A Central Role for Bid in Granzyme B-induced Apoptosis*

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Granzyme B, a protease released from cytotoxic lymphocytes, has been proposed to induce target cell death by cleaving and activating the pro-apoptotic Bcl-2 family member Bid. It has also been proposed that granzyme B can induce target cell death by activating caspases directly, by cleaving caspase substrates, and/or by cleaving several non-caspase substrates. The relative importance of Bid in granzyme B-induced cell death has therefore remained unclear. Here we report that cells isolated from various tissues of Bid-deficient mice were resistant to granzyme B-induced cell death. Consistent with the proposed role of Bid in regulating mitochondrial outer membrane permeabilization, cytochrome c remained in the mitochondria of Bid-deficient cells treated with granzyme B. Unlike wild type cells, Bid-deficient cells survived and were then able to proliferate normally, demonstrating the critical role for Bid in mediating granzyme B-induced apoptosis.

Granzyme B is a serine protease contained within the granules of cytotoxic lymphocytes (CLs). Upon conjugation with their targets, CLs release their granule contents into the synaptic cleft. Granzyme B then enters the target cell by endocytosis and induces apoptotic death via a perforin-dependent mechanism. The importance of CL-mediated killing in the immune response to various pathogens has made it imperative to understand the mechanism of action of granzyme B.

Overexpression of the oncogenic Bcl-2 renders cells resistant to granzyme B-induced apoptosis (1, 2), and the cells maintain their ability to proliferate (2, 3). Bcl-2 is one of a large family of proteins that regulate mitochondrial outer membrane permeabilization (MOMP) during apoptosis (4). Pro-apoptotic Bcl-2 family members (such as Bid, Bax, and Bak) induce MOMP (5), whereas anti-apoptotic members (e.g. Bcl-2 and Bcl-XL) prevent MOMP (6). Following MOMP, several pro-apoptotic proteins are released from the mitochondrial intermembrane space. In the cytosol, these proteins facilitate the activation of caspases, proteases that orchestrate the death of a cell by apoptosis. One of these proteins, cytochrome c, initiates a complex with dATP, apoptotic protease-activating factor (APAF-1), and pro-caspase-9. This results in the activation of caspase-9, which in turn activates caspase-3 (7). A second protein SMAC/Diablo that is also released from the mitochondrial intermembrane space displaces inhibitor of apoptosis proteins (IAPs) from caspases, allowing them to become activated by autoprocessing (8, 9).

Granzyme B has been reported to induce MOMP by cleaving and activating the pro-apoptotic Bcl-2 family member Bid after residue Asp-75 (1, 10–12). Granzyme B has also been shown to cleave caspase-3 directly when mixed with cytosolic lysates (13, 14); however, in intact cells, granzyme B only appears to be capable of partially processing procaspase-3 to a p20 form that shows little activity in a cellular context (15). SMAC/Diablo, released following MOMP, then displaces the IAPs from the p20 form of caspase-3, allowing auto-processing and full activation. Regardless of the exact mechanism of caspase activation (SMAC/Diablo or cytochrome c), these models rely explicitly on granzyme B-induced MOMP.

In contrast, a recent study has suggested that murine embryonic fibroblasts (MEF) from Bid-deficient (bid−/−) mice were as sensitive to granzyme B as control cells expressing wild type Bid (bid+/+) (16). In a separate study, Metkar et al. (17) demonstrated that the granzyme B-induced loss of mitochondrial transmembrane potential (ΔΨm, used as a surrogate indicator of MOMP) does not occur in MCF7 cells that lack caspase-3. On this basis, it was predicted that caspase-3 is required for mitochondrial damage during granzyme B-induced apoptosis, placing caspase activation upstream of Bid cleavage and MOMP. These studies cumulatively suggested that neither Bid nor MOMP is required for granzyme B-mediated cell death and proposed that MOMP acts as an amplification loop that may be dispensable during granzyme B-induced apoptosis. Although these studies propose to provide insight into the molecular mechanism of granzyme B-induced cell death, they are inconsistent with a number of studies showing that cells overexpressing Bcl-2 are protected against granzyme B-induced apoptosis (1–3) and maintain their proliferative potential following exposure to cytotoxic concentrations of granzyme B (2, 3).

To directly investigate the role of Bid in granzyme B-induced cell death, we isolated various primary and transformed cells from bid−/− and bid+/+ mice and compared their sensitivity.
to granzyme B. We found without exception that cells isolated from bid−/− mice were resistant to granzyme B-induced apoptosis. Mitochondria from Bid-deficient cells maintained an intact outer membrane and their transmembrane potential following treatment with granzyme B/perforin. Importantly, bid−/− cells treated with granzyme B/perforin maintained similar proliferative potential as untreated cells, whereas the proliferative potential of bid+/+ cells treated with granzyme B/perforin was markedly reduced. These data demonstrate a critical role for Bid-mediated MOMP in granzyme B-induced cell death and are consistent with the ability of Bcl-2 to completely rescue cells from granzyme B-induced apoptosis.

**EXPERIMENTAL PROCEDURES**

**Materials**

$^{51}$Cr (as sodium dichromate) was from Amersham Biosciences. Annexin V-FITC was from Roche Applied Science. Tetramethylrhodamine ethylester (TMRE) was from Molecular Probes (Eugene, OR). Mouse anti-cytochrome c antibody (Clone 6H1B2) was from Pharmingen, and phycoerythrin-labeled anti-mouse Ig was from Silenus, Australia. All other chemicals were from Sigma. Cell culture reagents were from Invitrogen. Perforin was purified essentially according to Liu et al. (18). Granzyme B was purified as described by Sun et al. (19). Purified granzyme B was free of granzyme A activity and perforin.

**Cell Culture**

All cells used in this study were cultured at 37 °C in a humidified CO$_2$ incubator in medium supplemented with 2 mM glutamine and 10% fetal bovine serum. Splenocytes, tail fibroblasts, and bone marrow dendritic cells were freshly isolated from 7–12-week-old C57BL/6 mice expressing wild type bid+/+ or age-matched mice deficient in bid−/− that were backcrossed nine times onto the same genetic background (20). MEF were from similar mice backcrossed five times (20). Splenocytes were induced to proliferate by culture in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 50 µg/ml gentamicin, 200 µg/ml penicillin, 100 µg/ml streptomycin sulfate, 55 µM β-mercaptoethanol, 600 units/ml interleukin-2, and 2 µg/ml phytomheatagglutinin. Populations of dendritic cells (>95% CD11c$^+$) were obtained by culturing fresh femoral bone marrow in RPMI 1640 supplemented with 55 µM β-mercaptoethanol, 2.5 mM Hepes, 10% fetal calf serum, and 1 µg/ml interleukin-4 for 3 days. Tail fibroblasts were grown in Dulbecco’s modified Eagle’s medium. MEF were cultured in Iscove’s medium supplemented with 200 µg/ml penicillin, 100 µg/ml streptomycin sulfate, and 55 µM β-mercaptoethanol. At passage 5, MEF were transformed with myc (21).

**Generation of Eμ-myeloid bid−/− B-cell Lymphomas**

To generate lymphomas deficient in bid expression, we crossed Eμ-C57BL6/Eμ-myeloid transgenic mice with bid−/− mice that were backcrossed 10 times onto a C57BL6 background. Following intercrossing of Eμ-myeloid bid+/− mice, Eμ-myeloid bid−/− and littermate control Eμ-myeloid bid+/+ lymphomas were harvested from lymph nodes, and a single cell suspension was prepared. Lymphoma cells were cultured in 10%-supplemented Dulbecco’s modified Eagle’s medium supplemented with 1 mM l-asparagine, 200 µg/ml penicillin, and 100 µg/ml streptomycin sulfate and 55 µM β-mercaptoethanol.

**Assays for Cell Death**

Cells were assayed for death by specific $^{51}$Cr release, annexin V binding, the loss of Δψm (TMRE staining), cytochrome c release, or growth arrest. For Specific $^{51}$Cr Release—1 $\times$ 10$^6$ cells were incubated in 100 µl of medium with $^{51}$Cr (75 µCi) for 1 h at 37 °C. Cells were washed three times in Hanks’ balanced salt solution containing 0.3% bovine serum albumin and 20 mM Hepes to remove the unincorporated $^{51}$Cr and resuspended at 2 $\times$ 10$^6$ cells/ml. Cells were incubated with granzyme B/perforin for 4 h at 37 °C, and the supernatant was harvested using a supernatant collection system (Molecular Devices Corp., Sunnyvale, CA). $^{51}$Cr released (cpm) into the supernatant was detected using a Wallac Wizard 1470 automatic γ counter (PerkinElmer Life Sciences). In each case, the spontaneous release of radiolabel over the time of the assay was no higher than 10% of the total incorporated radioactivity.

For Annexin V Binding—Following exposure to granzyme B and perforin, cells were incubated for 5 min at 37 °C in 100 µl of annexin V buffer (10 mM Hepes NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 1.8 mM CaCl$_2$) containing 0.5 µl of annexin V-FITC. Annexin V-FITC fluorescence was detected by flow cytometry in FL-1.

Results

**Bid-deficient MEF Are Resistant to Granzyme B-induced Cell Death**—Our previous experiments using transformed cell lines, together with studies performed in other labs, strongly suggested a critical role for Bid in regulating granzyme B-induced apoptosis (1, 10–12, 23, 24). In contrast, one recent study that used very high concentrations of granzyme B to induce cell death reported that bid−/− MEF were as sensitive to granzyme B-induced cell death as wild type cells (16). To directly determine the requirement for Bid during granzyme B-induced apoptosis, we treated bid+/+ and bid−/− fibroblasts isolated from mouse embryos with a broad range of concentrations of granzyme B (1–1000 nM) and sublytic concentrations of perforin (1 nM, producing <10% specific release of $^{51}$Cr). We found that 75 nM granzyme B was sufficient to induce death in 50–60% of bid+/+ MEF within 4 h as measured by the release of $^{51}$Cr that was preloaded into the cells (Fig. 1A). Indeed, a concentration of granzyme B as low as 8 nM was sufficient to induce significant death (20–30%) in bid+/+ MEF. In contrast, less than 10% (levels comparable with perforin alone) $^{51}$Cr release was observed when bid−/− MEF were treated with concentrations of granzyme B up to 75 nM. Bid−/− MEF were similarly resistant to native granzyme B purified from human NK cells (25 nM), whereas this concentration of granzyme B induced significant death in bid+/+ cells (not shown). The requirement for Bid in granzyme B-induced death was not absolute as high concentrations of granzyme B (250 nM) were able to induce some degree of cell death (~20%) in bid−/− cells (Fig. 1A), and 1000 nM granzyme B was sufficient to induce death in a similar percentage of bid−/− and bid+/+ MEF within 4 h (Fig. 1A, inset). The level of death observed in bid−/− cells treated with 250 nM granzyme B (~20%) was similar to that observed when bid+/+ MEF were treated with 8 nM granzyme B, demonstrating that bid+/+ cells are around 30 times more sensitive to granzyme B than bid−/− cells. Bid+/+ and bid−/− cells were equally sensitive to perforin-induced lysis (not shown) and to actinomycin D, a cytotoxic drug that does not require Bid for efficient apoptosis (not shown).

It has been suggested that the true role of Bid in granzyme B-induced cell death is to amplify the death pathway and that in bid−/− cells, granzyme B can cleave alternate death substrates and kill cells via a delayed, alternate pathway that was not detected in a $^{51}$Cr assay. To address this possibility and observe cell death directly, we treated bid+/+ or bid−/− MEF...
with granzyme B (25 nM) and perforin (1 nM) and observed cell morphology after 24 h (Fig. 1B). We found that the majority of bid+/+ MEF that were treated with granzyme B/perforin had rounded appearance and a collapsed morphology and were detached from the culture flask. In contrast, the morphology of bid−/− cells treated with granzyme B/perforin was unchanged from cells treated with perforin alone (Fig. 1B).

**Primary Cells Deficient in Bid Are Resistant to Granzyme B-induced Cell Death**—To determine whether Bid is universally required for granzyme B-induced apoptosis or whether the requirement for Bid was restricted to transformed MEF, we isolated a number of primary cell types from bid+/+ and bid−/− mice and compared their sensitivity with granzyme B-induced apoptosis. We found that splenic lymphocytes induced to proliferate with phytohemagglutinin (Fig. 2A), tail fibroblasts (Fig. 2B), or bone marrow-derived dendritic cells (Fig. 2C) isolated from bid−/− mice were resistant to granzyme B (25 nM)/perforin, whereas the same concentration of granzyme B/perforin killed 20–40% of the equivalent cells isolated from bid+/+ mice. Similar to what we observed in MEF, we found that 8 nM granzyme B was sufficient to kill a significant number (25–30%) of bid+/+ bone marrow-derived dendritic cells; however, ~30 times that concentration (250 nM) was required to kill a similar percentage of bid−/− cells.

To exclude the possibility that the bid−/− cells had died by apoptosis but had failed to release 51Cr, we stained cells with annexin V to detect phosphatidylserine exposed on the outer leaflet of the plasma membrane. We found that around 45% of granzyme B-treated bone marrow dendritic cells (Fig. 3) from bid+/+ mice stained strongly positive with annexin V; however, bid−/− cells remained annexin V-negative following granzyme B/perforin treatment (Fig. 3). Again, bid+/+ and bid−/− dendritic cells were equally sensitive to perforin-induced lysis and actinomycin D (not shown). These data clearly show that bid−/− primary cells isolated from diverse tissues are resistant to granzyme B-induced death.

**Bid Is Required for Granzyme B-induced MOMP and Loss of ΔΨm**—Caspase-cleaved Bid is believed to promote apoptosis via MOMP. Having clearly established the importance of Bid in granzyme B-induced apoptosis, we wished to examine the effect of granzyme B on mitochondria of bid−/− cells. We therefore assayed mitochondrial depolarization (the loss of mitochondrial transmembrane potential as measured by TMRE staining) and mitochondrial outer membrane permeabilization (cytochrome c release) in bid+/+ and bid−/− cells treated with 25 nM granzyme B and 1 nM perforin. As expected, and consistent with the data shown on Figs. 2C and 3, a distinct population (~40%) of bid+/+ dendritic cells treated with granzyme B/perforin had depolarized mitochondria (Fig. 4A), and cytochrome c had been released into the cytoplasm (Fig. 4B). In contrast, the majority of bid−/− cells treated with granzyme B (25 nM)/perforin maintained ΔΨm (~85%) (Fig. 4A) and maintained cytochrome c in the mitochondria as well as cells treated with perforin alone (Fig. 4B). These data strongly support a critical role for gran-
Bone marrow dendritic cells were treated to granzyme B-induced apoptosis. Marrow dendritic cells are resistant 4 h and assayed for 51Cr release. Data are presented as the mean ± S.D. of triplicate samples and are representative of two independent experiments performed on cells isolated from two different mice. Although untreated Eμ-myc transgenic mice with bid−/− mice to generate B-cell lymphomas that expressed myc but were devoid of bid expression. Eμ-myc/bid+/+ and Eμ-myc/bid−/− cells were equally sensitive to etoposide as syngenic Eμ-myc lymphomas expressing wild type bid (not shown). We found that 4 h following treatment with perforin/granzyme B (25 nM), ~30% of Eμ-myc/bid+/+ cells had released 51Cr (Fig. 6A), and ~50% Eμ-myc/bid−/− cells stained positive with annexin V (Fig. 6B). Similar results were also obtained using native granzyme B. In addition, a significant population of perforin/granzyme B-treated cells showed morphological signs of cell death after 24 h and stained positive with propidium iodide (Fig. 6C). In contrast, Eμ-myc/bid−/− cells did not release 51Cr or become annexin V-positive within 4 h of treatment with perforin/granzyme B. Moreover, these cells maintained normal morphology 24 h after exposure to granzyme B/perforin and did not take up propidium iodide. To determine the proliferative potential of these cells, we seeded untreated cells, cells treated with perforin alone, or cells treated with perforin/granzyme B in individual wells of a 96-well plate, and viable cells were counted after 24 h (Fig. 7). Although untreated Eμ-myc/bid+/+ cells and Eμ-myc/bid−/− cells proliferated at similar rates and survived perforin exposure equally well, we consistently found that the number of Eμ-myc/bid+/+ lymphomas treated with perforin/granzyme B was around half the number of similarly treated Bid-deficient cells. These data demonstrate that Bid-deficient cells survive and proliferate following treatment with concentrations of per-
forin and granzyme B that are sufficient to kill a significant number of E/H9262-
myc/bid/H11001/ lymphoma cells.

**DISCUSSION**

It is well documented that granzyme B induces caspase-dependent apoptosis; however, the mechanism by which granzyme B activates caspases, and specifically, the role of mitochondria in this process, are contentious. Bcl-2 blocks granzyme B-induced cell death (1–3) and maintains the proliferative potential of the treated cells (2, 3), suggesting that mitochondria are critical targets for a granzyme B substrate. Consistent with this hypothesis, granzyme B has been shown to cleave and activate the pro-apoptotic Bcl-2 family member Bid (1, 10, 11, 24), which facilitates caspase activation by releasing proteins from the mitochondrial intermembrane space (23). Although a recent study did agree that cells from Bid-deficient mice show no signs of MOMP, it was claimed that these cells were equally as sensitive to granzyme B-induced apoptosis as their wild type counterparts (16). A further study using the MCF-7 tumor cell line argued that caspase-3 was apparently required for the loss of mitochondrial transmembrane potential during granzyme B-induced apoptosis (17). On this basis, it was proposed that mitochondria are more appropriately seen as an amplification loop in the pathway to granzyme B-mediated apoptosis. These data challenge the hypothesis that Bid is required for granzyme B-induced cell death (16, 17) and is inconsistent with studies that have shown a role for mitochondria in this pathway (1–3).

In this study, we have presented data that demonstrate a critical role for Bid-mediated regulation of MOMP in granzyme B-induced cell death. We observed that both primary and transformed Bid-deficient cells from a variety of tissues were resistant to granzyme B-induced cell death. Of further consequence, we found that bid/mice treated with granzyme B maintained their proliferative potential, whereas there was a marked loss of proliferative potential in Bid-proficient cells treated with a similar concentration of granzyme B. These data are completely consistent with the ability of Bcl-2 to block granzyme B-induced cell death and allow the proliferation of granzyme B-treated cells. Our results contrast with those of Thomas et al. (16), who reported that bid−/− MEF are as sensitive to granzyme B-induced cell death as their wild type counterparts. Following careful titration of granzyme B, we found that low nanomolar concentrations of granzyme B were sufficient to induce death in bid+/+ cells; however, much higher concentrations of granzyme B (greater than 30-fold) could induce the death of bid−/− cells. Since the study by Thomas et al. (16) utilized very high concentrations of gran-

**FIG. 4.** Bid is required for granzyme B-induced MOMP. Bone marrow dendritic cells were treated with 25 nM granzyme B and 1 nM perforin for 4 h and assayed for the loss of ΔΨm (TMRE+) (A) or the release of cytochrome c from the mitochondria (B). Cells with low ΔΨm or cytoplasmic cytochrome c have lower fluorescence than control cells (indicated by M). The profiles are from one of two experiments performed on cells isolated from two different mice. *Numbers* indicate the percentage of cells with low ΔΨm or cytoplasmic cytochrome c. PE, phycoerythrin.

**FIG. 5.** Bid-deficient bone marrow dendritic cells survive following treatment with granzyme B/perforin. Bone marrow dendritic cells treated with 25 nM granzyme B and 1 nM perforin for 4 h were seeded in tissue culture plates at 2 × 10⁴ cells/well and were counted again after 3 days. Data are representative of three separate experiments on cells isolated from three individual mice.
FIG. 6. Bid-deficient Eμ-myc B-lymphoma cells are resistant to granzyme B/perforin-induced apoptosis. A, Eμ-myc lymphoma cells labeled with 51Cr were treated with 25 nM granzyme B and/or 1 nM perforin. Supernatants were harvested at 4 h and assayed for 51Cr release. Data are presented as the mean ± S.D. of triplicate samples and are representative of two independent experiments. B, Eμ-myc lymphoma cells treated with 25 nM granzyme B and/or 1 nM perforin were harvested at 4 h and assayed for annexin V binding. Numerals indicate the number of annexin V-positive cells indicated by the gate (M). Data are presented as the mean ± S.D. of triplicate samples and are representative of four independent experiments isolated from two individual mice. C, Eμ-myc lymphoma treated with 25 nM granzyme B and/or 1 nM perforin for 1 h were plated in 96-well tissue culture plates. Cells were stained with propidium iodide (PI) (500 ng/ml), and images were taken after 24 h. The data presented are representative of two independent experiments. DIC, differential interference contrast.
zyme (in the micromolar range), it is not surprising that they found that bid−/− cells appeared to be as sensitive to granzyme B as bid+/+ cells.

It has been suggested by us and others that granzyme B may directly cleave caspases under certain conditions (13–15). The data reported here support the hypothesis that if granzyme B can cleave caspases directly, they are not activated to a sufficient level to reach an apoptotic threshold (15), and that Bid-dependent MOMP is required for effective caspase activation. It also remains possible that granzyme B cleaves other (non-caspase) cellular substrates; however, these require MOMP for full effect, contribute to death only after granzyme B-induced MOMP, or are not sufficient to induce apoptosis but may contribute to the immune function of cytolytic lymphocytes in other ways.

Combining the results of various studies, we conclude that at low concentrations, granzyme B specifically targets Bid and only cleaves alternate substrates effectively at much higher concentrations of granzyme B. It is currently not possible to determine the effective concentration of granzyme B delivered to a target cell, and in any event, this may be influenced by a number of co-incident factors. Recently, it has been shown that induction of granzyme B expression in a population of activated CD8+ T-cells is stochastic; therefore, different T cells may be capable of secreting quite different amounts of granzyme B (25, 26). It is also known that CL can kill multiple targets in rapid succession, indicating that CLs do not secrete their entire granule contents onto one target. Even if it were possible to determine the concentration of granzyme B delivered to the synapse, the concentration of granzyme B taken up by a target cell is likely to differ from one type of cell to another. For example, cells expressing mannose-6-phosphate receptor or dynamin take up granzyme B more efficiently than cells deficient in these proteins (27). The concentration of granzyme B ultimately delivered to the target cell cytosol may also depend on its rate of endocytosis/exocytosis, the sensitivity of the cells to perforin, cell shape and size, and particularly, by the expression of direct granzyme B inhibitors by the target cell. Protease inhibitor 9, the adenovirus protein L100K, and the cow pox virus protein cytokine response modifier A (CrmA) have all been shown to block granzyme B activity to varying extents (28–31). The development of tools that can accurately estimate the concentration of free active granzyme B that reaches the cell cytosol will ultimately prove extremely valuable for understanding the mechanism of granzyme B-induced apoptosis in an vivo immune response.

In conclusion, although it is not possible to determine whether Bid is the exclusive granzyme B substrate, our data support the hypothesis that Bid is the preferred and often the critical substrate for granzyme B during apoptosis induced by CL.

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