A Role of the Mitochondrial Apoptosis-Inducing Factor in Granulysin-Induced Apoptosis

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Granulysin is a cytolytic molecule released by CTL via granule-mediated exocytosis. In a previous study we showed that granulysin induced apoptosis using both caspase- and ceramide-dependent and -independent pathways. In the present study we further characterize the biochemical mechanism for granulysin-induced apoptosis of tumor cells. Granulysin-induced death is significantly inhibited by Bcl-2 overexpression and is associated with a rapid (1–5 h) loss of mitochondrial membrane potential, which is not mediated by ceramide generation and is not inhibited by the general caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone. Ceramide generation induced by granulysin is a slow event, only observable at longer incubation times (12 h). Apoptosis induced by exogenous natural (C_{18}) ceramide is truly associated with mitochondrial membrane potential loss, but contrary to granulysin, this event is inhibited by benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone. Ceramide-induced apoptosis is also completely prevented by Bcl-2 overexpression. The nuclear morphology of cells dying after granulysin treatment in the presence of caspase inhibitors suggested the involvement of mitochondrial apoptosis-inducing factor (AIF) in granulysin-induced cell death. We demonstrate using confocal microscopy that AIF is translocated from mitochondria to the nucleus during granulysin-induced apoptosis. The majority of Bcl-2 transfected are protected from granulysin-induced cell death, mitochondrial membrane potential loss, and AIF translocation, while a small percentage are not protected. In this small percentage the typical nuclear apoptotic morphology is delayed, being of the AIF type at 5 h time, while at longer times (12 h) the normal apoptotic morphology is predominant. These and previous results support a key role for the mitochondrial pathway of apoptosis, and especially for AIF, during granulysin-induced tumoral cell death.

fast and the other slow, both of which implicate the participation of mitochondria. The fast pathway (1–5 h) is independent of ceramide generation and is associated with a caspase-independent rapid loss of mitochondrial membrane potential (ΔΨm). This ΔΨm loss is associated with the translocation of the mitochondrial apoptosis-inducing factor (AIF) to the nucleus. We also show that the AIF apoptotic pathway remains active in the presence of caspase inhibitors. Enforced overexpression of Bcl-2 prevented granulysin-induced loss of ΔΨm and AIF translocation, correlating to the observed protection from cell death. The slow pathway (≥12 h) is associated with ceramide generation, and although ceramide also mediates cell death through the mitochondrial pathway, ΔΨm loss induced by ceramide is inhibited by Z-VAD-fmk. Previous results has shown that although cytochrome c is also released from mitochondria upon granulysin treatment, caspase-9 activation is not detected during granulysin-induced apoptosis (13). Taken together, these data point to the release of AIF from mitochondria as an important apoptotic effector during granulysin-induced cell death.

Materials and Methods

Cells
The Jurkat T cell leukemia (American Type Culture Collection, Manassas, VA; clone E6.1) was used as target in the granulysin- and ceramide-induced cytotoxicity assays. Jurkat cells were cultured in RPMI 1640 medium (Life Technologies, Barcelona, Spain) supplemented with 5% FCS, 1-glutamine, and penicillin/streptomycin (hereafter, complete medium) using standard cell culture procedures. Jurkat cell lines stably overexpressing the viral protein CrmA, J-CrmA, and the murine Bcl-2 protein, J-Bcl2, as well as vector-transfected cells, J-vector, were gifts from Dr. D. Johnson (Pittsburgh University, Pittsburgh, PA) and were generated as previously described (14). Transfected cell lines were maintained in complete medium containing 0.5 mg/ml G418 (Life Technologies). All cell lines used were Mycoplasma free, as routinely controlled by PCR.

Materials
MTT, carbonyl cyanide m-chlorophenylhydrazone (CCCP), ceramide type III from bovine brain, and dodecane were products of Sigma (Madrid, Spain). Ac-DEVD-CHO and benzoylcarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) were obtained from Bachem (Bubendorf, Switzerland). 3,3′,4′-Diethylxacyrbocyanine iodide, 3,3′,4′-diethylxacyrboxyacarbocyanine iodide (DiOC6(3)), was obtained from Molecular Probes (Leiden, The Netherlands), and [1-14C]palmitic acid was purchased from Amersham (Madrid, Spain). The rabbit anti-mouse AIF polyclonal antiserum, which largely cross-reacts with human AIF, was generated as previously described (15).

Expression and purification of recombinant functional granulysin
Recombinant granulysin was expressed and purified essentially as described previously (10). Briefly, a portion of the initial 519 cDNA, coding for glycine residue 63 through the natural stop codon after leucine residue 145 of PS20 (16), was cloned into the pET28a vector (Novagen, Madison, WI) to express polystyrene-taggged recombinant 9-kDa granulysin in Escherichia coli BL21 (DE3). The recombinant protein was expressed and purified under denaturing conditions according to Novagen’s instructions. After purification, the granulysin was reduced by the addition of 1 mM DTT, allowed to refold in the presence of oxidized DTT, and dialyzed against Tris-buffered saline. The histidine tag was removed by thrombin treatment, and proteins were purified by gel filtration chromatography in a Sephadex G-25 column. Protein content was determined by the Bradford method, with BSA as a standard. Protein purity was always >95%, as assessed by SDS-PAGE in 15% polyacrylamide gels and Coomassie blue staining.

Cytotoxicity assays
For granulysin cytotoxicity testing, 2 × 105 cells/ml (100 μl/well) were resuspended in a serum-free medium composed of RPMI 1640, DMEM, and Ham’s F-12 medium (2/1/1, v/v/v), supplemented with BSA (1 mg/ml), sodium selenite (4.3 ng/ml), ethanolamine (1.53 μg/ml), transferrin (10 μg/ml), insulin (5 μg/ml), glutamine (2 mM) and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml) (17). Cells were incubated for different times (30 min to 16 h) in the presence of the absence of 20–50 μM recombinant granulysin. Natural (C12) ceramide (50–200 nM) was first dissolved in ethanol-dodecane (90:2, v/v), and then added to the serum-free culture medium, as indicated previously (18, 19). At the doses used, ethanol and dodecane (final concentration of solvent, 0.2–0.5%) had no effect on cell proliferation. Ac-DEVAD-CHO, an inhibitor of the caspase-3 subfamily, or Z-VAD-fmk, a general caspase inhibitor (20), were used at 600 and 100 μM, respectively. Peptide inhibitors were first dissolved in DMSO, then diluted in culture medium (final DMSO concentration, 0.4%), v/v). Cells were preincubated for 1 h with the protease inhibitors before adding the toxic stimuli to assure a sufficient incorporation by cells. Cell growth was determined by a modification of the MTT reduction method of Mosmann (21) and expressed as a percentage of growth in control cultures. Cell death was determined by microscopic inspection of trypan blue-stained cells.

Evaluation of mitochondrial membrane potential (ΔΨm) by flow cytometry
To evaluate ΔΨm, the cationic lipophilic fluorescein diocyanate (DiOC6(3)) was used (22). Cells (1.5 × 106 in 100 μl) were incubated with 20 nM DiOC6(3) for 15 min at 37°C. DiOC6(3) was prepared from a 40 μM stock solution in DMSO. This solution was diluted with sterile PBS, pH 7.4, to a 400 nM working solution, followed by a further dilution with the medium containing cells. As a negative control, cells were treated in parallel cultures with the protonophore-uncoupling agent CCCP at a final concentration of 50 μM (stock solution, 10 mM in ethanol). Cells were diluted with PBS to a final volume of 1 ml and analyzed by flow cytometry.

Ceramide and sphingomyelin determinations
Ceramide and sphingomyelin levels were assayed essentially as described previously (11, 19). Jurkat cells (2 × 105 in 10 ml) were labeled for 48 h with 5 μCi [1-14C]palmitic acid bound to fatty acid-free serum albumin in 1 M granulysin for different periods of time (30 min–16 h). The cells were harvested and washed with cold RPMI medium. Cell viability was determined in an aliquot of the corresponding cell suspensions by the trypan blue exclusion method, and total cell lipids were extracted at 4°C with chloroform-methanol (2:1, v/v) as previously described (19). Radioactivity in aliquots from chloroformic phases was determined by liquid scintillation counting, and equal amounts of radioactivity for each sample were applied to TLC silica gel G plates (Scharlau, Barcelona, Spain). Plates were prewashed with chloroform-methanol (1:1, v/v) and heat activated at 110°C for 1 h. A first development of TLC-loaded samples was performed with chloroform-methanol-water (60:30:5, v/v/v) up to 10 cm from the bottom of the plate, and a second development was performed to its full length with hexane-diisopropyl ether-acetic acid (98:2:2, v/v/v). Plates and radiolabeled bands were located by film autoradiography (Hyperfilm β-max; Amersham Pharmacia) at room temperature for 2–3 days. Then, sample lanes were covered with glass, allowing the marker lanes to be iodine stained. Authentic standards of ceramide, sphingomyelin, cholesterol, phosphatidylcholine, and phosphatidylserine (Sigma) were used as markers. The corresponding radiolabeled lipids were scraped and transferred to vials, to which 4 ml scintillation mixture (Normasint 11; Scharlau) containing 10% (v/v) methanol in ethyl alcohol was added. Radioactivity in samples was determined by liquid scintillation counting, and results are expressed as ceramide-sphingomyelin ratios.

Fluorescence and confocal microscopy
Morphological evaluation of apoptosis was monitored by labeling cell nuclei with oxidized p-phenylenediamine (PPDA) in glycerol and visualized by fluorescence microscopy (23). Briefly, cells were washed with PBS, pH 7.4, and fixed at room temperature with 1% paraformaldehyde in PBS for 15 min. Fixed cells were washed with PBS, centrifuged onto polylysine-coated coverslips in wells of a 24-well plate, mounted on a glass slide over a drop of PPDA stain (10 mg PPDA in 1 ml PBS mixed with 9 mL oxidized glycerol), and photographed.

Translocation of AIF from mitochondria to the nucleus during apoptosis was analyzed by confocal microscopy, as indicated previously (15). Briefly, control cells or cells treated with granulysin for the times indicated were collected, washed with PBS, and fixed in a solution of 4% paraformaldehyde in PBS. Cell suspensions were then placed onto round coverslips previously treated with t-polylysine, in wells of a 24-well plate, and centrifuged 5 min at 1200 rpm. Coverslips were then washed once...
with PBS, placed onto a drop of a 0.1% solution of saponin in PBS containing 5% goat serum and a 1/500 dilution of the anti-AIF antiseraum, and incubated for 30 min at room temperature in a humidified chamber. Coverslips were then washed once with a 0.1% solution of saponin in PBS and incubated in the same way with a 1/200 dilution of an anti-rabbit IgG Ab labeled with FITC (Caltag, Barcelona, Spain). Finally, coverslips were washed sequentially with 0.1% saponin, PBS, and distilled water; mounted onto the glass slides using a drop of Mowiol (Calbiochem, Madrid, Spain); and stored at 4°C in the dark until observation. Preparations were observed in a Zeiss 310 confocal microscope, and analyzed using LSM 3.95 software. Cells were observed in 10 successive focal planes, separated by 1 μm, and adjusted from the bottom to the top of the cell. The pictures shown correspond to the central part of the cell, normally the fifth or sixth focal plane.

Results

Effect of CrmA and Bcl-2 on granulysin and ceramide-induced apoptosis

Two main pathways of apoptosis have been elucidated to date: one is triggered by death receptors, and the other is the mitochondrial pathway, the main pathway used for stress-induced apoptosis (25, 26). In the first pathway the recruitment of adaptors to engaged receptors leads to activation of apical caspases, such as caspase-8 (27), while in the second pathway, the liberation of apoptogenic factors from mitochondria, such as cytochrome c, leads to the activation of caspase-9 (28). As a final step in both pathways, executioner caspases of the caspase-3 subfamily are activated. The death receptor pathway is blocked by CrmA, a viral serpin that preferentially binds and inhibits caspase-8 (29), while the Bcl-2 protein blocks the mitochondrial apoptotic pathway (30).

To analyze the relative involvement of each pathway in granulysin-induced cell death, we evaluated the cytotoxic potential of granulysin overexpressing CrmA or Bcl-2 (14). As shown in Fig. 1, CrmA offered some protection from granulysin-induced cell death (30% protection); the protection offered by Bcl-2 is more pronounced, although not complete (65% protection). This result points to a main implication of the mitochondrial apoptotic pathway in granulysin-induced apoptosis.

Since ceramide generation is also involved in granulysin-induced cell death (11), we tested in parallel the effect of CrmA or Bcl-2 overexpression on natural (C18) ceramide-induced apoptosis. As shown in Fig. 1, CrmA protection was limited (35% protection), while Bcl-2 protection from ceramide-induced apoptosis was complete (>90% protection). Similar results were obtained in previous studies using short chain ceramides (31–33). Therefore, the contribution of ceramide generation to granulysin-induced cell death seems to be strictly mediated through the mitochondrial apoptotic pathway.

Loss of ΔΨm induced by granulysin is independent of caspase activation and ceramide generation

To further assess the role of mitochondria in granulysin-induced cell death, we tested whether granulysin caused a loss in ΔΨm using the cationic lipophilic fluorochrome DiOC6(3) (22). It has been reported that this fluorochrome is sensitive to changes not only in mitochondrial, but also in plasma membrane potential (34). However, the contribution of plasma membrane potential to DiOC6(3) fluorescence is small, as demonstrated in a recent study (35). We have also shown by fluorescence microscopy that DiOC6(3) staining follows a punctate cytoplasmic pattern typical of a mitochondrial localization, with no detectable contribution of the plasma membrane. Treatment with the protonophore uncoupling agent CCCP or with the cytotoxic drug doxorubicin induces the complete loss of DiOC6(3) staining, while incubation in the presence of 0.15 M KCl, which abolishes plasma membrane potential, does not affect the fluorescence labeling (data not shown), in agreement with the results previously shown by flow cytometry (35). Therefore, DiOC6(3) can be properly used to estimate the changes in ΔΨm produced during apoptosis.

Granulysin treatment of Jurkat cells induced a rapid and progressive loss of ΔΨm, already detected after 1 h of incubation (compare Fig. 2, A and C), in agreement with our previous results (13). The percentage of cells exhibiting a low ΔΨm was about 75% after 12 h of incubation (Fig. 2G). In Jurkat cells, the loss of ΔΨm induced by several apoptotic signals, like the death receptor Fas/CD95 or the cytotoxic drug doxorubicin (25, 36), is dependent on the previous activation of Z-VAD-fmk-sensitive caspases. However, the ΔΨm loss induced by granulysin was not inhibited by Z-VAD-fmk at any time point tested (1 h, Fig. 2D; 5 h, Fig. 2F; or 12 h, Fig. 2H).

Since ceramide mediates apoptosis through the mitochondrial pathway, as indicated by the complete inhibition by Bcl-2 (Fig. 1), we performed a time-course analysis of granulysin-induced ceramide generation to estimate whether the observed rapid loss in ΔΨm induced by granulysin could be mediated by ceramide. We did not observe an increase in the Cer/SM ratio until 12 h of incubation with granulysin, in agreement with our previous study, in which this was the only time point analyzed (Table I) (11). No increase was observed after 1 or 5 h of incubation, although at 5 h the cytotoxicity of granulysin was already significant (Table I). These data do not support a role for ceramide generation in the rapid (1–5 h) ΔΨm loss induced by granulysin. In addition, the prominent loss in ΔΨm induced by natural ceramide (compare Fig. 3, A and B) was almost completely prevented by coincubation with the general caspase inhibitor Z-VAD-fmk (Fig. 3C). These data and those shown in Fig. 2 clearly distinguish between the apoptotic mechanisms initiated by granulysin and ceramide.
Bcl-2 effect on ceramide- and granulysin-induced loss of ΔΨm

Although protection from granulysin-induced cell death by Bcl-2 was significant, it was not as complete as that observed for ceramide-induced apoptosis (Fig. 1). We further examined the mechanism of the residual toxicity induced by granulysin in Bcl-2-overexpressing Jurkat cells (J-Bcl2).

Similar to that observed with Z-VAD-fmk (see Fig. 3C), Bcl-2 overexpression almost completely prevented ceramide-induced ΔΨm loss (90% protection; Fig. 3D). Granulysin-induced ΔΨm loss was detectable in J-vector cells after 5 h of incubation (compare Fig. 4F and B). This ΔΨm loss was also observed in J-Bcl2 cells (Fig. 4E), although to a much lesser extent than in control cells (60% protection). The ΔΨm loss induced by granulysin in J-vector cells after 12 h was prominent (Fig. 4E). At this time point the protection from ΔΨm loss offered by Bcl-2 overexpression was higher than that observed at the 5 h point, although it was not complete (Fig. 4F; 70% protection). Therefore, the protection offered by Bcl-2 on ΔΨm loss correlates with the protection from cell death shown in Fig. 1 for both ceramide- and granulysin-induced apoptosis.

Nuclear morphology of granulysin-treated cells in the presence of caspase inhibitors

In our previous study (11) we showed that while the inhibitor of the caspase-3 subfamily Ac-DEVD-CHO had almost no effect on granulysin-induced cell death, the general caspase inhibitor Z-VAD-fmk had some protective effect. We also showed that the processing of caspase-3 was somewhat limited during granulysin-induced apoptosis. However, granulysin induced the typical nuclear apoptotic morphology, which in most cases has been described to be dependent on caspase-3-mediated activation of the...
 FIGURE 5. Nuclear morphology of granulysin-treated cells. Jurkat (A–D) or J-Bcl2 (E and F) cells were left untreated (A) or were incubated in serum-free medium with 50 μM granulysin for 5 h (E) or 12 h (B and F) or with granulysin and either 600 μM Ac-DEVD-CHO (C) or 100 μM Z-VAD-fmk (D) for 12 h. Cell nuclei were stained with PPDA and photographed under epifluorescence illumination. Original magnification, ×400. The pictures shown are representative of at least three different experiments for each experimental condition. The percentage of cell death, estimated in parallel by trypan blue staining, and that of fragmented (E, F) and condensed (C, D) nuclei in the experiment shown are indicated in each case.

caspase-activated DNase (37). To test whether the nuclear apoptotic morphology observed was dependent on this subtle caspase-3 activation, we performed PPDA nuclear staining of cells treated with granulysin in the presence of Ac-DEVD-CHO and also of Z-VAD-fmk for comparison. As previously shown, granulysin induced the chromatin condensation and nuclear fragmentation typical of apoptosis (compare Fig. 5, A and B). However, this nuclear apoptotic morphology was no longer observed in cells treated with granulysin in the presence of Ac-DEVD-CHO (Fig. 5C) or Z-VAD-fmk (Fig. 5D). The nuclear morphology of cells dying in the presence of both caspase inhibitors was very similar: the chromatin condensed partially and was marginated to the edges of the nucleus, but there was no sign of nuclear fragmentation.

We also analyzed the nuclear morphology of J-Bcl2 cells treated with granulysin at different time points. After 5 h of incubation with granulysin, approximately 30% cell death was observed in Jurkat or J-vector cells (not shown), while this percentage was reduced to 12% in J-Bcl2 cells. A careful inspection of PPDA nuclear staining of J-Bcl2 cells treated in this way allowed localization of those cells with abnormal nuclear morphology, which were no >13% (Fig. 5E). Nevertheless, we were unable to detect any cell with the typical, fragmented nuclear apoptotic morphology. This abnormal nuclear morphology was similar to that observed for granulysin-treated Jurkat cells in the presence of caspase inhibitors (compare Fig. 5, C and D). When J-Bcl2 cells were incubated for 12 h with granulysin, cell death increased to 20%, while the percentage of cells with apoptotic nuclear morphology increased to a maximum of 22%. However, at this time point all the apoptotic nuclear morphologies detected were characterized by extensive chromatin condensation and nuclear fragmentation (Fig. 5F).

The nuclear morphology observed in Fig. 5, C–E, is reminiscent of that induced by AIF, a protein released from mitochondria in addition to cytochrome c during apoptosis which exerts its proapoptotic activity in a caspase-independent manner (15, 38).

AIF implication in granulysin-induced apoptosis

Therefore, we tested the possible involvement of AIF in granulysin-induced cell death. During apoptosis induction by a variety of stimuli, AIF is released from mitochondria and rapidly translocates to the nucleus, where it induces peripheral chromatin condensation and large-scale (50-kbp) DNA fragmentation (15). This translocation can be followed by confocal microscopy using a specific antiserum. As shown in Fig. 6A, AIF was almost exclusively localized in the cytoplasm of control Jurkat cells. Given the small size of the cytoplasm in these cells, it was difficult to distinguish the punctate staining corresponding to a typical mitochondrial localization. However, when cells were treated with granulysin for 5 h (Fig. 6B) or 12 h (Fig. 6C), the...
The means by which granulysin induces this caspase-independent, here, with AIF translocation from mitochondria to nucleus (Fig. 6). This correlated well with the observed protection from cell death of a total of 300 J-Bcl2 cells counted in different aleatory fields, only 20% of the cells showed nuclear staining for AIF. This correlated well with the observed protection from cell death (Fig. 1) and ΔΨm loss (Fig. 4) as a result of Bcl-2 overexpression. These data clearly demonstrate a role for AIF in granulysin-induced apoptosis and suggest that this is the apoptotic pathway remaining active in the presence of caspase inhibitors.

**Discussion**

The data obtained in the present work together with our previous results (11, 13) delineate a biochemical pathway involved in granulysin-induced cell death of tumor cells (Fig. 7). We showed previously that sphingomyelinase activation and ceramide generation were involved in granulysin-induced apoptosis (11), but the present data indicate that this pathway is only active at late time points (≥12 h), while granulysin toxicity is detectable at much earlier time points. This agrees with the observation that granulysin toxicity is maintained in cells depleted of sphingomyelin (SM) and ceramide by prolonged incubation with the ceramide synthase inhibitor fumonisin B1 (11). Together these data indicate the existence of a “fast,” ceramide-independent pathway of granulysin toxicity (Fig. 7).

The fast pathway of granulysin toxicity is associated with a rapid (~1 h) ΔΨm loss, which is caspase independent (Fig. 2). Granulysin-induced ΔΨm loss is associated with the release of cytochrome c from mitochondria (13) and also, as demonstrated here, with AIF translocation from mitochondria to nucleus (Fig. 6). The means by which granulysin induces this caspase-independent, rapid ΔΨm loss remains unclear. In a cell-free system, it has been shown that granulysin is unable to directly disrupt ΔΨm of isolated mitochondria (13), indicating that ΔΨm loss induced by granulysin is due to the action of a cytoplasmic mediator. Similarly to amebapore and NK-lysin, bioactive granulysin seems to exert its effects by association and insertion into lipidic domains of the plasma membrane (39). While the amebapore structure favors a pore-forming mechanism of action, granulysin and NK-lysin structures favor a membrane perturbation mechanism of action (39). In fact, it has been postulated that NK-lysin gets inserted into membranes affecting just the outer leaflet of the bilayer, but that this perturbation is sufficient to alter ionic equilibrium and the traffic of small molecules through membranes (40). Given the structural similarity between granulysin and NK-lysin (39), it is possible that granulysin exerts its effects on the plasma membrane by a similar mechanism (13). Previous results from our group demonstrated that granulysin is able to lyse negatively charged liposomes and to immediately induce cytoplasmic calcium concentration spikes in treated Jurkat cells (13). The increase in cytoplasmic calcium concentration is known to induce ΔΨm loss (41, 42) and apoptotic cell death through caspase-9 and caspase-3 activation (43–45). In consequence, the rapid ΔΨm loss induced by granulysin could be mediated by the cytoplasmic calcium concentration increase due to plasma membrane perturbation induced by granulysin insertion.

Although granulysin induces the rapid release of cytochrome c from mitochondria, the activation of caspase-9 has not been detected during granulysin-induced apoptosis (13). However, a somewhat limited caspase-3 activation, more easily detectable at early time points, was detected during granulysin-induced apoptosis (11, 13). It is possible that unprocessed procaspase 9, when complexed with cytochrome c and dATP, can activate downstream caspases, as previously proposed (46), or, alternatively, that the observed activation of caspase-3 may occur by a caspase-9-independent mechanism. In agreement with the described pathway for nuclear fragmentation (37), caspase inhibition by Ac-DEVD-CHO or Z-VAD-fmk prevents the typical apoptotic nuclear morphology (Fig. 5). In both cases the nuclear morphology detected resembled that induced by AIF in the absence of caspase and caspase-activated

![Hypothetical scheme for granulysin-induced apoptosis. Once inserted into the plasma membrane, granulysin facilitates calcium entry into the cytoplasm. An increased Ca2+ concentration would then lead to mitochondrial permeability transition, ΔΨm loss and release of cytochrome c (cyt c) and AIF. In addition, granulysin induces a slow activation of sphingomyelinas causing ceramide accumulation and the activation of a Z-VAD-sensitive caspase that induces mitochondrial permeability transition and ΔΨm loss. Apaf-1, apoptotic protease activation factor 1.](http://www.jimmunol.org/ Downloaded from)
different experiments. A recent study points to a role for ceramide generation (11, 50). A recent study points to a role for ceramide generation (11, 50). A recent study points to a role for ceramide generation (11, 50). This could also be the case for granulysin-induced apoptosis, since, similar to Bcl-2 protection from granulysin-induced apoptosis is not complete. This is in contrast with Bcl-2 protection from ceramide-induced cell death, which is complete (Fig. 1). The residual toxicity of granulysin on J-Bcl2 cells (~25%) correlates well to the extent of protection from AIF release (Fig. 6), indicating that the fraction of J-Bcl2 cells that remain sensitive to granulysin lose their ΔΨm and release AIF from mitochondria. The reason why protection from ceramide-induced apoptosis is more efficient than protection from granulysin should be related to the different mechanisms by which both apoptosis inducers cause ΔΨm loss. Ceramide-induced ΔΨm loss is mediated by a Z-VAD-sensitive caspase, while granulysin is able to induce ΔΨm loss through a caspase-independent pathway, possibly mediated by an increase in the intracellular calcium concentration. A caspase-mediated mechanism for ΔΨm loss has been characterized in some apoptotic processes, such as that mediated by Fas ligation. In this case, once caspase-8 is activated by recruitment via FADD to the engaged receptors, Bid, a proapoptotic molecule from the Bcl-2 family, is cleaved, and the fragments generated are able to insert in the mitochondrial outer membrane, producing by themselves, or in association with other molecules, mitochondrial permeability transition and ΔΨm loss (52, 53). It would be tempting to place caspase-8 as the Z-VAD-sensitive caspase responsible for ceramide-induced ΔΨm loss through Bid cleavage. However, this possibility is not supported by the poor protection offered by CrmA on ceramide-induced apoptosis, since the main target of CrmA antiapoptotic effect is caspase-8 (29). In the case of granulysin, the induction of mitochondrial permeability transition by disruption of the cellular ionic equilibrium seems to be rapid and efficient, probably because it does not need additional molecular intermediates. This could be the reason for the relative inefficiency of Bcl-2 protection from this pathway of ΔΨm disruption. The presence of a population of J-Bcl2 cells that is still sensitive to granulysin-induced apoptosis does not seem to be due to the existence of two populations of J-Bcl2 cells with different level of Bcl-2 expression, as analyzed by flow cytometry in permeabilized J-Bcl2 cells (data not shown). However, it seems that in the small percentage of J-Bcl2 cells that are still sensitive to granulysin-induced apoptosis, the typical apoptotic process is somewhat delayed. In these circumstances, AIF seems to be released before cytochrome c from mitochondria, and its particular effect on the nucleus is the only detected at early time points (Fig. 5E). At later time points, and presumably in the same cells in which AIF was previously released from the mitochondria, the caspase pathway should be activated at least in part, resulting in the typical nuclear fragmentation (Fig. 5F). This is in agreement with the recent observation that AIF translocation to the nucleus precedes temporally cytochrome c release during staurosporine-induced apoptosis (38).

In conclusion, granulysin-induced cell death may constitute an additional molecular mechanism mediated by CTL degranulation against tumor development. Knowledge of the biochemical mechanisms of apoptosis induction activated by granulysin should be useful in future cancer therapies using granulysin or granulysin-derived bioactive peptides (54).

Acknowledgments

We gratefully acknowledge Drs. Pilar Lasierra and Luis Larrad, Servicio de Immunología, Hospital Clínico Universitario, Universidad de Zaragoza, and contrary to the fast pathway of granulysin-induced apoptosis, the ΔΨm loss induced by natural (C18) ceramide is completely prevented by Z-VAD-fmk (Fig. 3). This places a still unidentified caspase up-stream of mitochondrial permeability transition during ceramide-induced apoptosis, in accord with the complete protection from ceramide-induced cell death, at least in Jurkat cells, by the general caspase inhibitor Z-VAD-fmk (11). The role of ceramide generation as part of the apoptotic execution machinery is controversial (48). Although ceramide generation is normally observed during apoptotic cell death, it is usually a late event that could constitute a safeguard mechanism to assure the completion of the apoptotic process (49, 50). This could also be the case for granulysin-induced apoptosis, since, similar to Fas-induced apoptosis, both apoptotic processes can take place in the absence of ceramide generation (11, 50). A recent study points to a role for ceramide generation more related to the changes that take place in the plasma membrane during apoptosis and linked to phosphatidylserine exposure in the outer leaflet of the plasma membrane and the formation of apoptotic blebs (51).

The data on the significant protection from granulysin-induced cell death by Bcl-2 reinforce the central involvement of the mitochondrial pathway of apoptosis in this CTL effector mechanism. It is noteworthy that, although important (~75%), Bcl-2 protection from granulysin-induced apoptosis is not complete. This is in contrast with Bcl-2 protection from ceramide-induced cell death, which is complete (Fig. 1). Table I. Time course of ceramide generation during granulysin-induced apoptosis.a

<table>
<thead>
<tr>
<th>Ceramide:SM ratio</th>
<th>1 h</th>
<th>6 h</th>
<th>12 h</th>
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<tbody>
<tr>
<td>Controlb</td>
<td>0.19</td>
<td>0.12</td>
<td>0.11</td>
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<tr>
<td>Granulysin50 μM</td>
<td>0.14</td>
<td>0.13</td>
<td>0.57</td>
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<tr>
<td>% cell deathc</td>
<td>5 ± 2</td>
<td>26 ± 4</td>
<td>85 ± 10</td>
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* Jurkat cells were labeled with [1-14C]palmitic acid and treated with 50 μM granulysin for the times indicated; lipids were then separated and analyzed as indicated in Materials and Methods.

* Results are expressed as ceramide SM ratio and are the mean of at least three different experiments.

* Results are expressed as percent of cell death, estimated by trypan blue staining.
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9.[indexPathPoint.x, indexPathPoint.y, indexPathPoint.width, indexPathPoint.height]