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Caspase Dependence of Target Cell Damage Induced by Cytotoxic Lymphocytes

Apurva Sarin,1 Elias K. Haddad, and Pierre A. Henkart2

Since the CTL secreted granule protease granzyme B can activate multiple target caspases, it has been proposed that this pathway is responsible for CTL-induced cytolysis of Fas-negative targets. However, target lysis via the granule exocytosis pathway is completely resistant to caspase inhibitors. To test the possibility that granzymes trigger a postcaspase cytoplasmic apoptotic pathway leading to lysis, we have examined the caspase dependence of several cytoplasmic changes associated with apoptotic death. Rapid prelytic phosphatidylserine externalization was induced in Jurkat target cells by both the Fas ligand (FasL)/Fas and the granule exocytosis effector pathways. This was specifically blocked by peptide ketone caspase inhibitors when induced by the former, but not by the latter, pathway. A rapid prelytic loss of target mitochondrial $\psi_m$ was also induced by both CTL effector pathways, and this was also specifically blocked by caspase inhibitors when induced by the FasL/Fas, but not by the granule exocytosis, pathway. Similarly, target membrane blebbing induced by CTL via the FasL/Fas, but not via the granule exocytosis, effector pathway was specifically blocked by caspase inhibitors. In contrast to the above nonnuclear damage, CTL-induced target staining by the lipid probe FM1–43 reflecting plasma membrane endocytosis was blocked by caspase inhibitors. Thus, when caspase activation is blocked, the granule exocytosis pathway triggers several parameters of target apoptotic damage in addition to lysis, suggesting that granzymes directly trigger a postcaspase cytoplasmic apoptotic death pathway. The Journal of Immunology, 1998, 161: 2810–2816.

Cytotoxic lymphocytes rapidly kill target cells in vitro using two effector pathways that are triggered by membrane receptors recognizing target Ag. One is a secretory pathway involving receptor-triggered exocytosis of preformed secretory granules, while the use other receptors-induced surface membrane expression of Fas ligand (FasL),3 which cross-links Fas on target cells (1). While CTL use both of these effector pathways to kill target cells, NK cells chiefly use the granule exocytosis pathway (2), which postulates a vital role for the granule protein perforin (also known as cytolyisin) in permeabilizing target membranes (3). The in vivo importance of this effector pathway has been confirmed by the development of perforin-deficient deficient, which show a seriously compromised ability to resist infection by noncytopathic viruses as well as other immunologic defects (4).

Since target cells killed by cytotoxic lymphocytes in vitro typically display characteristic apoptotic features (5), an appealing hypothesis has been that a common apoptotic death pathway existing in a latent form in all cells is triggered by these effector cells (6). Rapid progress has been made recently in identifying a pro tease family termed caspases as central molecular mediators of apoptotic cell death (7). The 10 currently identified human caspases are widely expressed in an inactive precursor form in many cells and have the hallmark specificity of cleaving protein substrates with aspartic acid residues at the P1 position. Their activation occurs as a result of proteolytic processing of procaspases at aspartic acid residues, so that caspases can autoactivate and process each other in an activation cascade (8). Despite the general consensus that caspases play a central role in apoptotic death, important questions remain unanswered. One of these is the nature of postcaspase cell destruction pathway leading to the various characteristic features of apoptotic cell death, including phosphatidyl serine (PS) externalization and lysis.

Recent experiments have shown that both effector pathways used by cytotoxic lymphocytes activate caspases. Cross-linking of target cell Fas by FasL on CTL membranes leads to activation of caspases 8 and probably 10 via the adaptor molecule FADD (9). These caspases can process and activate downstream caspases that, in turn, trigger cell destruction. We have shown that rapid target cell lysis and apoptotic nuclear damage by this CTL effector pathway are blocked by two classes of caspase inhibitors, confirming the predicted functional importance of caspases in this effector pathway (10).

For the granule exocytosis pathway, the requirement for caspases in target cell death is less clear. Caspase activation via the granule exocytosis pathway is predicted because the granule serine protease granzyme B recognizes a sequence motif compatible with caspase activation (11) and has been shown to initiate processing and activation of several caspases (12). CTL targets undergo rapid caspase-3 processing as predicted by these in vitro experiments (13), thus providing an explanation for apoptotic nuclear damage induced by granzyme B in the presence of sublytic doses of perforin (14). However, while CTL from mice lacking granzyme B induce target nuclear damage somewhat more slowly, their potency and rate of target lysis via the granule exocytosis pathway are unaffected by the loss of granzyme B (15). (Lysis by activated NK cells from mice lacking granzyme B is partially defective
Materials and Methods

Reagents

The protease inhibitors Chx-Val-Asp(O-methyl)-fluoromethyl ketone (ZVAD-FMK), Boc-Asp(O-methyl)-fluoromethyl ketone (BD-FMK), and Chx-Phe-Ala-fluoromethyl ketone (ZFA-FMK) were purchased from Enzyme Systems Products (Dublin, CA), made up as stock solutions of 50 mM in DMSO, and stored at −70°C. IgG anti-Fas mAb DX2 was obtained from Pharmingen (San Diego, CA). FM1−43, DiIC16, and DiOC6(3) were obtained from Molecular Probes (Eugene, OR) made up as 1 mM stocks in DMSO, and stored at 4°C. Annexin V-FITC was obtained from Trevigen (Gaithersburg, MD). PMA, ionomycin, and Hoechst 33342 were stock in DMSO, and stored at 4°C. Annexin V-FITC was obtained from Sigma (St. Louis, MO).

Target and effector cells

The human lymphoblastoid cell line Jurkat and the erythromyeloid leukemia K562 were maintained in complete medium (RPMI 1640 supplemented with 10% FCS, 100 IU penicillin, and 10 μg/ml streptomycin) and used as target cells. To distinguish these cells from effector cells by flow cytometry, they were pretreated with the protease inhibitors Chx-Val-Asp(O-methyl)-fluoromethyl ketone (ZVAD-FMK), Boc-Asp(O-methyl)-fluoromethyl ketone (BD-FMK), and Chx-Phe-Ala-fluoromethyl ketone (ZFA-FMK) were purchased from Enzyme Systems Products (Dublin, CA), made up as stock solutions of 50 mM in DMSO, and stored at −70°C. IgG anti-Fas mAb DX2 was obtained from Pharmingen (San Diego, CA). FM1−43, DiIC16, and DiOC6(3) were obtained from Molecular Probes (Eugene, OR) made up as 1 mM stocks in DMSO, and stored at 4°C. Annexin V-FITC was obtained from Trevigen (Gaithersburg, MD). PMA, ionomycin, and Hoechst 33342 were obtained from Sigma (St. Louis, MO).

Test assays of target damage

All experiments assessing the CTL granule exocytosis pathway used primary cultures of allo-CTL redirected to TNP target cells with 100 ng/ml αCD3×α-CD28 to TNP heteroclonally as previously described (17). In these incubations the Fas cytotoxicity pathway was blocked by addition of 10 μg/ml of IgG anti-Fas mAb DX2, which completely blocked Jurkat lysis by d11S CTL. Incubations were conducted in flat-bottom microtiter wells containing 10^6 target cells, which, when indicated, were pretreated with peptide-FMK caspase inhibitors for 1 h before addition of effector cells. The indicated number of effector cells was added and centrifuged at 300 rpm. The plates were then placed in a 37°C CO2 incubator at time zero. At the indicated times wells were harvested by pipetting, and target damage was assessed by flow cytometry after pooling an appropriate number of wells.

Results

Target nuclear damage, but not lysis, induced by human NK cells is blocked by peptide caspase inhibitors

Human PBL were tested for their ability to lyse and induce target nuclear damage in the classic NK target K562. As shown in Figure IA, a 4-h incubation of normal PBL with K562 target cells at an E:T cell ratio of 20 resulted in both lysis and apoptotic nuclear damage of these targets, confirming previous results with these apoptosis-resistant BCR-overexpressing target cells (19). The dependence of these cytotoxic effects on caspases was tested using the cell-permeable peptide-based caspase inhibitors ZVAD-FMK and BD-FMK, which were previously shown to block a variety of death readouts in lymphocytes treated with various apoptogenic agents (20). These caspase inhibitors caused a dose-dependent inhibition of K562 apoptotic nuclear damage, while the control reagent ZFA-FMK slightly enhanced such damage. In contrast, none

![FIGURE 1. Peptide caspase inhibitors block target nuclear damage but not lysis induced by NK cells. The human leukemia K562 cell line was used as a target for cytotoxicity by human PBMC, which were used directly (A; E:T cell ratio = 20) or after overnight activation with IL-2 (B; E:T ratio = 5). Four-hour incubations were conducted in the presence of peptide-FMK reagents: ZVAD-FMK (triangles), BD-FMK (squares), or ZFA-FMK (diamonds). Target lysis was measured by 51Cr release (filled symbols) in triplicate wells, and target nuclear damage (open symbols) was assessed microscopically on DiIC16 prelabeled K562 cells using Hoechst 33342 in parallel wells in the same plate, as described in Materials and Methods.

To assess nuclear morphology and formation of membrane blebs, cells were stained with 5 μg/ml Hoechst 33342, and DIIC16-positive cells were scored for apoptotic nuclear morphology or the presence of blebs by fluorescence microscopy. Plasma membrane recycling was measured by incubation of the cell suspension with 5 mM FM1−43 for 10 min and washing three times in complete medium before analysis on the flow cytometer. Because of the fluorescence properties of FM1−43, it was not possible to identify target cells with DiIC16, and in these experiments target cells were selectively analyzed using a scatter gate (two-dimensional forward and side scatter) defined by running effectors and targets alone. This approach limited these experiments to modest E:T cell ratios.
of these peptide-FMK reagents had a significant effect on target lysis. These results are strikingly parallel to those previously obtained with mouse CTL using the granule exocytosis effector pathway (10).

NK cells activated by overnight culture with IL-2 give an enhanced cytotoxic activity, and the caspase dependence of this activity was similarly tested in the experiment shown in Figure 1B. The considerably more potent cytotoxic activity of these activated NK cells showed a similar pattern of blocking by caspase inhibitors as fresh NK cells, i.e., potent blocking of nuclear damage by ZVAD-FMK, slightly weaker blocking by BD-FMK, and no effect of ZFA-FMK, while there was no effect on target lysis. In other experiments we further confirmed these findings using adherent activated mouse spleen NK cells with mouse YAC tumor targets (data not shown).

The CTL FasL/Fas effector pathway induces a caspase-dependent target cell PS exposure before lysis

Because our objective was to examine the nonnuclear apoptotic events induced in target cells by cytotoxic lymphocytes before lysis, we have used flow cytometry to identify PI-negative target cells prelabeled with a lipid probe and followed a third marker of apoptotic damage. We have separately tested the granule exocytosis and FasL/Fas effector pathways, since these together account for rapid in vitro CTL-mediated target cell death (1). We first examined the surface exposure of PS by binding of FITC-annexin V. Figure 2A shows that the FasL-bearing effector cell d11S (21) mediates a rapid PS exposure on PI-negative Jurkat target cells at modest E:T cell ratios. Figure 2B indicates that this PS exposure as well as the induction of apoptotic nuclear morphology are specifically blocked by peptide-FMK caspase inhibitors. This result is predicted by a pathway involving Fas-induced cross-linking of caspase 8 or 10 via FADD and was expected in light of previous results showing that PS exposure induced by anti-Fas Ab is caspase dependent (22, 23).

The granule exocytosis effector pathway induces a rapid target cell PS exposure before lysis that is not blocked by peptide-FMK caspase inhibitors

Figure 3A shows that primary in vitro-generated CTL, in the presence of IgG anti-Fas to block the Fas killing pathway, mediate a rapid prelytic PS exposure in Jurkat target cells. In Figure 3B we tested the ability of ZVAD-FMK and BD-FMK to block this PS exposure and observed a dramatic contrast to the results presented in Figure 2B. In this case, peptide-FMK caspase inhibitors efficiently blocked apoptotic nuclear morphology (confirming our previous results (10)), but had no effect on PS exposure.

A similar experiment with IL-2-activated human NK cells and K562 target cells is shown in Figure 4. In this case PS exposure was extremely rapid, with >35% of target cells showing increased annexin V staining within 20 min at an E:T cell ratio of 10. However, this PS exposure was not detectably inhibited by peptide-FMK caspase inhibitors, although these inhibitors blocked apoptotic nuclear damage in the same experiment (Fig. 4B).
Peptide caspase inhibitors block prelytic target mitochondrial potential loss induced by the CTL FasL/Fas pathway, but not by the granule exocytosis pathway

Dying cells have been reported to undergo a rapid loss of mitochondrial potential, \( \psi_m \), as measured by DiOC\(_6\) fluorescence (24). To determine whether CTL targets undergo a rapid prelytic loss of mitochondrial potential, we used a similar analysis by flow cytometry. Figure 5A shows that the FasL-bearing d11S cells induce a loss of mitochondrial \( \psi_m \) that is detectable within 1 h, and Figure 6A shows that CTL using the granule exocytosis pathway show a generally similar activity. The ability of peptide-FMK caspase inhibitors to block this mitochondrial damage was then tested. As shown in Figure 5B, the prelytic mitochondrial \( \psi_m \) loss induced by the FasL-bearing d11S CTL was dramatically and specifically blocked by these inhibitors along with nuclear damage. In contrast, loss of \( \psi_m \) via the CTL granule exocytosis pathway was not detectably inhibited by the caspase inhibitors, although in the same cells apoptotic nuclear damage was effectively and specifically inhibited (Fig. 6B).

Caspase inhibitors block membrane bleb formation by the CTL FasL/Fas pathway, but not by the granule exocytosis pathway

Membrane blebbing has long been recognized as an early sign of CTL-induced target cell injury (25, 26) and is part of the classical syndrome of apoptotic death (27). We have found that the DiIC\(_6\) lipid probe used in the above experiments to distinguish target cells from CTL affords a reliable means of visualizing target plasma membrane blebs in the fluorescent microscope. Using Jurkat target cells and the CTL effector systems described above, we observed a rapid induction of blebs by both the granule exocytosis and FasL/Fas pathways, with 40 to 50% of the target cells showing clear membrane blebs by 90 min at modest E:T cell ratios (Fig. 7). When peptide-FMK caspase inhibitors were tested for the ability to block this bleb formation, they were found to specifically block that induced by d11S, but not primary CTL using the granule exocytosis pathway.

Caspase inhibitors block the CTL-induced increase in target membrane recycling by the granule exocytosis pathway

CTL have been reported to rapidly induce an increase in staining of target cells after a pulse exposure to the lipid probe FM1–43 (28). Such increased FM1–43 staining is considered a measure of endocytic membrane internalization and is found at neuronal synapses after transmitter release (29, 30). Figure 8 shows the rapid increase in FM1–43 staining in Jurkat target cells induced by the CTL granule exocytosis pathway. Figure 8A shows that 90 min after mixing CTL with target cells under conditions of TCR engagement there was a clear subpopulation of target cells with increased FM1–43 fluorescence. This increase was inhibited by the caspase inhibitors BD-FMK and ZVAD-FMK, but not by the control compound ZFA-FMK (Fig. 8B). Thus, increased FM1–43 staining is one measure of nonnuclear damage induced by CTL granule exocytosis that is blocked by peptide-FMK caspase inhibitors and thus appears to be similar to nuclear damage in its sensitivity to caspase inhibitors.
The experiments described here show that both cytotoxic lymphocyte effector pathways rapidly induce target cell death, which has a full spectrum of apoptotic properties. These include membrane damage, as seen by PS externalization and blebbing, as well as the fall in electrical potential across the inner mitochondrial membrane. These findings confirm that target death by both effector pathways is apoptotic, as previously revealed by nuclear damage, and show that nonnuclear target damage induced by cytotoxic lymphocytes is rapid compared with that caused by many other agents.

The above examples of target damage induced by CTL are not surprising in light of previous demonstrations that CTL activate caspases, since caspase inhibitors block all measurable apoptotic damage by a wide range of apoptotic triggers. However, the most interesting aspect of the experiments described here is that three of the four measurements of target damage induced by the granule exocytosis pathway were not blocked by caspase inhibitors that completely blocked nuclear damage in the same experiment. This means that the these nonnuclear events do not require the granzyme-induced caspase activation that leads to nuclear damage, part of which is probably attributable to the recently described caspase-activated DNase (31). Since these nonnuclear changes are typically associated with apoptotic death, the results strongly suggest that granzymes directly trigger a nonnuclear molecular death pathway that leads to mitochondrial depolarization, membrane PS externalization and blebbing, and lysis, as shown in Figure 9. The molecular nature of the postcaspase cell destruction pathway has not been defined in any system. While it is sometimes assumed that caspase-induced nuclear damage leads to cell death, this is clearly not the case for CTL, since enucleated target cells are rapidly lysed by both pathways (32). We find it plausible that granzymes and caspases have common substrates that initiate this downstream death pathway, as shown in Figure 9. While a number of nuclear substrates of caspases have been identified, the critical substrates that initiate the pathway leading to lysis remain unidentified.

The data obtained from the present experiments are not compatible with the view that target lysis via the granule exocytosis pathway occurs solely as a result of perforin-induced membrane damage while target apoptotic damage is attributable to granzyme-mediated activation of caspases. This view is sometimes expressed.
by others despite its failure to explain both the kinetics of perforin vs CTL-induced lysis and the granzyme dependence of the cytotoxic activity of RBL transfectants (33). The present data showing that some nonnuclear damage is not blocked by caspase inhibitors argue further against this model.

One limitation of our experiments is the use of caspase inhibitors whose ability to block intracellular caspase activation is incompletely defined. For that reason, we previously showed (6, 10) that CTL granule exocytosis-induced target lysis was not affected by baculovirus p35, which inhibits caspases 1 to 4 at a 1:1 molar ratio (34). We have also previously shown that the peptide FMK inhibitors used in this paper block the DEVDase activity characteristic of caspases 3 and 7 in extracts of apoptotic Jurkat cells (20). However, it is not clear how to compare the activities of such caspases in extracts with those in intact cells, and it is not established which of the 10 known caspases are directly inactivated by the reagent conditions used. Thus, it is quite possible that these peptide-FMK inhibitors block the Fas death pathway by selectively reacting with caspases 8 and 10, which may be the targets of granzyme B (12). One can postulate that the failure of peptide-FMK caspase inhibitors to block lysis and nonnuclear damage by the granule exocytosis pathway is due to their failure to block the relevant granzyme B-activated caspases. However, such an explanation does not address why these inhibitors so effectively block nuclear damage in this pathway, and one would thus need to postulate that nuclear damage requires caspases sensitive to these inhibitors but that lysis and other nonnuclear damage use caspases that are resistant. Granule exocytosis-triggered lysis of T lymphoid cells appears unique among apoptotic agents in its resistance to inhibition by peptide-FMKs, as we have shown that peptide-FMK caspase inhibitors effectively and specifically block lysis of Jurkat target cells induced by anti-Fas, TNF, staurosporine, vinblastine, and several DNA-damaging chemotherapeutic agents (10, 35). In this respect Jurkat cells reflect the apoptotic lysis of most T lymphoid cells induced by a wide variety of agents (20). Thus, while we cannot formally rule out a role for peptide-FMK-resistant caspases as described above, our experiments show that it is very likely that CTL-injected granzymes act by directly triggering a downstream apoptotic death pathway without the involvement of caspases.

While unusual, there are several other reported examples of apoptotic cell death which are resistant to caspase inhibitors, and it will be of interest to determine whether these deaths share molecular steps with death induced by lymphocyte granule exocytosis. In fibroblasts, ZVAD-FMK and BD-FMK retard, but do not, prevent apoptotic nuclear damage and cell death induced by several agents (36), and defects in the ubiquitin-activating enzyme E1 lead to an apparently caspase-independent death (37). Some examples of apoptotic death resistant to ZVAD-FMK have been reported (38, 39), but we have found cases where the analogous caspase inhibitor BD-FMK specifically blocks death (20).

Externalization of PS is a functionally important manifestation of apoptotic death, since this triggers one of the recognition systems used by macrophages to phagocytose apoptotic cells (40). This lipid is normally restricted to the inner membrane leaflet but becomes detectable externally before cytolysis during apoptotic death (41). For the rapid target death induced by cytotoxic lymphocytes it has not been previously shown that PS externalization occurs before lysis, and our findings suggest that cells killed by CTL in vivo are likely to be rapidly phagocytosed.

The molecular pathways leading to PS externalization during apoptosis are not well understood. In Jurkat cells treated with anti-Fas this process requires active caspases and calcium in the medium (42, 43). A plausible model for this would be that caspase activation results in an elevation of intracellular calcium (44), thus activating the lipid scramblase that mediates transbilayer phospholipid “flip-flop” (45). For the granule exocytosis death pathway, it is likely that perforin pores also allow leakage of external calcium across the target membrane, although this may be temporary due to membrane repair processes. Thus, additional granule components such as granzymes may also be required to trigger PS exposure, and its calcium dependence is not readily tested, since calcium is also required for degradation. It is clearly important to design future experiments to probe this issue and to establish whether target lysis is closely linked to PS exposure.

The prelytic collapse of mitochondrial potential ψ is associated with the mitochondrial permeability transition and may occur in both apoptotic and nonapoptotic death (46). An associated release of a mitochondrial apoptosis-inducing factor has been proposed to be a critical step in apoptotic signaling (47). As shown for the CTL-mediated Fas death pathway in Figure 5B, ZVAD-FMK blocks the anti-Fas-induced loss of mitochondrial electron transport in Jurkat cells (48), the UV B-induced loss of mitochondrial ψ in CEM cells (49), and some mitochondrial damage in apoptotic thymocytes (46). For death induced by the granule exocytosis pathway, induction of mitochondrial damage is one candidate functional role for granymes. Granymes could be envisioned to trigger the release of mitochondrial cytochrome c, thus activating procaspase 9 via Apaf-1 (50, 51), or triggering the permeability transition and release of apoptosis-inducing factor (47). However, both of these are proposed to trigger death via activation of downstream caspases, making such models unattractive in light of the present evidence for caspase independence.

The increase in FM1–43 pulse staining induced by the CTL granule exocytosis pathway (Fig. 8) confirms a previous report that both CTL effector pathways rapidly induce an enhancement in plasma membrane endocytosis into internal vesicles revealed by this technique (28). This fluorescent lipid probe rapidly and reversibly partitions into the outer bilayer leaflet from the medium, so that postpulsing washing removes it from the exposed plasma membrane but not from membrane that underwent endocytosis during the pulse. The increased rate of plasma membrane uptake is a property of other apoptotic death systems as well, including thymocytes treated with dexamethasone and etoposide in vitro (28) (E. K. Haddad, unpublished observations). While it seemed plausible to assume that such enhanced endocytosis was part of the apoptotic membrane dysregulation also resulting in blebbing and PS exposure, Figure 8 shows that for CTL granule exocytosis-induced target damage, this enhanced endocytosis is part of a caspase-dependent pathway and hence distinct from the other types of membrane damage. These results also show that nuclear damage is not the only caspase-dependent damage induced by the granule exocytosis pathway.

Further molecular definition of the postcaspase cell destruction pathway is clearly of interest. The present results are helpful in that regard because they suggest that identification of critical protein substrates cleaved by both granymes and caspases may lead to identification of critical players triggering this pathway.

Acknowledgments

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