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COMMENTARY

Granulysin

A NOVEL ANTIMICROBIAL PEPTIDE OF CYTOLYTIC T LYMPHOCYTES AND NATURAL KILLER CELLS

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ABSTRACT. Granulysin is a novel antimicrobial protein produced by human cytolytic T lymphocytes and natural killer cells. It is active against a broad range of microbes, including Gram-positive and Gram-negative bacteria, fungi, and parasites. The fact that it kills *Mycobacterium tuberculosis* is particularly important, since the current vaccine (Bacille Calmette-Guerin, BCG) is of limited efficacy and antibiotic resistance is increasing. Although functionally related to other antibacterial peptides, defensins and magainins, granulysin is structurally distinct. Like porcine NK lysin and amoebapores made by *Entamoeba histolytica*, granulysin is related to saposins, small lipid-associated proteins present in the central nervous system. The identification of this novel molecule indicates a broader and perhaps more significant role for T lymphocytes in both innate and acquired antimicrobial defenses. BIOCHEM PHARMACOL **59**;4:317–320, 2000. © 2000 Elsevier Science Inc.

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More than 10 years ago, we set out to identify gene products expressed by T lymphocytes "late" (3–5 days) after T cell activation. Four such mRNAs were identified in our laboratory (519, RANTES†, WP34, and Tactile) [1–4], and numerous others were identified by groups employing similar approaches [5–8]. Among these mRNAs was one, designated 519, expressed exclusively by NK cells and T lymphocytes 3–5 days after activation [1]. Subsequent studies identified two other transcripts, 520 and 522, which were results of alternative splicing of the same gene product [9].

The 520 cDNA was expressed in *Escherichia coli*, and antibodies were generated against the recombinant protein [10]. Immunofluorescence studies with these antibodies indicated that the 520 protein was localized to cytolytic granules within CTL and NK cells and that the protein was exocytosed after triggering T cells via the T cell receptor. Based upon its location in cytolytic granules and its lytic activity against a variety of mammalian target cells, including the human erythroleukemia K562 cell line, B lymphoblastoid cell lines, and the murine tumor YAK, the protein was renamed "granulysin."

STRUCTURE

A comparison of the amino acid sequence of granulysin with known proteins in the National Center for Biotechnology Information database showed homology with a family of SAPLIP named for four small proteins (saposins) involved in sphingolipid catabolism in the central nervous system (Table 1) [11]. Important members of the family from mammalian sources include pulmonary surfactant protein-B (SP-B), which lowers surface tension at the liquid–gas interface in the lung [12], and the lipid hydrolases acyloxyacyl hydrolase [13] and acid sphingomyelinase [14]. Proteins of this family all interact with lipids. Most closely related to granulysin are amoebapores A–C, produced by *Entamoeba histolytica* [15], and NK lysin [16], isolated from pig intestines. Amoebapores are present within cytolytic granules of the protozoan parasite and are released directionally to kill bacterial prey. NK lysin was isolated in 1994 based upon its antibacterial activity as a heat-stable protein in pig intestinal extracts. These findings suggest an ancient, evolutionarily conserved, cytotoxic mechanism related to innate immunity.

All of the SAPLIP family members identified to date, with the exception of granulysin, share a common spacing of six cysteine residues, which appear to determine their structure [17]. Granulysin contains a tyrosine instead of a cysteine at the first position (Fig. 1). Hydrophobic residues throughout the sequence also are conserved among the family members.

ANTIMICROBIAL ACTIVITY

Because of its structural homology with members of the SAPLIP family, NK lysin, and amoebapores, we explored whether isolated recombinant granulysin could kill microbial targets [18]. With Robert Modlin and colleagues, we showed that granulysin was lytic against a broad range of microbes. It caused concentration-dependent growth inhi-

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† *Abbreviations*: CTL, cytolytic T lymphocytes; NK, natural killer; RANTES, regulated upon activation normal T cell expressed and secreted; and SAPLIP, saposin-like proteins.

TABLE 1. SAPLIP (saposin-like protein) family members and their proposed functions

Family member	Function	Identity to 9-kDa granulysin (%)	Similarity to 9-kDa granulysin (%)
Saposin A	Sphingolipid hydrolase activator	21	46
Saposin B	Sphingolipid hydrolase activator	19	50
Saposin C	Sphingolipid hydrolase activator	19	53
Saposin D	Sphingolipid hydrolase activator	20	46
Pulmonary surfactant protein B	Lipid organization in pulmonary surfactant	19	53
Acyloxyacyl hydrolase	Phagocytic cell lipase	22	50
Acid sphingomyelinase	Sphingolipid hydrolase	13	42
Amoebapore A	Pore-forming *E. histolytica* granule protein	16	47
Amoebapore B	Pore-forming *E. histolytica* granule protein	13	42
Amoebapore C	Pore-forming *E. histolytica* granule protein	18	47
NK-lysin	Lytic porcine T and NK cell granule protein	35	66
Granulysin	Lytic human CTL and NK cell granule protein	100	100

bition of both Gram-positive and Gram-negative bacteria, with greater than 1000-fold reduction in colony forming units for *Salmonella typhimurium*, *Listeria monocytogenes*, *E. coli*, and *Staphylococcus aureus*. Granulysin also killed fungi and parasites, including *Cryptococcus neoformans*, *Candida albicans*, and *Leishmania major*. Perhaps most startling was its activity against *Mycobacterium tuberculosis*, one of the pathogens most resistant to the human immune response. Recombinant granulysin killed more than 90% of extracellular *M. tuberculosis*, but was ineffective in killing intracellular organisms. However, the combination of recombinant granulysin and purified perforin, a pore-forming protein contained in the same cytolytic granules as granulysin, was highly effective at killing intracellular *M. tuberculosis*. Thus, proteins present in cytolytic granules of CTL and NK cells synergize to kill intracellular *M. tuberculosis*, and at least the combination of granulysin and perforin is capable of mimicking the activity of whole cells. This suggests an entirely new pathway for antimicrobial responsiveness, especially with regard to lysis of intracellular pathogens.

Although recombinant granulysin is highly potent in solution, mimicking interactions in the extracellular space, it is likely that granulysin is unable to reach the intracellular compartment alone. It appears to require perforin, or another pore-forming protein, for access across the outer cell membrane or perhaps for entry into endocytic vesicles themselves. Scanning electron microscopy showed that incubation of granulysin with *M. tuberculosis* caused the formation of distinct protrusion-like lesions on the surface of the mycobacteria [18]. Since other members of the

FIG. 1. Sequence comparison of granulysin with other SAPLIP family members. Family members shown are human granulysin (huGran), porcine NK lysin (pNK1) and its translated cDNA (pNK2), amoebapores (AP) A–C, human pulmonary surfactant protein-B (huPSP-B), human saposins (huSAP) A–D, residues 38–121 of human acyloxyacyl hydrolase (huAOAH), and residues 85–169 of human acid sphingomyelinase (huASM). All of the SAPLIP family members except granulysin contain six cysteines, numbered 1–6 at the bottom of the figure and contained within boxes. Connecting lines indicate the disulfide binding pattern between cysteines 1 and 6, 2 and 5, and 3 and 4. Conserved hydrophobic sequences are underlined. The five helices of the NK lysin structure are shown at the top. Reprinted with permission from Hanson DA and Krensky AM, In: *Cytotoxic Cells: Basic Mechanisms and Medical Applications*, pp. 213–227. Copyright (2000) Lippincott Williams & Wilkins. [Ref. 7].

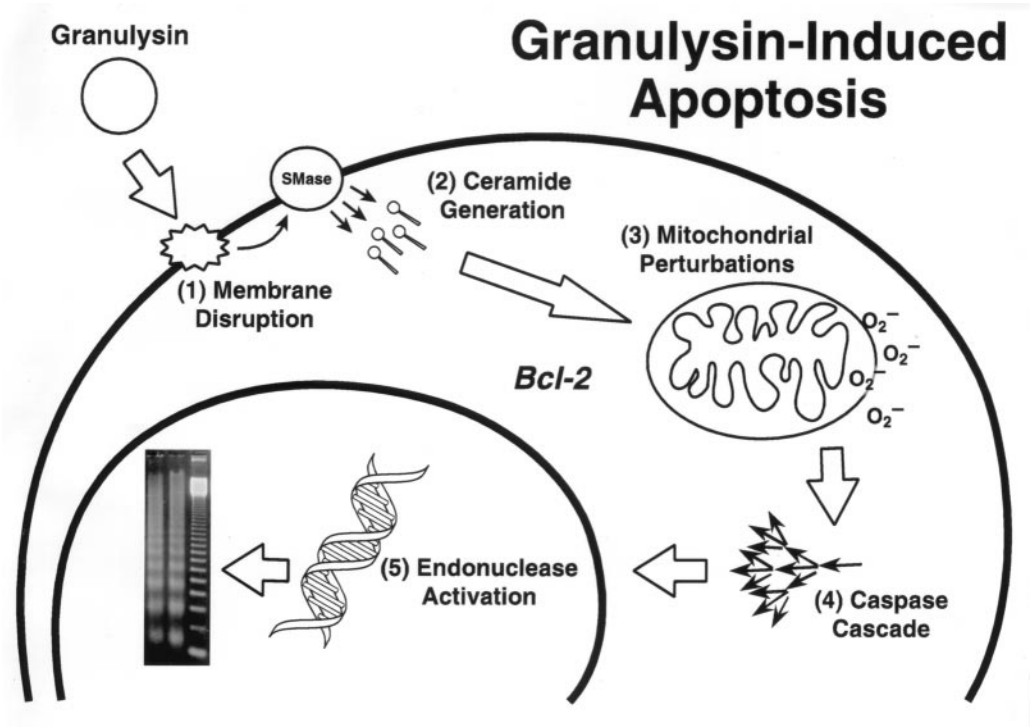

FIG. 2. Mechanism of action of granulysin in apoptosis of Jurkat cells. Granulysin-induced apoptosis includes several steps. Although the kinetics are not yet fully elucidated, the five steps shown are clearly involved in granulysin-mediated apoptosis of Jurkat T cells. The sphingomyelinase is shown as surface-associated, but it is not known presently if ceramide is generated from a plasma membrane neutral sphingomyelinase or a lysosomal acid sphingomyelinase (see Gamen *et al.* [20]). Overexpression of the anti-apoptotic gene *Bcl-2* protects Jurkat cells from granulysin-mediated apoptosis.

SAPLIP family interact with lipids, we hypothesize that granulysin interacts directly with lipids in the bacterial cell wall, leading to degradation evident as ultrastructural lesions.

MECHANISM OF ACTION

No other data regarding the mechanism of action of granulysin as an antibiotic are known, but considerable progress in understanding its interaction with liposomes and its effects on a human tumor cell line (Jurkat) has been made (Fig. 2) [19]. We now understand that granulysin directly affects the cell membrane. Although it does not appear to form pores *per se*, it allows leakage of intracellular dye from liposomes within seconds. Negatively charged phospholipids are required for lysis. Maximum leakage of a fluorescent probe (ANTS) was seen with pure phosphatidylglycerol (POPG) or 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC)/cardiolipin. Lowering pH to 5 markedly decreased lysis. This suggests that the acidic environment of the cytolytic vesicle protects the cell from autolysis. Also of note, recombinant granulysin does not lyse red blood cells.

In collaboration with Alberto Anel and colleagues, we showed that granulysin induces apoptosis in Jurkat cells [20]. Nuclear disintegration is accompanied by increased generation of ceramide from sphingomyelin, suggesting a direct action of granulysin on a sphingomyelinase. Since ceramide alone can induce apoptosis, this finding may help explain the cytolytic activity of granulysin. Nevertheless, an inhibitor of this pathway failed to eradicate granulysin-induced lysis, suggesting additional pathways as well. We have further shown that granulysin affects mitochondrial potential and that inhibitors of granzymes also inhibited granulysin-induced cytolysis. Thus, as depicted in Fig. 2, numerous links in an apparent pathway of granulysin-mediated cell death have been identified in the Jurkat model system. The precise relevance of any or all of these findings to its antimicrobial activity remains to be evaluated.

CLINICAL AND BIOLOGICAL IMPLICATIONS AS AN ANTIMICROBIAL

The identification and characterization of granulysin as an antimicrobial product of T lymphocytes and natural killer cells suggest a broader, and perhaps more important, role for these cell types in the ongoing war against microbes. Other immune cells, with phagocytic function, generally have been implicated as the important lines of defense against bacteria. The roles of CTL and NK cells have been confined more narrowly to antiviral immunity and certain specific bacterial and parasitic infections.

Tuberculosis is one of the most important public health problems in the world today [21]. As much as one-third of the world's population is infected with *M. tuberculosis*, and up to 3,000,000 people die each year from tuberculosis.

Although the threat is greatest in less developed nations, the rise of acquired immunodeficiency from AIDS, cancer, and chemotherapy (immunosuppressive drugs) has increased the risk in industrialized nations as well. At the same time, the efficacy of BCG (Bacille Calmette-Guerin), the currently used vaccine, is unproven, and antibiotic resistance is on the rise. There is a great need for additional therapies against this important human pathogen.

The control of *M. tuberculosis* and other intracellular pathogens is likely dependent on recognition and destruction of infected cells. Evidence from both murine and human systems implicates CTL and, in some cases, NK cells in protection against intracellular organisms. Granulysin may be of broad importance in protection against such organisms. Granulysin may lyse organisms extracellularly and/or in various intracellular compartments. *L. monocytogenes* and *Trypanosoma cruzi* escape from phagocytic vacuoles into the cytoplasm of infected host cells and may be destroyed outside of vesicles. The recent experiments with granulysin and perforin suggest that these proteins, acting together, provide a mechanism by which CTL also may control organisms that remain localized within phagosomes, including *S. typhimurium*, *E. coli*, and *M. tuberculosis*.

Because granulysin appears to have a novel mechanism of action against bacteria that persist intracellularly, it may be the prototype for a new class of antibiotics, with lesser or different side-effects, toxicities, and/or potential for emergence of resistance.

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