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Granulysin Induces Cathepsin B Release from Lysosomes of Target Tumor Cells to Attack Mitochondria through Processing of Bid Leading to Necroptosis

Honglian Zhang, Chao Zhong, Lei Shi, Yuming Guo, and Zusen Fan

Granulysin is a killer effector molecule localized in cytolytic granules of human NK and CTL cells. Granulysin exhibits broad antimicrobial activity and potent cytotoxic action against tumor cells. However, the molecular mechanism of granulysin-induced tumor lysis is poorly understood. In this study, we found that granulysin causes a novel cell death termed necroptosis. Granulysin can target lysosomes of target tumor cells and induce partial release of lysosomal contents into the cytosol. Relocalized lysosomal cathepsin B can process Bid to active tBid to cause cytochrome c and apoptosis-activating factor release from mitochondria. Cathepsin B silencing and Bid or Bax/Bak deficiency resists granulysin-induced cytochrome c and apoptosis-activating factor release and is less susceptible to cytolysis against target tumor cells. The Journal of Immunology, 2009, 182: 6993–7000.

Granulysin (GNLY) is a killer effector molecule localized in cytolytic granules along with a pore-forming protein, perforin (1, 2). GNLY has two major forms, 15 and 9 kDa. The 9-kDa form is processed from the larger precursor 15-kDa form and only the 9-kDa GNLY exerts cytolytic activity (3). GNLY is a member of the saposin-like protein family of lipid-binding proteins that includes amoeboapores, saposins, and NK lysin (4–6). GNLY exhibits broad-spectrum antimicrobial activity against both Gram-positive and -negative bacteria, fungi, and parasites (7–9). Recent reports showed that cytotoxic CD8+ T cells and CD4+ T cells use GNLY, rather than perforin, to kill Cryptococcus neoformans (10, 11).

Besides its antimicrobial function, GNLY also harbors potent cytotoxic activity against tumor cells (12). GNLY can lyse Jurkat cells with apoptotic features in vitro. This apoptosis is associated with an increase of intracellular calcium and a decrease of intracellular potassium. Increasing intracellular calcium causes mitochondrial damage to release cytochrome c (cyt c) and apoptosis-inducing factor (AIF) leading to mitochondria-dependent cell death (13–17). Mitochondria-deficient RBC were not lysed by GNLY even at a very high concentration. Krensky and colleagues (18) generated GNLY-transgenic mice and exhibited their significant protection against lethal tumor challenge. However, the molecular mechanism of GNLY-induced tumor lysis is less defined.

Lysosomes have been considered to be solely involved in necrotic and autophagic cell death for many years. Likewise, lysosomal proteases were believed to be limited to nonspecific intracellular protein degradation in the lysosome. However, recent accumulating evidence strongly demonstrated that lysosomes participate in the induction of apoptosis beyond that of simple “garbage disposals.” A complete destruction of lysosomes with release of high concentrations of lysosomal enzymes into the cytosol results in unregulated necrosis, whereas partial, sequential permeabilization triggers programmed cell death (PCD) (19). Several lysosomal proteases known as cathepsins, were released into the cytosol of target cells as a result of moderate lysosomal disruption (20, 21). Cathepsin B (Cath B) from lysosomes enhances mitochondrial release of cyt c and subsequent caspase activation in TNF-treated hepatocytes (22). Cathepsin D triggers Bax activation resulting in selective AIF release in T lymphocytes in the early commitment phase to apoptosis. An important cytotoxic factor, Bid, was reported to be cleaved by some cathepsins and translocated to the mitochondria following lysosomal disruption by lysosomotropic agents (23). Thus, a lysosomal pathway is connected with a mitochondrial pathway to initiate apoptosis through these Bcl-2 family proteins. In this study, we found that GNLY can target lysosomes of target tumor cells and induce partial release of lysosomal contents. Relocalized lysosomal Cath B can process Bid to active tBid to cause cyt c and AIF release from mitochondria. Bid or Bak/Bax deficiency resists GNLY-induced cyt c and AIF release and necroptosis.

Materials and Methods

Cell lines, Abs, and reagents

Jurkat cells were grown in RPMI 1640 and HeLa cells in DMEM supplemented with 10% FCS, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. Mouse embryo fibroblasts (MEFs) were cultured in DMEM supplemented with 10% FCS, 100 μM asparagine, and 50 μM 2-ME. Bid wild-type (WT) MEFs and Bid knockout MEFs were provided by Dr. A. Strasser (The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia) and Bax/Bak double knockout MEFs were provided by Dr. Q. Chen (Institute of Zoology, Chinese Academy of Sciences, Beijing, China). Human PBMC and RBC were obtained from healthy donors.

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volunteers and isolated by a lymphocyte separation kit. Commercial Abs were goat polyclonal to Cath B (Santa Cruz Biotechnology) for confocal and mouse anti-Cath B (Millipore) for Western blotting, rabbit polyclonal to LAMP-1 (Abcam), mouse anti-cyt c (BD Pharmingen), rabbit anti-AIF (BD Pharmingen), monoclonal anti-β-actin (Sigma-Aldrich), HRP-conjugated sheep anti-mouse IgG and HRP-conjugated sheep anti-rabbit IgG (Santa Cruz Biotechnology), Alexa Fluor 488-conjugated donkey anti-mouse IgG (Molecular Probes), and Alexa Fluor 594-conjugated donkey anti-rabbit IgG (Molecular Probes). Rabbit antiserum against Bid was provided by Dr. A. Krensky (Stanford University, Stanford, CA). Propidium iodide (PI), acridine orange (AO), and Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride were obtained from Sigma-Aldrich. Mitotracker was from Molecular Probes. Z-FA-fmk and FITC-annexin V were purchased from BD Pharmingen.

**Expression and purification of recombinant GNLY**

pET28a-GNLY (a gift from A. Krensky) was expressed in Escherichia coli BL21-AI and the soluble supernatant was purified on a nickel column. Then the His tag was removed by thrombin treatment. Reverse-phase HPLC was used to further purification. After lyophilization, the protein was suspended in PBS. GNLY concentration was determined by the bicinchoninic acid assay (Pierce).

**GNLY-mediated lysis of Salmonella**

Lysis of Salmonella was measured as previously described (24). Briefly, a log-phase culture of Salmonella (OD600 = 0.5) was diluted to 2 × 10^7/ml in 10 mM sodium phosphate buffer (pH 7.4) containing 0.03% Luria-Bertani broth. Twenty-five-microliter aliquots of Salmonella were incubated with 25 µl of different doses of recombinant GNLY at 37°C for 3 h. The mixture was diluted 100-fold with the cold phosphate buffer and plated on Luria-Bertani plates that were incubated at 37°C overnight. Bacterial colonies were counted.

**Hemolytic assay**

RBC of healthy volunteers were washed three times with PBS buffer. The washed RBC were suspended in PBS. Two percent RBC (v/v) were treated with GNLY at the indicated concentrations at 37°C for 4 h. H2O-treated RBC were used as a positive control of complete hemolysis. Supernatant absorbance at 540 nm was measured for determination of hemolysis.

**Electron microscopy**

HeLa cells (1 × 10^6) were treated with GNLY at 37°C for 6 h. Cells were washed twice with PBS and fixed with 2% glutaraldehyde at 4°C for 1 h and postfixed with 2% osmium tetroxide. Cells were dehydrated with sequential washes in 50, 70, 80, 90, and 100% ethanol and then embedded in Spurr’s resin. Ultrathin sections were obtained, mounted in copper grids, counterstained with uranyl acetate and lead citrate, and examined in a JEM-100 CX transmission electron microscopy.

**DNA fragmentation assay**

Jurkat (5 × 10^5) cells were treated with different doses of GNLY at 37°C for 6 h. Genomic DNA was extracted and analyzed by agarose gel electrophoresis.

**DNA nicking assay**

Jurkat or HeLa cells (2 × 10^5) were incubated with 50 µM GNLY at 37°C for 6 h. The cells were harvested and washed twice with ice-cold PBS and resuspended in lysis buffer (20 mM Tris (pH 7.5), 0.5% Nonidet P-40, 25 mM KCl, and 5 mM MgCl2). The nuclear pellet was harvested and incubated with 5 U of a Klenow fragment of DNA polymerase I and 10 µCi of [32P]dATP at 37°C for 1 h. The nuclei were then washed completely and deproteinized for alkaline gel electrophoresis.

**Laser-scanning confocal microscopy**

HeLa cells were grown overnight to subconfluency at 37°C in 8-well chamber slides coated with rat collagen I (BD Falcon). Cells were treated with 50 µM GNLY for the indicated times, washed, and fixed with 4% paraformaldehyde for 30 min at room temperature and permeabilized with 0.1% Triton X-100 for another 10 min, and the cells were incubated at room temperature for 1 h with anti-GNLY mAb and anti-LAMP-1 pAb (for Cath B release, incubated with anti-Cath B). The cells were further stained with Alexa Fluor 488-conjugated donkey anti-mouse IgG and Alexa Fluor 594-conjugated donkey anti-rabbit IgG. WT or BID−/− MEFs were grown overnight to subconfluency and incubated with 500 nM MitoTracker (Molec-

**FIGURE 1.** Recombinant GNLY possesses killing activity against Salmonella and target tumor cells. A. Purified recombinant GNLY protein was detected by Coomassie brilliant blue staining and Western blotting. B. Recombinant GNLY exhibits lytic activity against Salmonella. Bacterial colonies were counted after treatment by different doses of GNLY. C. Recombinant GNLY did not induce hemolysis of human RBC. Two percent RBC (v/v) were treated with GNLY at the indicated concentrations at 37°C for 4 h, and H2O-treated cells were used as 100% hemolysis. D. GNLY showed similar killing activity against Jurkat and HeLa cells. Human PBMC were isolated from healthy human peripheral blood by a lymphocyte separation kit. Jurkat cells, HeLa cells, and PBMC were treated with different doses of GNLY for 6 h and stained by annexin V and PI. Data are representative of at least four separate experiments and are shown as mean ± SD.

**Cellular Cath B activity assay**

Cath B activity was measured as described elsewhere (25). Briefly, Jurkat cells were pretreated with the inhibitor Z-FA-fmk for 30 min before GNLY treatment. The treated cells were incubated with an extraction buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, and 1 mM pefabloc, pH 7.5) containing 15 µg/ml digitonin for 15 min on ice. The total cellular Cath B activity was obtained by the extraction buffer containing 200 µg/ml digitonin. The supernatants were added to 1 vol of 100 µM Z-RR-AMC in cathepsin reaction buffer (50 mM sodium acetate, 4 mM EDTA, 8 mM DTT, and 1 mM pefabloc, pH 6.0). The mixture was incubated at 37°C for 1 h. Vmax of the liberation of amido-4-methylcoumarin hydrochloride (AMC) was measured through a fluorescence spectrophotometer F-4500.

**Cleavage assay**

GNLY-treated HeLa cells or Jurkat cells were lysed with 0.5% Nonidet P-40 lysis buffer and probed by anti-Bid Ab or caspase 3 for immunoblotting. In brief, 0.5 µM BH3 was incubated at 37°C for 2 h with different concentrations of Cath B in 20 µl of cleavage buffer (50 mM sodium acetate, 4 mM EDTA, and 8 mM DTT, pH 7.0).

**Flow cytometry analysis**

Cells treated with GNLY were stained with annexin V and PI or PI alone and analyzed by flow cytometry using a FACSCalibur (BD Biosciences).
FIGURE 2. GNLY induces necroptosis with ssDNA nicks. A and B, HeLa cells were incubated with 50 μM GNLY or 10 μg/ml STP or 20 mM H₂O₂ at 37°C for 6 h and detected by a high-magnification transmission electron microscopy (X1700) (A) or stained by PI and followed by confocal microscopy (B). C, GNLY does not cause dsDNA fragmentation. Jurkat cells were treated with 50 μM GNLY or 10 μg/ml STP or 20 mM H₂O₂ at 37°C for 6 h. Genomic DNA was extracted and visualized by 2% agarose gel electrophoresis with ethidium bromide staining. D, GNLY triggers ssDNA nicks. The nuclei of the above treated Jurkat cells were extracted and radiolabeled through Kleinow DNA polymerase. The DNA nicks were detected by denaturing alkaline gel electrophoresis. E, Caspase 3 was less processed by GNLY. Jurkat cells (2 × 10⁴) were treated as above and probed with anti-caspase 3 serum. β-Actin was unchanged as a loading control. F, Jurkat cells (2 × 10⁴) were incubated with 50 μM GNLY or 10 μg/ml STP or 20 mM H₂O₂ at 37°C for the indicated times and stained by annexin V and PI. Data are representative at least three separate experiments.

Cath B silencing, Bid silencing, and overexpression of Bcl-2

HeLa cells were transfected with pSUPER-siBid or pSUPER-LacZi plasmid (a gift from Dr. X. Jing, Cornell University Weill Graduate School of Medical Sciences, New York, NY) for Bid silencing control. pEF-Bcl-2 or pEF vector (a gift from Dr. A. Strasser, The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia) was transfected for Bcl-2 overexpression. RNA sequences against Cath B for RNA interference were designed based on pSUPER system instructions (Oligoengine) and cloned into pSUPER-puro. Cath B short hairpin RNA (shRNA)-encoding sequences were as follows: sense, 5'-H11032-GAGCTACTTGAAGAGGCTA-3' and antisense, 5'-TAGCCTCTTCAAGTAGCTC-3'. HeLa cells were transfected by using Lipofectamine 2000 (Invitrogen) as described. Cath B silenced, Bid-silenced, or Bcl-2 overexpressed stable cell lines were selected with puromycin.

Results

Recombinant GNLY possesses killing activity against Salmonella and target tumor cells

Recombinant 9-kDa GNLY was expressed in E. coli and purified by nickel affinity chromatography. The purified GNLY was determined with Coomassie brilliant blue staining and Western blotting (Fig. 1A). The recombinant GNLY showed strong killing activity to Salmonella in a dose-dependent manner (Fig. 1B), which is consistent with a previous report (25). Ten micromolar GNLY can completely inhibit the growth of 1 × 10⁶/ml Salmonella. In contrast, GNLY lacked lytic activity against RBC, even with a high dose of 50 μM GNLY for 4 h (Fig. 1C). The results were in agreement with a previous observation (26). We also found the lytic effects of GNLY to human PBMC were comparable to the RBC (Fig. 1D). Previous studies demonstrated that GNLY induces cytolysis of Jurkat cells (13). In this study, we showed that GNLY can also kill HeLa cells with similar dynamics of those for Jurkat cells (Fig. 1D).

GNLY triggers necroptosis with ssDNA nicks

A previous report showed that GNLY induces nuclear morphology of typical apoptosis through staining with oxidized p-phenylene diamine (14). However, we found that GNLY-treated HeLa cells showed incomplete, lumpy chromatin condensation (Fig. 2A). These observed effects are consistent with cell death. PI staining exhibited similar morphological nuclear changes as shown in Fig.
These observations are unlike caspase-dependent strong chromatin compaction to crescent-shaped masses (27). Staurosporine (STP)-treated HeLa cells showed strong chromatin condensation as the apoptotic control (Fig. 2A and B). Twenty micromolar H$_2$O$_2$-treated cells had no apparent nuclear compaction as the necrotic control. To determine the form of DNA damage in GNLY-induced death, genomic DNA was extracted from GNLY-treated cells. H$_2$O$_2$-treated cells did not show DNA ladders, while STP-treated cells exhibited typical DNA fragmentation. The DNA polymerase Klenow labeling was used to assess ssDNA breaks as previously described (28). GNLY caused increased [$^{32}$P]dATP incorporation and triggered ssDNA nicks (Fig. 2D). Although STP and H$_2$O$_2$ caused weak [$^{32}$P]dATP incorporation, STP-induced incorporation appeared to be DNA ladders. Caspase 3 activation is a key event in the induction of apoptosis. A previous report showed that caspase 3 was activated in GNLY-mediated cell death (15). However, we found that GNLY caused little caspase 3 processing (Fig. 2E). Most caspase 3 was activated in STP-treated cells, while H$_2$O$_2$ did not activate caspase 3. To further characterize the death features of GNLY-induced death, phosphatidylserine externalization was detected by using annexin V-FITC and PI staining. Phosphatidylserine externalization is an early hallmark of apoptosis. We found that GNLY-treated cells were both annexin V and PI positive even in early times (Fig. 2F). Most of the STP-treated cells were annexin V positive but PI negative. H$_2$O$_2$-treated cells caused annexin V and PI double staining as expected for the necrotic control. Taken together, GNLY induces necroptosis with ssDNA nicks.

**GNLY targets lysosomes of target tumor cells**

GNLY is a saposin-like protein and interacts with lipids to cause the disruption of membranes. Lysosomes are an organelle with lipid membranes. To determine whether GNLY enters into lysosomes of target cells, HeLa cells were treated with 50 μM GNLY or STP and probed by anti-Cath B Ab. GNLY staining green fluorescence is shown on the left, LAMP-1 staining red in the middle, and the merged image on the right. LAMP-1 staining green fluorescence is shown on the left, LAMP-1 staining red in the middle, and the merged image on the right.

**FIGURE 3.** GNLY induces lysosomal membrane permeabilization. A, GNLY can target lysosomes of target cells. HeLa cells were treated with 50 μM GNLY for 6 h and probed with anti-GNLY mAb and anti-LAMP-1 rabbit Ab, followed by staining with the second Alex Fluor 488-conjugated donkey anti-mouse Ab and Alex Fluor 594-conjugated donkey anti-rabbit Ab. GNLY staining green fluorescence is shown on the left, LAMP-1 staining red in the middle, and the merged image on the right. B, GNLY can trigger release of lysosomal contents into the cytosol. HeLa cells were incubated with AO and treated with 50 μM GNLY for 6 h. AO shows green (nucleus and cytosol; left) and red (lysosome; middle). The merged image is on the right.

**FIGURE 4.** GNLY induces gradual release of Cath B from lysosomes. A, GNLY induces Cath B release to the cytosol. HeLa cells were treated with 50 μM GNLY or STP and probed by anti-Cath B Ab. B, Digitonin (15 μg/ml) did not induce Cath B relocation. HeLa cells were treated with 15 μg/ml digitonin or mock treated and probed by anti-Cath B Ab. C, Cytosolic Cath B activity induced by GNLY is dose dependent. Jurkat cells (2 × 10$^5$) were treated with different doses of GNLY at 37°C for 6 h. The Cath B activity in the supernatant was detected by a fluorescence spectrophotometer. Two hundred micrograms of digitonin per ml of lysis buffer was used to lyse cells as total release. The protease substrate pan inhibitor Z-FA-fmk can block Cath B activity. D, GNLY induced gradual release of Cath B from lysosomes. Jurkat cells (2 × 10$^5$) were treated with 50 μM GNLY or STP at 37°C for the indicated times and analyzed Cath B activity (left) or the treated cells were stained with annexin V and PI for death analysis (right). These data represent at least four separate experiments.
target cell after treatment by GNLY. STP did not cause the release of Cath B (Fig. 4A). Similar results were obtained for cathepsin L (data not shown). It indicates that GNLY initiates the release of lysosomal contents into the cytosol of target cells.

To quantitate the extent of lysosomal permeabilization, we measured the activity of Cath B in GNLY-treated cytosolic fractions through using the fluorescence substrate Z-RR-AMC. We detected that 15 μg/ml digitonin extraction buffer was an optimal concentration for permeabilizing cell membranes without disruption of lysosomes (Fig. 4B). Approximately 10% of Cath B was released from the lysosome to the cytosol after GNLY treatment for 2 h. Cytosolic Cath B activity increased over time. About half of Cath B was released to the cytosol for 6 h or even longer. The cytosolic Cath B activity was measured in STP-treated cells as a control. Although the rates of cell death induced by 10 μg/ml STP and 50 μM GNLY were almost similar, the cytosolic Cath B activity treated by STP for 6 h was still very low (Fig. 4D). The release of Cath B from lysosomes induced by GNLY was in a dose-dependent manner (Fig. 4C). Cysteine protease pan inhibitor Z-FA-fmk (100 μM) completely blocked Cath B activity. These confirm that GNLY triggers release of Cath B into the cytosol of target cells.

**FIGURE 5.** Bid can be processed by Cath B. A and B, Bid is cleaved in GNLY-treated target cells. HeLa cells or Jurkat cells (1 × 10⁶) were treated with different doses of GNLY for 6 h (A) or HeLa cells with 50 μM GNLY for the indicated times and probed with anti-Bid serum (B). β-Actin was unchanged as a loading control. C, Liver-purified Cath B can cleave Bid to tBid. In brief, 0.5 μM rBid was treated with the indicated concentrations of Cath B at 37°C for 2 h in a neutral pH buffer. D, Z-FA-fmk can block Bid cleavage. HeLa cells were pretreated with 100 μM Z-FA-fmk for 1 h before GNLY treatment. β-Actin was probed as a loading control. E, Z-FA-fmk decreases TNF-α/CHX-induced death. Jurkat cells (2 × 10⁵) were pretreated with 100 μM Z-FA-fmk for 60 min before 100 ng/ml TNF-α along with 10 μg/ml CHX treatment and stained by annexin V and PI. Dead cells were calculated as double annexin V and PI and are shown as mean ± SD. *, p < 0.05. F, Z-FA-fmk decreases GNLY-induced death. Jurkat cells (2 × 10⁵) were pretreated with 100 μM Z-FA-fmk for 30 min before GNLY treatment and stained by PI. The data represent at least four separate experiments. *, p < 0.05.

**FIGURE 6.** Cath B silencing reduces GNLY-mediated Bid processing and release of cyt c and AIF. A, Cath B silencing blocks GNLY-induced Bid processing. Cath B was silenced by transfection with pSUPER-Cath B-shRNA. β-Actin was a negative control. Cath B-silenced HeLa cells were treated with 50 μM GNLY for 6 h and probed with anti-Bid serum. β-Actin was unchanged as a loading control. B, Cath B silencing reduces GNLY-induced release of cyt c and AIF. The supernatants and pellets of target cells were separated after above treatment and detected by immunoblotting. C, Cath B silencing is resistant to GNLY-induced cell death. The above treated HeLa cells were stained by PI. PI-positive cells were calculated as mean ± SD. *, p < 0.05. The data represent at least four separate experiments.

Cath B can process Bid to active tBid

Once Cath B was released from the lysosomes into the cytoplasm, it may cleave some target proteins to enhance cell death. A report showed that Cath B can cleave Bid to tBid at neutral pH in vitro (30). To determine whether Bid was degraded by GNLY, Bid was probed in GNLY-treated HeLa cells by anti-Bid Ab for immunoblotting. Bid was cleaved in a dose-dependent manner. Similar results were obtained in Jurkat cells (Fig. 5A). The cleaved products were undetectable maybe due to its low concentration or the Ab sensitivity. Bid was processed obviously within 4 h by GNLY treatment (Fig. 5B). β-Actin was probed as a loading control. To confirm that Cath B can process Bid at a neutral pH, rBid was treated with different concentrations of liver-purified Cath B at 37°C for 2 h in a cleavage buffer (pH 7.0). Cath B can directly cut rBid to produce an active 14-kDa tBid (Fig. 5C).

To evaluate whether released Cath B from the lysosome processes Bid, HeLa cells were pretreated with 100 μM Z-FA-fmk for 30 min before incubation with GNLY. The cysteine protease pan inhibitor Z-FA-fmk blocked Bid degradation (Fig. 5D). One hundred micromolar Z-FA-fmk can partially inhibit TNF-α/cycloheximide (CHX)-induced cell death (Fig. 5E), which is in agreement with a previous report (31). To determine whether Cath B contributes to GNLY-induced death, Z-FA-fmk-pretreated HeLa cells were incubated with GNLY and followed by flow cytometry. Z-FA-fmk significantly blocked GNLY-induced death (37.24 ± 3.2% vs 57.03 ± 2.93%, p < 0.01; Fig. 5F).

Cath B silencing blocks GNLY-mediated Bid processing and release of cyt c and AIF

To confirm the role of Cath B in GNLY-mediated cell death, we designed shRNA sequences against Cath B and constructed pSUPER-Cath B-shRNA plasmid to silence Cath B expression. pSUPER-Cath B-shRNA stable cell lines were established by puromycin selection. Cath B was silenced as shown in Fig. 6A. β-Actin was
unchanged as a control. Cath B-silenced and vector control-transfected HeLa cells were treated with GNLY at 37°C for 6 h. Cath B silencing blocked Bid degradation (Fig. 6A). Meanwhile, we found that Cath B silencing abolished GNLY-induced cyt c and AIF release (Fig. 6B). Moreover, Cath B-silenced HeLa cells were more resistant to GNLY-induced cytolysis compared with the vector control transfection (dead cells: 32 ± 3.8% vs 52 ± 1.2%, \( p < 0.01 \); Fig. 6C). These results indicate that Cath B contributes to GNLY-mediated cell death.

**Bid knockout or Bax/Bak-deficient MEFs decrease GNLY-induced death**

To further determine whether Bid processing was associated with GNLY-induced death, Bid WT and knockout MEFs were treated with GNLY at 37°C for 1 h and stained with PI. Bid knockout MEFs were more resistant to GNLY-induced cytolysis. PI-positive cells in Bid WT and knockout MEFs were as follows (10 μM: 36.02 ± 1.58% vs 31.1 ± 1.53%; 25 μM: 49.89 ± 2.97% vs 37.37 ± 2.64%; and 50 μM: 62.64 ± 1.24% vs 43.29 ± 1.55%; \( p < 0.01 \)). Time course experiments showed similar results to the above dose observations (Fig. 7A).

We next measured cell death of WT and Bax/Bak-deficient MEFs with the above-described GNLY treatment against Bid-deficient MEFs. Bax/Bak-deficient MEFs were still resistant to GNLY-induced death compared with WT MEFs (PI-positive cells: 10 μM, 20.28 ± 4.25% vs 41.13 ± 4.56%; 25 μM: 27.49 ± 1.76% vs 47.56 ± 2.38%; 50 μM: 39.71 ± 1.63% vs 60.76 ± 1.08%; \( p < 0.01 \); Fig. 7B). Mock-treated cells obtained background death (<10%). Similar results were observed in a time course assay. These results demonstrate that GNLY-induced Bid processing utilizes the Bax/Bak pathway to target mitochondria.

**Bid silencing or deficiency or Bcl-2 overexpression reduces GNLY-mediated cyt c and AIF release from mitochondria**

To test whether Bid silencing affects GNLY-induced cyt c and AIF release, Bid was silenced in the human tumor cell line HeLa cells by transfection of pSUPER-siBid or a control nonsense LacZ sequence plasmid pSUPER-LacZi. Bid-silenced stable cell lines were established by puromycin selection. Bid was almost undetectable through immunoblotting (Fig. 8A). Bid-silenced and control vector-transfected HeLa cells were treated with GNLY at 37°C at different time points. GNLY induced less cyt c and AIF release in Bid-silenced HeLa cells compared with those with transfection of the control vector (Fig. 8A). Similar results were achieved in Bid knockout MEFs (data not shown). These results were further confirmed by laser-scanning confocal microscopy. GNLY induced more cyt c release in WT MEFs than in Bid knockout MEFs (Fig. 8B). These data indicate that Bid processing can accelerate cyt c and AIF release into the cytosol of target cells.

Bcl-2 is an antiapoptotic protein in the Bcl-2 family. Bcl-2 suppresses apoptosis primarily by the inhibition of cyt c release. We wanted to look at whether Bcl-2 overexpression reduces cyt c and AIF release with GNLY treatment. pEF-Bcl-2 and a control empty vector plasmid were separately transfected into HeLa cells. Bcl-2-overexpressed HeLa cell lines were obtained via transfection with pEF-FLAG-Bcl2 and selected by puromycin. Bcl-2-overexpressed HeLa cells were treated with 50 μM GNLY for 6 h and analyzed as above. The data represent at least three independent experiments.

**Discussion**

PCD plays a very important role in the development, maintenance of multicellular organisms, and elimination of virally infected or transformed cells. Caspases have long been known as an essential role in apoptosis. Apoptosis is a kind of classical PCD, whose typical feature is the nuclear morphological changes with chromatin condensation and apparently simple geometric morphology.
(globular, crescent shaped). During apoptosis, caspases, in particular caspase 3, are strongly activated and cause caspase-activated DNase activation resulting in dsDNA fragmentation. However, many studies reported that caspase inhibition does not prevent death-domain receptor-induced or granzyme A or K-induced cell death (32–34). Cell death induced by such conditions lacks the typical features of apoptosis, which is termed necroptosis (35).

In necroptosis, the nuclear morphological changes are not with obvious chromatin condensation, but with chromatin clustering to speckles. In this study, we found that GNLY-induced cell death as a type of necroptosis. GNLY-treated nuclei showed incomplete and lumpy chromatin condensation, but not strong chromatin compaction. On the other hand, although GNLY treatment also activates caspase 3, the level of caspase 3 activity was significantly lower than that in STP-treated cell death (Fig. 2E). We first defined the type of GNLY-induced death as necroptosis with ssDNA nicks, which is not consistent with a previous report for a typical apoptosis (14).

As reported, some necrotic PCD is often classified as “aborted apoptosis” that is initiated by a standard apoptosis program, then limited at the level of caspase activation and finally replaced by caspase-independent routes (36). Additionally, increasing Ca2+ and reactive oxygen species are also important inducers of necrotic PCD. A previous report showed that GNLY induces apoptosis of Jurkat cells associated with a dramatic increase of the ceramide: sphingomyelin ratio (14). Caspase pan inhibitor Z-VAD-fmk or ceramide synthase inhibitor cannot block GNLY-induced apoptosis. This suggests that GNLY induced death is through ceramide-independent or caspase-independent cell death pathways. An increased Ca2+ level in the cytoplasm precedes depolarization of mitochondria (13). Increasing Ca2+ triggers loss of mitochondrial potential and release of cyt c and AIF. However, procaspase 9 is not processed to its active form in GNLY-induced death. Therefore, GNLY induces a novel death pathway whose mechanism is poorly understood.

GNLY belongs to the saposin-like protein family of lipid-binding proteins that interacts with cell membranes of target cells. Recently, Ziegler and colleagues (37, 38) found that the cholesterol content is very important for GNLY to destroy intracellular bacteria without damaging the infected cells. The content of cholesterol in the cell membrane of bacteria is low; therefore, it is very susceptible to GNLY. Another study (39) reported that cholesterol is enriched in the plasma membrane and in membranes of early endocytic organelles but it is nearly completely absent in inner lysosomal membranes. Surprisingly, we found that GNLY can enter into lysosomes and induce lysosomal contents release into the cytosol of target cells. The release of lysosomal contents is limited and partial. This result is consistent with the dynamic process of GNLY-induced necroptosis.

Lysosomal cystine proteases termed cathepsins represent the largest group of proteolytic enzymes in the lysosomes. Cath B and cathespin L are the most abundant in the lysosomes. Cathepsins have been implicated in the induction of apoptosis (40). Cath B-deficient hepatocytes are resistant to TNF-mediated apoptosis and mitochondrial release of cyt c. Cath B participates in caspase-independent cell death of WEHI-S cells (31). Bid activation may be a direct link between cathepsins and mitochondrial damage. Bid can be processed to active form by Cath B and cause cyt c release from mitochondria (19). Moreover, Cath B is more stable at physiological pH and less effectively inhibited by the major endogenous cysteine inhibitor cystatin B. Therefore, Cath B might play a critical role in lysosome-mediated Bid activation. In this study, we found that Cath B releases into the cytosol of target cells and Bid processing occurs with GNLY treatment. The cysteine protease pan inhibitor Z-FA-fmk can block Bid cleavage in GNLY-treated target cells, leading to a decrease in cell death. Moreover, Cath B silencing blocks Bid processing and releasing of cyt c and AIF from the mitochondria. Cath B silenced HeLa cells are resistant to GNLY-induced cell death. These data suggest that Bid may be processed by Cath B to initiate mitochondrial dysfunction.

Proapoptotic Bcl-2 proteins such as Bax/Bak induce a selective process of outer membrane permeabilization of mitochondria through the formation of channels or pores that allow the selective release of proteins soluble in the inner membrane space like cyt c. We found that Bid knockout or Bax/Bak deficiency indeed resists release of cyt c and AIF to decrease GNLY-induced death. This indicates that GNLY-mediated mitochondrial damage is Bid dependent. Bid knockout or Bax/Bak deficiency partially lowers GNLY-induced death. These results imply that other pathways involve this process such as increase of intracellular Ca2+. In this study, we first demonstrated that GNLY can target lysosomes to release Cath B, leading to Bid cleavage. Bid activation may be a direct link between cathepsin release and mitochondrial dysfunction in GNLY-induced necroptosis.

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Disclosures

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References


