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Mini review

Background, biology and significance of human granzymes

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Abstract

The human granzymes (Grz) are a highly conserved group of potent peptidases that are found, together with a pore forming protein-perforin in specialized granules of cytotoxic cells such as cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. Granule exocytosis (perforin/Grz) pathway is used by these cells to defend organism against virus-infected and tumor cells by inducing them to undergo apoptosis. While the pro-apoptotic functions of Grz have been well established, it has recently become apparent that Grz also possess important extracelular activities which are now being extensively investigated. Soluble Grz are found extracellularly in normal plasma suggesting their constitutive secretion in healthy individuals via a granule independent biosynthetic pathway. The potent activities of extracellular Grz appear to be controlled by highly abundant plasma derived serine peptidase inhibitors. However, unregulated activities of proteolytic Grz have been shown to result in disease pathology especially, in the absence of their corresponding inhibitors. To date, most of the studies have concentrated on the structure and function of granzyme A (GrA) and GrB while very little work has been done on the remaining Grz which include GrM, GrH and GrK in humans. In this report, we discuss the current knowledge of Grz biochemistry, biology, functions, activity regulation and their role in human pathology with special emphasis on the significance of human GrK in this field.

EVOLUTION HISTORY AND GENE ORGANIZATION OF GRANZYMES

The term granzymes or **gran***ule en***zymes** (Grz) was first employed almost 20 years ago by Jenne *et al. (1)* to denote a group of highly specific, evolutionary related serine peptidases, originally discovered in the secretory granules of murine Cytotoxic T lymphocytes (CTLs). Currently, Grz have been found only in humans and rodents. Characterization of Grz have identified Granzyme (Gr) A, B, H, K, and M in the granules of human cytotoxic cells and Gr A-G, K, L, M, and N in the granules of murine cytotoxic cells *(2, 3)*. The genes of these Grz are separated on three chromosomal loci. GrA and GrK *(tryptases)* are closely linked on chromosome 5 in humans and chromosome 13 in mice. GrB *(aspase)* and GrH *(chymase)* are linked to cathepsin G on human chromosome 14. The corresponding loci for Grz B, C, D, E, F, and G and cathepsin G in the mouse are located on chromosone 14. GrM *(metase)* is linked to neutrophil elastase (NE) and peptidase 3 on chromosome 19 in humans and chromosome 10 in mice *(2)*.

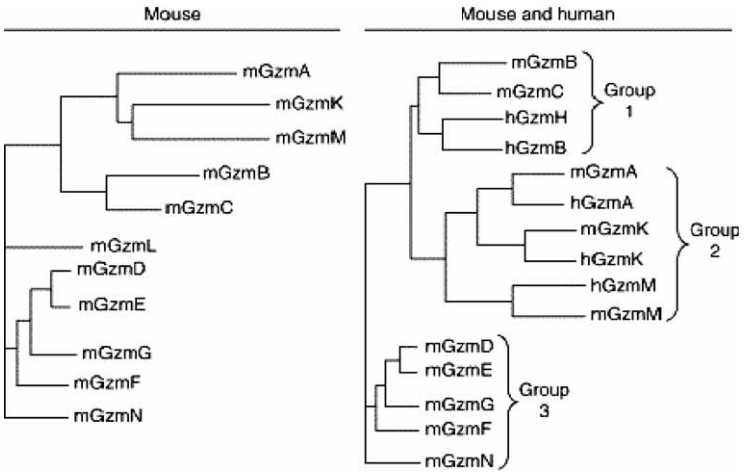

Figure 1. *Phylogenetic relationships among human and mouse Grz. Adopted from Grossman* et al. *2003 Curr Opin Immunol (4).*

Interspecies comparison of cDNA sequences displays a high degree of homology within the Grz of the same group, as revealed by phylogenic analysis and shown in Figure 1 *(36)*. The expression of human Grz is mostly restricted to CTLs and NK cells *(5)*. However, it has recently been reported that the genes of Grz are heterogeneously expressed across the different subsets of human cytotoxic cells *(6)*. How the expression of genes for these Grz is regulated under various physiological conditions yet remains unknown. At the amino acid level about 70% homology has been observed within any one Grz subfamily, e.g., between mouse, rat and human GrB. However, when Grz from different subfamilies are compared, e.g., GrA and GrB, similarity of amino-acid sequences falls to approximately 30–40%, even within the same species *(2)*.

GRANZYMES – A SERINE PEPTIDASES FAMILY

Functionally, Grz belong to the family of serine peptidases that are, after metallopeptidases, the second largest class of protein-cleaving enzymes in the body. The term »serine peptidase« indicates the importance of a serine residue in the active center of the enzyme *(7)*. A common, highly conserved catalytic mechanism utilized by serine peptidases is characterized by three key amino acids: *His*, *Asp* and *Ser* in their active site which are often referred to as a »catalytic triad«, and each of these amino acids play an essential role in cleaving ability of serine peptidases *(7, 8)*. Although the catalytic triad provides a unique mechanism by which to cleave substrate, it does not determine substrate specificity. Serine peptidases exhibit different substrate specificities because of their ability to selectively cleave peptide bonds by restricted amino acid residues. The cleavage site discrimination is achieved primarily through selective binding of the amino acid residue in the substrate that is N terminal to the scissile bond, (P1 position) (nomenclature of Schechter and Berger) *(9)*. For efficient catalysis the P1 residue of the substrate must tightly fit into the S1 substrate binding pocket of the enzyme *(9, 10)*. Interactions of other substrate residues (P2, P3,…) with binding sites (S2, S3,…) of the enzyme could amplify the substrate's binding *(8)*. Therefore, some serine peptidases, as for instance Grz, have an extended interaction site with the substrate whereas the others have a specificity restricted to the P1 substrate residue *(9, 10)*. Moreover, Grz were shown to exhibit divergent extended substrate specificities as determined by combinatorial libraries of their substrate binding pocket residues *(11)*. Valuable structural information regarding substrate binding sites have also been provided by molecular modeling of Grz *(8)* and by mutation analysis of their substrate binding pocket residues *(12)*. These studies have been initiated to determine optimum amino acid sequences that presumably represent natural protein sequence for Grz and thus, may help to identify physiologically relevant substrates for Grz. Initially, this correlation has been fulfilled for GrB. Nevertheless, the identification of natural Grz substrates has just begun and several important biological substrates of GrA and GrB have recently been identified *(8, 11)*.

GRANZYMES – TRYPSIN/CHYMOTRYPSIN-LIKE SUB-FAMILY

Serine peptidases share strong structural similarity and are grouped into sub-families that share close sequence homology. The Grz contain the catalytic triad: *His*57, *Asp*102 and *Ser*195 which is characteristics of chymotrypsin/trypsin sub-family *(8)* and exhibit one of four enzymatic activities: GrA and K (*tryptase*) cleaves after Arg or Lys, GrB (*aspase*) cleaves after Asp, GrM (*metase*) cleaves after Met or Leu, and GrH (*chymase*) cleaves after Phe, Tyr or Trp *(8,12)*. Although, Grz are highly similar to other chymotrypsin/trypsin-like enzymes *(8),* they also have several defining characteristics. All mature Grz have unique and conserved N-terminal Ile-Ile-Gly-Gly sequence and highly conserved residues at the positions 9–16 *(2)*. Their propeptide sequence is commonly

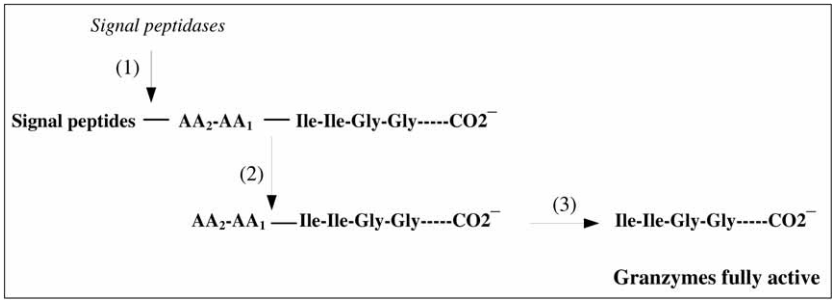

Figure 2. *The proteolytic activation of human Grz. A leader sequence consisting of 18-26 amino-acids residues (signal peptidases) of originally synthesized inactive pre-pro-granzymes is cleaved by signal peptidases (1) resulting in a pro-granzymes. The removal of the pro-dipeptide sequence (AA_2-AA_1) by DPPI (2) finally results in the fully active Grz (3) .*

Gly-Glu or Glu-Glu. They generally have three conserved disulfide bridges, although GrA and GrM have four. Only GrA has been characterized as a dimer consisting of two disulphide linked monomers *(2)*. Most importantly, unlike other peptidases of the chymotrypsin/trypsin sub-family such as trypsin, chymotrypsin and elastase that could cleave a very broad range of substrates, Grz have very distinctive substrate specificity which means that Grz also catalyze limited proteolysis *(2,12)*.

BIOGENESIS OF GRANZYMES

Grz are synthesized specifically by cytotoxic cells *(5)* with the transcription of their messages primarily by signaling initiated through binding of IL-2 or IL-15 and IL-12 *(13, 14)*. All Grz are initially translated as inactive pre-pro-enzymes that require two separate amino-terminal processing steps to become active (Figure 2). At first, in the endoplasmic reticulum, the leader signal peptide sequence is removed by a signal peptidase, leaving a N-terminal pro-sequence consisting of two amino-acid residues (pro-enzymes). Removal of this pro-dipeptide occurs on the way to and/or at the time of packaging into the secretory granules and requires activation by dipeptidyl peptidase I (DPPI; also known as cathepsin C, a cystein protease constitutively expressed in lysosomes) *(3, 5)*. The same activation process occurs for neutrophil serine peptidase and for mast cells chymase *(15)*. While there is no doubt that the activity of DPPI is crucial for activation of these serine peptidases *(2, 3)*, the precise compartment in which DPPI processes their pro-form is still uncertain *(16)*.

GRANULE DEPENDENT PATHWAY OF GRANZYMES BIOSYNTHESIS

During biogenesis Grz are directed and sorted into the secretory granules via the mannose-6-phosphate receptor (M6P-R) system (Figure 3). Therefore, in the Golgi apparatus Grz undergo an important M6P oligosaccharide modification of the N-linked glycans that allows their recognition and sorting into the granules by M6P-R *(3, 17)*. Although, neutrophil and mast cell serine peptidases share similar biogenesis, this M6P-R route appears to be specific for sorting of Grz *(15)*. Finally, the packaging of positively charged Grz within the granules is accomplished by non-covalent complex formation with negatively charged granule proteoglycan (PG). This PG has been called serglycin (SG) because it contains chrondroitin 4-sulfate glycosaminoglycans linked to Ser-Gly repeats in the central portion of the core protein *(3)*. It has been shown that approximately 30-50 Grz molecules bind to each of ~250 kDa SG making the complex as big as a viral particle *(18)*. Furthermore, it has recently been demonstrated that following activation of cytotoxic cells, Grz are released from their granules in macromolecular complexed form with SG. The same study showed that Grz-SG complexes are proteolytically active *(19)*. Nevertheless, the physiological role, if any, of such Grz-SG complexes necessitate further investigation.

GRANULE INDEPENDENT PATHWAY OF GRANZYMES BIOSYNTHESIS

An alternative, granule independent pathway of Grz biosynthesis has been postulated (Figure 3). As demonstrated by Isaaz *et al. (17)*, during biosynthesis Grz may not always enter secretory granules and therefore consequently are directly secreted via a constitutive, non--granule biosynthetic pathway. This study also showed that upon stimulation of CTLs, Grz could be secreted via regulated pathway from the scretory granules and also by the constitutive, non-granule pathway. In addition, Grz were found *de novo* synthesized by these cells. The majority of newly synthesized Grz happened to be constitutively extracellularly secreted while only minor part of the newly synthesized Grz re-filled the granules *(17)*. However, the exact nature of such constitutive secretion is not known, and ever since its recognition, this pathway has received no or little attention.

ACTIVITY REGULATION – INHIBITORS OF GRANZYMES

The potent activity of proteolytic Grz appears to be regulated in several ways. At first, even if Grz are stored inside granules as a fully active enzyme they still require a neutral environment to mediate their function. Therefore, the acidic pH inside granules prevents stored Grz

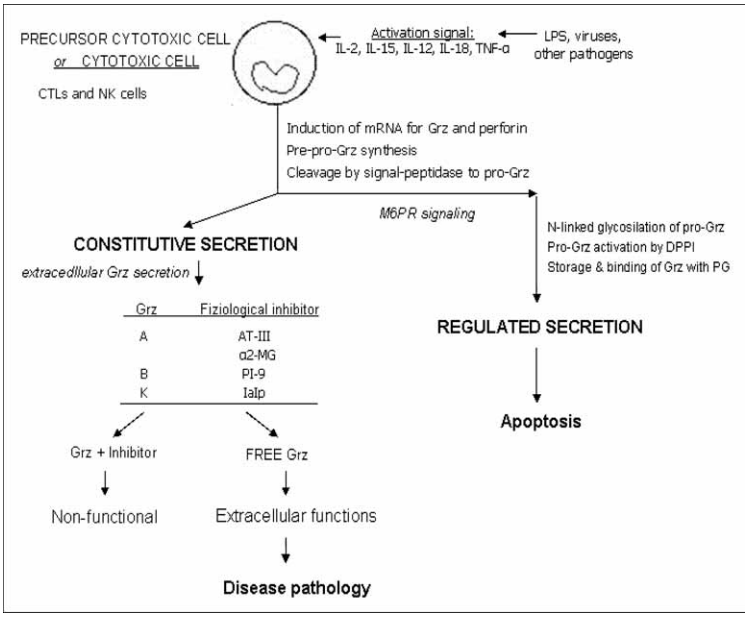

Figure 3. *Biogenesis of human Grz. Constitutive versus regulated secretion. Pro-apoptotic and extracellular Grz functions and its extracellular activities regulation by specific physiological inhibitors.*

from degrading any proteins localized inside the granules. Once released from the secretory granules into cytosol or extracellular space (neutral environment) Grz are maximally active . It has been shown that cytotoxic cells can synthesize their own intracellular serine peptidase inhibitors called serpins. These serpins are absent from the granules but present in the cytosol (pH around 7), and are thought to bind and neutralize mis-sorted or mis-packaged Grz thereby preventing so called self destruction of effector cell . One such intracellular serpin is PI-9 known to specifically inhibit GrB *(5)*. Recently, numerous novel intracellular serpins have been identified but their Grz specificities have to be characterized. Constitutive levels of Grz have been detected in human plasma *(5, 22)*. The exact nature of Grz release into extracellular environment is not fully understood. Theoretically, extracellular Grz may have originated from the immunological synapse during killing of the targets, from which they diffuse into the extracellular space as a free Grz or in complexes associated with PG *(20, 21)*. The appearance of such extracellular Grz potentially, may be a result of constitutive (non-specific) secretion by cytotoxic cells as observed after TCR (T cell receptor) triggering, or prolonged exposure to interleukin (IL-2) (*5, 22*). Furthermore, several studies have demonstrated that the matrix associated proteins vitronectin and fibronectin through interaction with CTL provide a co-stimulatory signal that could induce release of stored Grz *(5)*. The presence of bacteria has been shown to induce Grz release from lymphocytes via yet unknown mechanism *(23)*. However, under normal circumstances, there are physiological inhibitors (naturally occurring extracellular serpins) present in the plasma that bind to and form complexes with these extracellular Grz, and thereby inhibit their potent proteolytic activity (Figure 3). Serpins act as pseudosubstrates and exert their inhibitory effects by presenting their reactive site as an ideal substrate for the peptidase that leads to a reaction that traps the enzyme and distorts the catalytic site *(15)*. All members of the serpin family share a high degree of structural homology, but are highly specific when targeting serine peptidases. Some of these physiological inhibitors include antithrombin-III (AT-III) and α_2-macroglobulin (α_2-MG) for GrA , and Inter-alpha-inhibitor proteins (IaIp) for GrK . Although alpha-1-antitrypsin was believed to be a physiological inhibitor of GrB, recent studies suggest that is not the case . It also appears that these inhibitors are not able to inhibit Grz activity when the Grz are complexed with PG *(5, 27)*. It has been observed that activity of Grz in the absence of corresponding inhibitors may result in disease pathology *(30, 31)* as will be discussed later.

BIOLOGICAL SIGNIFICANCE OF HUMAN GRANZYMES

Intracellular functions – granzymes and apoptosis

Ever since discovery of target cell killing by cytotoxic cells, much effort has been devoted to understanding the lytic mechanisms involved in this process. A variety of independent studies have supported the original concept that proteolytic Grz are critically involved in the cytotoxic event during granule exocytosis as shown in Figure 4 *(32)*. In brief, following recognition and conjugation with a target cell, the cyototxic granules migrate from their dispersed locations in the cytosol to the immunological synapse, where their membranes fuse with the cytotoxic cell plasma membrane, that enable them to

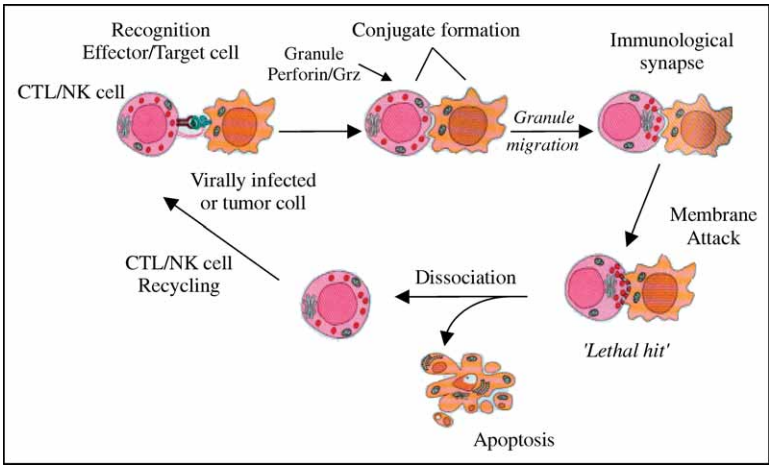

Figure 4. *The granule exocytosis pathway of cytotoxic cell killing. This pathway is not a single, but rather a sequence of events that includes several major steps as: conjugate formation, membrane attack, cytotoxic cell dissociation and target-cell destruction. Modified from Goldsby* et al. .

release their granular contents into the intracellular space *(18)*. Grz are than internalized by the target cell in a manner that remains controversial, but which results in their accumulation in endosome-like vesicles *(5)*. Cytoplasmic entry of Grz and induction of cell death, a process which is also known as membrane attack or 'lethal hit', is than critically dependent on the action of pore forming protein/perforin *(18)*. The 'lethal hit' happens very rapidly after contact with target cells. At this point cytotoxic cell can dissociate while the target cell is committed to die by apoptosis. Following dissociation, cytotoxic cells can seek another target up to twenty times, yet remain undamaged *(17)*.

The mechanisms of cell death induction have been extensively studied for only GrA and GrB. Several of apoptotic mechanisms induced by these Grz have been elucidated, and some of their intracellular substrates have been identified *(3, 22)*. The first and most studied effector molecule, GrB, is now recognized as the most powerful apoptosis inducing molecule. GrB initiates cell death rapidly by inducing an early DNA fragmentation, mainly through the caspase activation pathway. Many studies confirmed that GrB activates a number of procaspases (including -2, -3, -7-10) directly, but in most cases it involves indirect activation of caspases through activation of the mitochondrial pathway by the cleaving of a mitochondrial, pro-apoptotic Bcl-2 family member called Bid *(2)*. Recently, various caspase independent pathways have also been suggested for GrB and are yet to be more fully defined *(3, 34)*. Next to GrB, GrA effectively initiates cell death, however, via an alternative pathway that is clearly caspase independent *(5)*. The exact mechanisms by which GrA induces cell death are only partly understood and are currently being widely studied. The apoptotic effects of GrA were demonstrated by delivery of purified GrA into the target cell which resulted in apoptotic features such as DNA fragmentation, loss of mitochondrial transmembrane potential and generation of oxygen species *(18)*. GrA has also been shown to cleave several important nuclear proteins including lamins and histones. Recently, endoplasmic reticulum associated complex, known as the SET complex has been recognized as a special target for GrA possibly inducing a novel form of DNA damage *(18)*. The physiological relevance of GrA and GrB in inducing apoptosis has been confirmed in a study with mice deficient in GrA(-/-) and/or GrB(-/-). This study demonstrated that CTL derived from GrA(-/-) or GrB(-/-) mice similarly induced early proapoptotic features but with distinct kinetics. Consistent with *in vitro* findings, CTL from GrA(-/-) but not from GrB(-/-) mice activated caspase 3 and 9 *(34)*. Undoubtably, Grz are now well recognized as the principal effector molecules that play indispensable roles in initiating the process of apoptosis ('programmed' death of the cell) in virus-infected and tumor and/or other potentially harmful cells *(32)*. However, it is still not clear if the Grz-induced apoptotic features are perforin dependent *(18)*. Recent evidences derived from studies in perforin deficient mice strongly indicated that the proteolytic activities by which Grz initiate the process of apoptosis in cytosol and/or nucleus of the target cells are critically perforin dependent *(34)*. A clear mechanistic explanation supporting such perforin and Grz synergy remains undefined.

Extracellular functions of granzymes

Based on the findings that Grz also appear extracellularly *(5, 35)*, a number of important extracellular functions have been postulated for these peptidases. Grz were shown to cleave a number of extracellular matrix components (EMC) *(5, 36)*, to induce cytokine secretion and/or to directly activate cytokines *(20, 21, 37)*. Furthermore, the role of Grz in proteolytic activation of the peptidase-activated receptor (PAR) family has been postulated *(5)*. Together, these observations strongly suggest that extracellular Grz possess important pro-inflammatory properties which may result in pathological consequences. Thus, these extracellular activities of Grz are now being widely investigated.

Cleavage of extracellular matrix proteins

The involvement of Grz in disrupting extracellular matrix integrity was demonstrated by the ability of GrA to cleave components of the extracellular matrix such as PG, collagen type IV, laminin, and fibronectin *(2, 5, 36)*. In addition, GrA has been shown to activate the proenzyme of urokinase-type plasminogen activator, which activates plasmin, an enzyme that is also very effective at degrading extracellular matrix *(5, 36)*. Recently, it has been illustrated that, like GrA, GrB can also cleave EMC. GrB degrades PG in matrix synthesized by chondrocytes, and specifically, the abundant structural PG of cartilage, aggrecan *(5)*. The number of extracellular substrates identified for human GrB continues to increase. Such disruption of EMC causes protein leakage, and thereby facilitates entry and migration of innate cells through tissues or extravasation *(36)*. More importantly, it has been recently reported that components generated by the cleavage of extracellular matrix could either directly or indirectly trigger the inflammatory response leading to systemic inflammation/sepsis and its related conditions *(25)*.

Cytokine secretion and/or activation

The inflammatory properties of Grz have also been demonstrated by their ability to exert other biological responses such as, the induction of cytokine production and secretion *(2)*. Next to thrombin, another peptidase with trypsin-like activity, purified human GrA has been shown to induce human lung fibroblasts and epithelial cell lines to produce IL-6 *(21)*. Furthermore, GrA was also shown to induce the production of IL-6, IL-8, and tumor necrosis factor (TNF)-α by human peripheral blood mononuclear cells and purified monocytes and to enhance the phagocytic activity of monocytes. Importantly, induction by thrombin resulted only in increased IL-8 production and enhanced phagocytosis, but did not induce IL-6 or TNF-a production *(20)*. Grz have also been implicated in proteolytic activation of certain cytokines. GrA has been shown to directly activate the proinflammatory cytokine IL-1β by cleaving off its propeptide *(37)*. In addition, GrA was suggested to activate PAR-2 receptor *(5)*. However, the precise role of Grz in such activation is unknown.

ORPHAN GRANZYMES

The current understanding of the molecular basis of apoptotic cell death, principally obtained with GrA and GrB, has revealed the complexity of intracellular events involved. The remaining Grz that are reffered to as »orphan Grz« have been largely neglected and for a long time considered functionally redundant. The role of these Grz in apoptosis became evident from the studies in which cytotoxic cells derived from mice with complete absence of both, GrA and GrB (double knockout) retained ability to kill certain susceptible targets . Although apoptotic pathways and target cellular substrates of orphan Grz are largely unknown, it may well be that different Grz induce independent cell death pathways in host response to circumvent targets. Likewise, these remaining Grz may also exhibit an array of potentially different extracellular activities which are yet to be discovered.

EXTRACELLULAR GRANZYMES AND DISEASES

Grz and enzymes that regulate their activity have been associated with certain human pathological conditions. As cells involved in the major defense of higher organisms, cytotoxic lymphocytes have been acknowledged to detect and eliminate potentially harmful cells by inducing them to undergo apoptosis *(22)*. However, it has been observed that upon cytotoxic cells activation a significant amount of Grz appeared in human plasma *(5, 23)*. As previously mentioned, peptidase inhibitors are usually present in the body at very high concentrations and their proposed function is to inhibit the potential bystander effects of these peptidases thus, preventing them from damaging normal structures. Under certain conditions, a lack of specific peptidase inhibitors can result in disease pathology when serine peptidases are present *(38)*. For example, hypersensitivity pneumonitis (HP) results from uninhibited GrA and GrB in the lung as a result of accumulation of activated alveolar CTLs and NK cells in response to allergens. The fact that neither one of the three main serine peptidase inhibitors of the lung, namely α1-antitrypsin, secretory leukocyte peptidase inhibitor, and elafin could inhibit GrA or GrB activity clearly indicates that HP occurs as a result of uncontrolled proteolysis that leads to tissue damage and inflammation *(29)*. This finding is further supported by the observation that individuals that have an inherited deficiency of α_1-antitrypsin activity show an accelerated and enhanced development of emphysema. The basis for the development of emphysema is that the inactive α_1-antitrypsin present in the lung is unable to inhibit the activity of NE that consequently results in damaging the lung tissue *(30, 31)*. Furthermore, elevated plasma levels of GrA and GrB have been associated with systemic inflammation/sepsis. The injection of LPS (lipopolysaccharide), believed to be a major trigger of sepsis, into human volunteers was found to induce a 5 fold increase in soluble GrA that peaked at 2 hrs after injection and also resulted in an increase of soluble GrB that peaked at 6 hrs *(23)*. Examination of patients with melioidosis indicated that GrA levels were significantly elevated in patients with bacterimic melioidosis compared to controls. In these patients, levels of GrA correlated with mortality. GrB levels were significantly higher in patients with bacterimic melioidosis, but there was no difference between the group of survivors and nonsurvivors *(23)*. These investigators went on to show that addition of heat killed bacteria to whole blood resulted in a significant increase in the levels of GrA and GrB. Direct measurement of Grz also indicated that levels of GrA and GrB were elevated in a subset of septic patients *(39)*. High levels of GrA and GrzB have been observed in

plasma of infectious mononucleosis patients *(40)*. Moreover, soluble GrA and GrB levels were elevated in an array of pathophysiological situations such as infection with intracellular pathogens, graft versus host disease, susceptibility to transplantable and spontaneous malignancies, the lymphoid homeostasis, rheumatoid arthritis and autoimmune disease *(5, 12)*. Molecular mechanism of Grz function and regulation in such situations are under intense investigation.

FUNCTIONAL SIGNIFICANCE OF GRANZYME K

To date, there had been little investigation into the role of human GrH, GrM and GrK although, their involvement in immune responses is being recognized *(4)*. Most recently, our laboratory provided new evidence concerning functional importance of GrK. Similar to GrA and GrB, we observed that low constitutive levels of GrK existed extracellularly in normal plasma. Importantly, our data also revealed that plasma GrK levels correlated with the stage of sepsis and that free, uninhibited GrK molecule appeared in sepsis patients along with the loss of its respective physiological inhibitor, IaIp in plasma *(35)*. Thus, our findings strongly indicate that the presence of unhibited GrK, as a potent peptidase may contribute to the development of sepsis and related inflammatory conditions. In addition, functional significance of GrK has been highlighted by several other studies. GrK mediates tryptic activity in complete absence of second Grz-tryptase, GrA as demonstrated in mice in which GrA was genetically deleted. At the same time, in these mice GrK expression was unimpaired. This same study illustrated minimal cytotoxic defect in these GrA deficient mice that could be due to the persistent expression of additional tryptase, GrK *(41)*. Furthermore, the potential of GrK to induce apoptosis has been reported *(41, 42)*. Once delivered into the target cell along with sublytic dose of perforin, GrK has been shown to induce cell death by late release of DNA in a fashion similar to GrA. However, it does not cause typical apoptotic nuclear morphology observed with GrA and GrB, suggesting that GrK may use a unique pathway in killing susceptible targets *(18)*. In the recent study, Bade *et. al.* demonstrated that GrK expression and regulation pattern differs from that of other Grz across different subsets of cytotoxic cells *(22)*. This study further suggests that GrK might exhibit potentially significant functions under certain physiological conditions. In addition, significantly elevated GrK levels were observed in patients with several viral infections suggesting that GrK actively participates in innate responses against certain viruses. However, the target cellular substrate for GrK and the molecular mechanism of its function remain unknown.

BACKGROUND AND CURRENT BIOLOGY OF HUMAN GRANZYME K

Granzyme K, a 26 kDa protein was originally discovered as *granzyme 3* in granules of human lymphokine activated killer cells *(28)*. It is most similar to GrA. Both proteins are tryptases and are encoded by closely linked genes located on the same human chromosome, (5q11-12) *(43)*. However, GrK differs from GrA on the basis of its amino acid sequence and monomeric structure *(44)*. Human recombinant GrK (*rec*GrK) has been expressed in *Escherichia coli* as a pro-Grz and was obtained catalytically active following cleavage by DPPI *(28)*. Hirata *et al.* used the same recombinat technology and obtained catalytically active *rec*GrK without refolding or di-peptide deletion *(45)*. Recently, we obtained catalytically active native human GrK from NK 92MI cells secretory granules by using a method for rapid isolation of granular proteases *(35)*. In addition, GrK appeared extracellularly secreted by these NK cells thus suggesting the existence of a constitutive, non-granule biosynthetic pathway (unpublished data). While it is reasonable to assume that GrK as well is sorted to the secretory granules via M6P recognition signal, the nature of the mechanism responsible for its constitutive secretion yet remains unknown. During biogenesis, GrK might escape a M6P oligosaccharide modification of the N-linked glycans, then consequently fails to acquire the M6P receptor granule-targeting signal and thus may be directly secreted. Previous data support a similar scheme for constitutive secretion of GrB but not GrA by TCR triggered CTLs *(17)*. An alternative biosynthetic pathway, commonly suggested for hematopoietic serine peptidases, is the absence of DPPI during biosynthesis and processing that then results in direct secretion of the pro-enzyme forms *(15, 46)*. In any case, such constitutive secretion supports the idea that GrK fulfils additional extracellular functions apart from its pro-apoptotic role in the well regulated lytic events occurring during NK and/or CTL lysis of target cells *(41)*. In analogy to GrA, as the closest structural and functional relative of GrK *(43)*, it could be anticipated that GrK might cleave extracellular matrix proteins or might proteolytically process as of yet, unidentified mediators of inflammation *(5, 20, 21)* . Although, both are tryptases, GrK might differ from GrA on the basis on its structural characteristics and its distinctive substrate cleaving specificity *(2, 11)*. Furthermore, the proteolytic activities of these, though similar Grz-tryptases is differentially regulated. IaIp of human plasma had been shown to effectively inhibit extracellular GrK activity *(28),* while the major physiological inhibitor of GrA, AT-III was ineffective in inhibiting GrK *(28)*.

In summary the current findings demonstrate that different Grz exhibit distinctive substrate specificities, that are heterogeneously expressed across different cytotoxic cell subsets and may induce multiple independent cell death pathways indicating, that each one of the Grz might play specific functions under certain physiologic situation. It become evident that Grz also possess extracellular activites and that the levels of extracellular Grz are elevated in a number of diseases. However, what are the exact physiological functions; and the pathological consequences of extracellular Grz in diseases and, how their activities are regulated in specific situations remains to be defined. In addition, an insight into GrK

biology and function is now being clearly revealed. Thus, detail characterization of human GrK is required to define the exact physiological substrate and the molecular mechanism(s) of its actions in human physiology and pathology.

REFERENCES

1. JENNE D E, TSCHOPP J 1988 Granzymes, a family of serine proteases released from granules of cytolytic T lymphocytes upon T cell receptor stimulation. *Immunol Rev 103: 53-71*
2. TRAPANI J A 2001 Granzymes: a family of lymphocyte granule serine proteases. *Gen Biol 2:* 3014.3011-3014.3017
3. RUSSELL J H, LEY T J 2002 Lymphocyte-mediated cytotoxicity. *Annual Rev Immunol 20: 323-370*
4. GROSSMAN W J, REVELL P A, LU Z H, JOHNSON H, BREDEMEYER A J, LEY T J 2003 The orphan granzymes of humans and mice. *Curr Opin Immun 5: 544-552*
5. BUZZA M S, BIRD P I 2006 Extracellular granzymes: current perspectives. *Biol Chem 387: 827-837*
6. HANNA J, BECHTEL P, ZHAI Y, YOUSSEF F, MCLACHLAN K, MANDELBOIM O 2004 Novel Insights on Human NK Cells' Immunological Modalities Revealed by Gene Expression Profiling. *J Immunol 173: 6547-6563*
7. CARTER P, WELLS J A 1988 Dissecting the catalytic triad of a serine protease. *Nature 332: 564-568*
8. KAM C M, HUDIG D, POWERS J C 2000 Granzymes (lymphocyte serine proteases): characterization with natural and synthetic substrates and inhibitors. *Biochem Bioph Acta 1477: 307-323*
9. SCHECHTER I, BERGER A 1967 On the size of the active site in proteases.I. Papain. *Biochem Biophys Res Comun 27: 157-162*
10. SCHECHTER I, BERGER A 1968 On the active site of proteases 3. Mapping the active site of papain; specific peptide inhibitors of papain. *Biochem Biophys Res Comun 32: 898-902*
11. MAHRUS S, CRAIK C S 2005 Selective Chemical Functional Probes of Granzymes A and B Reveal Granzyme B is a Major Effector of Natural Killer Cell-Mediated Lysis of Target Cells. *Chem & Biol 12: 567-577*
12. SMYTH M J, KELLY J M, SUTTON V R, DAVIS J E, BROWNW K A, SAYERS T J, TRAPANI J A 2001 Unlocking the secrets of cytotoxic granule proteins. *J Leukoc Biol 70: 18-29*
13. DEBLAKER-HOHE D F, YAMAUCHI A, YU C R, HORVATH-ARCIDIACONO J A, BLOOM E T 1995 IL-12 synergizes with IL-2 to induce lymphokine-activated cytotoxicity and perforin and granzyme gene expression in fresh human NK cells. *Cell Immunol 165: 33-43*
14. LIU K, CATALFAMO M, LI Y, HENKART P, WENG N P 2002 IL-15 mimics T cell receptor crosslinking in the induction of cellular proliferation, gene expression, and cytotoxicity in CD8+ memory T cells. *Proc Natl Acad Sci USA 99: 6192-6197*
15. PHAM C T N 2006 Neutrophil serine proteases: specific regulators of inflammation. *Nat Rev Immunol 7: 541-550*
16. TRAN T V, ELLIS K A, KAM C M, HUDIG D, POWERS J C 2002 Dipeptidyl peptidase I: importance of progranzyme activation sequences, other dipeptide sequences, and the N-terminal amino group of synthetic substrates for enzyme activity. *Arch Biochem and Bioph 403: 160-170*
17. ISAAZ S, BAETZ K, OLSEN K, PODACK E, GRIFFITHS G M 1995 Serial killing by cytotoxic T lymphocytes: T cell receptor triggers degranulation, re-filling of the lytic granules and secretion of lytic proteins via a non-granule pathway. *Eur J iImmunol 25: 1071-1079*
18. LIEBERMAN J 2003 The ABCS of granule-mediated cytotoxicity: new weapons in the arsenal. *Nat Rev Immunol 3: 361-370*
19. METKAR S S, WANG B, EBBS M L *et al.* 2003 Granzyme B activates procaspase-3 which signals a mitochondrial amplification loop for maximal apoptosis. *J Cell Biol 160: 875-885*
20. SOWER E L, FROELICH C J, ALLEGRETTO N, ROSE P M, HANNA W L, KLIMPEL G R 1996 Extracellular Activities of Human Granzyme A. Monocyte Activation by Granzyme A Vesrus a-Trombin. *J of Immunol 156: 2585-2590*
21. SOWER L, KLIMPEL G, HANNA W, FROELICH C 1996 Extracellular activities of human granzymes. I. Granzyme A induces IL6 and IL8 production in fibroblast and epithelial cell lines. *Cell Immunol 171: 159-163*
22. BADE B, BOETTCHER H E, LOHRMANN J, SCHAUER C H, BRATKE K, JENNE D E, VIRCHOW J C, LUTTMANN W 2005 Differential expression of the granzyme A, K and M and perforin in human peripheral blood lymphocytes. *Int Immunol 17: 1419-1428*
23. LAUW F N, SIMPSON A J H, HACK C E, PRINS J M, WOLBINK A M, DEVENTER S J H, CHAOWAGUL W, WHITE N J, POLL T 2000 Soluble Granzymes Are Released during Human Endotoxemia and in Patients with Severe Infection Due to Gram-Negative Bacteria. *J Infect Dis 182: 206-213*
24. BOONTHAM P, CHANDRAN P, ROWLANDS B, EREMIN O 2003 Surgical sepsis: dysregulation of immune function and therapeutic implications. *Surgeon 4:187-206*
25. BRUNN G J, PLATT J L 2006 The etiology of sepsis: turned inside out. *TRENDS in Mol Med 12: 10-16*
26. MASSON D, TSCHOP J 1988 Inhibition of lymphocyte protease granzyme A by antithrombin III. *Mol Immunol 25: 1283-1289*
27. SPAENY-DEKKING E, KAMP A, FROELICH C, HACK C 2000 Extracellular granzyme A, complexed to proteoglycan, is protected against inactivation by protease inhibitors. *Blood 95: 1465-1472*
28. WILHARM E, PARRY M A A, FRIEBEL R, TSCHESCHE H, MATSCHINER G, SOMMERHOFF C P, DIETER J E 1999 Generation of Catalytically Active Granzyme K from *Escherichia coli* Inclusion Bodies and Identification of Efficient Granzyme K Inhibitors in Human Plasma. *J Biol Chem 274: 27331-27337*
29. TREMBLAY G M, WOLBINK A M, CORMIER Y, HACK C E 2000 Granzyme Activity in the Inflamed Lung Is Not Controlled by Endogenous Serine Proteinase Inhibitors. *J Immunol 165: 3966-3969*
30. CARRELL R, LOMAS D 2002 Alpha1-antitrypsin deficiency – A model for conformational diseases. *N Engl J Med 346: 45-53*
31. LOMAS D, MAHADEVA R 2002 a_1-antitrypsin polymerization and the serpinopathies: pathobiology and prospects for therapy. *J Clin Invest 110: 1585-1590*
32. SMYTH M J, TRAPANI J A 1995 Granzymes: exogenous proteinases that induce target cell apoptosis. *Immunol Today 16: 202-206*
33. GOLDSBY R A, KINDT T J, OSBORNE B A 1999 Kuby's Immunology. 4th (*ed*) Freeman WH & Co, New York, NY.
34. PADRO J, BOSQUE A, BREHM R, WALLICH R, NAVAL J, MULLBACHER A, ANEL A, SIMON M M 2004 Apoptotic pathways are selectively activated by granzyme A and/or granzyme B in CTL-mediated target cell lysis. *J Cell Biol 167: 457-468*
35. RUCEVIC M, FAST L D, JAY G D, RESPALCIOS F M, SUCOV A, SIRYAPORN E, LIM Y P 2007 Altered Levels and Molecular Forms of Granzyme K in Plasma from Septic Patients. *Shock 27: 488-493*
36. SIMON M M, KRAMER M D, PRESTER M, GAY S 1991 Mouse T-cell associated serine proteinase 1 degrades collagen type IV: A structural basis for the migration of lymphocytes through vascular basement membranes. *Immunol Rev 73: 117-119*
37. IRMEL M, HERTIG S, MACDONALD H R, SADOUL R, BECHERER J D, PROUDFOOT A, SOLARI R, TSCHOPP J 1995 Granzyme A is an interleukin 1 beta-converting enzyme. *J Exp Med 181: 1917-1922*
38. CHEN C, ARROW A L, QI J, D'ANDREA M R, ANDRADE-GORDON P 2003 A novel serine protease predominately expressed in macrophages. *Biochem J 374: 97-107*
39. ZEERLEDER S, HACK C E, CALIEZI C *et al.* 2005 Activated cytotoxic T cells and NK cells in severe sepsis and septic shock and their role in multiple organ dysfunction. *Clin Immunol 116: 158-165*
40. SPAENY-DEKKING E H A, HANNA W L, WOLBINK A M, WEVER P C, KUMMER A J, SWAAK A J G, MIDDELDROP J M, HUISMAN H G, FROELICH J C, HACK E 1998 Extracellular Granzymes A and B in Humans: Detection of Native Species During CTL Responses *in vitro* and *in vivo*. *J Immunol 160: 3610-3616*
41. SHRESTA S, GODA P, WESSELSCHMIDT R, LEY T J 1997 Residual cytotoxicity and Granzyme K expression in Granzyme A-deficient cytotoxic lymphocytes. *J Biol Chem 32: 20236-20244*
42. SHI L, KAM C M, POWERS J C, AEBERSOLD R, GREENBERG A H 1992 Purification of three cytotoxic lymphocyte granule serine proteases that induce apoptosis through distinct substrate and target cell interactions. *J Exp Med 176: 1521-1529*
43. HINK-SCHAUER C, ESTEBANEZ-PERPINA E, WILHARM E, FUENTES-PRIOR P 2002 The 2.2-A Crystal Structure of Hu-

man Pro-granzyme K Reveals a Rigid Zymogen with Unusual Features. *J Biol Chem 277: 50923-50933*

44. SATTAR R, ALI S A, ABBASI A 2003 Bioinformatics of granzymes: sequence comparison and structural studies on granzyme family by homology modeling. *Biochem Bioph Res Com 308: 726-735*
45. HIRATA Y, HIROFUMI I, TAKAKO S, LI Q, NAGAHARA N, MINAMI M, KAWADA T 2005 Expression of enzymatically active human granzyme 3 in *Escherichia coli* for analysis of its substrate specificity. *Arch Biochem and Bioph 446: 35-43*
46. SKOLD S, ZEBERG L, GULLBERG U, OLOFSSON T 2002 Functional dissociation betwen proforms and mature forms of proteinase 3, azurocidin, and granzyme B in regulation of granulopoiesis. *Exp Hematol 30: 689-696*
47. SCHAEFER U, BRUCKER B, ELBERS A, NEUGEBAUER E 2004 The capacity of α_2-macroglobulin to inhibit an exogheneous protease is significantly increased in critically ill and septic patients. *Schock 22: 16-22*