The Journal of Immunology

Apoptosis Induced by Granzyme B-Glycosaminoglycan Complexes: Implications for Granule-Mediated Apoptosis In Vivo

John P. Galvin, Liesbeth H. A. Spaeny-Dekking, Baikun Wang, Prem Seth, C. Erik Hack and Christopher J. Froelich

J Immunol 1999; 162:5345-5350; ;
http://www.jimmunol.org/content/162/9/5345

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Apoptosis Induced by Granzyme B-Glycosaminoglycan Complexes: Implications for Granule-Mediated Apoptosis In Vivo

John P. Galvin,* Liesbeth H. A. Spaeny-Dekking,† Baikun Wang,* Prem Seth,‡ C. Erik Hack,† and Christopher J. Froelich[1]*

Lymphocyte granule-mediated apoptosis occurs by perforin-mediated intracellular delivery of granule-associated serine proteases (granzymes). A granule-associated proteoglycan, namely serglycin, that contains chondroitin 4-sulfate (CS) glycosaminoglycans is present in the granules of cytotoxic cells. Serglycin acts as scaffold for packaging the positively charged granzymes and probably chaperones the proteases secreted extracellularly. To learn how the interaction of granzyme B (GrB) with serglycin might influence the apoptotic potential of this proteases, we have evaluated a model system where desalted CS is combined with isolated human granzyme. CS-GrB complexes were very stable, remaining undissociated in salt concentrations upwards to 500 mM (pH 7.4). On the basis of a capture enzyme immunoassay that accurately detects GrB, equivalent amounts of active free and CS-GrB, delivered by perforin or adenovirus, efficiently induced apoptosis in Jurkat cells and produced a similar time-dependent increase in caspase-3-like activity. CS-GrB processed isolated caspases-3 and -7 less efficiently than free granzyme. However, when added to cytosolic extracts, rates of processing were nearly equivalent for the two forms, suggesting cationic GrB may nonspecifically bind cytosolic proteins, leading to reduce proteolytic activity. Finally, GrB was found to be exocytosed from lymphocyte-activated killer cells as a neutral, high macromolecular weight complex, which possessed apoptotic activity. Collectively, the results indicate that neutral, high m.w. GrB has the capacity to induce cell death and will be useful to study the mechanism of cytotoxic cell-mediated apoptosis in vitro. ***The Journal of Immunology*****, 1999, 162: 5345–5350.**

The immune system uses lymphocyte granule-mediated cytotoxicity to protect the host from invasion by intracellular pathogens and tumor cells. Apoptosis is induced by perforin (PFN),[3] and the granzymes where granzyme B (GrB) plays a pivotal role (1). Granzymes are highly cationic and, similar to the mast cell proteases, are compartmentalized within the secretory granule on a scaffold of anionic chondroitin A sulfate proteoglycan, called serglycin. In contrast to other proteoglycans, serglycin is highly glycosylated and resistant to proteolysis. Various serglycins have been characterized in distinct cell types, differing primarily on the basis of the glycosaminoglycan side chains: serosal mast cells—heparin sulfate; bone marrow mast cells and megakaryocytes—chondroitin 6-sulfate (CS). Serglycin ensures the proper packaging of granule constituents and acts as extracellular carrier for many of the granule components after secretion (2). For example, all mast cell proteases except mast cell tryptase (murine mast cell protease) are secreted as a macromolecular complex into the extracellular space (3).

We have proposed that polymerized PFN pores and GrB are internalized into endosomes of the target cell during granule-mediated cytotoxicity. PFN then permeabilizes the vesicles delivering the granzyme to the cytosol. Subsequently, GrB induces cell death by activating the caspase cascade (4). Experimental models designed to characterize the biochemical events that occur during granule-mediated apoptosis have relied on the free granzyme. However, the cationic nature of the serine protease may lead to nonspecific interactions with both intercellular and intracellular anionic membranes as well as artifactual vesicular trafficking. As a consequence, results obtained from studies using isolated granzymes may not provide a valid view of granule-mediated apoptosis in vivo. We describe here a model system consisting of CS-GrB complexes. This system has been used to study the capacity of the neutral, high m.w. form of the granzyme to induce apoptosis after delivery by PFN or replication-deficient adenovirus type 2 (AD) as well as process the preferred caspase subtrates. In addition, we report preliminary evidence that a high m.w. form of GrB secreted by cytotoxic cells also has potent apoptotic activity.

Materials and Methods

Cell lines

Jurkat cells were maintained in RPMI 1640/10% heat-inactivated FCS supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 50 μg/ml streptomycin, and MCF-7 stably expressing procaspase-3 (5) were cultured in Iscove's modified DMEM/10% heat-inactivated FCS plus antibiotics.

Reagents

Human GrB and perforin were purified to homogeneity from a human NK cell line (YT) (6), and a nonreplicating strain of adenovirus type 2 (AD)

*Evanston Northwestern Healthcare Research Institute, Northwestern University, Evanston, IL 60201; †Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and the Laboratory for Clinical and Experimental Immunology, University of Amsterdam, Amsterdam, The Netherlands; and ‡Medicine Branch, National Cancer Institute, National Institute of Health, Bethesda, MD 20892

Received for publication September 29, 1998. Accepted for publication February 8, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the National and Greater Chicago Chapter of the Arthritis Foundation and Eisenberg Foundation.

[2] Address correspondence and reprint requests to Dr. Christopher Froelich, Evanston Hospital, Research Department, WH Building, Room B643, 2650 Ridge Avenue, Evanston, IL 60201. E-mail address: granzyme@merle.acns.nwu.edu

[3] Abbreviations used in this paper: PFN, perforin; AD, replication-deficient adenovirus type 2; ac-DEVD-afc, carbobenzoxy-DEVD-methylcoumarin; CS, chondroitin sulfate; GrB, granzyme B; EIA, enzyme immunoassay.

0022-1767/99/$02.00

was cultured and isolated as described (7). The peptidic substrate, carbobenzoxy-DEVD-methylcoumarin (ac-DEVD-afc), was supplied by Kamiya Biomedical (Spokane, WA).

Generation of CS-GrB complexes

A neutral form of GrB was produced by mixing desalted CS (Calbiocem, San Diego, CA) and GrB (3:1 w/w) in hypotonic Tris buffer (25 mM NaCl, pH 7.4). Thereafter, the mixture was applied to a cation-exchange column (Mono S, Pharmacia, Uppsala, Sweden) followed by a continuous NaCl gradient (0–2.0 M). The amount of GrB in the flow-through (electrochemically neutral GrB, hereafter called CS-GrB) and in the eluate (free cationic GrB) was determined by enzyme immunoassay (EIA) (8). When titrated with a specific active site inhibitor (anti-GraB), ~80% of free GrB represents active enzyme (9). Using an esterolytic assay (see below), the sp. act. of CS-GrB recovered in the flow-through was found to be comparable to the free form of the granzyme.

GrB EIA

Purified mAb GB11 was incubated at 2 μg/ml in 0.1 M sodium carbonate/bicarbonate buffer, pH 9.6, for 16 h at 4°C in microtiter plates (100 μl/well; Nunc Maxisorb Immunoplate, Roskilde, Denmark). The plates were then washed with PBS/0.02% (w/v) Tween 20. An identical washing procedure was performed after each incubation step, which consisted of 100 μl, except for the blocking step (150 μl). After coating, plates were blocked with PBS/2% (v/v) cow milk for 45 min. Samples and standards (free GrB at different concentrations) were diluted in proprietary ELISA buffer (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands) and incubated for 1 h. Next, the plates were incubated with an excess of biotinylated GB10 mAb (0.5 μg/ml) together with 1% (v/v) normal mouse serum for 1 h. The plates were incubated for 30 min with streptavidin-polymerized horseradish peroxidase (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service), after which bound peroxidase was visualized by incubation with a solution of 100 μg/ml 3,3′,5,5′-tetramethyl-benzidine (Merck, Darmstadt, Germany) and 0.003% (v/v) H_2O_2 in 0.11 M sodium acetate buffer, pH 5.5. The reaction was stopped by the addition of an equal volume of 2 M H_2SO_4 to the wells. Finally, the absorbance at 450 nm was read on a Titertek Multiscan plate reader (Labsystems, Helsinki, Finland). Duplicate samples were measured, and the mean value is reported where SDs did not exceed 10%.

Measurement of GrB esterolytic activity

Using Boc-Ala-Ala-Asp-thiobenzyl ester (0.1 mM) diluted in reaction buffer (0.2 M HEPES, 0.3 M NaCl, 1 mM EDTA, 0.5% Triton X-100 (v/v), pH 7.0) (6), GrB activity was measured by colorimetric absorbance changes using a UVmax microplate reader (Molecular Device, Menlo Park, CA). GrB activity was defined as units where 1 U of esterolytic activity equaled the amount of enzyme that hydrolyzed 1 nmol of substrate per minute.

Delivery of free and CS-GrB to target cells with AD or PFN

Jurkat cells (1×10^6/ml) were treated with an equivalent amount of active free GrB or CS-GrB (1 μg/ml) and AD (100 pfu) in 1 ml microfuge tubes containing RMPI 1640 supplemented with 0.5% BSA (9). For PFN delivery, target cells were treated with either free GrB or CS-GrB for 15 min and then exposed to a predetermined sublytic concentration of the pore-forming protein (9).

Apoptosis: morphologic analysis

Apoptosis was assessed at 4 h with Hoescht stain and defined as either nuclear condensation or fragmentation. Cells were fixed with 0.5% paraformaldehyde for 15 min, cytospun to microscope slides, and stained with Hoescht 33342 (1 μg/ml) for 15 min. Using a Zeiss Fluorescent microscope, the percentage apoptotic cells among 300 cells was determined in duplicate samples. Mean values were reported where SDs did not exceed 10%.

Apoptosis: measurement of caspase-3-like fluorogenic activity in whole cells

Following delivery of free or CS-GrB, Jurkat cells were washed (RPMI 1640/0.5% BSA) and resuspended in new microfuge tubes. Anti-GraB, an anti-chymotrypsinogen engineered to react specifically with the granzyme (5×10^5 molecules/cell), was added to each tube to avoid artifactual in vitro cleavage of caspases after lysis. Lysates (100 μl) were added, in duplicate, to each microwell (Nunc Maxisorb Immunoplate) followed by a solution of ac-DEVD-afc (100 μl). Fluorescent values were determined in duplicate every 10 min over a defined period using a Fluorimeter plate reader (Cambridge Technologies, Cambridge, MA).

Table I. *Influence of pH on the formation of CS-GrB complexes*[a]

pH	NaCl (mM)	GrB in Flow-Through (%)	GrB Bound to Mono S (%)
7.4	150	63	37
5.5	150	85	15

[a] CS-GrB complexes were produced by mixing a threefold excess of desalted CS with isolated GrB at the assigned pH in Tris buffer. Thereafter, the samples were submitted to cation-exchange chromatography, and the amount of granzyme in the flow-through and bound to the column were determined by EIA. The percent GrB was calculated by dividing the amount of the granzyme bound or in flow-through by the total granzyme recovered. Results are representative of two experiments.

Cleavage of procaspases-3 and -7 by free and CS-GrB

Caspase-3 and -7 were encoded on vectors under the control of a T7 RNA polymerase promoter (10–13). The [^{35}S]methionine-labeled proteins were prepared from these vectors using a T7-coupled reticulocyte lysate transcription translation (TnT) system (Promega, Madison, WI). The cleavage assay consisted of 20 μl of TnT reaction mix and a volume of reaction buffer (100 mM HEPES, pH 7.5, 20% glycerol, 0.5 mM EDTA, 5 mM DTT) that contained equivalent amounts of active free or CS-GrB (10 nM). Samples were incubated at 37°C, and 30-μl aliquots were removed at various times between 0 and 90 min. Proteolysis was terminated by adding SDS buffer (5 μl) to the samples and heating to 100°C for 5 min. Samples were then submitted to SDS-PAGE using 15% Tris-Tricine minigels (Bio-Rad, Hercules, CA). Gels were then dried and imaged on film.

Degranulation and enrichment of GrB complexes from lymphocyte-activated killer cells (LAK)

IL-2-stimulated PBMC were harvested after 6 days of stimulation. The harvested cells, lymphocyte activated killer cells (LAK), were washed in Iscove's modified DMEM supplemented with BSA (0.02%) and resuspended at 1×10^6 cells/ml in Iscove's modified DMEM plus BSA (0.02%) containing three mAbs directed against CD2 (4B2, 6G4, and Hic 27, 1 μg/ml per mAb) and phorbol myristic acetate (1 ng/ml). Cell were then incubated in tissue culture flasks for 5 h. Supernatants were collected, centrifuged for 8 min at 1500 rpm to remove LAK cells and debris, and stored at −20°C until GrB levels were measured by EIA and esterolytic assay.

Western Blotting of procaspase-3 in cell lysates

Detection of processed procaspase-3 in MCF-7 cell lysates was performed as previously described (5). After treatment with either free or CS-GrB, the lysates (10^6 cell equivalents) were resolved by SDS-PAGE (15%) and transferred to nitrocellulose. Rabbit anti-caspase-3 (supplied by V. Dixit, Genentech, San Francisco, CA) was used at a dilution of 1:2500 followed by incubation with anti-rabbit Ig-horseradish peroxidase (1:10,000) (Amersham, Arlington Heights, IL), and signals were visualized with an enhanced chemiluminescence kit (Amersham).

Results

Generation of CS-GrB complexes

A nonionic form of GrB was produced by complexing the granzyme with CS glycosaminoglycan. Commercially available CS was first extensively dialyzed against water to remove sodium ions. Then a threefold excess of CS was added to isolated GrB (3:1 w/w). The generated CS-GrB complexes were separated from residual free granzyme by cation-exchange chromatography where neutral complexes resided in the flow-through and free GrB bound to the matrix and was eluted during performance of linear NaCl gradient. The amount of free and CS-GrB was then determined by a recently described capture EIA (8). Using this strategy, the influence of pH on the formation of CS-GrB complexes was determined (Table I). When dialyzed CS and free GrB were mixed at pH 5.5 and 7.4 (NaCl 150 mM), 85% and 63% of the granzyme, respectively, associated with the glycosaminoglycan.

Table II. *CS-GrB complexes are stable under physiologic conditions*[a]

Concentration of NaCl (mM) Used to Dissociate CS-GrB Complexes (pH 7.4)	GrB in Flow-Through (%)	GrB Bound to Mono S (%)
200	99	<1
300	99	<1
400	99	<1
500	99	<1

[a] CS-GrB complexes (10 μg granzyme by EIA) were mixed with the designated NaCl solution for 1 h at room temperature. The samples were submitted to cation-exchange chromatography and the percent GrB in the flow-through and bound to the column were determined as described in Table I. Results are representative of two experiments.

We have previously reported that cationic GrB binds tightly to a cation-exchange matrix eluting at ~590 mM NaCl (6). On the basis of this observation, it was predicted that once GrB and CS associated, NaCl concentrations below mM 590 would be unable to disrupt the ionic interaction. To examine the strength of this ionic interaction, CS-GrB complexes were mixed in NaCl solutions ranging from 200 to 500 mM Tris-HCl, pH 7.4, and the amount of dissociated GrB was measured after elution from a cation-exchange column. Regardless of the salt concentration, >99% of GrB remained associated with the glycosaminoglycan (Table II). This result demonstrated the remarkable stability of the ionic interaction and suggests that once the granzyme binds to granule-associated anionic CS side chains of serglycin a stable interaction would be maintained in vivo.

Comparison of the processing of preferred caspase substrates by GrB and CS-GrB in vitro

Caspase-3 and -7 are both efficiently processed by GrB, where cleavage occurs at the IXXD sequence connecting the large and small subunits (14, 15). To determine whether the cationic charge might influence the rate of cleavage of the zymogens, procaspase-3 and -7 were expressed by TnT and rates of proteolysis were determined in the presence of equal amounts of active free and CS-GrB. The time required to consume the zymogen provides a qualitative estimate of the Vmax (16). Caspase-7 was almost completely processed by free GrB at 30 min while the CS-GrB complex processed caspase-7 to the same extent after 60 min (Fig. 1*a*). Caspase-3, on the other hand, was minimally cleaved at 90 min by CS-GrB, while free GrB processed about 50% of the zymogen by this time (Fig. 1*b*). Clearly, processing of both procaspase-3 and -7 is diminished significantly when GrB is complexed with CS.

CS-GrB induces apoptosis when delivered into Jurkat cells

We have shown previously that AD effectively delivers GrB because the granzyme appears to bind to the target cell in a specific, saturable fashion (9). This interaction allows the granzyme to be internalized and delivered to the cytosol by the endosomolytic action of AD. However, because the studies were performed with the cationic granzyme, it was impossible to exclude the possibility that a portion of the observed binding to the plasma membrane was charge dependent. To address this issue, we asked whether equivalent amounts of active free and CS-GrB could induce similar levels of apoptosis after delivery to Jurkat cells by AD. Jurkat cells were treated with either free or CS-GrB plus AD, and the percentage of cells undergoing apoptosis was visualized by Hoescht stain and fluorescent microscopy. Both forms of the granzyme produced comparable levels of apoptosis, suggesting CS-GrB does not have a reduced capacity to bind and be internalized by the target cell.

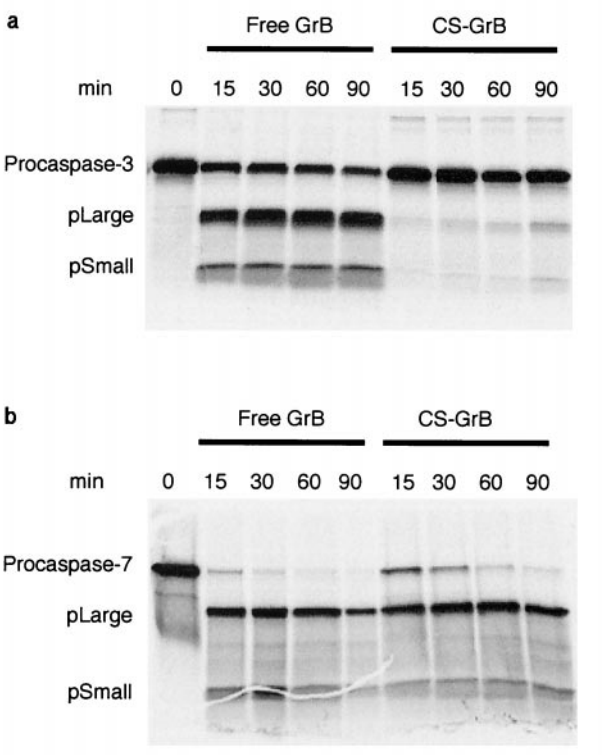

FIGURE 1. Comparison of kinetics of proteolysis in vitro that free and CS-GrB manifest against caspase-3 and -7. The [^{35}S]caspases were generated and isolated as described (12). In a total volume of 320 μl, translated caspases were mixed with either free GrB or CS-GrB (30 nM) for the times indicated and analyzed by SDS-PAGE and autoradiography: *a*, kinetics of [^{35}S]caspase-3 (~250 ng) processing; *b*, kinetics of [^{35}S]caspase-7 (~300 ng) processing.

(Fig. 2*a*). To ensure that the apoptotic activity of CS-GrB was not unique to the AD delivery system, Jurkat cells were pulsed with either free GrB or CS-GrB complexes and treated with a sublytic concentration of PFN (9). Both free GrB and CS-GrB induced similar levels of cell death when delivered by PFN (Fig. 2*b*).

CS-GrB is equivalent to free GrB in generating caspase-3-like activity

Assuming similar amounts of free GrB and CS-GrB were bound and delivered by AD and on the basis of the reduced rate of proteolysis that CS-GrB displayed against caspase-3, it could be predicted that CS-GrB should generate lower levels of caspase-3-like activity in whole cells with delayed kinetics. Using the fluorogenic substrate, ac-DEVD-afc, to quantify levels of caspase-3-like activity, a dose-response analysis was performed comparing delivery of equivalent amounts of active free and CS-GrB. As shown in Fig. 3*a*, the levels of ac-DEVD-afc activity were similar for both forms of the granzyme. Furthermore, the delivery of equivalent amounts of free and CS-GrB complexes resulted in similar time-dependent increases in DEVD-afc activity (Fig. 3*b*).

Processing of the caspases in cytosolic extracts

We have recently provided unequivocal evidence that GrB initiates the caspase cascade in whole cells by activating caspase-3 (5). Because CS-GrB processed isolated caspase-3 less efficiently than free GrB, the similar time-dependent increase in ac-DEVD-afc activity mediated by the two forms of the granzyme was unexpected. To account for this discrepancy, cationic GrB, once delivered intracellularly, might bind to negatively charged cytosolic proteins,

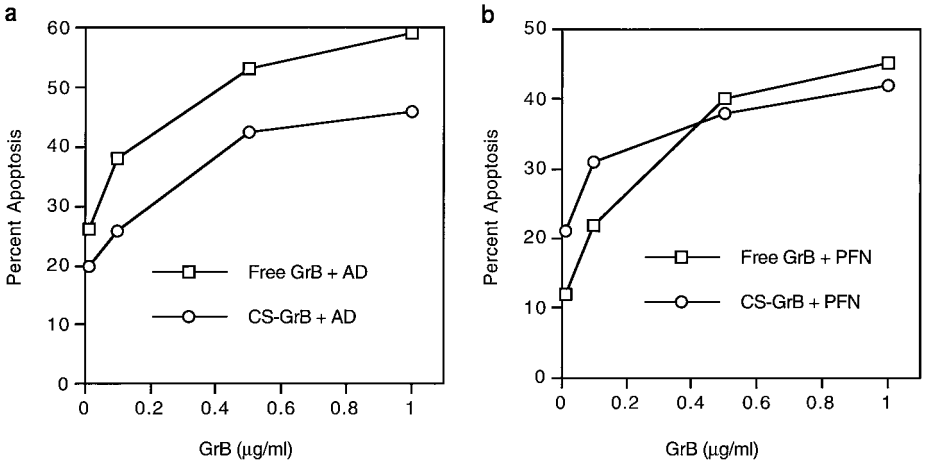

FIGURE 2. Comparison of the capacity of free GrB and CS-GrB to induce apoptosis when delivered into Jurkat cells with either AD or PFN. *a*, Jurkat cells were exposed simultaneously to AD and equivalent amounts of either free GrB or CS-GrB, and apoptosis was determined by Hoescht stain 4 h later. *b*, Targets were pulsed with the indicated amounts of free or complexed GrB, washed, and then exposed to sublytic PFN. Four hours later the number of apoptotic cells were enumerated.

reducing the amount of granzyme able to process procaspase-3. To partially mimic conditions in whole cells, we compared the rates of proteolysis of procaspase-3 that free and CS-GrB produced after the addition to MCF-7 cytosolic extracts. In comparison to the results that showed free GrB cleaved isolated procaspase-3 more rapidly than CS-GrB (Fig. 2*a*), the capacity of free GrB to process the caspase in cell lysates was at least similar if not somewhat less than the rate observed for CS-GrB (Fig. 4). Therefore, the equivalent rates of ac-DEVD-afc activity generated by delivery of free and CS-GrB to intact cells may be, in part, attributed to binding of the cationic free granzyme to cytosolic proteins, leading to a reduction in the amount of protease available for processing the endogenous procaspase-3.

GrB is secreted from cytotoxic cells as a macromolecular complex that has apoptotic activity

Because GrB appears to tightly bind to CS, the prediction was made that GrB exocytosed by stimulated cytotoxic cells should be released in a high m.w. form. Following stimulation with IL-2 for 5 days, LAK cells were induced to undergo granule exocytosis by exposure to phorbol myristic acetate and anti-CD2 mAbs in media supplemented with BSA. Combining two approaches, we determined whether the granzyme was secreted as a neutral, high m.w. complex. First, no cationic free GrB was detected in the supernatant analyzed by cationic-exchange chromatography. Then the levels of free and complexed GrB were estimated by EIA after filtration of the supernatant through a 100,000-kDa membrane. The vast majority of the granzyme was retained by the membrane, suggesting the presence of a macromolecular complex (>99%) (Fig. 5). Having shown the supernatant from stimulated LAK cells contained only a form of granzyme that exceed 100,000 kDa, we then compared the apoptotic potential of the GrB derived from the LAK cells to free GrB. Jurkat cells were pulsed with equivalent amounts of active free and LAK cell-derived GrB, washed, and treated with AD. As shown in Fig. 6, the free and the high m.w. form of GrB were able to induce similar levels of cell death after delivery by AD.

Discussion

Granule-mediated apoptosis is divisible into 3 steps: 1) signal-dependent exocytosis of the granule components; 2) intracellular

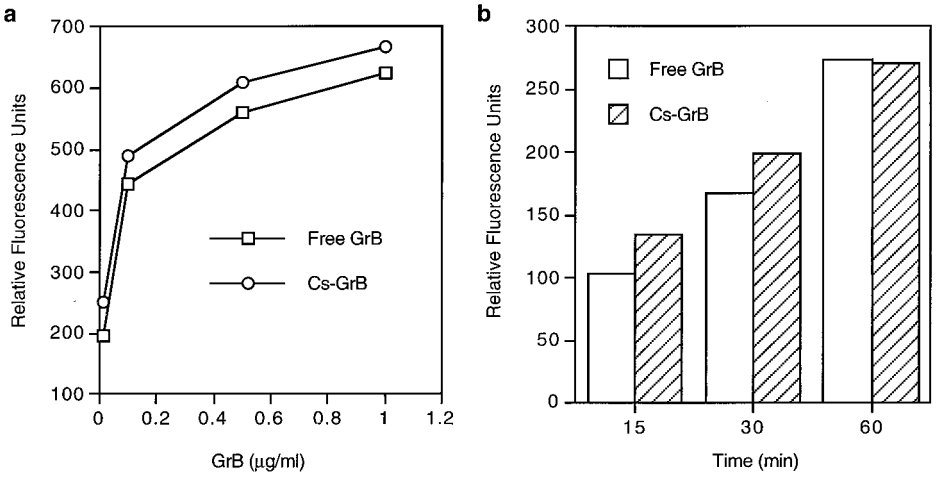

FIGURE 3. Comparison of the capacity of free GrB and CS-GrB to induce caspase-3-like activity in Jurkat cells. *a*, In the presence of AD, Jurkat cells were exposed to increasing amounts of the two forms of the granzymes, and caspase-3-like activity was measured at 1 h. *b*, Jurkat cells were treated with equivalent amounts of either free GrB or granzyme complexed with CS (500 ng/ml), and caspase-3-like activity was measured at times indicated.

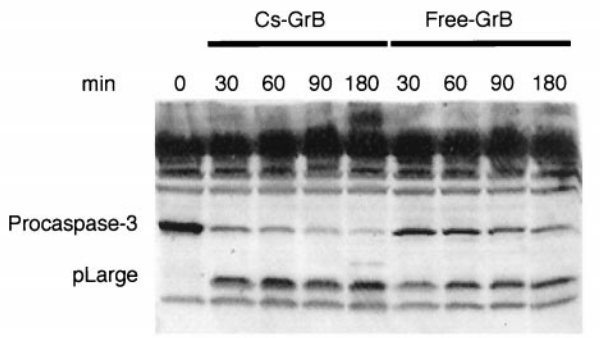

FIGURE 4. Comparison of kinetics of proteolysis in vitro that free and CS-GrB manifest against caspase-3 in cytosolic extracts. Cytosolic extracts were obtained from MCF-7 cells as previously described (5). In a total volume of 600 μl, extracts were mixed with either free GrB or CS-GrB (30 nM) for the times indicated and analyzed by SDS-PAGE. The kinetics of procaspase-3 processing was determined by enhanced chemiluminescence and autoradiography.

delivery of the granzymes by PFN; and 3) induction of apoptotic pathways by the serine proteases. The signaling pathways that initiate granule secretion and the mechanism underlying GrB-mediated apoptosis have slowly been elucidated. However, the biochemical characteristics of the secreted granzymes and the mechanism of granzyme delivery are largely unknown. Using a recently described EIA that more accurately measures GrB than available chromogenic assays (8), it has become possible to examine the biochemical behavior of preformed, neutral CS-GrB complexes and to learn whether native granzyme is secreted from cytotoxic cells in a macromolecular form.

Our results extend studies described for the tryptase-like activity identified in the granules, which is primarily attributable to GrA. The tryptase activity of cloned NK cells was found to associate with a proteoglycan, presumably serglycin. On the basis of esterolytic activity, murine granzyme A, likewise, has been shown to remain complexed upon exocytosis from cytotoxic cells (17–19). We show here: 1) GrB remains tightly bound to CS glycosaminoglycans under physiologic conditions; 2) while the cationic charge of GrB apparently enhances the proteolytic efficiency of GrB against isolated caspases, complex formation with CS protects the granzyme from nonspecific inhibition in the presence of complex cytosolic extracts; 3) CS-GrB has the capacity to induce cell death when delivered by AD or PFN generating levels of caspase-3-like activity similar to equivalent amounts of free GrB; and 4) GrB is secreted from stimulated LAK cells in a macromolecular form (>100 kDa), which also induces apoptosis after intracellular delivery. Taken together, the evidence suggests that a neutral, high m.w. complex form of GrB induces apoptosis in vivo.

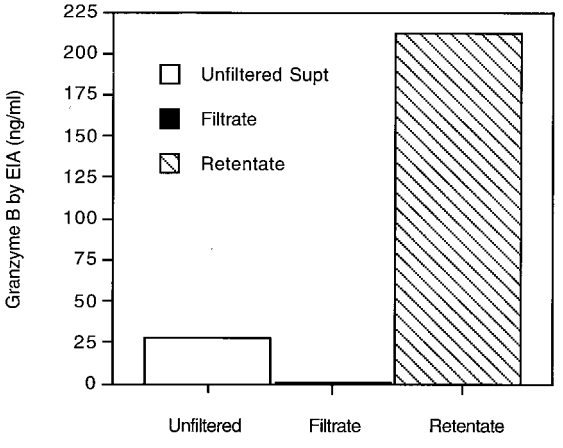

FIGURE 5. Granzyme B secreted from LAK cells is released as a macromolecular complex exceeding 100,000 kDa. IL-2-stimulated PBMC were resuspended at a concentration of 1×10^6 cells/ml in Iscove's modified Dulbecco's medium/0.02% (w/v) BSA containing three mAbs directed against CD2 (4B2, 6G4, and Hic 27) each at 1 μg/ml and phorbol myristic acetate at a final concentration of 1 ng/ml. Unstimulated cells were resuspended in Iscove's modified Dulbecco's medium/0.02% BSA. Cell suspensions were placed in flasks and incubated for 4 h. Supernatant were collected, centrifuged for 8 min at 1500 rpm to remove intact calls, and stored at −20°C until used. The free and complexed GrB were separated using a 100,000-kDa cut-off membrane (Centricon, Beverly, MA), and the levels of active GrB were determined in the unfiltered, the flow-through, and retained fractions.

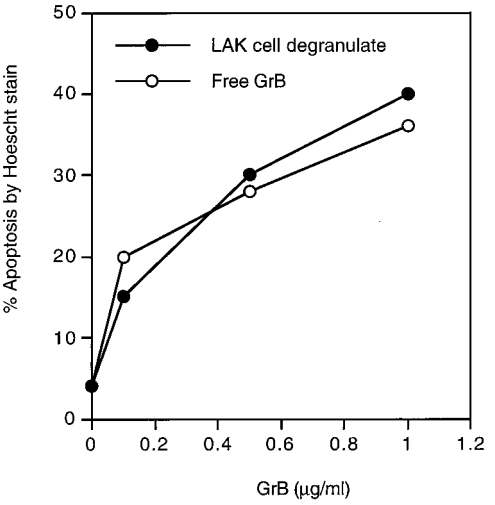

FIGURE 6. High m.w. GrB is able to induce apoptosis. Jurkat cells were pulsed for 1 h with an equivalent amount of either free GrB or the high m.w. fraction derived from the supernatant of stimulated LAK cells. After the pulse, cells were washed, treated with AD, and apoptotic changes were assessed at 4 h by Hoescht stain.

Numerous reports have described the biochemical and biologic effects of isolated GrB (1, 4, 20). However, little attention has been given to the concept that the physiologically relevant form may represent a neutral macromolecular complex. Although the final outcome, cell death, occurs regardless of the form of GrB delivered to the target cell, studies performed with the cationic granzyme may not provide insights to the mechanism of apoptosis that occurs in vivo. In this regard, it has been reported that GrB readily binds and enters the nucleus of the target cell after delivery by PFN (21, 22). Because nuclear translocation appears to require the interaction of free GrB with an endogenous cytosolic factor (23), this process may not occur when complexed GrB is delivered intracellularly.

We have recently provided convincing evidence that GrB activates the executioner caspases-3 and -7 through a novel two-step process. After intracellular delivery, GrB first processes procaspase-3 even though this zymogen is not the most preferred caspase substrate (5). As verified here with CS-GrB, the granzyme cleaves caspase-7 more rapidly than caspase-3. However, during GrB-mediated apoptosis the caspase-7 propeptide is removed first, then cleavage occurs between the subunits. Strikingly, caspase-7 is unprocessed in caspase-3-deficient MCF-7 cells exposed to GrB (5). Transfection with caspase-3 restores the removal of the caspase-7 propeptide and the capacity of GrB to cleave the caspase-7 between the large and small subunits. Thus GrB initiates the death pathway by processing the accessible caspase-3, and caspase-7 propeptide regulates transactivation of the zymogen by granzyme. As a consequence, two proteases, caspase-3

and GrB, are required to activate procaspase-7. It will be important to learn whether complexed GrB activates these executioner caspases in a similar manner.

In addition to influencing how free GrB processes caspases in vivo, studies that employ cationic granzyme may not accurately portray the kinetics of cell death. The addition of free GrB to cytosolic extracts appeared to decrease the rate of proteolysis of caspase-3 compared with CS-GrB. As a consequence, the K_{cat} that we have reported for free GrB against isolated caspase-3 (15) may be an overestimate of the reaction rate that is likely to occur in vivo. This finding could explain why both forms of the granzyme produced equivalent levels of caspase-3-like activity after intracellular delivery. The reduction in caspase activation by free GrB could be due to two mechanisms: the free GrB interacts with cytosolic membranes reducing the amount available for cleaving the caspases or soluble cytoplasmic proteins could bind the granzyme and act as nonspecific competitive inhibitors. The latter issue becomes important for the interpretation of studies where cells are transfected with anti-apoptotic proteins. For example, Bcl-2 has been reported to protect cells treated with GrB and PFN but not against the rapid cell death mediated by cytotoxic T cells. (24). The inability of free GrB to induce death might be due to a protein-protein interaction of the granzyme with overexpressed Bcl-2, resulting in a nonspecific reduction in GrB-mediated processing of caspase-3.

In comparison to free tryptase-like granzyme, GrA-proteoglycan complexes are relatively resistant to inactivation by antithrombin III, a major plasma inhibitor of this granzyme.[4] The protease inhibitor-9, unlike extracellular serpins, has been reported to efficiently inactivate free GrB (25). Protease inhibitor-9 has been postulated to protect the cytotoxic cell from misdirected GrB released during granule exocytosis. However, protease inhibitor-9 is expressed at highest levels in the cytosol of cytotoxic cells. The location of protease inhibitor-9 suggest that the serpin might selectively inhibit free GrB that is inadvertently targeted and activated in the cytosol and not block granzyme complexed with serglycin. Therefore, GrB/serglycin complexes might offer a host advantage against cells infected with viruses that manufacture similar anti-granzyme serpins.

The cationic charge of free GrB would also favor nonspecific binding to the anionic plasma membrane. We reported that GrB appears to specifically interact with Jurkat cells (9). The work here found that CS-GrB as well as GrB secreted from LAK cells induced cell death after intracellular delivery. Because PFN- and AD-mediated delivery requires interaction of the granzyme with the plasma membrane, the results suggest that the nonionic form of GrB also binds to cells. This finding provides further evidence that the granzyme interaction with the cell membrane is not dependent on cationic charge. Although the stoichiometric interaction of GrB with serglycin remains to be determined, it is intriguing to speculate that a single serglycin protein might bind multiple granzyme molecules (26). Serglycin could then serve to focus GrB-receptor interactions as well as provide a multigranzyme proteolytic complex that enhances the processing of preferred caspase and non-caspase substrates after intracellular delivery.

Acknowledgments

We thank X. Yang for assistance in the preparation of the in vitro-translated procaspase-3 and procaspase-7.

[4] E. H. A. Spaeny-Dekking, A. M. Kamp, Y. van Hensbergen, W. van Haren, C. J. Froelich, and C. E. Hack. Extracellular granzyme A, complexed to proteoglycans, is protected against inactivation by protease inhibitors. *Submitted for publication.*

References

1. Darmon, A. J., and R. C. Bleackley. 1998. Proteases and cell-mediated cytotoxicity. *Crit. Rev. Immunol. 18:255.*
2. Matsumoto, R., A. Sali, N. Ghildyal, M. Karplus, and R. L. Stevens. 1995. Packaging of proteases and proteoglycans in the granules of mast cells and other hematopoietic cells. *J. Biol. Chem. 270:19524.*
3. Ghildyal, N., D. S. Friend, R. L. Stevens, K. F. Austen, C. F. Huang, J. F. Penrose, A. Sali, and M. F. Gurish. 1996. Fate of two mast cell tryptases in V3 mastocytosis and normal BALB/c mice undergoing passive systemic anaphylaxis: prolonged retention of exocytosed mMCP-6 in connective tissues, and rapid accumulation of enzymatically active mMCP-7 in the blood. *J. Exp. Med. 184:1061.*
4. Froelich, C. J., V. M. Dixit, and X. Yang. 1998. Granule mediated apoptosis: a matter of viral mimicry. *Immunol. Today 19:30.*
5. Yang, X., H. R. Stennicke, B. Wang, D. R. Green, R. U. Jänicke, A. Srinivasan, P. Seth, G. S. Salvesen, and C. J. Froelich. 1998. Granzyme B mimics apical caspases: description of a unified pathway for trans-activation of executioner caspases-3 and -7. *J. Biol. Chem. 273:34278.*
6. Hanna, W. L., X. Zhang, J. Turbov, U. Winkler, D. Hudig, and C. J. Froelich. 1993. Rapid purification of cationic granule proteases: application to human granzymes. *Protein Expr. Purif. 4:398.*
7. Seth, P. 1994. A simple and efficient method of protein delivery into cells using adenovirus. *Biochem. Biophys. Res. Commun. 203:582.*
8. Spaeny-Dekking, E. H. A., W. L. Hanna, A. M. Wolbink, P. C. Wever, J. A. Kummer, A. J. G. Swaak, J. M. Middeldorp, H. G. Huisman, C. J. Froelich, and C. E. Hack. 1998. Extracellular granzymes A and B: detection of native species during CTL responses in vitro and in vivo. *J. Immunol. 160:1360.*
9. Froelich, C. J., K. Orth, J. Turbov, P. Seth, B. M. Babior, R. A. Gottlieb, G. M. Shah, R. C. Bleackley, V. M. Dixit, and W. L. Hanna. 1996. New paradigm for lymphocyte granule mediated cytotoxicity: targets bind and internalize granzyme B but a endosomolytic agent is necessary for cytosolic delivery and apoptosis. *J. Biol. Chem. 271:29073.*
10. Hugunin, M., L. J. Quintal, J. A. Mankovich, and T. Ghayur. 1996. Protease activity of in vitro transcribed and translated *Caenorhabditis elegans* cell death gene (*ced-3*) product. *J. Biol. Chem. 271:3517.*
11. Ghayur, T., M. Hugunin, R. V. Talanian, S. Ratnofsky, C. Quinlan, Y. Emoto, P. Pandey, R. Datta, Y. Y. Huang, S. Kharbanda, H. Allen, R. Kamen, W. Wong, and D. Kufe. 1996. Proteolytic activation of protein kinase C δ by an ICE/CED 3-like protease induces characteristics of apoptosis. *J. Exp. Med. 184:2399.*
12. Orth, K., K. O'Rourke, G. S. Salvesen, and V. M. Dixit. 1996. Molecular ordering of apoptotic mammalian Ced-3/ICE proteases. *J. Biol. Chem. 171:.*
13. Muzio, M., A. M. Chinnaiyan, F. C. Kischkel, K. O'Rourke, A. Shevchenko, C. Scaffidi, J. D. Bretz, M. Zhang, J. Ni, R. Gentz, M. Mann, P. H. Krammer, M. E. Peter, and V. M. Dixit. 1996. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD-95 (FAS/Apo-1) death-inducing signaling complex (DISC). *Cell 86:817.*
14. Zhou, Q., and G. S. Salvesen. 1997. Activation of pro-caspase-7 by serine proteases includes a non-canonical specificity. *Biochem. J. 324:361.*
15. Talanian, R. V., X. Yang, J. Turbov, P. Seth, T. Ghayur, C. A. Casiano, and C. J. Froelich. 1997. Granule-mediated cell killing: mechanisms of granzyme B initiated apoptosis. *J. Exp. Med. 186:1323.*
16. Poe, M., R. L. Stein, and J. K. Wu. 1992. High pressure gel-permeation assay for the proteolysis of human aggrecan by human stromelysin-1: kinetic constants for aggrecan hydrolysis. *Arch. Biochem. Biophys. 298:757.*
17. Stevens, R. L., K. Otsu, J. H. Weis, R. V. Tantravahi, F. Austen, P. A. Henkart, C. Galli, and C. W. Reynolds. 1987. Co-sedimention of chondroitin sulfate-A glycoaminoglycans and proteoglycans with the cytolytic secretory granules of rat large granular lymphocyte (LGL) tumor cells, and identification of a mRNA in normal and transformed LGL that encodes proteoglycans. *J. Immunol. 139:863.*
18. Gromkowski, S. H., T. C. Brown, D. Masson, and J. Tschopp. 1988. Lack of DNA degradation in target cells lysed by granules derived from cytolytic T cells. *J. Immunol. 141:774.*
19. Peters, P. J., H. J. Geuze, H. A. Van der Donk, and J. Borst. 1990. A new model for lethal hit delivery by cytotoxic T lymphocytes. *Immunol. Today 11:28.*
20. Medema, J. P., R. E. M. Toes, C. Scaffidi, T. S. Zheng, R. A. Flavell, C. J. M. Melief, M. E. Peter, R. Offringa, and P. H. Krammer. 1997. Cleavage of FLICE (caspase-8) by granzyme B during cytotoxic T lymphocyte-induced apoptosis. *Eur. J. Immunol. 27:3492.*
21. Pinkoski, M. J., M. Hobman, J. A. Heiben, K. J. Tomaselli, L. Feng, P. Seth, C. J. Froelich, and R. C. Bleackley. 1998. Cytosolic granzyme B is necessary and sufficient to induce apoptotic cell death. *Blood 92:1044.*
22. Trapani, J. A., P. Jans, M. J. Smyth, C. J. Froelich, E. A. Williams, V. R. Sutton, and D. A. Jans. 1998. Perforin-dependent nuclear entry of granzyme B precedes apoptosis, and is not a consequence of nuclear membrane dysfunction. *Cell Death Differ. 5:488.*
23. Trapani, J. A., K. A. Brown, M. J. Smyth, and D. A. Jans. 1996. Immunolocalization of granzyme B to nucleus: implication for lymphocyte mediated apoptosis. *J. Biol. Chem. 271:4127.*
24. Sutton, V. R., D. L. Vaux, and J. A. Trapani. 1997. Bcl-2 prevents apoptosis induced by perforin and granzyme B, but not that mediated by whole cytotoxic lymphocytes. *J. Immunol. 158:5783.*
25. Sun, J., C. H. Bird, V. Sutton, L. McDonald, P. B. Coughlin, T. A. De Jong, J. A. Trapani, and P. I. Bird. 1996. A cytosolic granzyme B inhibitor related to the viral apoptotic regulator cytokine response modifier A is present in cytotoxic cells. *J. Biol. Chem. 271:27802.*
26. Pejler, G., and L. Berg. 1995. Regulation of rat mast cell protease 1 activity—protease inhibition is prevented by heparin proteoglycan. *Eur. J. Biochem. 233:192.*