Extracellular Matrix Remodeling by Human Granzyme B via Cleavage of Vitronectin, Fibronectin, and Laminin*

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Abstract

Human granzyme B (GrB) released from cytotoxic lymphocytes plays a key role in the induction of target cell apoptosis when internalized in the presence of perforin. Here we demonstrate that GrB also possesses a potent extracellular matrix remodeling activity. Both native and recombinant GrB caused detachment of immortalized and transformed cell lines, primary endothelial cells, and chondrocytes. Cell detachment by GrB induced endothelial cell death (anokis). GrB also inhibited tumor cell spreading, migration, and invasion in vitro. Investigation into the underlying mechanism revealed that GrB efficiently cleaves three proteins involved in extracellular matrix structure and function: vitronectin, fibronectin, and laminin. In vitronectin, GrB cleaves after an Arg-Lys-Asp (RGD) motif, which is part of the integrin-binding site found in matrix proteins. We propose that targeting of the integrin-extracellular matrix interface by GrB may allow perforin-independent killing of target cells via anokis, restrict motility of tumor cells, facilitate lymphocyte migration, or directly reduce virus infectivity. It may also contribute to tissue destruction in diseases in which extracellular GrB is evident, such as rheumatoid arthritis and atherosclerosis.

Cytotoxic lymphocytes (CLs) play a key role in the eradication of virally infected and malignant cells. Specialized lyso- somes (granules) within these cells contain multiple cytotoxins, including the pore-forming molecule, perforin. These cytotoxins are stored in complex with the proteoglycan, serglycin (1, 2). Release of cytotoxic granule contents from CLs into the target cell results in the rapid induction of apoptosis and is the primary mechanism used to destroy abnormal cells (3).

The major cytotoxins present in human CL granules are the serine proteases, granzyme A and granzyme B, and both contribute to the induction of target cell death (reviewed in Ref. 4). These molecules are internalized with perforin, which is essential for the cytosolic release of the granzymes (5, 6). Granzyme B (GrB) is involved in the rapid induction of target cell apoptosis during granule-mediated killing, via proteolysis of key intracellular substrates. Although the exact order of events remains controversial, the unusual “Asp-ase” activity of GrB allows it to cleave and activate effector caspases, disrupt mitochondrial function via cleavage of the Bcl-2 family member Bid, and also induce rapid DNA degradation by cleaving key nuclear repair proteins (reviewed in Refs. 7 and 8). Granzyme A (GrA) also participates in the induction of target cell death but in a mechanism that is caspase-independent (9).

Most work has focused on the role of GrB in apoptosis induction and the identification of intracellular substrates. However, there is emerging evidence that GrB may function extracellularly and may have a role outside the immune system. For example, GrB is produced in the absence of perforin by cells of the reproductive system, in developing spermatocytes and in placental trophoblasts (10). GrB is also produced by granulosa cells of the human ovary, where it is up-regulated in response to follicle-stimulating hormone (11). In addition, GrB has been detected in a subset of primary human breast carcinomas (12) and in chondrocytes of articular cartilage (13). Within the immune system, non-cytolytic cells have also been reported to produce GrB in the absence of perforin. GrB is produced by a subset of human dendritic cells, where it is up-regulated upon cell activation (14), and migrating immature thymocytes also express GrB (15).

Extracellular granzymes are observed in various diseases that elicit a CL-mediated immune response, such as atherosclerosis, vascular transplant disease, and rheumatoid arthritis (16, 17). Plasma granzymes are also elevated during viral infections (human immunodeficiency virus-1, Epstein-Barr virus) and certain bacterial infections (18, 19). These extracellular granzymes may arise via constitutive (nonspecific) secretion after CLs degranulate (20) or may escape the immunological synapse as the CL degranulates, disengages, and moves onto another target (21, 22). Interestingly, a number of extracellular functions have been defined for the other major CL granzyme, GrA. GrA cleaves fibronectin, collagen, and basement membrane proteoglycans and induces intracellular signaling via cell surface receptors (23–26).

A limited number of studies have shown that GrB has the ability to cleave extracellular substrates. GrB degrades the cartilage proteoglycan, aggrecan (27), and is thought to contribute to joint destruction in rheumatoid arthritis (17, 28). GrB can also cleave the cell surface neuronal glutamate...
receptor, implicating it in the generation of auto-antigens (29). Finally, rat GrB was originally identified via its ability to induce rounding and aggregation of adherent tumor cell lines in the absence of perforin (30), an effect that has not been further investigated.

Thus, it is evident that GrB may have a perforin-independent extracellular role. Here we demonstrate that human GrB can detach primary and transformed human cell lines via a potent ECM remodeling activity. Three novel extracellular substrates of granzyme B are identified: the ECM components vitronectin, fibronectin, and laminin. GrB cleaves vitronectin after the RGD integrin-binding motif, explaining the ability of GrB to disrupt adhesion to ECM. Biologically, modulation of cell adhesion by GrB induces anoikis of primary endothelial cells and inhibits tumor cell spreading, migration, and invasion. This has important implications for the function of GrB and in the pathogenesis of diseases in which extracellular GrB is elevated, such as rheumatoid arthritis and atherosclerosis (17, 31).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The breast adenocarcinoma cell line MCF-7, the MDAMB-231 breast carcinoma cells (provided by T. Brown, Monash University), and the LIM1215 colon carcinoma cell line (derived from a patient with inherited non-polyposis colorectal cancer (32)) were all routinely cultured in standard RPMI 1640 medium containing 10% fetal calf serum and 2 mM glutamine (Invitrogen). Primary human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described (33) and were used between passages 3 and 6. Primary bovine chondrocytes were isolated and cultured according to Ref. 34 and were used at the first passage. In experiments performed under serum-free conditions, hybridoma-serum free medium containing 2 mM glutamine (H-SFM, Invitrogen) were used for MCF-7, HUVEC, and chondrocytes, whereas for LIM1215 cells, RPMI serum-free medium containing 2 mM glutamine and serum albumin, and 2 μg/ml iron-saturated transferrin (RPMI-SFM) were used.

**Reagents and Antibodies**—Matrix proteins used were: human plasma fibronectin (In Vitrogen), human plasma vitronectin (Promega), type I and II bovine collagen (provided by M. Rowley, Monash University), and human placental laminin (Sigma). Recombinant human lamin-10 was produced in human embryonic kidney cells (HEK 293 cells) and human placental laminin (Sigma). Recombinant human GrB was produced in Pichia pastoris (40). Chondroitin-sulfate complexed GrB was produced as described previously (1). Recombinant, native, and chondroitin sulfate-complexed GrB all displayed equal enzymatic activity on the peptide substrate Abz-ITDSSMSEK-dnp, assessed as described previously (41).

**Cell Detachment Assays**—In initial experiments, MCF-7 cells (in standard RPMI growth medium containing 10% FCS) or HUVECs (in M199 media containing 20% FCS (33)) were plated onto 12-well glass slides (Tissuetek) (10×10^5 wells) or wells of a 96-well tissue culture tray (Nunc) (4×10^5 well) and allowed to adhere overnight. Cells were washed once in PBS and incubated in 50 μl of H-SFM containing the indicated concentration of GrB, Pro-GrB, GrBS183A active site mutant, native GrB, or chondroitin sulfate-complexed GrB. In most cases, cells were then incubated at 37 °C for 24 h, after which media were removed, and cells were washed once with PBS to remove non-adherent cells. Images of washed or unwashed cells in 96-well trays were captured using contrast using an Onyx microscope software. In a few cases on glass slides were fixed for 2 min in 50% acetone/methanol, and images were captured using MCID image analysis software. For quantitation of remaining adherent cells, cells were incubated with 100 μl of 0.5 mg/ml MTI (Sigma) in H-SFM for 4 h. Deposited formazan crystals were solubilized by addition of 100 μl of 0.05 μM HCl in isopropl alcohol and detected at A590. Mean and standard deviation (S.D.) from triplicate wells were used for statistical software. In some experiments, GrB was preincubated with the indicated molar ratio of heparin, heparan, or chondroitin sulfates for 30 min at 37 °C prior to exposing to cells.

**Detachment Assays on Pure Matrices**—Wells of a 96-well tray were coated overnight at 4 °C with 10 μg/ml (50 μl/well) vitronectin, fibronectin, types I or II collagen, or 2 μg/ml commercial laminin or recombinant laminin-10. Nonspecific binding sites were blocked with 2% BSA for 1 h at 37 °C. MCF-7, HUVEC, and LIM1215 cell suspensions were obtained non-enzymatically by incubation in 5 mM EDTA for 5–10 min at 37 °C, or chondrocyte suspensions were obtained by trypsinization. Cells were washed twice in PBS, resuspended in H-SFM, and 4×10^5 cells/well (MCF-7, HUVEC), or 1×10^5 cells/well (primary chondrocytes) were allowed to adhere to various matrices for 1.5–2 h at 37 °C. Alternatively, LIM1215 cells were resuspended in RPMI-SFM and plated at 1×10^5 cells/well. Less than 2% of these cells adhered to plastic, demonstrating specific adhesion to each matrix. Non-adherent cells were removed by washing twice in PBS. Cells were treated for 24 h with GrB as described above, except that LIM1215 cells were treated in RPMI-SFM. For MCF-7, HUVECs, and chondrocytes, MTT assays were performed directly to assess total cell viability, or non-adherent cells were removed to assess the number of adherent cells. For LIM1215 cells, adherent cells were quantified by adding 100 μg/ml 0.2 μg/ml calcine (Molecular Probes) for 45 min at 37 °C. Wells were washed with 3× Tris-buffered saline containing 1 mM MgCl2, 2 mM CaCl2, and lysed with 100 μl of 1% SDS. Fluorescence was measured at an excitation wavelength of 482 nm and emission wavelength of 520 nm using a FLUOstar OPTIMA (BMG Labtechnologies).

**Matrix Cleavage by GrB**—For cleavage of matrix proteins in solution, 100 ng GrB or Pro-GrB or GrBS183A was incubated with 200 ng of human vitronectin or fibronectin in 20 μl of H-SFM, and incubated at 37 °C for the indicated time. For cleavage of recombinant laminin-10, reactions contained 3.6 μg of laminin-10 and 100 ng GrB (equal molar) in 20 μl of PBS. Reactions were stopped by the addition of denaturing Laemmli sample buffer. For assessment of cleavage of proteins in the matrix, wells of a 24-well tray were coated with 500 ng of vitronectin or fibronectin in PBS overnight and washed with PBS prior to treatment with various forms of GrB. In some experiments, MCF-7 cells were plated on the matrix after blocking with BSA as described above, prior to GrB treatment. After the indicated time, supernatants were collected, cells (if present) were removed using 5 mM EDTA, and matrix proteins were obtained using matrix lysis buffer and scraping as described (42). Cleavage assays in solution, supernatants, and matrix proteins were run on SDS-PAGE gels and immunoblotted with anti-vitronectin antibodies (1:2000) or anti-fibronectin antibody (1:4000), or laminin-10 cleavage reactions were visualized by silver staining.

**Identification of GrB Cleavage Site in Vitronectin**—The GrB cleavage site was identified in human GrB after co-culturing with Pickia pastoris as described previously (39). Briefly, GrB was produced as a His-tagged inactive pro-enzyme (Pro-GrB), which is then activated by enterokinase. The active site mutant of GrB (designated GrBS183A) was generated by site-directed mutagenesis, replacing Ser183 with Ala, using the mutagenic oligonucleotide 5′-TTAAAGGGAGCCTGGGGGCCCCTTCTTG-GG-3′, and was produced as for wild type GrB. Native granzyme B was purified from human YT natural killer lymphoma granules as described (40). Chondroitin-sulfate complexed GrB was produced as described previously (1). Recombinant, native, and chondroitin sulfate-complexed GrB all displayed equal enzymatic activity on the peptide substrate Abz-ITDSSMSEK-dnp, assessed as described previously (41).
smaller pieces, loaded on to an Applied Biosystems (Foster City, CA) Procise N-terminal peptide sequencer, analyzed using a “pulsed liquid polyvinylidene difluoride protein” method, and set for 10 sequence cycles. The data were analyzed using the Model 610 software program supplied by Applied Biosystems.

Cell Migration, Spreading, and Invasion Assays—For wound healing assays, wells of a 24-well tray were coated with 1/100 g of fibronectin and blocked with 2% BSA as described above. MDA-MB-231 cells were obtained using 5 mM EDTA and washed, and 4 × 10⁶ cells/well were allowed to adhere for 2 h in H-SFM. A wound was created using a 1-ml pipette tip, and the well was washed extensively with RPMI-SFM. Medium was then replaced with 300 μl of RPMI medium containing 2% FCS (required for cell migration but not allowing cell proliferation) and 100 nM GrB. Images of the wound were taken at day 0, and the same area (identified by markings) was photographed after 24 h. For cell spreading assays, wells of a 96-well tray were coated with 50 μl of RPMI medium containing 2% FCS (required for cell migration but not allowing cell proliferation) and 100 nM GrB. Medium was then replaced with 300 μl of RPMI-SFM containing 2% FCS (required for cell migration but not allowing cell proliferation) and 100 nM GrB. Images of the wound were taken at day 0, and the same area (identified by markings) was photographed after 24 h. For invasion assays, 1 × 10⁵ MDA-MB-231 cells were plated in RPMI-SFM containing 0.1% BSA into Transwells® (6.5-mm diameter, 8-μm pore size (Costar)) that had been precoated with 50 μl of fibronectin (10 μg/ml) or Matrigel (1 mg/ml, BD Biosciences). Cells were allowed to adhere for 2 h and were washed, and medium was replaced with 100 μl of RPMI-SFM containing the indicated concentration of GrB. Cells were allowed to migrate toward 5% FCS for 24 h, after which cells on the underside of the membrane were fixed and stained with hematoxylin.

RESULTS

GrB Detaches Adherent Human Cells—To examine potential extracellular functions of human GrB, various human cell lines were exposed to recombinant human GrB in the absence of perforin. When primary endothelial cells (HUVECs) were exposed to GrB alone, a striking morphology change (rounding and balling up) was observed within 2–4 h (data not shown), which was followed by a loss of adherent cells (Fig. 1 A, top panel). As a control, cells were also treated with GrB zymogen (ProGrB), which still has the N-terminal propeptide attached. ProGrB treatment of HUVECs induced no morphological changes or loss of cells from the monolayer (Fig. 1A, top panel). As a control, cells were also treated with GrB zymogen (ProGrB), which still has the N-terminal propeptide attached. ProGrB treatment of HUVECs induced no morphological changes or loss of cells from the monolayer (Fig. 1A, top panel). As a control, cells were also treated with GrB zymogen (ProGrB), which still has the N-terminal propeptide attached. ProGrB treatment of HUVECs induced no morphological changes or loss of cells from the monolayer (Fig. 1A, top panel). As a control, cells were also treated with GrB zymogen (ProGrB), which still has the N-terminal propeptide attached. ProGrB treatment of HUVECs induced no morphological changes or loss of cells from the monolayer (Fig. 1A, top panel).

Matrix Remodeling Function of Human GrB

![Matrix Remodeling Function of Human GrB](image-url)
Matrix Remodeling Function of Human GrB

It was unclear whether GrB induces apoptosis followed by cell detachment or whether GrB acts extracellularly to induce cell detachment, which is followed by death. To clarify this, the effect of GrB on a breast adenocarcinoma cell line (MCF-7) that exhibits anchorage-independent cell growth was examined. GrB treatment of MCF-7 cells also resulted in a dramatic loss of the monolayer (Fig. 1A, middle panel). Treatment with ProGrB or the GrB active site mutant (GrBS183A) had no effect on cellular morphology or adhesion. Fig. 1A (lower panel) shows a dose-response experiment in which the morphology of MCF-7 cells being detached during exposure to GrB was examined. At lower concentrations of GrB, cells round up but remain attached, whereas at higher concentrations, the cells detach and clump together. The morphology of the detached cells suggests that GrB is able to disrupt cell-matrix adhesion but not cell-cell interactions.

The loss of adherent MCF-7 cells induced by GrB is not a result of cell death as detached MCF-7 cells are viable by replating experiments (data not shown) and MTT assay. As shown in Fig. 1B, treatment of cells with GrB (and not the pro-enzyme) resulted in a dramatic loss of adherent cells. However, neither enzyme greatly affected total cell viability. These results demonstrate that extracellular GrB mediates cell detachment (not death), an effect that requires its protease activity. As shown in Fig. 1C, GrB induced cell detachment is dose-dependent and occurs at physiologically relevant concentrations, with an EC50 of ~15 nM.

To confirm that GrB acts extracellularly, GrB detachment activity on MCF-7 cells stably expressing the intracellular GrB inhibitor PI-9 (clone 16.11, described in Ref. 43) was assessed. MCF-7 cells expressing PI-9 were equally as sensitive to GrB-mediated detachment (data not shown), demonstrating that GrB indeed acts extracellularly to induce cell detachment. Numerous other cell lines, including the continuous placental trophoblast cell lines ED27 and ED77 and the transformed cell lines BeWo (choriocarcinoma), COS-1 (monkey kidney), and HTB (chondrocytic), all exhibited morphology changes or loss of adherence upon treatment with GrB (data not shown).

Physiological Forms of GrB Induce Cell Detachment—It was important to demonstrate that the detachment activity is not specific to recombinant GrB and that native GrB has the same activity. Therefore, the detachment activity of recombinant GrB (rGrB) was compared with that of native GrB (nGrB) purified from natural killer granules (40) and also with GrB in complex with chondroitin sulfate (CSGrB) (1). CS-GrB mimics serylgin-bound GrB that is released from CLs. Both nGrB and CS-GrB induced similar morphology changes to MCF-7 cells (Fig. 2A), which resulted in cell detachment (Fig. 2A, graph). rGrB and nGrB detachment activity were almost identical.

There was a slight reduction in the efficiency of cell detachment induced by CS-GrB (Fig. 2A), which prompted us to examine whether the interaction of GrB with various glycosaminoglycans influences cell detachment. As shown in Fig. 2B, preincubation of GrB with heparin and heparan sulfates abolished detachment activity, with complete inhibition occurring with heparin sulfate at a 1:1 molar ratio. By contrast, detachment was only significantly inhibited by chondroitin sulfates when used at very high molar ratios (100:1). Together, these results suggest that: 1) native GrB released from CLs possesses detachment activity and 2) GrB may bind a heparin-like molecule to mediate cell detachment as the interaction of GrB with glycosaminoglycans does not interfere with enzymatic activity (data not shown and Ref. 2).

GrB Detachment Activity Is Matrix-specific—All previous detachment experiments were performed after plating cells in medium containing 10% FCS. Serum contains a complex mixture of adhesive proteins; however, the matrix proteins vitronectin and fibronectin are the major adhesive factors (44). Therefore, cell detachment activity was assessed after plating cells onto matrices comprising purified vitronectin, fibronectin, or collagen (a major component of basement membranes). As...
shown in Fig. 3A, GrB specifically induced morphology changes and detachment of MCF-7 cells and HUVECs plated on vitronectin and fibronectin, whereas cells plated on collagen were unaffected. (No difference was observed regardless of whether type I or type II collagens were employed.)

GrB-mediated Detachment Induces Anoikis—Primary endothelial cells undergo apoptotic cell death (anoikis) as a result of cell detachment (45). This consequence of HUVEC cell detachment is evident from both the cell morphology and the viability after GrB treatment (Fig. 3A). There was a strong correlation between GrB-induced detachment of HUVECs and the induction of cell death, with death of up to 75% of cells detached from vitronectin. Cell detachment and therefore cell death was slightly less efficient when cells were plated on fibronectin; however, a strong correlation remained. HUVECs remained viable after GrB treatment when plated on collagen. These results demonstrate that GrB can induce anoikis in the absence of perforin. By contrast, MCF-7 cells were more resistant to detachment-induced death, with a maximum 25% cell death observed on vitronectin or fibronectin.

Given the elevated levels of GrB that occur in synovial fluid of rheumatoid arthritis patients, we also examined the effect of extracellular GrB on primary chondrocytes isolated from bovine cartilage. GrB efficiently mediated detachment of chondrocytes from fibronectin; however, no cell death was observed even after 48 h of treatment (Fig. 3B).

GrB Efficiently Cleaves the Matrix Protein Vitronectin at the Integrin-binding Site—Cell adhesion to vitronectin and fibronectin is mediated by binding of cell surface integrins to a specific sequence (RGD) on each of these matrix proteins (reviewed in Refs. 46 and 47). To determine whether GrB acts on integrins or directly on matrix proteins, the effect of pretreating the cells or the matrix with GrB was examined. Pretreatment of the matrix with GrB clearly inhibited MCF-7 cell adhesion, whereas pretreatment of the cells had no effect on cell adhesion (data not shown), indicating that GrB acts on the matrix to induce cell detachment. Therefore, direct cleavage of matrix proteins by GrB was assessed, and both vitronectin and fibronectin were identified as novel GrB substrates (Figs. 4 and 5).

Vitronectin is an adhesive glycoprotein present at high concentrations in plasma and incorporated into the ECM (see reviews in Refs. 48 and 49). Under reducing conditions, soluble vitronectin from human plasma appears in two forms, full-length (75 kDa) and a 65-kDa form in which the disulfide-linked C-terminal fragment generated by endogenous cleavage has been removed (Fig. 4A, also indicated in Fig. 4B by arrows). GrB slowly cleaves both the 65-kDa form and the 75-kDa form of soluble vitronectin, as shown by the loss of full-length proteins and the generation of initial cleavage fragments over 24 h (Fig. 4B, initial fragments indicated by *). ProGrB and the active site mutant (GrBS183A) do not cleave vitronectin (Fig. 4B). By contrast, GrB cleavage of vitronectin plated as a matrix on plastic is extremely efficient (Fig. 4C). Here cleavage products were detected with as little as 3 nM GrB within 15 min. GrB also cleaved the matrix form of vitronectin in the presence of adherent MCF-7 cells (Fig. 4D), with the majority of full-length protein degraded within 1 h.

The initial cleavage of vitronectin by GrB resulted in the loss of 5–10 kDa from both the 65-kDa form and the 75-kDa form, suggesting a cleavage site near the N terminus. N-terminal sequencing of the initial cleavage product derived from the 65-kDa form (Fig. 4, fragment indicated by #) yielded the sequence VFTMPDE. Therefore, the primary GrB cleavage site in vitronectin is at the integrin-binding (RGD) site (14TRGD18VFTM18), explaining the ability of GrB to disrupt integrin-dependent adhesion. The presence of a secondary cleavage site is suggested by the appearance of additional fragments when higher GrB concentrations were used or prolonged incubations were performed (Fig. 4).

GrB Cleaves the Matrix Protein Fibronectin—Fibronectin is a complex ECM glycoprotein, composed of two similar but non-identical 250-kDa disulfide linked subunits (reviewed in Refs. 50–52). GrB cleavage of human fibronectin in solution is shown in Fig. 5A. Although leukocyte elastase was able to efficiently cleave soluble fibronectin, even 100 nM GrB for 24 h had no effect on fibronectin in this state. By contrast, GrB cleaved fibronectin when it was coated onto plastic (Fig. 5, B and C), demonstrating that a matrix-specific conformation of fibronectin is critical for GrB recognition and cleavage. GrB cleavage of matrix fibronectin released fragments into the supernatant (Fig. 5B) and was very efficient, with fragments detected within 10 min (Fig. 5C). ProGrB and the GrB active site mutant (GrBS183A) did not cleave matrix fibronectin (Fig. 5C). GrB cleavage sites within fibronectin are yet to be determined; however, the sizes of the two of the major cleavage products (Fig. 5B, indicated by *) suggest cleavage at Asp4195, which comprises the RGD site of fibronectin. The generation of other fragments also suggests the presence of a secondary GrB cleavage site.

GrB Cleaves the Matrix Protein Laminin and Inhibits Cell Spreading—The identification of vitronectin and fibronectin as novel GrB substrates prompted us to examine whether GrB also cleaves another common ECM component, laminin. Cell detachment was assessed using both a commercial preparation of laminin from human placenta (comprising a mixture of several laminin isoforms (53, 54)) and purified recombinant laminin-10, an isoform found ubiquitously expressed in basement membranes of many adult tissues (55–57). GrB induced detachment of LIM1215 colon carcinoma cells from both laminin preparations but more efficiently from recombinant laminin-10 (Fig. 6, A and B). As the commercial laminin preparation contains mainly trimmed forms of laminin (37), we used recombinant laminin-10 (35) to assess GrB cleavage. Laminin-10 is composed of three disulfide linked chains, α5, β1, and γ1 (55), with apparent molecular masses of 360, 210, and 200 kDa, respectively (37, 58). GrB efficiently cleaved the α5-chain of laminin-10, generating major cleavage products within 30 min (Fig. 6C, indicated by arrows). Upon prolonged incubation, further cleavage products were evident (indicated by *), which may result from cleavage of the β-chain. Cleavage sites within laminin-10 are yet to be determined; however, the α5-chain possesses the cell-binding RGD sites, and fragment sizes are consistent with cleavage at one of these sites, in addition to a secondary (non-RGD) site.

Laminin-10 has been observed at the leading edge of colon cancers (37, 59) and is known to induce cell spreading (including the presence of pseudopod-like extensions) and increase motility of LIM1215 cells in the presence of epidermal growth factor (58, 60). Although GrB pretreatment of laminin-10 matrix had no effect on the initial adhesion of LIM1215 cells (data not shown), cell spreading was significantly inhibited in the presence of GrB (Fig. 6D).

GrB Disrupts the Integrin-dependent Processes of Tumor Cell Migration and Invasion—Integrin-dependent adhesion to matrix proteins is also critical for cellular migration and invasion (reviewed in Ref. 61). We therefore examined whether GrB can influence these processes. The highly motile and invasive breast carcinoma cell line, MDA-MB-231, was used in an in vitro migration (wound-healing) assay on fibronectin. As shown in Fig. 7A, GrB inhibited migration without inducing detachment. GrB also inhibited cell migration without detachment on the more complex matrix Matrigel (major components: lami-
GrB detaches cells from vitronectin (Vn) and fibronectin (Fn) and induces endothelial cell death. A, wells were coated with 10 μg/ml vitronectin, fibronectin, or type II collagen (Col). MCF-7 cells or HUVECs were plated onto matrices in H-SFM and allowed to adhere for 1.5 h. Any non-adherent cells were removed, and cells were treated with increasing concentrations of GrB for 24 h in H-SFM. Images show live
was incubated with 100 nM GrB, ProGrB, GrBS183A active site mutant, analyzed by N-terminal sequencing.

The integrin-binding site (RGD), endogenous cleavage site, and disulfide bond (S-S) are shown. N, N terminus; C, C terminus. B, 200 ng of human plasma vitronectin was incubated with 100 nM GrB, ProGrB, GrBS183A active site mutant, or no enzyme (--) in H-SFM for 24 h. Reactions were stopped by the addition of Laemmli sample buffer, and proteins were resolved on a reducing 10% SDS-PAGE gel and immunoblotted with mouse anti-vitronectin antibody. The 75- and 65-kDa forms of plasma vitronectin are indicated by arrows. C, wells of a 24-well tray were coated with 500 ng of vitronectin and treated with the indicated concentration of GrB in H-SFM for 15 min, supernatants were removed, and matrix proteins were collected and immunoblotted as described for B, except that a rabbit polyclonal antibody was used for immunoblotting. D, GrB cleavage of matrix vitronectin in the presence of MCF-7 cells. Wells of a 24-well tray were coated with 500 ng of vitronectin, MCF-7 cells in SFM were allowed to adhere for 1.5 h, and non-adherent cells were removed. Cells were then treated with 100 nM GrB for the indicated time, after which any adherent cells were non-enzymatically removed, and the matrix was collected and immunoblotted with the rabbit polyclonal antibody. * indicates initial cleavage products, and # indicates product analyzed by N-terminal sequencing.

Unwashed cells after treatment with 100 nM GrB (original magnification ×400). The graphs represent the percentage of adherent and the percentage of dead cells after treatment, as assessed by MTT assay. Values represent mean ± S.D. from triplicate wells. B, primary bovine chondrocytes plated onto wells coated with 10 μg/ml fibronectin were treated with 100 nM GrB in H-SFM for 24 or 48 h. Images are of live unwashed cells (original magnification ×400) after 24 h of treatment. The graph represents the percentage of viable and adherent (Adh) cells of triplicate wells (mean ± S.D.), as assessed by MTT assay at the indicated time.

GrB Cleavage of ECM Proteins—GrB cleavage of vitronectin at the RGD site is, to our knowledge, the first demonstration of a mammalian proteinase cleaving this critical sequence. Several other proteases are known to cleave vitronectin; however, this occurs at sites near the C terminus, and in marked contrast to GrB, does not depend on vitronectin attaining a specific conformation (62, 63). Soluble vitronectin is mainly in a monomeric globular conformation, but when incorporated into an ECM, it forms multimers in which binding sites for other proteins are more accessible (64–66). In fact, it has been shown that the RGD site in soluble plasma vitronectin is buried and unable to bind inte-
S.D. from triplicate wells.

fide-linked heterotrimers containing different combinations of isoforms identified to date. Each isoform is composed of disul-
both in solution and in a matrix. Laminin-10 is one of 15 membrane protein, laminin, is an efficient substrate of GrB (71, 72).

altered by incorporation into the matrix or bound to a surface further exposed when plasma fibronectin is conformationally
integrins (70), is part of the cell-binding region that becomes
ism can be determined, given its ability to efficiently disrupt inte-
grin-mediated adhesion to this matrix, GrB probably cleaves
this protein at its RGD site. Two of the major cleavage products
are consistent with cleavage after the Asp1495 of mature fi-
bronectin. This site, which is known to interact with several
integrins (67), explaining the inefficient cleavage by GrB until its
exposure upon binding to a surface.

Like vitronectin, soluble fibronectin assumes a closed globu-
lar conformation but forms insoluble multimeric fibrils when
incorporated into the ECM (68, 69). In fibronectin, this confor-
mational change appears to be critical for GrB recognition and
cleavage. Although the GrB cleavage sites in fibronectin are yet
to be determined, given its ability to efficiently disrupt inte-
grin-mediated adhesion to this matrix, GrB probably cleaves
this protein at its RGD site. Two of the major cleavage products
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integrins (70), is part of the cell-binding region that becomes
further exposed when plasma fibronectin is conformationally
altered by incorporation into the matrix or bound to a surface
(71, 72).

Unlike vitronectin and fibronectin, the major basement
membrane protein, laminin, is an efficient substrate of GrB
both in solution and in a matrix. Laminin-10 is one of 15
isoforms identified to date. Each isoform is composed of disul-
fide-linked heterotrimers containing different combinations of
α(1–5), β(1–2), and γ(1–3) chains (73, 74). The α5 chain is
efficiently cleaved by GrB and contains the cell-binding region
(including RGD sites), thus explaining the ability of GrB to
disrupt cell adhesion to this matrix (55, 73, 74). The α5 chain is
also present in laminin-11 and -15, which suggests that GrB
will cleave other laminin isoforms. This appears likely as GrB
detached cells from a preparation of laminin containing a mix-
ture of several isoforms.

GrB detachment activity is inhibited by the glycosaminogly-
can, heparin sulfate, but not by chondroitin sulfate. It is well
established that cationic GrB interacts with both chondroitin
sulfate and heparin sulfate (30, 75–77). Heparin sulfate does
not inhibit GrB enzymatic activity; thus, it probably prevents
GrB binding to the ECM. Since vitronectin, fibronectin, and
laminin are not proteoglycans, it is likely that heparin, but not
the less anionic chondroitin sulfate, blocks an electrostatic
interaction between GrB and negatively charged surface do-
 mains on these proteins. Indeed, vitronectin possesses a well
characterized anionic domain immediately downstream of the
RGD site (see review in Ref. 48).

**Extracellular GrB Function in Lymphocytes**—The disruption of integrin-mediated adhesion by GrB may constitute a per-
forin-independent killing mechanism of cytotoxic lymphocytes
via the induction of anoikis of susceptible cells. GrB may also
inhibit tumorigenesis via inducing anoikis of susceptible tumor
cells and by inhibition of tumor cell spreading, migration, or
invasion, all of which are integrin dependent processes (see
review in Ref. 61). It will be interesting to determine whether
GrB can also disrupt tumor angiogenesis.

The ECM remodeling activity of GrB may also facilitate mi-
gration of activated lymphocytes through tissues. It is known
that newly synthesized GrB is nonspecifically secreted after CLs
are triggered to degranulate (20), and several studies have de-
monstrated that the interaction of cytotoxic T lymphocytes with
vitronectin and fibronectin but not collagen stimulates prolifer-
eration, activation, and degranulation (78–80). Indeed, it has been
proposed that GrA plays a role in lymphocyte migration through
basement membranes due to its ability to degrade collagen, fi-
bronectin, and matrix proteoglycans (23, 24).

**FIG. 6.** GrB cleaves laminin, inducing cell detachment and inhibiting cell spreading. A and B, LIM1215 cells were plated into wells of
a 96-well tray previously coated with 2 μg/ml commercial laminin or recombinant laminin-10. Cells were allowed to adhere for 2 h, after which the
medium was replaced with SFM containing the indicated concentration of GrB and incubated at 37 °C for 24 h. Images shown are after treatment
with 100 nM GrB (A, original magnification ×200). B, the graph represents the remaining adherent cells assessed by calcein uptake, with mean ±
S.D. from triplicate wells. C, 3.6 μg of recombinant laminin-10 was incubated with 100 nM GrB in PBS for the indicated time or with no enzyme
(−) for 24 h. Reactions were stopped by the addition of Laemmli sample buffer, and samples were resolved on a 3–10% reducing SDS-PAGE gel
followed by silver staining. Positions of α, β, and γ chains are indicated. Arrows indicate primary cleavage products, and the asterisk indicates
secondary cleavage products. D, LIM1215 cells were allowed to adhere to wells coated with 2 μg/ml recombinant laminin-10 that had been
pretreated with 100 or 200 nM GrB, in SFM also containing the indicated concentration of GrB, and epidermal growth factor. After 6 h, the number
of spread/non-spread cells was counted. The graph represents mean ± S.D. from triplicate wells, after counting a minimum of 150 cells/well, and
is representative of two independent experiments.
via induction of matrix metalloproteinase, collagenase, and stromelysin expression by chondrocytes and fibroblasts (94, 95). They also induce monocyte and neutrophil chemotaxis, disrupt chondrocyte cell adhesion, and enhance proliferation of CD4+ T cells (reviewed in Ref. 93). We have shown that GrB degrades fibronectin and detaches primary chondrocytes cultured on this matrix. In contrast to primary endothelial cells that died as a result of GrB-mediated detachment, chondrocytes remained viable. This is consistent with previous observations and is likely due to the continued synthesis of their own complex ECM (96, 97). The characterization of the fibronectin fragments generated by GrB, and their presence in the synovial fluid of rheumatoid arthritis patients, should provide further insight into the contribution of GrB to this disease.

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