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Granzyme B Activity in Target Cells Detects Attack by Cytotoxic Lymphocytes

Beverly Z. Packard,‡ William G. Telford, Akira Komoriya, and Pierre A. Henkart

Lymphocyte-mediated cytotoxicity via granule exocytosis operates by the perforin-mediated transfer of granzymes from CTLs and NK cells into target cells where caspase activation and other death pathways are triggered. Granzyme B (GzB) is a major cytotoxic effector in this pathway, and its fate in target cells has been studied by several groups using immunodetection. In this study, we have used a newly developed cell-permeable fluorogenic GzB substrate to measure this protease activity in three different living targets following contact with cytotoxic effectors. Although no GzB activity is measurable in CTL or NK92 effector cells, this activity rapidly becomes detectable throughout the target cytoplasm after effector-target engagement. We have combined the GzB substrate with a second fluorogenic substrate selective for caspase 3 to allow both flow cytometry and fluorescence confocal microscopy studies of cytotoxicity. With both effectors, caspase 3 activity appears subsequent to that of GzB inside all three targets. Overexpression of Bcl-2 in target cells has minimal effects on lysis, NK- or CTL-delivered GzB activity, or activation of target caspase 3. Detection of target GzB activity followed by caspase 3 activation provides a unique readout of a potentially lethal injury delivered by cytotoxic lymphocytes. The Journal of Immunology, 2007, 179: 3812–3820.

Lympocyte-mediated cytotoxicity is a major immune effector function required for normal defense against pathogens, control of neoplasms, and regulating immune responses (1, 2). Cytotoxic lymphocytes utilize two molecular pathways to kill targets: the perforin-dependent granule exocytosis pathway and the Fas ligand/Fas pathway (3). As shown in studies with perforin-defective mice and humans, the perforin-dependent pathway plays an important and generally dominant role both in vitro and in vivo (4). It is now clear from in vitro studies that perforin’s main role is to permeabilize target membranes to allow entry of proteases that also originate in secretory granules of effector cells (5). These effector proteases are granzymes, a subclass of serine proteases with diverse proteolytic specificities (6).

Granzyme B (GzB) is a major component of cytotoxic lymphocyte granules and has received the most attention as a cytotoxic mediator because its protease activity is quite similar to caspases (7). GzB can trigger an apoptotic caspase cascade either directly via its ability to process procaspases or indirectly via its ability to cleave the Bcl-2 family member BID (8). GzB-truncated Bid, tBid, exhibits strong proapoptotic activity by promoting permeabilization of the mitochondrial outer membrane to allow release of Apaf-1 and other apoptotic mediators into the cytoplasm. In many cells, a similar “mitochondrial amplification loop” involving Bid cleavage greatly enhances apoptosis via receptors bearing death domains that activate procaspase 8, particularly Fas. However, the presence of Bcl-2 has been reported to block caspase activities and apoptosis via multiple pathways including that induced by purified perforin and GzB (9).

Most experiments designed to examine the role of granzymes in cytotoxicity have relied on anti-granzyme Abs to localize granzymes after fixation or to immunoprecipitate granzymes after extraction; these approaches have led to difficulties in interpretation because of uncertainty about whether immunoreactive granzymes were proteolytically active. We have thus designed a selective fluorogenic GzB substrate that can be used with living cells to probe the activity of this protease in situ during cell-mediated cytotoxicity (10). The protease substrate design strategy used in the current study is based on insertion of peptide-recognition motifs (typically 7–8 aa around the cleavage site) derived from physiologic macromolecular protease targets into a peptide backbone of 20 aa (11). Covalent labeling near both ends of the peptide with the same fluorophore results in 90–99% fluorescence quenching due to non-covalent intramolecular cyclization. Proteolytic cleavage relieves the quenching, resulting in a fluorescent signal. Because the intact substrates are permeable to the membranes of living cells and the fluorogenic proteolytic fragments are well retained, these substrates have proven useful for the detection of intracellular proteolytic activities, including caspases (12–15) and elastase (10). In the current study, we have used this approach to study protease activities in target cells attacked by effector cells, with substrates for both GzB (using its DNA-dependent protein kinase catalytic subunit cleavage site -VGPD ↓ FGR-) and caspase 3 (using its poly(ADP-ribose) polymerase cleavage site -DEVD ↓ GI-). By using two fluorophores with nonoverlapping signals simultaneously, this approach permits monitoring of both activities in parallel. The ability to detect GzB activity within target cells allows confirmation that the effector cell has delivered a potentially lethal injury, while subsequent caspase activation combined with morphologic signs of target cell apoptosis further define death in individual target cells.

Materials and Methods

Materials

RPMI 1640 medium and FCS were purchased from HyClone and horse serum from Sigma-Aldrich. The following mouse anti-human mAbs with

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2 Abbreviations used in this paper: GzB, granzyme B; PI, propidium iodide.

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isotype-matched controls were used: anti-Fas (CD95) IgM (catalog no. IM2387) from Beckman Coulter, fluorescein-conjugated anti-Fas (CD95) IgG1 (catalog no. FAB142F) from R&D Systems, and PE-conjugated anti-Bcl-2 IgG1 (catalog no. 556535) from BD Pharmingen. OKT3 monoclonal purified from the supernatant of the hybridoma CRL 8001 from the American Type Culture Collection by a protein A/G column from Genex was labeled with TFP-PEO-biotin from Pierce. Recombinant human IL-2 (rIL-2) was obtained from Cetus/Chiron, staurosporine from EMD, human trypsin from Sigma-Aldrich, propidium iodide (PI) from Molecular Probes, TFL4 from OncolImmun, streptavidin and sulfo-NHS-LC biotin from Pierce (catalog no. 21335), recombinant human GzB from Kamiya, and recombinant human caspase 3 from BD Pharmingen. Jurkat cells obtained from Beckman Coulter as well as Ramos and Daudi were grown in RPMI 1640 plus 10% FCS and NK92 obtained from the American Type Culture Collection in IL-2-containing (200 U/ml) RPMI 1640 plus 10% FCS and 10% human serum. The CD8+ and 641 tumor-infiltrating lymphocytes, respectively (16), were established from human melanoma masses in the presence of IL-2 and have been propagated in IL-2-containing (200 U/ml) RPMI 1640 plus 10% FCS. The Bcl-2 and LacZ (vector)-transfected Jurkat cells, a gift from Drs. C. Walsh (University of California, Irvine, CA) and S. Hedrick (University of California San Diego, LaJolla, CA), were passaged in RPMI 1640 plus 10% FCS and 500 μg/ml geneticin. All cells used in this study were of human origin.

Protease substrates

The reagents and methods used for peptide synthesis and derivatization have been described in detail previously (10). Briefly, peptides were synthesized using both an automated peptide synthesizer and by manual solid-phase methodology and subsequently purified by reverse-phase HPLC. Each purified peptide was derivatized with the appropriate fluorophore and characterized as previously described (12). For activation with 488-nm laser lines and detection with FITC-type filters, peptides were homodually labeled with rhodamine-110, whereas for studies with krypton or 561 diode laser lines labeling was with tetramethylrhodamine. Protease activity in solution was measured using a Photon Technology International fluorometer with FeliX software. Substrates were dissolved at 1 μM in PBS (pH 7.2) for GzB and in 50 mM HEPES (pH 7.2) containing 10 μM DTT for caspase 3 activity determinations. All measurements were made at 37°C with excitation set at 505 ± 2 nm and emission at 530 ± 2 nm. Final concentrations of GzB and caspase 3 were 200 and 100 nM, respectively.

Apoptosis experiments

Cells were suspended at 1 × 10^5/ml in RPMI 1640 containing 10% FCS plus either 1.5 μM staurosporine or 1 nM anti-Fas Ab and incubated at 37°C for 3 or 6 h, respectively. Immediately following apoptotic induction, incubations with 10 μM fluorogenic substrates were conducted at 37°C for 40 min as described previously (12, 13) and fluorescence intensity due to the cleavage of these probes was measured by flow cytometry as described below. For lymphocyte-mediated cytotoxicity, target cells were incubated with 400 nM TFL4 in PBS at 37°C for 30 min, washed twice with 10-fold excess medium and then counted. Redirection cytotoxicity was performed as described elsewhere (17), with the following modification: Biotin labeling of target cells was with 50 μM sulfo-biotin-NHS at 4°C for 15 min. Effector and TFL4-labeled target cells were added to individual wells of a 96-well round-bottom tissue culture plate either alone or at E:T ratios shown in the figures. After centrifugation and decantation of the supernatant, cells were resuspended in RPMI 1640 containing 10 μM fluorogenic substrates. Following a brief centrifugation, incubations were conducted at 37°C for the indicated times.

Flow cytometry

For all experiments to which flow cytometric analysis was applied, a minimum of 10,000 gated events were collected; identical conditions were replicated at least three times each. Specifically, for apoptosis experiments 10,000 PI-negative cells were acquired as previously described (12) and for cell-mediated cytotoxicity studies, data acquisition continued until the signals from 10,000 target cells were collected. When measuring apoptosis induced in Jurkat cells via staurosporine or anti-Fas Ab, intracellular protease activation was measured exclusively in PI-negative cell populations; in all studies, the starting as well as vehicle control samples contained <5% PI-positive cells. For cell-mediated cytotoxicity experiments, protease activation was quantitated exclusively in TFL4-positive target cell populations using a single argon ion (488 nm) laser source and with simultaneous excitation from an argon (488 nm) and rhodamine 110-labeled substrate and a 561-nm diode laser (18) for simultaneous TFL4 and tetramethylrhodamine-labeled substrate) at the indicated times. 530/30-, 610/20-, and 675/20-nm bandpass filters were used to detect fluorescence from rhodamine 110, tetramethylrhodamine, and TFL4, respectively. Samples were analyzed on BD Biosciences (LSR II and FACScalibur) and Beckman Coulter (Epics XL) instruments. Data were acquired, analyzed, and plotted using FlowJo, CellQuest, System II software for Epics XL, and WinMidi (Dr. J. Trotter, Scripps Research Institute, LaJolla, CA) software.

Microscopy

Effector and target cells were placed in a temperature-controlled imaging chamber from Bioptechs and viewed on a Leica TCS SP2 laser scanning confocal microscope system using a ×63, 1.4 numerical aperture objective at 37°C. Samples were excited using 488 nm of argon, 568 nm of krypton, and 633 nm of red helium/neon laser lines. Differential interference contrast and fluorescence images were acquired as stacks of single optical sections of 1 μm in thickness; fluorescence image reconstruction of stacks was conducted using MetaMorph software (Molecular Devices).

Results

Selective cleavage of fluorogenic caspase 3 and GzB substrates

Fig. 1A shows that the substrates used in this study are highly selective for their intended proteases, with recombinant GzB cleaving its substrate but showing no activity on the caspase 3 substrate, while recombinant caspase 3 is active on its expected substrate but does not detectably cleave the GzB substrate. Peptide library studies suggest that the caspase 3 substrate may also be cleaved by caspase 7, but not by other caspases or GzB (19). We have previously presented specificity data for the caspase 3 substrate relative to other caspases and caspase substrates of the same design (12, 20).
Apoptotic Jurkat cells analyzed after a 3-h incubation treatment with staurosporine showed intracellular caspase 3-like activity when analyzed by flow cytometry (Fig. 1B), consistent with our previous studies (12–14). As expected for cells containing no GzB, the caspase/H11001 cells show no increase in fluorescence over background when monitored for GzB activity. Similarly, after Jurkat exposure to anti-Fas Ab followed by incubation with the two fluorogenic protease substrates, a bimodal distribution of caspase 3/H11001 and caspase 3/H11002 cells was observed (Fig. 1C), consistent with previous studies (13). However, again no fluorescence increase was observed with the GzB substrate. Thus, caspase activation via either the intrinsic (staurosporine) or extrinsic (anti-Fas Ab) pathway results in activation of intracellular caspase 3-like activity, but no evidence for GzB activity was observed, as expected from the solution specificity shown above.

Measurement of intracellular protease activities during cell-mediated cytotoxicity

To quantitate protease activities in target cells as a function of delivery and activation of protease activities by effector cells, targets were prelabeled with TFL4, whereas effectors were not. Thus, in all dot plots throughout this study target cells appear in the two upper quadrants and effectors in the lower two. In the left column of Fig. 2, the upper two panels show an ~1 log increase in fluorescence intensity due to loading of target cells with the GzB substrate (Fig. 2, A vs B); the lowest panel presents an additional ~1 log fluorescence increase of the TFL4-loaded targets due to GzB activation after coinubcation with CD8(H11001) effectors (Fig. 2C). An assessment of a possible increase in cell permeability during delivery and activation of GzB in target cells as measured by leakage of PI is shown in the middle panels of Fig. 2. It is noteworthy that PI(H11002) cells do not release 51Cr ions or 51Cr complexed with macromolecules. Thus, since these data indicate no major permeability

Table I. Fluorescence intensity inside target cells due to intracellular cleavage of the GzB substrate compared with possible leakage of cleaved GzB substrate fragments into targets

<table>
<thead>
<tr>
<th></th>
<th>Targets Alone</th>
<th>Targets plus Effectors</th>
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<tbody>
<tr>
<td>Intact Substrate</td>
<td>3.2%</td>
<td>58.1%</td>
</tr>
<tr>
<td>+10% fragments</td>
<td>0.4%</td>
<td>3.0%</td>
</tr>
<tr>
<td>+5% fragments</td>
<td>0.2%</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

of Fig. 2, the upper two panels show an ~1 log increase in fluorescence intensity due to loading of target cells with the GzB substrate (Fig. 2, A vs B); the lowest panel presents an additional ~1 log fluorescence increase of the TFL4-loaded targets due to GzB activation after coincubation with CD8(H11001) effectors (Fig. 2C). An assessment of a possible increase in cell permeability during delivery and activation of GzB in target cells as measured by leakage of PI is shown in the middle panels of Fig. 2. It is noteworthy that PI(H11002) cells do not release 51Cr ions or 51Cr complexed with macromolecules. Thus, since these data indicate no major permeability
perturbation by labeling with TFL4 or addition of effectors, the GzB+ target cells would not release $^{51}$Cr in the time frame (1 h) of these flow cytometry measurements. The panels in the right column of Fig. 2 present the three parameters together in individual plots.

To ensure that the putative GzB activity was indeed due to cleavage of the GzB substrate inside target cells rather than leakage from cleaved fragments into the extracellular space, the -VGPD-FGR- substrate was cleaved by recombinant GzB and the cleaved fragments were then added to targets with or without effectors present. The percentage of cleaved substrate in the medium after $50\%$ of target cells had been induced to show the putative intracellular GzB activity by flow cytometry was measured by HPLC to be $<2\%$; therefore, the cleaved fragments were added at 5 and 10% (v/v). The data in Table I show that addition of fragments did not result in the percentage of TFL4+ cells exceeding targets loaded with both TFL4 and the intact substrate in the absence of effectors. Thus, the increase in target cell fluorescence plotted on the abscissa of all dot plots following exposure to effectors is consistent with intracellular cleavage of the GzB substrate.

Fig. 3 shows protease activity data for three target cells (Jurkat, Ramos, and Daudi) in which the induction of GzB and caspase 3 activities were assessed following incubation with three different types of lymphocytes: the cytotoxic NK cell line NK92, the cytotoxic CD8+ CTL line 660, and the noncytotoxic CD4+ T cell line 641. In all targets, exposure to the NK92 and CTL lines resulted in induction of GzB activity followed by caspase 3 activity, while the noncytotoxic (CD4+) line displayed minimal effects. These results are in stark contrast to the lack of GzB activity in Jurkat cells following intrinsic and extrinsic apoptotic stimuli (Fig. 1) and show the effectiveness of active GzB delivery to the target cell.
Fig. 4A compares the acquisition of GzB and caspase 3 activities inside one of the targets (Jurkat) using identically labeled substrates run in parallel; thus, the comparison is between cleavage of peptides containing the -VGPD FGR- sequence for GzB and the -DEVD GIN- for caspase 3. In the top panel of Fig. 4A, it can be seen that the GzB activity inside the NK92 effector cells is overwhelmingly negative, similar to the GzB-negative Jurkat cells in Fig. 1. In this experiment, a very minor subfraction (<3%) of NK92 cells do show a significant signal in the GzB channel, but this background does not increase with time. Caspase 3 activity also increases in the TFL4-labeled Jurkat target cells during incubation with NK92 cells but the percentage of caspase 3+ target cells at each time point is lower than the percentage of GzB+ cells. Fig. 4B presents both GzB-
and caspase 3-like activities in TFL4-gated target cells incubated with NK92 cells. This criss-cross experiment used both combinations of substrate pairs at identical probe concentrations to rule out fluorophore bias in demonstrating the order of appearance of the two protease activities. The generally diagonal profiles show that individual cells acquire both activities, while the curvature in these plots indicates that GzB activity precedes caspase 3-like activity at all time points.

**Examination of intracellular protease activities by microscopy**

To further assess activation of the two classes of proteases inside target cells, TFL4-labeled Jurkat cells were examined by microscopy after coincubation with NK92 effectors in the presence of the same GzB and caspase 3-like substrates (Fig. 5) used for the above flow cytometry studies. It is important to note that all optical sections were acquired at 1 μm and the fluorescence above background in all images was confirmed to be of intracellular origin; beyond the E:T junction, no probe was observed to be associated with the extracellular face of the plasma membrane of any target cell. Furthermore, the fluorescent signal from cleavage of the GzB substrate is quite diffuse in the cytoplasm of target cells (Fig. 5, A and B); this is in contrast to the punctuate appearance of transferrin labeled with the same green fluorophore after endocytic entry into Jurkat cells (Fig. 5C). Since the objective again was to observe the order of activation of the two proteases, a criss-cross experiment similar to that above was conducted, again using identical probe concentrations. Fig. 5A shows that GzB activity is detectable in individual target cells attacked by cytotoxic cells before caspase 3-like activity (image acquisition for the lower panel is 15 min after the top panel). Fig. 5B shows a target cell with a prominent bleb, indicative of a significantly late time after effector-target interaction as well as strong signals from both protease activities. Moreover, all protease-positive cells eventually showed the blebbing and transient swelling characteristic of apoptosis (12). Thus, although the number of cells sampled by microscopy is limited relative to flow cytometry, these images are in accord with the flow cytometry data of target-effector engagement followed by target cell acquisition of GzB and then caspase 3 activities.
Determination of the presence of GzB activity inside activated effector cells

Although GzB is a known component of cytotoxic lymphocyte granules, the molecule has long believed to be in an inactive state due to the low intragranular pH. However, because of recent evidence for active GzB inside activated effector cells (21), a comparison of the GzB activity was made among CTLs and CD4+ T cells after 1 h under four conditions: in the presence and absence of OKT3 with or without activated Jurkat targets. As shown in Fig. 6A, intracellular GzB activity did not increase under these activation conditions in either T cell type as measured by flow cytometry. Additionally, Fig. 6B clearly shows GzB activity inside the Jurkat target and at the synapses between effectors and targets.

Effect of Bcl-2 overexpression on caspase and GzB activation

Caspase 3 activation by intracellular GzB can occur by direct pro-caspase 3 cleavage or via Bid cleavage, mitochondrial damage, and caspase 9 activation, and both of these possibilities are compatible with the above results. To probe this issue in further detail, we examined intracellular protease activity in Jurkat cell targets overexpressing Bcl-2. The Jurkat Bcl-2 transfectants used have significantly enhanced Bcl-2 protein expression (Fig. 7A), which does not inhibit their lysis by NK92 cells in 4 h (Fig. 7B). However, Bcl-2 expression dramatically inhibited caspase 3 activation after incubation with staurosporine (Fig. 7C) or anti-Fas Ab (Fig. 7D), consistent with a mitochondrial amplification loop in caspase activation via these two pathways (22). (Conditions in this set of experiments were identical to those in Fig. 1, B and C, i.e., 3 and 6 h, respectively.) Table II shows a comparison between the effect of Bcl-2 on activation of GzB and caspase 3 after a 1-h exposure to CD8+ lymphocytes with an E:T of 1:1. Virtually no effect on GzB activation was measured, whereas, Interestingly, the slight caspase 3 difference between the Bcl-2 and control vector line was consistent with the 51Cr release data of Fig. 7B. Moreover, the confocal images in Fig. 7E clearly show the Bcl-2-transfected targets (blue) following NK92 effector-target incubation undergoing the same morphologic signs of apoptosis as observed above.

Discussion

Apoptosis can be triggered by three distinct pathways: intrinsic, extrinsic, and GzB/perforin. Signaling through each involves the sequential activation of several caspases modulated by interactions with multiple ancillary molecules. Since the study of biologic molecules within cells demands specific detection techniques that allow quantitation and localization, labeled Abs have provided the major tool for such work. However, fixation and permeabilization are required for immunodetection of molecules within cells, excluding studies with living cells. More recently, probes created by fusing proteins of interest with naturally fluorescent proteins have allowed real-time visualization of specific molecules in living cells. Although this approach has provided dramatic insights into cellular activities, the probes may be expressed at nonphysiologic levels and their subcellular localization may not represent that of the molecule being studied. One novel approach to this problem is the direct detection of enzymatic function by fluorogenic substrates such as those used in this study. Most fluorogenic enzyme substrates were developed for cell-free biochemical measurements and are not suitable for use in living cells. The peptide substrates used here have been shown to be highly permeable to the membranes of living cells: the mechanism of their entry into live cells being consistent with passive diffusion, apparently due to the large hydrophobic surface of the two fluorophores in their quenched cyclic structure (12). After enzymatic cleavage of the peptide, the slow diffusion rate of the two water-soluble fluorescent peptide fragments across membranes allows detection of the protease activity within the latter’s physiologic compartment. Using cell-permeable fluorogenic substrates containing sequences to detect specific caspases, we have previously determined the order of activation of these proteases inside live cells undergoing apoptosis via both intrinsic and extrinsic pathways (12). We have also detected punctate, highly localized caspase activation foci in various cell types as an early stage of apoptosis (23). We have now applied this methodology to address the following questions regarding the GzB/perforin pathway: 1) inside injured target cells can GzB activity be clearly distinguished from caspase 3? 2) Does the expected order of GzB activity preceding caspase 3 activity prevail inside target cells? 4) Do GzB and caspase 3 activities colocalize? 5) Is GzB activity detectable inside effector cells? 6) Does Bcl-2 expression alter the protease activities in injured target cells?

GzB vs caspase 3 activity inside live cells

Although many fluorogenic enzyme substrates have been used extensively in biochemical studies, the multitude of enzyme environments within living cells may give rise to enzymatic properties different from those observed in standard solution enzymatic assays. Thus, although it was clear from Fig. 1A that the GzB substrate was not recognized by caspase 3 in a standard in vitro assay, it was important to test this specificity in situ by examining apoptotic cells with high levels of caspase 3 in the absence of GzB. Using this approach, Fig. 1, B and C, confirms the lack of reactivity of the GzB substrate within apoptotic cells expressing demonstrable caspase 3-like activity.

Thus, the presence of caspase 3 activity combined with the absence of GzB activity inside apoptotic cells induced via either the intrinsic or extrinsic pathway provided a good background for use of these probes in examining the predicted caspase activation subsequent to the introduction of active granzymes into target cell cytoplasm. In the current study, three target cell lines were chosen to monitor the appearances of these two protease activities following exposure to three different types of lymphocytes. It is important to note that activation of both proteases occurs in cells that are largely impermeable to PI (Fig. 2); thus, delivery of the lethal hit is into a highly viable cellular environment. As shown in Fig. 3, the cytotoxic NKs and CTLs produced GzB activity in targets before caspase 3; furthermore, with increasing E:T ratios the percentage of target cells expressing each enzyme increased. As expected, exposure to the noncytotoxic CD4+ T cells, which contain neither perforin nor GzB, did not result in either activity significantly above background (<10% of all targets were positive). Moreover, the time dependence of the two activities clearly is supported by both flow cytometry (Fig. 4) and microscopy (Fig. 5A) observations, and they are additionally reinforced by the crosssection use of fluorogenic substrates to rule out the possibility that detection of one substrate cleavage is inherently more sensitive than another. Therefore, the virtual absence of either protease activity in Jurkat cells alone (small upper panels, Fig. 4B) and appearance of measurable GzB activity in target cells only after engagement by effectors (larger lower panels, Fig. 4B) point to the latter’s essential...
GzB has not previously been detected in target cells attacked by cytotoxic lymphocytes, although its uptake from solution in the presence or absence of soluble perforin or other membrane-active agents has been studied in model systems by several laboratories (24–26). In these models, immunoreactive GzB was observed in endosomes in target cells. In contrast, in the current study Jurkat target cells showed relatively uniform cytoplasmic staining, with no evidence of an endosomal vesicular pattern (Fig. 5, A and B) in contrast to the punctuate appearance of fluorescently labeled transferrin following its receptor-mediated uptake by the same cells (Fig. 5C). We would expect our substrate to detect GzB in early endosomes, although not after the acidification characteristic of late endosomes. However, major differences may be expected when examining intracellular granzymes delivered by cytotoxic cells compared with those taken up after incubation of granzymes added to the medium. In the former case, the granzyme and perforin concentrations at the target membrane are locally high, due to effector exocytosis directed into the junctional region. These high local concentrations favor perforin pore formation and granzyme diffusion into the target cytoplasm as shown in Fig. 5B. In contrast, in the model systems, granzymes added to the medium are at much lower concentrations at the plasma membrane, favoring uptake after surface binding. Subsequent endocytosis results in a compartmentalized granzyme intracellular localization, similar to Fig. 5C.

The appearance of GzB activity exclusively in target cells that have been directly engaged by effectors supports a highly directed cell-cell transfer such as a perforin-mediated pore in the target plasma membrane. Additionally, many nonproductive effector-target interactions were observed before successful delivery of GzB activity with caspase activity appearing only in GzB\(^+\) targets, suggesting that target cell killing requires multiple points of effector activity.

Detection of GzB activity in effector cells

Examination of the effector cells in the presence of the GzB substrate by flow cytometry and microscopy shows no significant activity in the presence or absence of target cells. Furthermore, as the flow cytometry data in Fig. 6A indicate, the lack of change of fluorescence signaling from the GzB probe in both CD8\(^+\) and CD4\(^+\) T cells irrespective of activation by OKT3 or presence of target cells does not support the proposed proapoptotic role for GzB released into the effector cell cytosol (21). The difference between conclusions drawn in previous studies (27) and the present may reside in changes that take place during fixation and extraction vs direct measurement of protease activities in live cells.

The lack of detection of active GzB in effector granules is expected in light of the low granule pH and previous studies showing that granzymes are inactive below pH 7 (28). The substrates used here incorporate fluorophores derived from rhodamine, and their fluorescence is pH independent within the physiologic range, so that their cleavage in acidic compartments would be readily detectable. Indeed, the general lack of activity in living cells with either GzB or caspase substrates argues that these peptide substrates are resistant to the potent lysosomal proteases.

After effector exocytosis, perforin and granzymes have been postulated to be released into the synapse-like junctional region where the pH is neutral and full granzyme activity is expected. The GzB activity observed in the cytoplasm of the target cell and at the edges of the two effectors cells in Fig. 6B support this model.

Effect of target Bcl-2 on proteases and cytotoxicity

GzB entry into target cells can trigger apoptosis by one of two pathways: Bid cleavage and mitochondrial damage or direct cleavage of procaspases, particularly procaspase 3. If mitochondrial damage is required for caspase 3 activation, increased Bcl-2 expression would be expected to block caspase activation as observed in Fig. 7, C and D for staurosporine and anti-Fas, respectively. However, the minimal effect of Bcl-2 overexpression in targets (Fig. 7A) attacked by NK92 cells (Table II) suggests that Bid cleavage and the mitochondrial amplification loop are not required for cell death. Although one group has presented similar results using whole cells, the same study found that apoptosis induced in a model system with soluble perforin and GzB was inhibited by Bcl-2 overexpression (9). Moreover, other groups have found that target Bcl-2 expression did suppress lymphocyte-mediated killing via granule exocytosis (29, 30). These differences could be due to distinct levels of Bcl-2 expression or it may well be that different death pathways dominate in various target cells. However, serious skepticism must be applied to conclusions drawn from measurements of extracts since protease activation or deactivation may occur during the extraction process (31). In the current study where protease activities were measured in live cells, overexpression of Bcl-2 failed to inhibit the activation of caspase 3 following entry of GzB into Jurkat cells by either NKs or CTLs (Table II), and this is in clear contradistinction to apoptotic initiation from either intrinsic or extrinsic apoptogens (Fig. 7, C and D). Thus, the pathway for activation of caspases when GzB is delivered via cytotoxic lymphocytes is distinct from the intrinsic and extrinsic pathways. Presumably, in nontransfected cells, both direct activation of procaspases and the mitochondrial amplification loop are available; redundancy in the caspase network allows blockage of one pathway to have a minimal effect on delivery of a lethal hit by GzB from effectors cells (Fig. 7E).

Although cytotoxic lymphocytes clearly provide one means by which the immune system can combat viral infections and tumors, there are many examples in which demonstrable immune responses fail to achieve this desired result. One well-documented mechanism is the evasion of recognition by the immune system by altering expression of MHC molecules or their loading of antigenic peptides. Another less-studied mechanism is the postrecognition resistance of some cells to the injury inflicted by cytotoxic lymphocytes. A clear example of this can be found in studies showing CTL fail to kill some cells displaying recognizable alloantigens and viral Ags, as shown by specific cold target inhibition (32).

Molecular mechanisms proposed for such target resistance include cytoplasmic protease inhibitors, particularly serpins, that block granzymes (32), surface proteases (33), or altered cytoskeletal organization (34). Further studies of this important area will be greatly enhanced by the availability of techniques that monitor target cell injury inflicted by lymphocytes. The intracellular GzB substrate we have described above provides the only currently available approach to measure such injury and its use should elicit insight into the mechanism of target cell defenses.

Disclosures

Drs. Packard and Komoriya are the inventors of the substrates used in this study and the owners of the company holding the patents on these substrates.

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