Granule-mediated cytolysis is the major pathway for killer lymphocytes to kill pathogens and tumor cells. Little is known about how granzyme K functions in killer lymphocyte-mediated cytolysis. We previously showed that human GzmK triggers rapid cell death independently of caspase activation with single-stranded DNA nicks, similar to GzmA. In this study we found that GzmK can induce rapid reactive oxygen species generation and collapse of mitochondrial inner membrane potential (ΔΨm). Blockade of reactive oxygen species production by antioxidant N-acetylcyesteine or superoxide scavenger Tiron inhibits GzmK-induced cell death. Moreover GzmK targets mitochondria by cleaving Bid to generate its active form tBid, which disrupts the outer mitochondrial membrane leading to the release of cytochrome c and endonuclease G. Thus, we showed herein that GzmK-induced caspase-independent death occurs through Bid-dependent mitochondrial damage that is different from GzmA.

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Granzyme K Targets Mitochondria in Bid-dependent Way

**MATERIALS AND METHODS**

**Cell Lines, Antibodies, and Reagents**—Cells were grown in RPMI 1640 (Jurkat) or Dulbecco’s modified Eagle’s medium (HeLa) supplemented with 10% fetal calf serum, 50 μM β-mercaptoethanol, and 100 μg/ml penicillin and streptomycin. Commercial antibodies were mouse monoclonal antibody against cyt c (BD Pharmingen), β-actin (Sigma-Aldrich), and CoxIV (Molecular Probes). Rabbit Bid and EndoG antisera were kindly gifts of Dr. X. Wang. DIOC6 (3) and HE were from Molecular Probes (Eugene, OR). NAC and Tiron were from Sigma. The Pro-Ject™ (P) protein transfection reagent kit was from Pierce, and Annexin V-fluorescein isothiocyanate was from BD Pharmingen.

**Loading GzmK with Pro-Ject, Adenovirus, or Perforin**—Cells were washed three times in Hank’s balanced salt solution and resuspended in loading buffer (Hanks’ balanced salt solution with 0.5 mg of BSA per ml, 1 mM CaCl₂, and 1 mM MgCl₂). HeLa cells or Jurkat cells (2 × 10⁵) in 50 μl of loading buffer were incubated at 37 °C for the indicated times with different concentrations of GzmK, S-AGzmK, or GzmB in 20-μl cleavage buffer (50 mM Tris-HCl, pH 7.5, 1 mM CaCl₂, 1 mM MgCl₂). The reaction products were boiled in SDS loading buffer before SDS-PAGE. For in vivo cleavage assay, cells loaded with GzmK and P were lysed with 0.5% Nonidet P-40 lysis buffer. The lysates were probed by immunoblotting.

**Mitochondrial Isolation**—Murine liver mitochondria were isolated as described (19). Briefly, the murine liver was homogenized in ice-cold mitochondrial isolation buffer (MIB containing 250 mM mannitol, 0.5 mM EGTA, 5 mM HEPES, 0.1% BSA, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.2), and the pellets were removed after centrifugation at 600 × g for 10 min at 4 °C. After centrifugation at 10,000 × g for 10 min at 4 °C, the mitochondria pellet was resuspended in 4 ml of MIB and loaded onto a continuous Percoll gradient that consisted of 30% Percoll, 225 mM mannitol, 25 mM HEPES, 0.5 mM EGTA, and 0.1% BSA (pH 7.2). The suspension/gradient was centrifuged at 40,000 × g for 1 h at 4 °C. The mitochondria were recovered from the brownish band at 1.10 g/ml with a pipette. Then the mitochondrial pellets were washed with MIB by centrifugation at 6300 × g for 10 min at 4 °C. The mitochondria were resuspended in the buffer containing 400 mM mannitol, 10 mM KH₂PO₄, 50 mM Tris-HCl (pH 7.2) with 1 mg/ml BSA.

**Cytochrome c Release**—Murine mitochondria were incubated with GzmK or GzmB in the presence or absence of recombinant Bid in a final volume of 50-μl reaction buffer (220 mM mannitol, 68 mM sucrose, 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) at 30 °C for 30 min. After incubation, the reaction mixture was centrifuged at 12,000 × g for 5 min. SDS buffer (5×) was added to the supernatants and the mitochondrial pellets. The samples were boiled for 5 min and loading onto the 12% SDS-PAGE for Western blot analysis using cyt c antibody.

**RESULTS**

GzmK Targets Mitochondria—We previously demonstrated GzmK induces rapid caspase-independent cell death with externalization of phosphatidylserine, nuclear morphological changes, and single-stranded DNA nicks (9). GzmK-induced death is through targeting all the three GzmA substrates of the SET complex, including SET, Ape1, and HMG2 (17, 20–22).
We compared delivery reagents for loading granzymes into the intact cells and found the cationic lipid reagent PJ can substitute for perforin or Ad to transport granzymes into target cells (9, 23). Granzymes loaded with PJ induce similar kinetics of death by loading with perforin or Ad. Mitochondrial damage is an initial step for apoptosis. One previous study showed rat GzmK induced an increase of ROS (10). We wanted to investigate whether GzmK provokes morphological changes in mitochondria. GzmK and S-AGzmK were produced with pET26b vector in *Escherichia coli* or *Pichia pastoris* system described in our previous report (9). 1 μM GzmK was loaded with PJ into HeLa cells at 37 °C for 4 h and visualized by electronic microscopy. GzmK plus PJ treatment induced profound mitochondrial swelling with loss of cristae structure (Fig. 1). The mitochondria of mock treated cells showed normal features with a condensed state and narrow cristae, identical to the mitochondria of cells treated with S-AGzmK plus PJ or GzmK alone (Fig. 1 and not shown).

**GzmK Causes Loss of ΔΨ**—Mitochondrial functions, including protein import, ATP generation, and lipid biogenesis, are dependent on the maintenance of ΔΨ (24). Loss of ΔΨ is likely to contribute to the death of cells (25). To assess whether GzmK causes ΔΨ collapse, Jurkat cells were treated with GzmK plus Ad and analyzed by FACS analysis. Cells loaded with GzmK plus Ad showed a dose-dependent decrease of ΔΨ, assayed by the change in fluorescence of the sensitive dye DiOC6, which targets to the negatively charged environment of the mitochondrial matrix at a low concentration in intact cells (Fig. 2A). 0.5 μM GzmK started to initiate reduction of ΔΨ within 1 h. Inactive S-AGzmK plus Ad or mock treated cells were without effect. An uncoupler of oxidative phosphorylation CCCP was used to treat cells as a positive control. GzmK-induced ΔΨ reduction also occurred in a time-dependent manner (Fig. 2B). 1 μM GzmK plus Ad initiated a dramatic decrease in ΔΨ within 1 h. By 4 h, ΔΨ was completely collapsed. GzmK and Ad alone or S-AGzmK plus Ad had little effect. GzmK-induced ΔΨ rupture requires enzymatic activity of GzmK.

**GzmK Triggers a Rapid Increase of Intracellular ROS**—ROS have been proposed as critical regulators of apoptosis (12, 26). Superfluous ROS cause mitochondrial damage as well as nuclear DNA damage. Mitochondria are the major source of intracellular ROS (27). ROS can be generated after damage to the mitochondrial inner membrane accompanying the permeability transition, which may be one route for releasing proapoptotic factors from the intramembrane space (28).

To detect ROS accumulation in GzmK-induced death, Jurkat cells were treated with GzmK plus Ad as measured by detection of the conversion of hydroethidine (HE) to fluorescent ethidium. 0.2 μM GzmK began to trigger ROS production within 50 min of treatment as seen by the increase in mean fluorescence intensity of the ROS indicator dye (Fig. 3A). GzmK augmented ROS generation with the increasing.

**FIGURE 1.** GzmK triggers mitochondrial swelling with loss of cristae structure. HeLa cells were incubated with 1 μM GzmK and PJ at 37 °C for 4 h and detected by a transmission electron microscopy. GzmK plus PJ-treated cells show distinct mitochondrial swelling with loss of cristae structure. The mitochondria of mock treated cells show normal features with a condensed state and narrow cristae, similar to the mitochondria of cells treated with S-AGzmK plus PJ. The normal and inflated mitochondria are indicated with arrows.

**FIGURE 2.** GzmK initiates loss of mitochondrial transmembrane potential. GzmK plus Ad induced dose- and time-dependent loss of mitochondrial ΔΨ. Jurkat cells were treated with the indicated doses of GzmK for 4 h (A) or 2 μM GzmK for the indicated times (B) in the presence of Ad (100 pfu/ml) at 37 °C. The classic uncoupler of oxidative phosphorylation carbonyl cyanide m-chlorophenylhydrazone (CCCP, 10 μM) was used as a positive control. Changes in mitochondrial transmembrane potential (ΔΨ) were determined by using 20 nM DiOC6 (3). These data are representative of at least three independent experiments.
concentrations. 2 \mu M GzmK reached a high peak for ROS production (mean fluorescence intensity, 86). ROS accumulate rapidly after GzmK treatment. Within 10 min, GzmK began to generate ROS, and ROS increased over time (Fig. 3B). GzmK, Ad alone, or S-AGzmK with Ad at the same time points all had comparable levels. \( \mathrm{H}_2\mathrm{O}_2 \) treatment was used as a positive control. To further verify ROS generation induced by GzmK, time-lapse microscopy was used to visualize ROS production at a single cell level. HeLa cells stained with 2',7'-dichlorofluorescin diacetate (DCF-DA) were treated with GzmK plus Ad, and followed by a time-lapse fluorescence microscopy. Images were captured every 2 min. 0 min and 42 min time intervals show the representative process of ROS increase (C). The curves represent the dynamics of ROS increase in the cells selected with arrows (D). The data are representative of three independent experiments.

Blockade of ROS Production Inhibits GzmK-induced Cell Death—We next wanted to detect whether reagents that react with oxidative intermediates can decrease GzmK-induced ROS. The antioxidant NAC or superoxide scavenger sodium 4,5-dihydroxybenzene 1,3-disulphonate (Tiron) pretreatment inhibited GzmK-induced ROS accumulation (Fig. 4A). This is consistent with the observations of GzmA by Martinvalet et al. (15). Even high concentrations of NAC and Tiron we used were nontoxic as determined for each reagent through staining by Annexin V and propidium iodide (not shown). To further determine the consequence of ROS on cell death, Jurkat cells were preincubated with 4 mM NAC or 50 mM Tiron before being loading with GzmK by Ad, and cell death was analyzed by Annexin V or Hoechst staining. NAC treatment can inhibit GzmK-induced death (Annexin V+ cells: 22 ± 2% versus 54 ±...
2.1\%, \; p < 0.01; \; \text{Hoechst staining: } 25 \pm 3.6\% \text{ versus } 70 \pm 5.1\%, \; p < 0.01 \) (Fig. 4B). Tiron treatment gave similar results (Annexin V+: cells: 22\% \pm 2\% \text{ versus } 54 \pm 2.1\%, \; p < 0.01; \; \text{Hoechst staining: } 25 \pm 3.6\% \text{ versus } 70 \pm 5.1\%, \; p < 0.01 \) (Fig. 4C). Similar results were found for propidium iodide staining (data not shown). Six control groups were used: GzmK, Ad, NAC, Tiron alone, NAC plus Ad, or Tiron plus Ad. These groups resulted in comparable cell death.

GzmK Directly Processes Bid to Produce Its Active Form tBid in Its Recombinant or Native Form of Lysates and Intact Cells—Bid is a BH3-only protein of the Bcl-2 family that plays an important role in mitochondria-dependent apoptosis. Undergoing apoptosis, Bid can be processed to a truncated Bid (tBid) that potently attacks the outer mitochondrial membrane to initiate release of cyt c and other proapoptotic factors (19, 29). To investigate whether Bid is processed by GzmK, 0.5 \mu M rBid was incubated with different concentrations of GzmK and probed by anti-Bid antibody. Bid began to degrade at 0.5 \mu M GzmK (Fig. 5A). The full-length Bid was completely processed at a high concentration of 2 \mu M GzmK. By 30 min, Bid started to degrade after GzmK treatment and was completely hydrolyzed within 4 h. Even with high concentration of 2 \mu M S-AGzmK, Bid was uncleaved. To assess whether GzmK degrades native Bid, Jurkat cell lysates (2 \times 10^5 cell equivalents) were incubated with different doses of GzmK. Bid was degraded by GzmK in a dose-dependent fashion (Fig. 5B). The same blot was stripped and probed with \beta-actin. \beta-Actin was unchanged as a negative control. After 2 h, Bid was dramatically degraded by 2 \mu M GzmK and almost completely processed by 4 h. Similar results were obtained in HeLa cells (data not shown). To further verify Bid processing is physiologically relevant, Jurkat cells were treated with 1 \mu M GzmK in the presence of PJ. Cytosolic Bid degradation occurred after 2 h (Fig. 5C). tBid production increased over time. The size of tBid produced by GzmK was similar to that processed by GzmB. Cells loaded with GzmB showed stronger enzymatic activity for Bid cleavage than GzmK. S-AGzmK was without effect. \beta-Actin was unchangeable at all time points as a loading control.

GzmK Induces cyt c and EndoG Release in a Bid-dependent Way—cyt c release from mitochondria appears to be an early event during apoptosis induced by a variety of stimuli (30, 31).
Cytosolic cyt c, together with Apaf-1 and procaspase-9, in an ATP-dependent manner, forms apoptosome, which causes caspase activation that orchestrates the biochemical execution of cells (32, 33). EndoG is a proapoptotic DNase that is released from mitochondria and translocates to the nucleus where it damages chromatin DNA without caspase activation during apoptosis, which represents a caspase-independent apoptotic pathway initiated from the mitochondria (34). We next wanted to investigate whether GzmK initiates the release of cyt c and other proapoptotic factors and whether these events are Bid-dependent. Murine liver mitochondria were isolated and treated with GzmK in the presence or absence of rBid at 37 °C for 30 min. rBid- or GzmB plus rBid-treated mitochondria were used as controls. cyt c and EndoG release were confirmed in GzmK-loaded intact HeLa cells by confocal microscopy. cyt c and EndoG released quickly from mitochondria in HeLa cells after GzmK loading with perforin and is considered as a slowing granzyme. The same group also demonstrated that remaining in mitochondria (8, 37) showed that rat GzmK disrupts ΔΨ but does not initiate release of cyt c (10). We previously showed that human GzmK induces rapid cell death without caspase activation (9). The features of death are characterized by rapid externalization of phosphatidylserine, nuclear morphological changes, and single-stranded DNA release of SMAC and AIF was also required for the presence of rBid (data not shown). These results were further confirmed by laser scanning confocal microscopy (Fig. 6B). GzmK loading with PFP induced cyt c and EndoG release in HeLa cells. GzmB plus PFP was used as a positive control. Similar results were found in GzmK-treated HeLa cells by delivery of PJ or Ad (data not shown).

**DISCUSSION**

It has not been defined how GzmK functions in CTL- and NK-cell induced cytolysis. Shi et al. (8, 37) showed that rat GzmK induces DNA fragmentation without nuclear morphological changes after GzmK loading with perforin and is considered as a slowing granzyme. The same group also demonstrated GzmK disrupts ΔΨ but does not initiate release of cyt c (10). We previously showed that human GzmK induces rapid cell death without caspase activation (9). The features of death are characterized by rapid externalization of phosphatidylserine, nuclear morphological changes, and single-stranded DNA.
GzmK triggers rapid ROS production and loss of inner mitochondrial membrane (10, 38). We showed that early events in apoptosis that involve the opening by PTP of the mitochondrial membrane space. Increase of ROS and loss of membrane permeability to ions and solutes, followed by physiological disruption of its outer membrane, and physiological disruption of its outer membrane, with the consequent release of proteins to the intermembrane space. Increase of ROS and loss of ΔΨ are indicative of mitochondrial dysfunction, hallmarks of apoptotic mitochondrial damage (12). It is postulated that ROS and ΔΨ collapse are early events in apoptosis that involve the opening by PTP of the inner mitochondrial membrane (10, 38). We showed that GzmK triggers rapid ROS production and loss of ΔΨ. Our data suggest that GzmK damages the inner mitochondrial membrane thus resulting in mitochondrial solutes to the intermembrane space.

Whether generation of ROS is critical to apoptosis or only a side effect of mitochondrial dysfunction has not been well defined. ROS induce the opening of PTP through oxidation-dependent mechanisms and are potent inducers of apoptosis (12, 39). Lieberman’s group (15) showed that ROS is critical in GzmA-induced caspase-independent death. ROS generation induced by GzmA is not inhibited by Bcl-2 overexpression or by pan caspase inhibitors. We found that GzmK can initiate rapid ROS generation and confirmed this at a single cell level. Both the antioxidant NAC and the superoxide scavenger Tiron blocked ROS production, which protected cells from GzmK-induced death. By contrast, Martinvalet et al. (15) reported that NAC is less effective than Tiron at neutralizing ROS and is unable to protect GzmA-induced death. We previously showed that GzmK can cleave Ape1/Ref-1 leading to disruption of its redox function as GzmA (9, 22). A primary function of Ape1/Ref-1 is maintaining reduced thiols and DNA binding of transcription factors, such as AP-1 and NF-κB. Ape1/Ref-1 can inhibit intracellular ROS accumulation. We found that GzmK can degrade tumor suppressor p53.4 A recent study showed that p53 protects genome stability from oxidation by ROS (40). Down-regulation of p53 results in excessive oxidation of DNA that is prevented by NAC treatment. Dietary supplementation with NAC prevents frequent lymphomas characteristic of p53-deficient mice.

cyt c release from mitochondria has been defined as an important event in the activation of downstream caspases and apoptosis (31). Once released to the cytosol, cyt c acts as a cofactor in conjunction with Apaf-1, procaspase 9, and ATP/dATP to form an active holoenzyme that processes and activates downstream caspase 3 (30, 32, 33). The signals to induce cyt c release are often propagated through proapoptotic Bcl-2 family members. Translocation from the cytoplasm to mitochondria during apoptosis has been reported for Bid, Bak, Bak, Bad, and Bim (19, 41–45). These proteins are inactive in the cytoplasm and are translocated to the mitochondria after a death signal. Following Fas ligation and recruitment of Fas-associated death domain to the trimeric receptor complex, caspase 8 is activated and then degrades cytosolic Bid to tBid, which translocates to mitochondria to cause the release of cyt c (19). GzmB directly cleaves Bid to produce a 14-kDa GzmB-truncated Bid (called gtBid), which is different from caspase 8-truncated 15-kDa Bid (36). gtlBid recruits Bax to mitochondria through a caspase-independent mechanism where it is integrated into the mitochondrial membrane and initiates cyt c release. We showed here that Bid is processed by GzmK, which appears to have the same pattern as GzmB. GzmK-truncated Bid might mimic gtBid to recruit Bax leading to caspase-independent mechanism of mitochondrial damage, which is different from GzmA. Moreover, GzmK can induce the release of other proapoptotic molecules, including EndoG, SMAC, and AIF, which enhance GzmK-induced death.

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