin expression in NK cells did not affect mitochondrial membrane depolarization or ROS generation in YAC-1 target cells (Fig. 3A–D). Similar results were obtained using EL4 and EL4.F15 target cells, suggesting that Fas signaling is not involved in granulysin-induced cell death pathways (Supplemental Fig. 3A–D). In NK cells lacking granzyme B, expression of granulysin did not alter either mitochondrial membrane potential (ΔΨm) loss or ROS generation, despite the fact that gzmB−/−GNLY+/+ NK cells had higher cytolytic activity than did gzmB−/− NK cells (Fig. 3A–D). However, treatment of YAC-1 cells with recombinant 9-kDa granulysin resulted in ΔΨm and ROS generation, as shown earlier with Jurkat cells (25, 27, 28) (Fig. 3E). These findings indicate that recombinant 9-kDa granulysin activates apoptosis through the mitochondrial signaling pathway, whereas granulysin delivered by cytolytic granules in conjunction with perforin mediates apoptosis by a different pathway.

Granulysin delivered by NK cells mediates DNA fragmentation that is caspase dependent but does not involve caspase-3 or caspase-9

Because DNA fragmentation is a hallmark of apoptosis (33), we measured DNA fragmentation by TUNEL assay. GNLY+/+ NK cells, compared with WT cells, caused a small but significant increase in DNA fragmentation in YAC-1 targets. (Fig. 4A). We also

**FIGURE 1.** Granulysin expression in murine NK cells. A, Intracellular staining and flow cytometry analysis of granulysin expression kinetics in IL-15–activated splenocytes from GNLY+/+ mice over 12 d of culture. B, Expression of granulysin in NK1.1+ and CD8+ cells after 7 d of culture of GNLY+/+ and WT splenocytes with IL-15. C, Confocal microscopy of granulysin expression in IL-15–activated NK cells. NK cells were isolated from IL-15–activated splenocytes by negative selection using magnetic beads (Miltenyi). Nuclei are stained with DAPI shown in blue; cells were stained with either preimmune serum (left panel) or anti–9-kDa granulysin Ab (green; center and right panels). Data are representative of three independent experiments.
observed that the percentage of TUNEL-positive target cells was reduced when \textit{gzmb}^{-/-} NK cells were employed (31 to 23% for WT to \textit{gzmb}^{-/-} effectors, respectively). \textit{gzmb}^{-/-} GNLY+/2 NK cells caused a level of DNA fragmentation equivalent to that of WT NK cells. A pan-caspase inhibitor, Q-VD-OPH, inhibited DNA fragmentation mediated by all effectors. Therefore, both granzyme B and granulysin cause DNA fragmentation that is caspase dependent.

We previously showed that recombinant 9-kDa granulysin activates caspase-3 in Jurkat cells (25). Therefore, we examined the role of caspase-3 and its upstream initiator caspase-9 in the cell-based apoptosis assay. Both WT and GNLY^{+/+} NK cells caused activation of both caspase-3 and caspase-9 in YAC-1 cells, but expression of granulysin did not alter the level of activated caspase-3 or caspase-9 (Fig. 4B,4C). NK cells lacking granzyme B were less efficient at activating both caspase-3 and caspase-9 in targets, but again, expression of granulysin did not change the amount of active caspase-3 or caspase-9 in target cells (Fig. 4B,4C). Thus, target cell death mediated by granulysin delivered via cytotoxic cells does not involve mitochondrial damage and its downstream signaling via caspase-9 and caspase-3.

Granulysin causes ER disruption and enhanced caspase-7 and caspase-12 activation

Because DNA fragmentation caused by NK cell-delivered granulysin involved neither caspase-3 nor caspase-9, the role of other executioner caspases was evaluated. Caspase-3 and caspase-7 are structurally similar (34), and they share many substrates, so we next tested involvement of caspase-7. YAC-1 target cells incubated with \textit{gzmb}^{-/-} NK cells had elevated levels of activated caspase-7, and \textit{gzmb}^{-/-} GNLY^{+/+} NK cells induced even more activated caspase-7 (Fig. 4D). In a 2-h assay at different E:T ratios, \textit{gzmb}^{-/-} GNLY^{+/+} NK cells caused significantly more caspase-7 activation in target cells, compared with \textit{gzmb}^{-/-} NK cells (Fig. 4E), indicating that cytotoxic cell-delivered granulysin activates caspase-7 rather than caspase-3 and/or caspase-9.

Although caspase-3 and caspase-7 show similar activity toward many synthetic peptide substrates, Walsh et al. (35) recently observed that the percentage of TUNEL-positive target cells was reduced when \textit{gzmb}^{-/-} NK cells were employed (31 to 23% for WT to \textit{gzmb}^{-/-} effectors, respectively). \textit{gzmb}^{-/-} GNLY^{+/+} NK cells caused a level of DNA fragmentation equivalent to that of WT NK cells. A pan-caspase inhibitor, Q-VD-OPH, inhibited DNA fragmentation mediated by all effectors. Therefore, both granzyme B and granulysin cause DNA fragmentation that is caspase dependent.

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reported that caspase-3 and caspase-7 exhibit differential activity toward multiple substrate proteins, including Bid, XIAP, gelsolin, caspase-6, and the co-chaperone p23. Furthermore, in CHO cells, caspase-7 localizes to the ER and physically interacts with glucose-regulated protein 78 (GRP78), an ER resident chaperone (36). During ER stress, activated caspase-7 interacts with and activates caspase-12, which is also an ER-localized caspase (37). Because the involvement of caspase-7 in ER stress has been well documented and granulysin activates caspase-7, but not caspase-3, in target cells, we evaluated ER stress pathways in target cells. In YAC-1 target cells, both gzmb+/− and gzmb−/−GNLY4/− NK cells caused ER membrane damage after a 1-h incubation, and gzmb+/−,4/− effectors, compared with gzmb−/− effectors, caused enhanced ER disruption (Fig. 5A). When YAC-1 cells were incubated with recombinant 9-kDa granulysin (Supplemental Fig. 4A, 4C). However, ER membrane disruption was unaffected by recombinant 9-kDa granulysin (Supplemental Fig. 4B, 4D).

To further delineate the pathways activated by cytotoxic cell-delivered granulysin, we analyzed the status of caspase-12, an ER-resident protein that is involved in apoptotic pathways induced by ER stress. Caspase-12 activation was detected using an Ab specific for the cleaved form of caspase-12. Incubation of YAC-1 cells with gzmb−/−GNLY4/− NK cells produced a higher amount of activated caspase-12 than did gzmb−/− NK cells (Fig. 5C), further supporting the role of cell-delivered granulysin in ER disruption.

GRP78 and C/EBP homologous protein/growth arrest DNA damage-inducible gene 153 are two other ER markers whose expression is upregulated during ER stress. Shinkai et al. (38) have reported that knockdown of GRP78 leads to enhanced cell death. Analysis of YAC-1 targets incubated with either gzmb−/−GNLY4/− or gzmb−/− NK cells did not reveal any change in expression of C/EBP homologous protein/growth arrest DNA damage-inducible gene 153 (Supplemental Fig. 5B). However, expression of GRP78 was reduced in YAC-1 cells following incubation with gzmb−/−GNLY4/− NK cells, compared with gzmb−/− NK cells (Supplemental Fig. 5A).
It has been reported that cytochrome c release and activation of caspase-9 also cause ER stress (37). Although we found that the amount of activated caspase-9 in targets was similar regardless of whether the NK effector cells express granulysin (Fig. 4C), we evaluated cytochrome c release from target cells after a 2-h incubation with NK cells. Cytochrome c release was similar in YAC-1 cells incubated with either gzmb<sup>−/−</sup> or gzmb<sup>−/−</sup>GNLY<sup>+/−</sup> NK cells (Fig. 5D). Thus, granulysin delivered by effector cells does not damage mitochondria but does cause ER damage.

Granulysin-mediated cell death in human targets

Although the availability of GNLY<sup>+/−</sup> mice allowed us to use cytotoxic cells from these animals to study the role of granulysin in cell-mediated apoptosis, granulysin is normally expressed in human, but not in mouse, NK cells. Therefore, the observed mechanistic differences between NK cell-mediated lysis of murine targets and the previously described apoptosis of human tumor cells by recombinant 9-kDa granulysin may reflect species-specific differences in the target cells. To address this, we used human K562 and THP-1 cell lines as targets for WT and GNLY<sup>+/−</sup> effector cells. In a 3-h apoptosis assay, GNLY<sup>+/−</sup> NK cells, compared with WT effectors, showed marked increase in both K562 and THP-1 target cell death (Fig. 6A, 6B). Perforin is required for apoptosis of human target cells because WT and GNLY<sup>+/−</sup> NK cells, but not perf<sup>−/−</sup> or perf<sup>−/−</sup>GNLY<sup>+/−</sup> NK cells, caused apoptosis of K562 and THP-1 target cells (Supplemental Fig. 6A, 6B). Thus, cytotoxic cell-delivered granulysin requires perforin for apoptosis of both human and mouse target cells.

We next analyzed DNA fragmentation of human targets, using WT and GNLY<sup>+/−</sup> effector cells. GNLY<sup>+/−</sup> NK cells were much more effective at inducing DNA fragmentation in human targets than were WT NK cells (Fig. 6C, 6D), suggesting that some species-specific differences do exist. The pan-caspase inhibitor Q-VD-OPH almost completely inhibited DNA fragmentation in both target cells.

The role of ER stress in human target cell killing was also assessed. GNLY<sup>+/−</sup> NK cells induced a higher level of ER disruption in both K562 and THP-1 targets than did WT NK cells (Fig. 6E, 6F). Taken together, these findings indicate that the presence of perforin is critical for cell-delivered granulysin—facilitated cell death in either human or mouse target cells. Furthermore, apoptosis mediated by cell-delivered granulysin involves ER stress, DNA fragmentation, and apoptosis in both mouse and human target cells (Fig. 7).

Discussion

Granulysin is expressed by activated CTLs and NK cells and is present in cytolytic granules with perforin and granzymes (17). To date, our understanding of the apoptotic pathways activated by granulysin comes from in vitro assays using recombinant 9-kDa granulysin protein (24, 25, 27, 28). In the current study, we investigated the apoptotic pathways activated by granulysin delivered to target cells by effector cells. We crossed mice expressing granulysin with mice lacking perforin or granzyme B activated splenocytes from these animals to produce in vitro activated NK cells. In the absence of perforin, no lysis of targets cells was observed. However, granulysin could substitute for granzyme B to cause target cell death. Investigation of intracellular events following effector–target cell interaction revealed that NK cell-delivered granulysin and recombinant granulysin activate distinct pathways resulting in apoptosis: Recombinant granulysin activates apoptosis through mitochondrial damage and caspase-3, whereas cell-delivered granulysin induces cell death through ER stress and caspase-7.

The granulysin transgenic mice used for this work were generated using a bacterial artificial chromosome containing the human GNLY gene and its 5′ and 3′ flanking regions (23). Granulysin is expressed in leukocytes from transgenic mice with kinetics similar to what is found in PBMCs from humans: Granulysin is constitutively expressed in NK cells and, following activation through the TCR, appears in T lymphocytes 8–10 d after activation. This is significantly later than perforin and granzyme B, which are detectable by 2 d after activation (23).

CTLs and NK cells mediate target cell lysis by two independent pathways: exocytosis of preformed granules and ligation of death receptors on target cells with their respective ligands on effector cells (2–8). Both of these pathways lead to target cell apoptosis and membrane disruption. Membrane disruption is chiefly mediated by perforin, whereas both granzymes A and B are crucial for granule-mediated nucleolysis, with granzyme B being the main contributor (39). We found that IL-15–activated NK cells from perf<sup>−/−</sup> splenocytes did not induce apoptosis or DNA fragmentation regardless of whether they express granzyme B and/or granulysin. In contrast, our group previously reported that recombinant 9-kDa granulysin is able to enter and cause death of both murine and human tumor cells (16, 27). Those earlier experiments used 50–100 μM recombinant granulysin, implying that at high concentrations, granulysin can cause membrane damage and enter cells as predicted by its crystal structure (26). Although we do not know the actual concentration of granulysin in cytolytic granules, the concentration may not be high enough to cause membranolysis, thus requiring synergy with perforin.

Recombinant 9-kDa granulysin (50 μM) causes apoptosis of mammalian cells through a well-defined series of events: An initial
increase in cytosolic Ca\(^{2+}\) and a decrease in K\(^+\) trigger disruption of mitochondria and ROS production, which results in release of cytochrome c and AIF, leading to activation of caspase-3 and DNA fragmentation (24, 25, 27, 28). However, cytotoxic cell-delivered granulysin does not mediate additional mitochondrial damage, regardless of the presence of granzyme B. These findings indicate that, in the absence of other cytolytic molecules, micromolar granulysin is able to cause ionic imbalance and mitochondrial depolarization, but when granulysin is delivered in cytolytic granules, mitochondria are not the primary target.

NK cells expressing granulysin (in the presence or absence of granzyme B) mediate increased DNA fragmentation, as measured by TUNEL assay, and this DNA fragmentation is almost completely blocked by a pan-caspase inhibitor. Previously, we reported that recombinant 9-kDa granulysin activates caspase-3 (25). Effector cells from WT or GNLY\(^{+/+}\) animals activated both caspase-3 and caspase-9 with similar efficiency, as measured by Abs specific for the cleaved forms of each caspase. These data again suggest that under different experimental conditions, granulysin activates different apoptotic pathways. A similar dichotomy was reported for granzyme B. Addition of purified murine granzyme B to cells activates apoptosis through caspase-3 (11, 40), whereas delivery of granzyme B by cytotoxic cells activates both caspase-3 and Bid cleavage. However, in target cells lacking Bid, murine granzyme B\(^+\) CTLs did not elicit any cytochrome c release, whereas in targets lacking caspase-3, murine granzyme B\(^+\) CTLs caused normal cell death. These findings indicate that 1) exogenous granulysin B activates different pathways than does granulysin B delivered by CTLs, and 2) as yet undetermined pathways are involved when granulysin B is delivered through CTL granules (41).

We observed that NK cells lacking granzyme B were less efficient than WT NK cell at activating both caspase-3 and caspase-9, but there was no change in caspase-3 or caspase-9 activation when NK cells from gzmb\(^{-/-}\)GNLY\(^{+/+}\) animals were tested, indicating again that granulysin does not activate caspase-3 or caspase-9. Pardo et al. (42) reported that ex vivo virus-immune CD8\(^+\) cells from gzmb\(^{-/-}\) animals do not induce activation of caspase-3 or caspase-9 in target cells.

Granulysin delivered by effector cells causes an increase in DNA fragmentation that is blocked by pan-caspase inhibitors but does not involve mitochondrial damage or activation of caspase-3. Therefore, we sought to identify alternative apoptotic pathways activated by granulysin. Caspase-7 is another executioner caspase that is closely related to caspase-3, with highly similar structure and function (34). We observed that NK cells expressing granulysin in the absence of granzyme B mediated enhanced activation of caspase-7, implicating it in granulysin-induced cell-mediated apoptosis. It has been reported that caspase-7 is localized to the ER along with other ER chaperones and is involved in ER stress. In the ER, GRP78 forms a complex with caspase-7 and caspase-12, preventing release of caspase-12 from the ER. In ER stress, caspase-7 associates and activates caspase-12, resulting in increased cell death (36, 37). We found that gzmb\(^{-/-}\)GNLY\(^{+/+}\) NK cells induce more ER stress and cleavage of caspase-7 than do gzmb\(^{-/-}\) effectors. We also observed enhanced activation of caspase-12 in YAC-1 cells after incubation with gzmb\(^{-/-}\)GNLY\(^{+/+}\) NK cells. GRP78 blocks caspase-7 activation, and reduced expression of GRP78 leads to enhanced cell death under ER stress conditions (36, 38). We also detected reduced expression of GRP78 and enhanced expression of activated caspase-7 in targets incubated with gzmb\(^{-/-}\)GNLY\(^{+/+}\) NK cells. The results obtained with the cell-based cytotoxicity assay suggest that granulysin triggers ER but not mitochondrial stress, resulting in the activation of the caspase-7 pathway, leading to DNA fragmentation and cell death. In contrast, recombinant 9-kDa granulysin causes mitochondrial but not ER damage, indicating that the context of granulysin delivery is central to the apoptotic pathways activated.

The effector cells used in our study contain murine perforin and granzymes along with human granulysin, giving rise to potential issues of species-specific substrates. Previously, we showed that recombinant 9-kDa granulysin causes apoptosis in both mouse and human targets with similar efficiency (16, 25). Recently Rosen and colleagues (43) investigated the substrate specificities of human and mouse granzyme B. The primary sequence of these two molecules is well conserved, but the substrate specificities differ significantly. Human and mouse granzyme B cleave species-specific procaspase-3 more efficiently than the unmatched substrate. Moreover, human granzyme B efficiently cleaves human or mouse Bid, but neither human nor mouse Bid is cleaved by mouse granzyme B. To begin to address potential species-specific differences in our system, we used the human lymphoid cell lines K562 and THP-1 as targets for the mouse NK cells. In support of our
findings using murine target cells, we found that murine NK cells expressing granulysin caused significantly more apoptosis, DNA fragmentation, and ER stress than did NK cells without granulysin. Similar to mouse targets, DNA fragmentation in human target cells induced by effector cells (with or without granulysin) is largely caspase dependent.

In conclusion, to our knowledge, this study is the first investigation of the intracellular mechanisms by which granulysin induces cell death during the cytotoxic cell–target cell interaction. Granulysin activates an apoptosis pathway that involves ER stress and caspase-7, a series of events that are distinct from those activated by recombiant 9-kDa granulysin (Fig. 7). In contrast, granzyme B activates a pathway that involves mitochondrial damage and caspase-3 and caspase-9 activation (Fig. 7). Activation of multiple nonredundant pathways by different molecules delivered by cytolytic effector cells may enable the host defense system to counteract the multiple escape mechanisms employed by pathogens or tumor cells.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Colocalization of granulysin and granzyme B in \(GNLY^{+/−}\) NK cells. NK cells were isolated from IL-15 activated splenocytes by negative selection using MACS isolation kits. Nuclei were stained with DAPI shown in blue, cells were stained with anti-9 kDa granulysin antibody (green) (left panel) and with anti-mouse granzyme B antibody (red, center panel) (R & D systems). The two images were then superimposed (right panel) to show colocalization of granulysin and granzyme B (yellow). Data are representative of three independent experiments.
Supplemental Figure 2. Flow based killing assay of EL4 and Fas resistant EL4.F15 cells. IL-15 activated splenocytes were incubated with EL4 (A) and EL4.F15 (B) cells for 4 hours at various E:T ratios. 7-AAD incorporation was assayed in CFSE-labeled target cells by flow cytometry. *p < 0.05, **p < 0.005. Results are the mean of three independent experiments. Error bars represent SEM.
Supplemental Figure 3. Signaling through Fas does not affect mitochondrial polarization and ROS generation in target cells. EL4 and EL4.F15 cells were incubated with effectors for 2 hours at a 20:1 E:T ratio and ΔΨᵣ loss (A and C) and ROS generation (B and D) were analyzed by staining cells with DiOC₆(3) or 2-HE, respectively, and analyzed by flow cytometry after gating on DDAO-SE+ cells. Data presented are the mean of at least three independent experiments ± SEM.
Supplemental Figure 4. Granulysin delivered by NK cells disrupts ER. A and C, EL4 and Fas resistant EL4.F15 cells were incubated with either wild type or GNLY<sup>−/−</sup> cytotoxic cells and ER disruption was measured by BFA BODIPY staining. B and D, EL4 and EL4.F15 cells cells were incubated with either medium (grey histograms), thapsigargin (10 μM) or 9 kDa granulysin (25 μM) and ER disruption was measured by BFA BODIPY staining. BFA BODIPY<sub>low</sub> cells are indicated by the horizontal bars and the percent is shown by number. *p < 0.05, **p < 0.005. Data presented are the average of three independent experiments ± SEM.
**Supplemental Figure 5.** Cytotoxic cell delivery of granulysin reduces expression of GRP78 but not CHOP/GADD153. YAC-1 targets were incubated with the indicated effector cells for 1 h and stained for intracellular expression of GRP78 (A) and CHOP/GADD153 (B) (Santa Cruz Biotechnology). *p < 0.05. Results are expressed as the mean ± SEM for three independent experiments.
Supplemental Figure 6. Cytotoxic cell delivered granulysin requires perforin for lysis of human target cells. IL-15 activated splenocytes from WT, GNLY^{+/−}, perf^{−/−} and perf^{−/−} GNLY^{+/−} were incubated with K562 (A) and THP-1 (B) target cells at 10:1 E:T ratio for 3 h. 7-AAD incorporation was assayed in CFSE-labeled target cells by flow cytometry. Data presented are the mean of at least three independent experiments ± SEM.