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Mechanisms of granule-mediated cytotoxicity

Editorial overview

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The members of Judy Lieberman's laboratory study cytotoxic T lymphocytes and their role in antiviral immunity. In addition to deciphering the cell death pathway induced by granzyme A, they are interested in how CD8⁺ T-cell function is regulated, especially in the setting of chronic infection and with a particular emphasis on HIV. They also try to apply their understanding to immunotherapy and vaccine development. More recently, they have been investigating the potential of harnessing RNA interference for treating human disease.

Abbreviations

CTL cytotoxic T lymphocyte
NK natural killer
TCR T-cell receptor

Granule-mediated cytotoxicity is undoubtedly the most important effector function of CD8⁺ T cells and natural killer (NK) cells. After a killer cell recognizes its target, the cytotoxic granules move to the immunological synapse, where their membranes fuse with the killer cell plasma membrane, and they release their contents to induce target cell apoptosis. The principal death effectors are the serine proteases (called granzymes, for granule enzyme) and the membrane perturbing proteins, perforin and granulysin. These are bound to a proteoglycan matrix. Although killer cells can also destroy their targets by engaging death receptors such as fas, studies using perforin knockout mice have clearly shown the importance of the granule exocytosis pathway for controlling viral infection and tumors. The same basic mechanisms are used by all killer cells, whether they are CD4⁺ or CD8⁺ cytotoxic T lymphocytes (CTLs), or NK cells, although the granzymes they contain may vary between the killer cells. Although most of the cytotoxic molecules were identified in the mid-1980s as molecular biology tools became widely available, a molecular understanding of their mechanisms of action lagged behind. Granzyme B, which activates the caspase pathway of apoptosis, was the first cytolytic molecule to be widely studied. Research to delineate the molecular basis for granule-mediated cell death has been very active over the past few years. We now have a better understanding of the cell biology of granule exocytosis and the delivery of cytolytic effector molecules. Newly discovered mechanisms show how killer cells might be protected from their own weapons of mass destruction. The actions of granzyme B have been elaborated upon with more precision and detail, whereas studies of the other granule components have made it clear that killer cells are armed with more than one way to destroy their targets. This section of *Current Opinion in Immunology* dissects the recent progress in understanding the anatomy of the murder perpetrated by killer cells via release of cytotoxic granules.

The review by Clark and Griffiths [1] describes the cell biology behind the formation and release of cytotoxic granules, which are specialized secretory lysosomes. The cytolytic molecules, including granzymes, perforin, granulysin and fas Ligand, need to be sorted and stored in these acidic granules in a way that protects the killer cell from self-destruction. A number of human and mouse genetic diseases have been described that have defects in the sorting, movement or secretion of cytotoxic granules and other secretory

lysosomes in hematopoietic cells and melanocytes. These mutations have been used to describe the pathways for granule trafficking and to highlight differences between these secretory cells. After killer cell recognition of an appropriate target, the cytotoxic granules attach and move along microtubules under the direction of the microtubule organizing center to the immunological synapse. In a secretory zone of the synapse, distinct from the signaling zone that contains the T-cell receptor (TCR) or NK-cell receptor (NKR) and accessory signaling molecules, the cytotoxic granule membrane fuses to the killer cell plasma membrane and releases its contents.

The next step in the story, reviewed by Catalfano and Henkart [2], is the delivery of the cytotoxic effector molecules to the target cell via perforin. The key role of perforin in delivering the lethal hit for protection against intracellular pathogens and tumors is highlighted by studies in the perforin knockout mouse and by the identification of perforin mutations in a subset of patients with familial hemophagocytic lymphohistiocytosis (FHL). The original model of perforin action was that perforin polymerized in the target cell plasma membrane to make holes to facilitate the direct entry of the granzymes and deliver the lethal hit. This model was revised when it was shown that granzymes are endocytosed into cells in a perforin-independent manner. One pathway for endocytosis is the cation-independent mannose-6-phosphate receptor (MPR), but other pathways for endocytosis clearly exist. Perforin is nonetheless required for release of the granzymes from the endosome. A clear mechanistic explanation for how this occurs is lacking. Studies of perforin have been hampered by the lack of an active recombinant molecule, despite decades of attempts by a number of laboratories, and the inability to image perforin in the target cell. Henkart's group has recently solved one of the important puzzles in the field [3]: how is the killer cell protected from perforin released into the immunological synapse? As discussed in their review, they showed that cathepsin B in the cytotoxic granule membrane relocates to the killer cell plasma membrane after granule membrane fusion, and cleaves and inactivates any perforin that is redirected toward the killer cell. Recent studies have also shown that killer cells and some other immunologically privileged cells and sites are also protected from granule-mediated cell death by expression of serine protease inhibitors, called serpins.

In an associated opinionated article, Raja, Metkar and Froelich [4] discuss the importance of the granule proteoglycan serglycin for delivery of granzymes and perforin. Within the granule the positively charged perforin and granzymes are bound by noncovalent charge interactions to serglycin in a >300 kDa complex that is as big as a viral particle. Until recently, studies of granule-mediated apoptosis have used isolated purified granzymes or recombinant granzymes delivered with purified

perforin or endosomolytic agents, such as streptolysin or adenovirus, to study the mechanism of cell death induction. In the original view, serglycin served as an inert carrier, important only in the safe storage of the cytolytic molecules within the killer cell; the granzymes and perforin dissociated from serglycin when the pH changed from acidic to neutral after granule exocytosis. This group has argued, however, using detailed biophysical studies, that the whole complex is internalized and delivered to the target cell, and that the physiologically relevant complex needs to be considered as a whole to truly understand cell death induction. If this is the case, the problem of perforin-facilitated delivery of the granzymes becomes even bigger — from delivery of a 25–50 kDa granzyme molecule (already too big to fit through the small perforin polymeric channels visualized in old electron microscopy studies) to delivery of a much larger complex. The definitive proof of this hypothesis would be to identify serglycin within granzyme-containing endosomes and the cytosol of target cells.

We now move on to reviews of the death mechanisms activated by each of the granule effector molecules. The best studied of these molecules is granzyme B. Trapani and Sutton [5] dissect the pathways activated by the 'smart Aspase' granzyme B, which cleaves terminal caspases as well as caspase pathway substrates. Recent studies suggest that granzyme B has a central role in activating the mitochondrial pathway by cleaving bid to initiate the release of cytochrome c together with HtrA2/Omi and Smac/DIABLO, the inhibitors of the inhibitor of apoptosis proteins. Although it was previously thought that granzyme B directly and fully activates the effector caspase, caspase 3, it now seems likely, from recent studies of the Bleackley and Trapani groups [6,7], that granzyme B only partly activates caspase 3 and requires mitochondrial involvement to complete activation. This explains why bcl-2 overexpression, a tumor strategy for caspase evasion, severely inhibits granzyme B-mediated apoptosis. However, a recent study by the Froelich group [8] questions whether bid cleavage is truly the initiating event of granzyme-B-induced apoptosis under physiological conditions. Granzyme B (in addition to granzymes A and C) also induces cell death and mitochondrial damage with loss of the mitochondrial transmembrane potential and increase in reactive oxygen species independently of bid or other cytosolic mediators. Nothing is known about this alternative mitochondrial cell death pathway. This review also discusses a recently described and not infrequent granzyme B human polymorphism, mammalian serpin inhibitors of granzyme B, and some recently described viral inhibitors of granzyme B.

The other abundant granzyme is granzyme A. The review by Lieberman and Fan [9] describes a novel cell death pathway induced by granzyme A. Although the target cells have all the morphological features of apoptosis, the

pathway does not involve the caspases and the form of DNA damage is uniquely single stranded. Targets that can evade caspase- or granzyme-B-mediated cell death, such as by bcl-2 overexpression, are susceptible to granzyme A. So far it has been discovered that granzyme A seems to focus on disrupting the nucleus, especially proteins in chromatin or that modify chromatin. Newly described targets of granzyme A include the histones, lamins, the base excision repair enzyme Ape1, the DNA bending protein HMG-2 and the nucleosome assembly protein SET. The granzyme A-activated DNase (GAAD) was recently identified as NM23-H1 in the SET complex. SET is the GAAD inhibitor; when SET is cleaved by granzyme A, the DNase is unleashed.

In addition to granzymes A and B, mammals, and especially mice, express other granzymes whose activity is largely not understood. However, knockout mice studies suggest that these other granzymes play significant roles in immune function and regulation. Ley and colleagues [10] review these neglected 'orphan' granzymes. Recent work suggests that there may be subsets of killer cells that express different subsets of granzymes, which may have distinct roles in immune protection. Furthermore, distinct activation stimuli may induce different patterns of granzyme expression, although this needs to be studied. The Ley laboratory recently opened the door to understanding the role of the orphan granzymes by showing that granzyme C activates a novel cell death pathway with morphological features of apoptosis [11]. This caspase-independent pathway has some features of the granzyme A pathway (such as single-stranded DNA nicking), but seems clearly distinct (as damaged DNA can be labeled with TUNEL staining). Granzyme C induces prominent direct mitochondrial damage with dramatic mitochondrial swelling. Delineating the caspase-independent cell death pathways induced by the granzymes is likely to be a fruitful research area in the next few years, furthering the understanding of the foolproof mechanisms available for immune protection from viruses and tumors that can evade caspase activation. Some of the orphan granzymes do not appear to activate cell death and may have other roles that need to be delineated.

The final review in this section, by Clayberger and Krensky [12], discusses another granule component,

granulysin, which can lyse bacteria as well as tumors. Granulysin, which is expressed in humans but not in mice, belongs to a family of proteins that inserts into, and disrupts, membranes, and this is its primary mechanism of action. The recent solution of the crystal structure of granulysin provides a clear model for its action. A small granulysin peptide mimics the membranolytic activity of the whole molecule, and it may be possible to develop small molecule stabilized analogs for therapeutic use. Targeted membranes include bacterial membranes and both mitochondrial and plasma membranes of mammalian cells. In addition to conventional bacterial targets, granulysin may also be important in defense against mycobacteria and fungi.

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