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Nuclear war: the granzyme A-bomb

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Granzyme A, a serine protease in the cytotoxic granules of natural killer cells and cytotoxic T lymphocytes, induces caspase-independent cell death when introduced into target cells by perforin. Granzyme A induces single-stranded DNA damage as well as rapid loss of cell membrane integrity and mitochondrial transmembrane potential through unknown mechanisms. Granzyme A destroys the nuclear envelope by targeting lamins and opens up DNA for degradation by targeting histones. A special target of the granzyme A cell death pathway is an endoplasmic reticulum-associated complex, called the SET complex, which contains three granzyme A substrates, the nucleosome assembly protein SET, the DNA bending protein HMG-2, and the base excision repair endonuclease Ape1. The SET complex also contains the tumor suppressor protein pp32 and the granzyme A-activated DNase NM23-H1, which is inhibited by SET. Granzyme A cleavage of SET releases the inhibition and unleashes NM23-H1. Cleavage of Ape1 by granzyme A interferes with the ability of the target cell to repair itself. The novel cell death pathway initiated by granzyme A provides a parallel pathway for apoptosis, important in destroying targets that overexpress bcl-2 or are otherwise invulnerable to the caspases.

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Abbreviations

CAD	caspase-activated DNase
CTL	cytotoxic T lymphocyte
GAAD	GzmA-activated DNase
IGAAD	inhibitor of GAAD
Gzm	granzyme
ICAD	inhibitor of CAD
IL	interleukin
NK	natural killer
PP2A	protein phosphatase 2A
ROS	reactive oxygen species

Introduction

Granzyme A (GzmA) is the most abundant protease in the cytotoxic granules of natural killer (NK) cells and cyto-

toxic T lymphocytes (CTLs) [1–4]. Its expression is constitutive in many NK cells and is activated approximately three to five days after naïve T cells are induced to differentiate into CTLs by encountering antigen. Similar to granzyme B (GzmB) and perforin, GzmA is stored in cytotoxic granules, which are specialized secretory lysosomes, and is released into the immunological synapse upon triggering by engagement with a target cell [5]. Unlike perforin and GzmB, GzmA continues to be expressed long after T-cell activation, perhaps even in long-term memory cells [4,6,7]. Until recently, GzmA was thought to induce a slow, secondary nonapoptotic cell death pathway because oligonucleotide fragments of DNA were not released from targeted cells until at least 16 hours after degranulation and then only to a limited extent [8,9]. However, GzmA-induced cell death occurs without delay, and DNA damage in the GzmA pathway involves single-stranded nicks, which result in large DNA fragments that are not detected by the usual apoptosis assays [8–10]. This review will describe recent studies that have begun to delineate the molecular basis for an alternative cell death pathway that is activated when GzmA is delivered into target cells by the membrane-perturbing protein, perforin [11–13]. In addition to its role in inducing cell death, GzmA also has extracellular effects. GzmA enhances inflammation by cleaving the propeptide from IL-1 β and activating macrophages through an unknown mechanism; it also inhibits clotting by activating pro-urokinase and inactivating the thrombin receptor via cleavage [14–17].

GzmA and GzmB independently and synergistically induce cell death in a perforin-dependent manner [18]. Perforin delivers the granzymes into the target cell cytosol, where they begin to do their damage. By a poorly understood mechanism, both granzymes also rapidly accumulate in the nucleus, where they can activate pathways of nuclear damage [19,20]. GzmA is a serine protease that cuts after the basic amino acids arginine or lysine, similar to trypsin. Unlike trypsin, however, it is a highly specific enzyme [21]. 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 probably provide the enzymatic specificity (see also Update; [22]).

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 the other granzymes [23–26]. 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 mousepox virus ectromelia and herpes simplex neuronal infections. Some cell types may also be more sensitive to one granzyme than another [10]. The other granzymes may not be able to compensate for the lack of GzmA in these viral challenge models because most NK cells, which are important in the initial innate immune response to infection, do not normally express GzmB or other cell-death inducing granzymes [27]. They do express GzmM, but this granzyme is not known to cause target cell death.

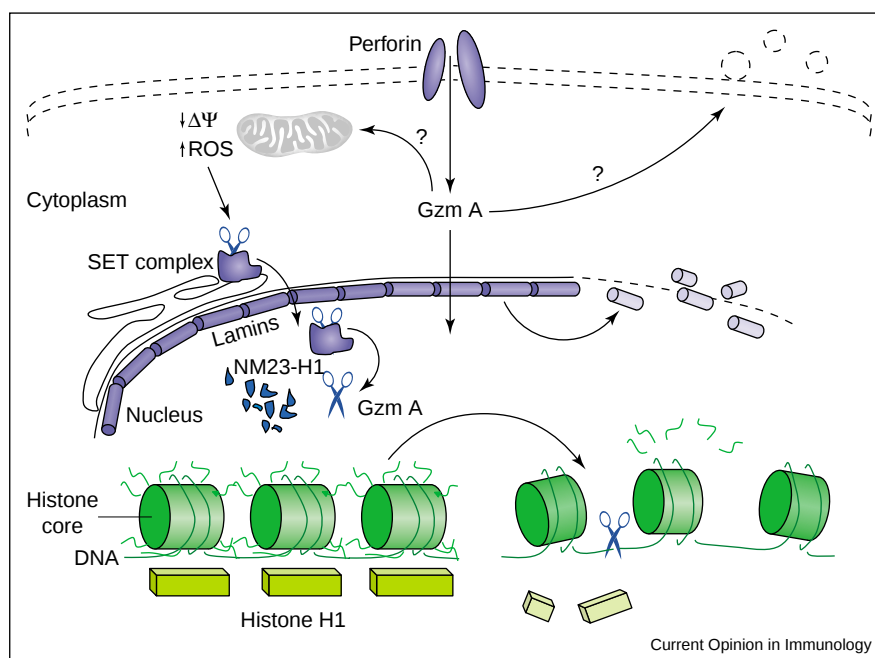
Although natural serine protease inhibitors of GzmB have been described in mouse and man, it is not known whether these serpins also inhibit GzmA [28,29]. Until recently, no natural inhibitors of GzmA had been identified. A recent report, however, identified pancreatic secretory trypsin inhibitor (PSTI) on mouse intraepithelial lymphocytes in the gut and showed that it is a potent GzmA inhibitor [30^{*}]. These authors postulated that PSTI might have a role in protecting cells in the small intestine from secreted GzmA. Extracellular GzmA inhibitors are physiologically relevant, as in conditions of inflammation or hyperactive immune stimulation, such as HIV infection or rheumatoid arthritis, GzmA can be

detected at nanomolar concentrations in the plasma or synovial fluid [31,32].

Features of granzyme A-induced cell death

Cell death induced by GzmA and perforin is rapid (Figure 1). Within minutes of perforin delivery, the integrity of the plasma membrane (as measured by chromium release or trypan blue inclusion) is disrupted and there is prominent membrane blebbing (PJ Beresford, J Lieberman, unpublished data; [11]). Target cells have all the morphological features of apoptosis. Chromatin condensation and nuclear fragmentation can be readily seen within a few hours of granzyme loading [11,33,34^{*}]. GzmA, however, does not activate the caspases or induce cleavage of most key caspase pathway substrates, such as bid or the inhibitor of caspase-activated DNase (ICAD; D Martinvalet, J Lieberman, unpublished data; [11,35^{**}]). Cells that are resistant to caspase-mediated cell death, including cells that overexpress bcl-2, are sensitive to the apoptotic effects of GzmA [11]. This may be important for immune defense against cancers and viruses that have devised strategies for evading caspase-mediated apoptosis. Although mitochondrial transmembrane potential ($\Delta\Psi$) and function are disrupted and reactive oxygen species (ROS) rapidly accumulate in GzmA-targeted cells, cytochrome c and other mitochondrial apoptotic mediators (such as apoptosis inducing factor [AIF], endonuclease

Figure 1



The mechanisms of cell death induced by GzmA and perforin. GzmA is introduced into cells through an incompletely understood mechanism involving perforin. Prominent features of the GzmA cell death pathway are disruption of mitochondrial function with loss of transmembrane potential ($\Delta\Psi$), increased reactive oxygen species (ROS) and disruption of plasma membrane with prominent blebbing. The mechanism of these actions is unknown (?). Increased ROS is hypothesized to cause the translocation of the SET complex, an important GzmA target, to the nucleus. In the nucleus, GzmA cleaves the lamins, histone H1, the tails of core histones and cleaves three components of the SET complex (SET, HMG2, and Ape1). GzmA disruption of the histones opens up chromatin to DNases. Cleavage of SET liberates the GzmA-activated DNase NM23-H1.

G [endoG], SMAC/Diablo or HtrA2/Omi) are not released (D Martinvalet, J Lieberman, unpublished data).

GzmA induces DNA damage by single-stranded nicks [11]. These nicks produce large multikilobase fragments that are not detected by conventional assays that measure apoptotic DNA damage. The DNA damage is not visualized on conventional agarose gels, cannot be labeled with terminal deoxynucleotidyl transferase and the fragments are too large to be readily released from dying cells. Nonetheless, nicked DNA can be visualized within a few hours by alkaline agarose gels that denature DNA into its separate strands and by labeling with the Klenow fragment of DNA polymerase I. Although nothing is known about the molecular basis for the plasma membrane and mitochondrial damage induced by GzmA, the novel mechanisms of DNA and nuclear damage are beginning to be understood and are the focus of this review.

The SET complex: a key granzyme A target

The key to unraveling the GzmA pathway was the identification of potential GzmA targets that bind to a recombinant mutant form of the enzyme, in which the active site Ser184 is mutated to Ala184 (S-AGzmA; [36]). A newly described 270–420 kDa multiprotein complex, termed the SET complex, binds to S-AGzmA and contains three GzmA substrates (Figure 2; [22,34*,37]). Most, but not all, of the components of the SET complex have been identified. They include the nuclear assembly protein SET, the DNA bending protein HMG-2, the base excision repair (BER) pathway apurinic endonuclease Ape1, the tumor suppressor protein pp32, and the transcriptional regulator and nucleoside diphosphate kinase

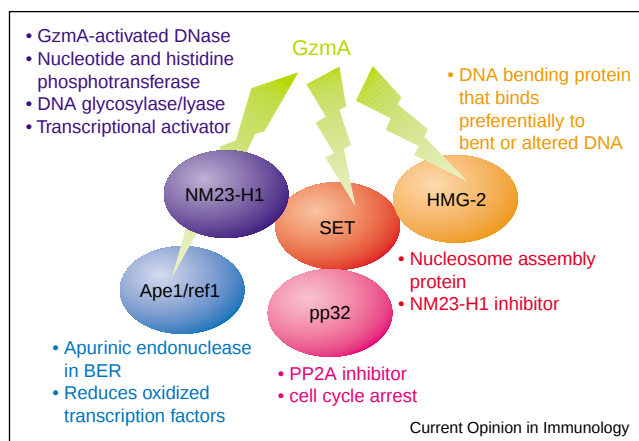
NM23-H1 [35**]. The first three proteins are physiologically relevant substrates of GzmA, and their cleavage by GzmA destroys all of their known functions. The inclusion of proteins in the SET complex is highly specific, as the close homologues of SET (TAF-1 α), HMG-2 (HMG-1), and NM23-H1 (NM23-H2) are not included. SET binds tightly to NM23-H1 and HMG-2. It also binds tightly to S-AGzmA, as a complex of the two proteins is not disrupted during SDS-PAGE [36].

The SET complex proteins are all highly expressed and ubiquitous in cells. Although a large fraction of these proteins appear to be part of the SET complex, they also participate in other cellular complexes and functions in cells. For example, a smaller complex that contains SET, pp32 and their homologues has been shown to play a role in modifying chromatin and regulating the half-life of some immediate early gene mRNAs [38–44]. Interestingly, pp32 may also play a role in caspase-mediated apoptosis [45*]. pp32 promotes caspase-9 activation in the apoptosome formed following the initiation of the mitochondrial apoptotic pathway and enhances subsequent activation of caspase-3.

The normal function of the SET complex, which is associated with the endoplasmic reticulum (ER) in many cells, but also shuttles to the nucleus, is not known, although the functions of its different components suggest that it is involved in regulating chromatin structure, integrity and gene expression. We hypothesized that the SET complex is involved in the repair response to oxidative stress and translocates to the nucleus in response to oxidative damage [34*]. In fact, as a reaction to oxidative stress, Ape1 is known to translocate to the nucleus, where it repairs the most prevalent form of oxidative DNA damage (abasic sites) and reduces and activates key proto-oncogenes, such as fos, jun, NF- κ B and myb, which are involved in the immediate early repair response [46–50]. Understanding how this complex functions in normal cells will, however, require further study.

The SET complex is probably important in preventing oncogenic transformation (possibly as a consequence of its repair properties), as three of the SET complex proteins have been heavily implicated in cancer. SET was initially identified in an undifferentiated leukemia as a translocated gene linked to a nucleoporin. The translocation probably disrupts SET's normal cellular location near the ER and places it in the nucleus [51]. SET is an ATP-independent nucleosome assembly protein (NAP), implicated in activating viral DNA replication and inhibiting histone acetylation and DNA demethylation [41,42,52–56]. The NAP activity of SET contributes to increasing chromatin accessibility [53]. SET also binds to the transcriptional coactivators CBP/p300 and to HRX/MLL/ALL-1, the human homolog of the *Drosophila* trithorax gene, a histone methyltransferase involved in regulating

Figure 2



The SET complex. The SET complex contains five known proteins, which function to modulate chromatin, repair DNA damage and regulate transcription. Several other proteins are in the complex, but their identification is still to be confirmed. GzmA cleaves and functionally cripples SET, HMG-2 and Ape1. The proteins that are touching have been shown to interact directly, although the interaction between SET and pp32 is weak. BER, base excision repair.

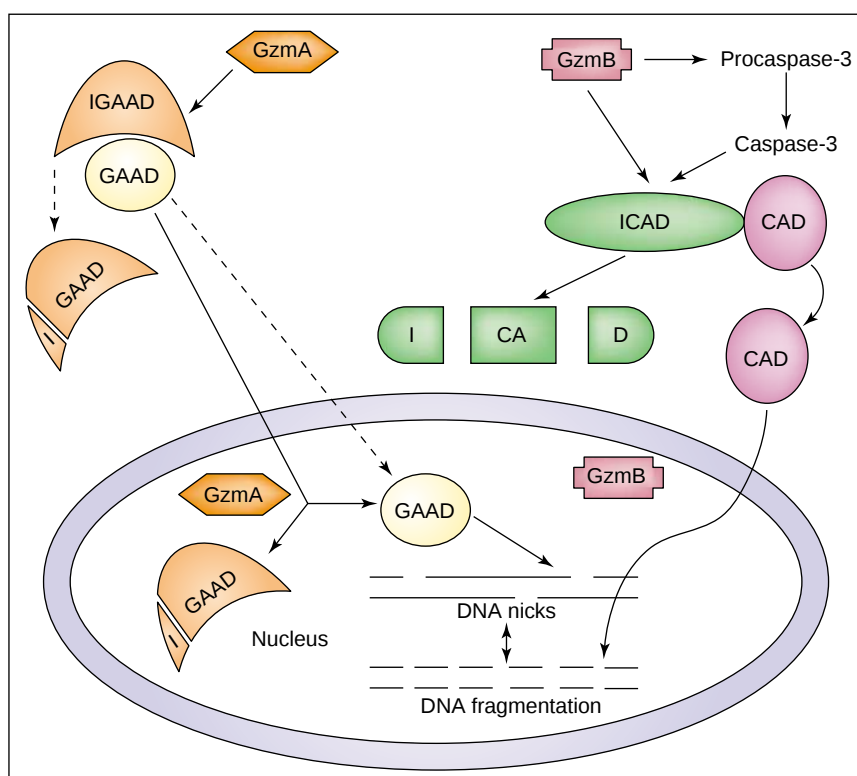
chromatin accessibility [39,57]. pp32 is a tumor suppressor gene whose overexpression causes cell cycle arrest by an unknown mechanism [58]. It acts as a regulatory subunit of protein phosphatase 2A (PP2A) and inhibits its activity [59]. SET also binds to and inhibits PP2A [57,60]. The role of the SET complex in oncogenic transformation may involve PP2A, which also binds to several of the polyoma virus transforming antigens, implicating it in oncogenesis [61]. NM23-H1 was first identified as a gene that is mutated or underexpressed in metastatic cancers [62]. It has also been implicated in regulating the expression of platelet-derived growth factor (PDGF; [63]). Although the evidence implicating NM23-H1 in cancer is extensive [64], a clear mechanistic understanding is still lacking. The identification of NM23-H1 in the SET complex may help to solve this mystery.

Mechanism of granzyme A-mediated DNA damage

GzmA interaction with the SET complex is the key to DNA damage in this novel apoptotic pathway [22,35^{**}]. GzmA activates DNA nicking in isolated nuclei only

in the presence of a small amount of cytoplasm. The essential cytoplasmic component is the SET complex. The SET complex contains the GzmA-activated DNase (GAAD) as well as an inhibitor (IGAAD). GAAD is NM23-H1, which was previously shown to nick DNA, and IGAAD is SET. When GzmA is introduced into target cells, the SET complex rapidly (within five minutes) translocates to the nucleus, perhaps as a consequence of the increase in ROS. GzmA cleavage of SET releases the inhibition of NM23-H1, which then begins to nick DNA. The importance of NM23-H1 and SET in GzmA-mediated cell death was confirmed by finding increased DNA damage and cell death in cells that overexpress NM23-H1 or have silenced SET and, conversely, by finding less cell death in targets with silenced NM23-H1 or enhanced SET expression. The mechanism used by GzmA to activate DNA cleavage by inactivating a DNase inhibitor is reminiscent of the activation of CAD by caspase or GzmB cleavage of its inhibitor ICAD (Figure 3; [65–69]). In both pathways, the sequestration of the DNase in the cytosol bound to a specific inhibitor guarantees that inappropriate DNA damage is not easily unleashed.

Figure 3



Mechanism of DNA damage by granzymes. GzmA and GzmB induce DNA damage by two distinct pathways, both of which involve a sequestered DNase in the cytosol bound to a specific inhibitor. The granzymes cleave the inhibitor to liberate the active DNase, which traffics to the nucleus. DNA damage by GzmA results in single-stranded nicks, whereas damage by GzmB results in blunt double-stranded breaks. In the case of GzmA, most of the cleavage probably occurs in the nucleus, as the whole complex rapidly translocates to the nucleus after GzmA loading. GzmB can act either directly or indirectly by activating caspases. CAD, caspase-activated DNase, also known as DFF40; GAAD, granzyme A-activated DNase (e.g. NM23-H1); ICAD, inhibitor of CAD (also called DFF45); IGAAD, inhibitor of GAAD (e.g. SET). Figure modified with permission from [35^{**}].

GzmA cleavage of the multifunctional repair protein Ape1 in the SET complex also plays an important role in preventing cell recovery via repair [34^{*}]. Many apoptotic stimuli induce repair pathways at the same time that they activate death. The ultimate life or death outcome depends on the relative strengths of these responses. GzmA cleavage of Ape1 destroys its endonuclease activity for DNA repair as well as its redox activity for activating the immediate early response. Cells engineered to express a mutant form of Ape1 resistant to GzmA cleavage are able to repair some of the GzmA-induced damage and are relatively refractory to cell death. Similarly, cells with silenced Ape1 expression are more susceptible to GzmA cell death induction. Therefore, targeting the SET complex has two pro-apoptotic effects — it activates the DNase NM23-H1 and blocks a key cellular repair pathway.

GzmA also interacts very strongly with the small heat shock protein hsp27, which is another participant in the stress response [70]. The interaction is so tight that strong denaturing agents, such as urea, are required to dissociate these two molecules. Hsp27 is not a substrate of granzyme A. Interestingly, CTLs have very low levels of this heat shock protein and it is not induced significantly when they are subjected to heat shock. The possible importance of hsp27 in CTL-mediated death remains to be elucidated.

Lamins and histones are also targets of granzyme A

GzmA attacks the integrity of the nuclear envelope and chromatin, possibly to facilitate the entry and activity of its DNase. The lamins are key intermediate filament proteins that constitute the structural framework beneath the nuclear envelope and provide anchor sites for chromatin and the nuclear pore complex [71]. The nuclear lamina dissolves during cell division and also during caspase-mediated apoptosis [72–74]. GzmA cleaves the lamins within 20 minutes of perforin-mediated GzmA loading [33]. Because lamin cleavage is a common feature of both caspase-dependent and -independent apoptosis, disruption of the nuclear lamina is probably requisite for inducing apoptosis by any means.

GzmA also has a profound direct effect on chromatin [75]. It completely degrades the linker histone H1, which anchors the DNA around the core histones. Removing linker histones opens up chromatin from a compacted solenoid to an extended state. GzmA also proteolyzes the tails from the core histones. This is likely to open up chromatin still more, as trypsinization (which also removes the tails but at distinct sites from GzmA) further unfolds chromatin. The proteolytic activity of GzmA in intact nuclei increases the susceptibility of DNA to exogenous nucleases. This probably enhances the activity not only of NM23-H1, but also of the caspase-activated

DNases, CAD and endoG. This may help to explain the known synergy of combined GzmA and GzmB in triggering DNA damage.

Conclusions

GzmA activates a novel caspase-independent apoptotic pathway that is just beginning to be uncovered with distinct substrates and a novel form of DNA damage. An important target in GzmA's nuclear war is a newly discovered complex, the SET complex, which is hypothesized to be important in the repair response to oxidative stress. By cutting three of the proteins in the SET complex, GzmA activates a DNase (NM23-H1) in the complex to make single-stranded DNA cuts and disables the cell's ability to repair the damage. In addition, GzmA dissolves the nuclear lamina and targets the histones to open up DNA to NM23-H1 and other DNases. GzmA also disrupts mitochondrial function and the integrity of the plasma membrane through unknown mechanisms.

Update

Two groups have recently solved high resolution crystal structures of the GzmA dimer [76,77]. The active sites on each monomer point in opposite directions. Nearby non-catalytic surfaces on the partner molecule appear to provide extended regions ('exosites') for GzmA binding to its substrates. Exosite binding may explain why the amino acids around the cleavage site do not determine specificity and may help explain the basis for the exquisite specificity of the protease.

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