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News and Commentary

Granzyme B-mediated apoptosis – the elephant and the blind men?

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Cytotoxic cell granule-mediated apoptosis is a unique form of cell signaling that entails the intracellular delivery of granule-associated serine proteases (granzymes). Recent work has suggested that the cytotoxic cell secretes macrocomplexes consisting of multiple granzymes and Perforin (PFN) bound to a scaffold proteoglycan named serglycin.[1] Among the granule proteases, the mechanism of apoptosis induction would seem most straightforward for granzyme B (GrB). GrB has been reported to cleave numerous caspases *in vitro* (-3, -6, -7, -8 -9 and -10)[2] stimulating the notion that the protease is able to initiate death by *activating* multiple family members *in vivo*. To complicate matters, the granzyme might also kill by digesting structural and regulatory proteins.[3,4] Finally, GrB has been reported to initiate death through a mitochondria-centered pathway by cleaving the BH3-only proapoptotic Bcl-2 family member, Bid.[5–10] Together the results imply that the granzyme has a multifaceted potential to ensure target cell death through mechanisms that are: (1) caspase-driven, (2) BH3 protein-driven, and (3) due to cleavage of crucial cellular proteins. These observations, however, require reconciliation with the finding that Bcl-2 reproducibly inhibits GrB-mediated apoptosis as defined by loss of clonogenic potential in the target cells. Bcl-2 is predicted to protect against a mitochondrial assault, but defense against apoptosis initiated through caspases as well as digestion of cellular proteins is more difficult to comprehend.

GrB-mediated apoptosis: an elephantine puzzle

Apoptosis induced by cytotoxic T cells (CTLs) was originally observed to be associated with proteolysis of procaspase-3.[11] It was not clear at the time whether the granzyme entered the target or engaged a transmembrane signal to activate a 'caspase cascade'. Complicating matters, microinjection of rat GrB into target cells failed to induce cell death.[12] Although the cytosolic delivery of GrB was inferred by studies where the granzyme was delivered intracellularly with adenoviral particles,[13] this critical issue was eventually clarified by microinjection of the more active human form by the Bleackley and Froelich labs.

The next logical step then was to identify the caspase(s) that were directly activated by the granzyme to initiate the death process. Since procaspase-3 was cleaved after CTL engagement, it was considered a prime contender. However, other procaspases had also been shown to undergo proteolytic reduction to the requisite large and small subunits *in vitro* providing an ample number of candidates. Therefore, evidence might be obtained by ranking proteolytic rates the granzyme displayed against the various family members – certainly the most reactive should be processed first after cytosolic entry. Procaspase-7 and -10 were cleaved extremely rapidly followed by procaspase-3 while the others tested (procaspase-4/5/6/8) were considered processed at nonphysiologic rates.[2] Consequently, proteolysis of procaspase-7 and -10 was evaluated in cells lacking a functional CASP-3 gene by standard immunoblotting. The results showed both zymogens were processed within minutes of delivery but, when precautions were taken to inactivate the granzyme prior to solubilization, neither zymogen was cleaved indicating that the apparent digestion was due to granzyme-mediated adventitious proteolysis. In targets expressing procaspase-3, on the other hand, procaspase-3 and -7 but not -10 underwent sequential proteolysis indicating that GrB initiated a death pathway by first processing the less preferred procaspase-3 and together both proteases contributed to maturation of procaspase-7.[14] Notably, these studies documented virtually tandem proteolysis within minutes of granzyme delivery, but intracellular activation in whole cells and associated signs of cell death were not reported.

These findings emphasize the difficulties in delineating intracellular events triggered by the granzyme. Foremost, susceptibility to proteolysis *in vitro* does not predict that a protein is processed similarly *in situ*. The likelihood that an intracellular substrate is cleaved depends on both k_{cat}/K_m as well as accessibility once the granzyme reaches the cytosol. Secondly, solubilization of GrB-treated targets for data acquisition liberates sequestered granzyme allowing extremely rapid cleavage of susceptible but otherwise inaccessible substrates. In the absence of a strategy to inactivate the granzyme (and subsequent rapidly activated caspases) prior to solubilization, the results may reflect events in the lysate rather than in the target cells undergoing cell death.

A major goal has been to distinguish the initial and subsequent proteolytic events mediated by the granzyme *versus* caspases. Attempts to answer this question have almost uniformly relied on soluble oligopeptide inhibitors. Unlike the forms of cells death where caspases undergo restrained autoactivation via protein–protein interactions, the cytosolic delivery of GrB presents the pool of procaspases with a renewable activator. This condition contributes to the

greater difficulty encountered in effectively blocking caspase activities in GrB-treated cells rather than in cells engaged to die through the mitochondrial apoptosome or death receptors.[2] The study of cells deficient in key procaspases offers a sound experimental approach, but circumstances may require use of soluble inhibitors. On the basis of the executioner caspases engaged, DEVD-fmk is a crucial choice, and effectiveness of inhibition should be standardized for the operative methodology. Furthermore, despite the use of DEVD-fmk, complementation with a broad-spectrum inhibitor is often necessary,[2] but VAD-fmk may be problematic due to its capacity to facilitate paradoxically necrotic cell death in certain cell types.

The agent used to deliver GrB may also have unpredictable consequences. In comparison to the focal release of PFN by a CTL, bathing targets in soluble PFN is not a physiologic surrogate. PFN treatment is associated with necrotic permeabilization of a substantial minority of target cells (>20% – *sublytic dose*). The adventitious proteolysis in the effected subset cannot be estimated by controls incubated with either PFN or granzyme alone. The adenoviral delivery system offers rapid loading of the granzyme without untoward necrosis[13] and has allowed us to appreciate the liabilities of PFN delivery by failing to verify that GrB directly cleaves PARP in target cells as we had previously reported. Finally, whenever possible, analysis of proteolytic digests (i.e. cell lysates) might be complemented by simultaneously assessing apoptotic indices in whole cells where the standard remains loss of clonogenic potential.

GrB woos mitochondria

Most forms of cell death are viewed to center on mitochondria, with caspases functioning downstream. Therefore, investigators considered the possibility that the GrB either directly or via intermediary proteins disrupted mitochondrial integrity. Arnold Greenberg first showed that GrB delivered by PFN was associated with reduction in mitochondrial membrane potential ($\Delta\Psi_m$). Although the mechanism (i.e. granzyme directly *versus* caspase) was unclear, the results established that mitochondrial disruption was a key event during GrB-mediated apoptosis.[10]

To learn whether the granzyme acted alone or in concert with other factors to perturb mitochondria, investigators used an *in vitro* cytochrome *c* (cyt. *c*) release assay consisting of isolated mitochondria, cytosol and granzyme. The addition of the granzyme resulted in the formation of truncated BID, which then interacted with Bax to permeabilize isolated mitochondria releasing cyt. *c*.[6] Contemporaneously, BID was reported to undergo cleavage in whole targets treated with GrB and PFN.[9] For both cyt. *c* release and BID cleavage either in cytosol or whole cells, precautions that minimized adventitious proteolysis were not reported preventing the clear distinction between GrB and granzyme-activated caspase-mediated effects. However, cyt. *c* release was firmly established to occur during GrB-mediated apoptosis by imaging intact cells. Pinkoski *et al*.[8] performed time lapse confocal microscopy and showed that release of labeled cyt. *c* from mitochondria was readily apparent. Others then showed that Bid/Bax/Bak is required for the release of cyt. *c* imaged in whole cells,[15] but viewed the process as caspase-independent because zVAD-fmk did not alter the outcome. On the basis of these studies, the granzyme was predicted to initiate a BID/Bax/Bak-dependent, caspase-independent mitochondrial disruption and cell death.

Does GrB initiate death through the caspases?

Due to concerns about the interpretation of experiments that depended on analysis of lysed granzyme-loaded targets, the physiologic effector, GrB-SG, was evaluated *in situ* using AD for delivery. Relying on measurement of apoptotic indices (active caspase-3, $\Delta\Psi$m and TUNEL) in cells lacking procaspases-3 (CASP-3 (−/−) MEFs) and deficient MCF-7 cells with a paired line overexpressing the zymogen (MCF-$7_{\pm casp\text{-}3}$), the presence of procaspase-3 appeared to be necessary for mitochondrial disruption.[16] Coupled with the observation that $\Delta\Psi$m was substantially reduced in a Bax/Bak- and caspase-dependent manner, the results suggested that the predominant GrB death pathway starts with intracellular delivery of the granzyme, continues with activation of procaspase-3 and is followed by caspase-mediated engagement of a BH3-only/Bax/Bak pathway. The BH3-only protein that is cleaved by caspase-3 remains unclear. If adventitious proteolysis is controlled, then BID cleavage was not readily detected in MCF-$7_{casp\text{-}3}$ cells by immunoblot but processing is detectable after the line is exposed to TRAIL[16] or staurosporine.[17] More sensitive *in situ* assays may reveal that truncated BID (or related protein) is generated to amplify GrB-mediated apoptosis through mitochondria. In addition, GrB does not seem to facilitate at least in MCF-7 cells DNA fragmentation by releasing the inhibitor of caspase-activated DNAse (ICAD).[4] Finally, in Bax/Bak-deficient cells, DNA fragmentation was delayed but reached levels comparable to wild-type controls. Therefore, as originally speculated by Greenberg,[10] mitochondria assist in optimizing but are not essential for apoptosis initiated by this granzyme (Figure 1).

The question remains whether the granzyme causes cell death through other caspases. Despite the absence of procaspase-3, MCF-7 targets gradually develop nuclear condensation over 20 h, which was inhibited by zDEVD-fmk. These results stress that other caspases such as procaspase-7 might also initiate death without mitochondrial dysfunction (Figure 1), an observation recently made also for ultraviolet-induced death in similar MCF-7 cells.[18] Finally, given the crucial amplifying role of mitochondria in GrB-induced death, Smac/DIABLO or Omi/HtrA2 released from damaged mitochondria would understandably modulate the intensity of the apoptotic response to the granzyme through inactivation of the inhibitor of apoptosis protein (XIAP).[19,20] This concept is emphasized by the recent report that melanoma cells lacking Smac/DIABLO become more sensitive to GrB after expressing a proform of the XIAP inhibitor which is activated by the granzyme after intracellular delivery.[21]

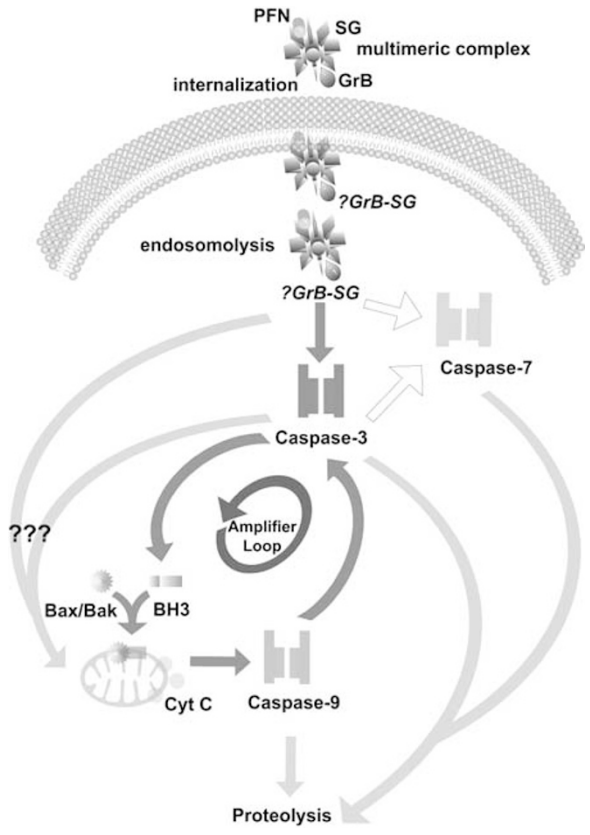

Figure 1 Pathways for GrB-mediated apoptosis. Following endocytosis, GrB is released to the cytosol where a portion of the intracellular stores of procaspase-3 is activated (dark gray arrows). At present, it is unclear whether the GrB–SG complex is internalized en masse or the granzyme enters in a monomeric form. The activated caspase then in a BH3-only/Bax/Bak-dependent manner facilitates mitochondrial permeabilization, releasing cyt. *c* and generating the caspase-9 apoptosome. Thereafter, caspase-9 completes the amplification loop (black arrow) by cleaving additional procaspase-3. Light gray arrows describe hypothetical pathways where the granzyme and caspase-3 perturb mitochondria in a BH3-indepedent manner. Finally, white arrows describe the postulated sequential activation of procaspase-7 by granzyme and caspase-3

Coming full circle – Bcl-2 blocks GrB-mediated death at the most proximal step

Bcl-2 has been used as a tool to underscore the importance of caspase-independent, mitochondria-centered, GrB-mediated killing. Since it is not entirely clear how Bcl-2 modulates this form of cell death, the interpretation of results remains difficult. For Bcl-2 to effectively restrain such an apparently multi-faceted killer, the antiapoptotic protein might be hypothesized to act at the most proximal aspect of the granzyme-mediated death pathway by inhibiting the activation of procaspase-3. This however remains to be formally demonstrated. Despite a marked reduction in active caspase-3 within intact cells expressing Bcl-2, the processed subunits were identified by immunoblot. Targets experiencing GrB delivery in the presence of DEVD-fmk[2] or cells overexpressing XIAPs show similarly processed procaspase-3.[19,20] Together, the results suggest that Bcl-2 could interfere with oligomerization of GrB processed subunits to active heterodimers akin to the described Bcl-2-associated sequestration of BH3-only domain proteins.[22]

Concluding remarks

GrB-mediated killing appears more unitary than originally predicted.[2] Clearly, granzyme-initiated, caspase-3-dependent recruitment of mitochondria leads to optimal obliteration of targeted cells, but additional work will be necessary to learn whether the granzyme also kills by activating less accessible caspases and by cleaving structural/regulatory proteins.[3] Effector–target cytotoxicity assays with killer cells containing only GrB should provide a physiologically relevant perspective for apoptotic pathways activated by the granzyme. Nevertheless, if GrB fails to be the predicted 'natural born killer', other serglycin-bound family members (e.g. granzyme A) should be capable replacements.

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