Review

Molecular Mechanisms of Lymphocyte-Mediated Cytotoxicity

Zusen Fan[1, 2] and Qixiang Zhang[1]

Granule-mediated cytotoxicity is the major mechanism for lymphocytes to kill viruses, intracellular bacteria and tumors. The cytotoxic granules move to the immunological synapse by exocytosis after recognition of a killer cell. The contents of the granules are delivered into target cells with the help of perforin by endocytosis. A group of serine protease granzymes cleave their critical substrates to initiate DNA damage and cell death. The most abundant granzymes are granzyme A and B. They induce cell death through alternate and nonoverlapping pathways. The substrates and functions of the majority of the orphan granzymes have not yet been identified. It is possible that the diversity of granzymes provides fail-safe mechanisms for killing viruses and tumor cells. *Cellular & Molecular Immunology*. **2005;2(4):259-264.**

Introduction

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are important effector cells in the immune response to viruses, intracellular bacteria and tumors. Much of our understanding of lymphocyte-mediated cytolysis has come from *in vitro* cultured cell experiments. These killer cells use the major mechanisms, Fas-FasL and granule-mediated apoptosis, to induce cell death in infected and transformed target cells (1). After a killer cell recognizes its target, the cytotoxic granules move to the immunological synapse by exocytosis, where their membrane fuses with the killer cell plasma membrane, then they release their contents into target cells by endocytosis (2, 3). Granule-mediated cytolysis is a principal pathway for tumor killing and intracellular infection. Cytotoxic granules contain a pore-forming protein perforin and a group of serine proteases called granzymes (Gzms), in a proteoglycan matrix (4, 5). Perforin was initially observed to form pore-like structures analogous to the C9 component of complement by polymerization in the presence of calcium. The Gzm is the name for a family of cytoplasmic granule-associated proteins that are released specifically into the target cell after killer-target contact. Some granzymes seem to be exclusively expressed in certain subsets of lymphocytes. Gzms are serine esterases and different from the poreforming and target membrane-disrupting perforin or granulysin present in the same granules. Gzms include GzmA, B, C, D, E, F, G, H, K and M. Nine mouse, eight rat and five human Gzms have been found. These Gzms are related to rat mast cell protease-1, mast cell protease-2, and cathepsin G, which induce cell apoptosis. The most abundant Gzms are GzmA and B. Transfection experiments and mice deficient in perforin, GzmA or B suggest that perforin and either Gzm is sufficient for granule-mediated lysis by CTL and NK cells (6). Mice deficient in perforin are severely immuno-compromised as humans with familial hemophagocytic lymphohistiocytosis (HLH) due to mutations in the perforin gene (7).

Researches to reveal the molecular mechanisms for granule-mediated cell death have been very active over the past few years. We have gotten deeper understanding of the cell biology of granule exocytosis and the delivery of cytolytic effector molecules. In this review I focus on the recent progress in understanding the molecular mechanisms of granule-mediated cytolysis.

Contents of the granule

Cytolytic lymphocytes utilize secretory granules for storage and exocytosis of their cytotoxic contents for their targets. Lytic granules hold many cytoxic molecules, including perforin, Gzms and other lysosomal proteins (8, 9). These organelles have an acidic pH of 5.1-5.4 and perform hydrolytic and degradative actions (10). Lytic granules are secretory lysosomes. Similar to conventional lysosomes, lytic

[1]National Laboratory of Biomacromolecules, Center for Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China;

[2]Corresponding to: Dr. Zusen Fan, National Laboratory of Biomacromolecules, Center for Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China. E-mail: zusenfan2001@yahoo.com.

granules are the end point of the endocytic pathway. The lytic granule is one of the best-studied examples of a secretory lysome. T cell receptor (TCR) stimulation promotes the synthesis of perforin and Gzms and other granule proteins. Lytic granule secretion can be triggered by activation of killer cells.

Perforin

Although perforin was cloned near twenty years, how perforin works still remains unclear. Perforin is homologous to the complement C9, which was originally thought to work by forming pores in the target cell plasma membrane that help Gzm entry (11). Pores of approximately 50 nm in diameter or smaller are formed when perforin homo-multimerizes in the plasma membrane in a Ca^{2+}-dependent manner (12). These pores can be visualized by electron microscopy. Perforin forms pores that can kill a cell by necrosis is a manner reminiscent of its homologue complement, at the sublytic concentrations required to deliver Gzms. The pores, if formed at all, may be too small to allow Gzms to enter. In fact when perforin is added at sublytic concentrations, the integrity of the plasma membrane is not breached and small molecule dyes do not enter the cell (13, 14). Perforin pores are too small to allow passage of globular molecules as big as Gzms.

All cytolytic cells can express perforin, including CD4^{+} CTL, CD8^{+} CTL, T cells and NK cells. Perforin expression appears to be regulated upon T cell activation and depends on activating the IL-2 pathway (15). The trans- cription factors that control perforin expression have not been clearly defined, but likely include Ets family member (16, 17). The biosynthesis and storage of perforin in killer cells is carefully designed to protect killer cells from the potentially lethal effect of perforin. Upon synthesis in the endoplasmic reticulum (ER), perforin probably binds to its inhibitor calreticulin (18). It is then transported, presumably bound to calreticulin, *via* the trans-Golgi to cytotoxic granules, which are modified secretory lysomes (19). The cytotoxic granules are acidic and contain in addition to perforin and Gzms, careticulin, the proteoglycan serglycin, possibly several other molecules whose function in the granules is poorly understood. Perforin is inactive at the acidic pH of the cyto-lytic granules, but perforin stability in the granules requires the acidic environment. Perforin needs to be activated by a cysteine protease to remove a C terminal glycosylated peptide (20). Proteolytic cleavage probably occurs in the granule since it requires an acidic environment, but during the exocytosis, perforin is released into the synaptic cleft and likely dissociates from calreticulin and serglysin in the synapse. Killer cells are protected from perforin damage by a recently found mechanism (21). The lysosomal and granule membrane protein cathepsin B, transferred to the killer cell plasma membrane when the cytotoxic granule membrane fuses to the plasma membrane, inactivates any perforin redirected towards the killer cell by proteolytic perforin cleavage.

Granulysin

Human CTLs and NK cells, not rodent cells, contain a second membrane-damaging protein known as granulysin. Granulysin is processed from a larger 15 kD precursor to a 9 kD protein in the dense cytotoxic granules of cytolytic lymphocytes (22). Granulysin is homologous to saposin-like proteins that are involved in membrane-lipid degradation. Recent studies showed granulysin was an effective anti-microbial agent, which kills microorganisms through increasing the membrane permeability of Gram-positive and -negative bacteria, mycobacteria and fungi (23). A recent crystal structure demonstrated that granulysin binds to the bacterial membrane by a cluster of positive residues and might aggregate and tunnel into the membrane through its hydrophobic core to lead to molecular electroporation. To lyze intracellular bacteria, the activity of granulysin could be facilitated by perforin (23). In addition, granulysin possesses tumoricidal and anti-viral functions. At high concentrations in a micromolar range, granulysin initiates cell death with apoptotic nuclear features. It suggests that granulysin may provide a back-up membrane-disrupting molecule for perforin in humans, just as the various granzymes provide alternate ways to trigger apoptosis (24). However, there is no experimental evidence to support this idea. Granulysin mediates a caspase-independent cell death. Similar to GzmC, granulysin abolishes the mitochondrial outer membrane, releasing proapoptotic factors such as AIF, cytochrome C and presumably EndoG. The importance of mitochondria in granulysin-induced cell death is indicated *via* its inhibition in Bcl-2-overexpressing cells. DNA damage might appear mainly through these mitochondrial mediators. At low concentrations in a nanomolar range, granulysin possesses a chemoattractant and activator of immune cells (25). Granulysin has multiple biological functions. In the immediate area, high concentrations of granulysin kill microbes, whereas further away, a chemoattractant gradient of granulysin triggers migration of immune cells toward the site of injury or infection and also causes release of additional immune factors, expanding the immune response timely by focusing the inflammatory infiltration. It indicates that granulysin not only contributes to innate immunity but also to adaptive immunity through attracting and activating human immune cells.

GzmA

GzmA, also called Gzm1, is the most abundant protease in the cytotoxic granules (26, 27). Its expression is constitutive in NK cells and activated about three to five days after naïve T cells are induced to differentiate into CTLs by encountering antigen. Like GzmB and perforin, GzmA is stored in cytotoxic granules, and released into the immunological synapse upon triggering by engagement with a target cell (8). Unlike perforin and GzmB, GzmA continues to be expressed long after T cell activation, perhaps even in long-term memory cells (28, 29). Until recently GzmA was

thought to induce a slow, secondary nonapoptotic cell death pathway because oligonucleotide fragments of DNA were not released from target cells until at least 16 h and then only to a limited extent (30, 31). However, GzmA-induced cell death measured by trypan blue inclusion occurs within minutes, and DNA damage in the GzmA pathway involves single-stranded nicks, which result in large DNA fragments not detected by the usual apoptosis assays (30-32). Recent studies have begun to delineate the molecular basis for an alternate cell death pathway activated when GzmA is delivered into target cells by the membrane perturbing protein, perforin (33-35).

GzmA is a serine protease that cuts after the basic amino acids Arg or Lys, like trypsin. However, unlike trypsin, it is a highly specific enzyme (36). Moreover, unlike the caspases, its substrate specificity is not defined by the short linear sequence around the enzyme cleavage site, but requires more extended interactions, which likely provide the enzymatic specificity (37-39). Mice in which GzmA has been genetically deleted are immunocompetent and have killer cells that can eliminate virus-infected or tumor cell targets *via* apoptosis induced by perforin delivery of other Gzms (40-43). Nonetheless, the importance of redundancy provided by multiple parallel apoptotic pathways, and of GzmA in particular, has been clearly demonstrated when GzmA-deficient mice are challenged with viral infection. GzmA-deficient mice are compromised in their ability to contain the mouse pox virus ectromelia and herpes simplex neuronal infection. Some cell types may also be more sensitive to one Gzm than another (32). The other Gzms may not be able to compensate for the lack of GzmA in these viral challenge models because most NK cells, important in the initial innate immune response to infection, do not normally express GzmB or other cell-death inducing Gzms (44).

GzmA, delivered into target cells *via* perforin, induces single-stranded DNA damage as well as apoptotic morphology and loss of cell membrane integrity (45, 46). GzmA destroys the nuclear envelope by targeting lamins and opens up DNA for degradation by targeting histones (33, 47). A special target of the GzmA cell death pathway is a 270-440 kD ER-associated complex, called the SET complex, which contains three GzmA substrates, the nucleosome assembly protein (NAP) SET, the DNA bending protein HMG-2 and the apurinic endonuclease Ape1 in base excision repair (BER) (48, 49). Ape1 is a multifunctional oxidative stress response protein and base excision repair endonuclease. GzmA cleavage of Ape1 interferes with the ability of the target cell to repair itself. The SET complex contains the GzmA-activated DNase, NM23-H1, which is inhibited by the GzmA substrate SET (50). The novel cell death pathway unleashed by GzmA provides a parallel pathway for apoptosis, important in destroying targets that overexpress Bcl-2 or are otherwise invulnerable to the caspases.

GzmB

GzmB, known as Gzm2 or serine protease B, is a caspase-like serine protease that cleaves substrates at the carboxyl side of acidic residues, particularly aspartic acid (46, 51). Unlike other forms of apoptosis, GzmB-induced cell death is imposed extrinsically and sufficient to overcome defects in the intrinsic suicide program. The execution of apoptosis by GzmB generally occurs by activation of the caspase family of cysteine proteases, which are central regulators of the apoptotic pathway for multiple inducers of cell death (52). Caspases are synthesized as a single polypeptide and function as a heterotetramer consisting of two large and two small subunits. Processing occurs at aspartic acid residues between the subunits and can be initiated by other caspases or by GzmB (53, 54). Although a number of caspases are processed by GzmB *in vitro*, caspase 3 and 7 are cleaved most rapidly in cells treated with perforin loaded GzmB (55, 56). Caspase 3, a central execution caspase, is directly processed by GzmB. It suggests that GzmB-mediated apoptosis is caspase-dependent. GzmB can also directly cleave some key caspase pathway substrates, such as Bid, ICAD, DNA-PK$_{cs}$, NuMa, PARP and lamin B, and apoptosis induced by GzmB can be blocked through pan caspase inhibitors (1, 51).

GzmB causes DNA fragmentation in cells through activating a nuclease called as caspase-activated DNase (CAD) *via* degradation of its inhibitor of CAD (ICAD) (57-59). CAD exists as a heterodimer with ICAD in the nucleus, where CAD is inactive. The mechanism used by GzmB to activate DNA fragmentation by inactivating a DNase inhibitor is reminiscent of the activation of nick-induced nuclease NM23H1 by GzmA cleavage of its inhibitor SET. Furthermore, ICAD is not cleaved in the induction of apoptosis *via* GzmB deficient CTLs (60, 61). ICAD deficient cells are resistant to GzmB-mediated killing.

Mitochondria play a key role in regulation of cell death. Mitochondrial function is disrupted early in apoptotic response and mitochondrial changes are critical in propagating and amplifying many death signals. Pro-apoptotic signals induce mitochondria to release cytochrome C and other proteins, disrupting mitochondrial function and activating other down stream events. GzmB can cleave Bid to tBid upon target cell entry and tBid translocates to the mitochondria. tBid interacts with its receptors Bak and Bax to cause cytochrome C release (62-64). GzmB can cause mitochondrial depolarization even in the absence of cytochrome C release through the permeability transition pore or the Bax/Bak receptors (1). It suggests that GzmB amplifies its death signal by cleaving Bid and causing mitochondrial dysfunction.

GzmC and GzmH

In addition to GzmA and B, other Gzm genes have also been described, but little is known about their functions, so called as "orphans" (65). The orphan Gzms include C, D, E, F, G, K, L, M and N in the mouse, H, K, and M in the human. The recent findings of nonredundant mechanisms of GzmA, B, C indicates the possible functions of these orphan Gzms in providing "fail-safe" mechanisms against target killing

(66-69). GzmC is one of the serine proteases located in the granules of CTL and NK cells in the rodent. The cDNA of GzmC is identical to a previously described serine protease, termed CCP2 (cytotoxic cell protease-2) of murine CTLs (70). The rat homolog was identified as RNKP-4. A recent study showed that GzmC is a potent inducer of another cell death pathway, which is distinct from that triggered by either GzmA or B (69). GzmC induces cell death quickly as other Gzms at the equal concentration and initiates cell membrane damage, Annexin-V staining, loss of the mitochondrial membrane potential and chromatin condensation. GzmC causes caspase-independent cell death with single-stranded DNA nicks, rather than oligonucleosomal DNA fragmentation similar to GzmA. The mitochondrial changes induced by GzmC are particularly apparent, with impressive mitochondrial swelling, cytochrome C release and opening a mitochondrial pore. Although cytochrome C is released, the caspases are not activated during GzmC-mediated death. The enzymatic specificity of GzmC needs to be determined. GzmH is a chymase within the GzmB cluster, which is most closely related to murine GzmC. Its actual function in granule-induced death has not yet been clarified.

GzmK

GzmK is another tryptase located in the same chromosome as GzmA. Similar to GzmA, GzmK purified from rat RNK-16 cell line initiates apoptosis of target cells in a late 14 h cytotoxicity assay (71). GzmK is much less abundant than GzmA. However, GzmK has twice the specific activity of GzmA. One report showed purified GzmK induces caspase-independent cell death without apoptotic nuclear morphology, but with disruption of the mitochondrial potential and dysfunction, as measured by the generation of reactive oxygen species (ROS). However, GzmK does not lead to the release of pro-apoptotic mitochondrial components and the typical apoptotic nuclear morphology (65). These results suggest that GzmK may induce a unique death pathway.

GzmM

GzmM is another serine protease in mice and humans that cleaves preferentially after methionine residues. It is not expressed by conventional CD4$^+$ or CD8$^+$ T cells, but preferentially expressed by cells participating in the innate immunity, including NK cells and γδ T cells. GzmM is highly expressed in NK cells (67). GzmM induces a novel caspase-independent cell death and mitochondrial disruption, which does not feature obvious DNA fragmentation. GzmM-deficient mice were generated and displayed normal NK cell/T cell development and homeostasis and intact NK cell-mediated cytolysis of tumor targets (72). GzmM-deficient mice showed increased susceptibility to murine CMV (MCMV) infection typified by the presence of more viral inclusions and transiently higher viral burden in the visceral of GzmM-deficient mice compared with WT mice. However, GzmM-deficient mice are as resistant as WT

control mice to mouse pox ectromelia infection and challenge with a lot of NK-sensitive tumors. These results suggest that GzmM plays a differential role in controlling some viral infections.

Conclusions

CTL-mediated killing pathway uses perforin to deliver the Gzms to target cells, where they cleave physiological substrates that initiate DNA damage and apoptosis. GzmA and B induce cell death in a perforin-dependent manner *via* independent and synergistic pathways. The orphan Gzms may provide "fail safe" mechanisms for target killing. The granule-mediated death is extremely intriguing and complex, which has yielded tremendous information to understand a variety of biological systems. The basic science to elucidate this pathway is still robust and will be got deep insight into the mechanisms against viruses and cancers, which will provide therapeutic approaches for clinical application against viral infection and tumors.

Acknowledgement

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