Purification, molecular cloning and mechanism of action of graminelysin I, a snake-venom-derived metalloproteinase that induces apoptosis of human endothelial cells

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Apoptosis, a programmed, physiological mode of cell death, is important in tissue homeostasis. Here we report that a new metalloproteinase, graminelysin I, purified from *Trimeresurus gramineus* venom, induced apoptosis of human endothelial cells as examined by electrophoresis and flow cytometry. Graminelysin I contains only a metalloproteinase domain. It is a single-chain proteinase with a molecular mass of 27020 Da. cDNA sequence analysis revealed that the disintegrin-like and cysteine-rich domains of the putative precursor protein of graminelysin I are likely to be processed post-translationally, producing the proteinase domain (graminelysin I). Graminelysin I cleaved the α chain of fibrinogen preferentially and cleaved the β chain either on longer incubation or at higher concentration. Graminelysin I inhibited the adhesion of human umbilical-vein endothelial cells (HUVECs) to immobilized fibrinogen and induced HUVECs detachment in a dose-dependent manner. These effects on

HUVECs were abolished when graminelysin I was pretreated with EDTA. However, graminelysin I did not inhibit the adhesion of HUVECs to immobilized collagen. HUVECs were susceptible to death after treatment with graminelysin I when they were cultured on immobilized fibrinogen. In contrast, HUVECs were rather resistant to treatment with graminelysin I if they were cultured on immobilized collagen. Furthermore, graminelysin I induced apoptosis of HUVECs in a dose-dependent manner. Similarly, its apoptosis-inducing activity was blocked if it was treated with EDTA. These results suggest that the catalytic activity of graminelysin I on matrix proteins contributes to its apoptosis-inducing activity.

Key words: DNA fragmentation, endothelial cell detachment, extracellular matrix, metalloprotease, reprotolysin.

INTRODUCTION

Apoptosis, a programmed, physiological mode of cell death, is important in tissue homeostasis. The characteristic morphological signs of apoptosis are cellular shrinkage, membrane blebbing, nuclear condensation and DNA fragmentation [1]. It has been increasingly recognized that apoptosis might be involved in several critical events occurring during normal development and might have a key role in a wide variety of diseases [2]. Evidence from recent studies suggest that apoptosis of vascular endothelial cells regulates angiogenesis and promotes vascular regression [3,4]. In addition, there are many vascular diseases, such as atherosclerosis and restenosis, that are associated with apoptosis of vascular cells [5].

Cell-cell and cell-matrix contacts are necessary for the maintenance of survival of anchorage-dependent cells such as endothelial cells [5]. Many signals from the extracellular matrices (ECMs) are relayed to the intracellular signalling machinery by integrins, which are heterodimeric transmembrane receptors with overlapping specificity towards ECM components [6]. For example, survival signals from fibronectin are transduced by the focal adhesion kinase ('FAK') whose phosphorylation leads to p53 inactivation, and maintain cell survival in endothelial cells and fibroblasts [7]. Interaction with ECM proteins via the integrin $\alpha_v\beta_3$ inhibits p53 activity, decreases the expression of p21 and

Bax and therefore promotes cell survival [3]. This is particularly important during the migration of endothelial cells in angiogenesis as well as in the maintenance of tumour vasculature [5].

Snake-venom metalloproteinases (SVMPs) belong to a reprotolysin subfamily of the metalloproteinases that contain a conserved zinc-chelating sequence, HEXXHXXGXXH (single-letter amino acid code). SVMPs can be divided into four groups, P-I to P-IV, on the basis of their molecular sizes [8]. *Trimeresurus gramineus* is a pit viper found in Taiwan; it has medical importance. Two metalloproteinases (formerly haemorrhagic principles HR1 and HR2) from *T. gramineus* venom have been identified [9]. In addition, a cDNA encoding the potent platelet aggregation inhibitor, trigramin, was cloned from *T. gramineus*. Sequence analysis suggested that trigramin is translated initially as a precursor protein and is further processed post-translationally [10]. However, only a few SVMPs have been characterized further and have had their cDNA isolated and completely sequenced. Previous studies revealed that SVMPs have the characteristics to induce haemorrhage, to inhibit platelet aggregation and to degrade extracellular proteins. For example, kistomin, an anti-platelet metalloproteinase, was purified in our laboratory from venom of *Calloselasma rhodostoma* [11]. Kistomin selectively cleaved human platelet glycoprotein Ib and inhibited thrombin- and ristocetin-induced platelet aggregation [12]. In addition, VAP1 and VAP2, purified from *Crotalus atrox*

Abbreviations used: ADAM, a disintegrin and metalloproteinase; BCECF/AM, 2',7'-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein acetoxymethyl ester; ECM, extracellular matrix; FBS, fetal bovine serum; HUVEC, human umbilical-vein endothelial cell; MMP-1, matrix metalloproteinase 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; SVMP, snake-venom metalloproteinase.

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venom, specifically induced apoptosis of human umbilical-vein endothelial cells (HUVECs) [13,14]. Protein sequence analysis revealed that VAP1 and VAP2 belong to the metalloproteinase family. cDNA sequence analysis further identified the structural organization of VAP1, including a proteinase domain, a disintegrin-like domain and a cysteine-rich domain [15]. However, few reports were focused on the action mechanisms of apoptosis induced by these metalloproteinases. ADAMs (a disintegrin-like and metalloproteinase-containing protein) are also zinc-dependent metalloproteinases with high amino acid sequence similarity and domain organizations similar to those of SVMPs [16]. Extensive studies indicate that ADAMs are involved in highly diverse biological processes, including fertilization, neurogenesis, myogenesis, embryonic release of transforming growth factor α and inflammatory response [17]. However, the function of most ADAM gene products is still unknown.

In the present study we have purified a new SVMP, graminelysin I, that induced the apoptosis of vascular endothelial cells. On the basis of the molecular mass and the amino acid and cDNA sequence, graminelysin I contains only a proteinase domain. Graminelysin I cleaved fibrinogen, inhibited the adhesion of HUVECs to immobilized fibrinogen and induced the detachment of HUVECs from the immobilized fibrinogen. These effects were abolished when graminelysin I was preincubated with EDTA. In addition, graminelysin I had different effects on the viability of HUVECs that were cultured on fibrinogen or collagen matrix. These results suggest that the degradation of ECM components by graminelysin I contributes to its apoptosis-inducing effect on endothelial cells.

EXPERIMENTAL

Materials

Freeze-dried powder of *T. gramineus* venom was produced in the laboratory of M.-Y. Liao. DEAE-Sephadex A50, Sephadex G-100 and Superdex 75 FPLC columns were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), and a reverse-phase C_8 column for HPLC was from Merck Chemical Co. (Darmstadt, Germany). Fibrinogen, propidium iodide, collagenase, BSA and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Medium 199, fetal bovine serum (FBS), agarose and all cultured reagents were purchased from Life Technologies (Grand Island, NY, U.S.A.) except that endothelial cell growth supplement was from Upstate Biotech (Lake Placid, NY, U.S.A.). 2',7'-Bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM) was purchased from Molecular Probes (Eugene, OR, U.S.A.). RNase A was from Amersco (Cleveland OH, U.S.A.).

Cell culture

HUVECs were prepared by the method described previously [18]. In brief, umbilical cord veins were cannulated and flushed with cord buffer to remove blood, then filled with 0.1% collagenase (type I) for 10 min at 37 °C. Isolated endothelial cells, identified as positively immunofluorescent staining for von Willebrand factor antigen, were maintained in Medium 199 containing 20% (v/v) FBS, 30 μ g/ml endothelial cell growth supplement, 4 mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin, and incubated at 37 °C in air/CO₂ (19:1). Cells between the second and fourth passages were used in this study.

Purification of graminelysin I from *T. gramineus* venom

Freeze-dried crude venom powder (0.6 g) dissolved in 0.05 M ammonium acetate, pH 8.0, was applied to a DEAE-Sephadex A50 chromatography column (3 cm $\times$ 70 cm) equilibrated with starting buffer (0.05 M ammonium acetate, pH 8.0). The column was eluted with a linear gradient of starting buffer (700 ml) and buffer B (0.25 M ammonium acetate, pH 6.0; 700 ml) at a flow rate of 0.375 ml/min. The second-stage elution was followed with a linear gradient of buffer B (350 ml) and buffer C (1 M ammonium acetate, pH 5.2; 350 ml). The crude venom was fractionated into 12 fractions and screened by azocaseinolytic assay as described previously [19]. The fraction with azocaseinolytic activity was applied to a Sephadex G-100 column and eluted with a buffer containing 0.02 M ammonium bicarbonate, pH 7.8. The major fraction obtained was further chromatographed on Superdex 75 FPLC column (HR 10/30) and eluted with a buffer containing 0.02 M ammonium bicarbonate buffer and 0.15 M NaCl, pH 7.8, at a flow rate of 0.75 ml/min. Fractions corresponding to a retention of 14–17 min were collected. The purity and molecular mass of graminelysin I were analysed by PAGE followed by staining with Coomassie Blue.

Digestion of alkylated graminelysin I with CNBr

Graminelysin I (200 μ g) was dissolved in digestion buffer [6 M urea/50 mM Tris/HCl (pH 8.0)]; dithiothreitol was then added to a final concentration of 10 mM and incubated for 1 h at 37 °C. The denatured graminelysin I was further alkylated by adding vinylpyridine to a final concentration of 20 mM and incubated for 2 h at room temperature. After precipitation with 90% (v/v) ethanol, the alkylated graminelysin I was dissolved in 30% (v/v) formic acid and reacted with CNBr at room temperature for 3 days. Formic acid and CNBr were removed by vacuum-drying; the cleaved fragments were subjected to further analysis.

Protein sequencing

The CNBr-cleaved fragments of S-pyridylethylated graminelysin I were subjected to HPLC and SDS/PAGE [15% (w/v) gel]. For HPLC, the cleaved fragments were dissolved in 0.1% (v/v) trifluoroacetic acid and filtered through a 0.22 μ m pore-size Millex®-GV filter (Millipore, Bedford, MA, U.S.A.). The supernatant was applied to a C_8 HPLC column (250 mm $\times$ 10 mm). Elution was performed with a linear gradient of 0–70% (v/v) acetonitrile over a period of 45 min. The major fraction was subjected to sequencing analysis. Sequence analysis was performed with a PerkinElmer protein sequencer. Fragments separated by PAGE were transferred to PVDF membrane before sequence analysis.

RNA isolation and cDNA library construction

Total RNA was isolated from *T. gramineus* venom glands with a BlueExtract kit (LTK BioLaboratories Co., Ltd, Linko, Taipei, Taiwan). cDNA was synthesized from 1.5 mg of the total RNA and used to construct a cDNA library in the Uni-ZAP XR vector (Stratagene, La Jolla, CA, U.S.A.). The *T. gramineus* cDNA library has an estimated amplified titre of 10¹⁰ plaque-forming units/ml, representing 10⁶ recombinants with an average insert size of 1.6 kb.

Amplification and cloning of graminelysin I precursor cDNA

To obtain cDNA of putative SVMP, recombinant lambda DNA species were prepared from the cDNA library and used as templates in PCR. Primer 1 [5'-CGAATTCAAAAT(G/C)-

TGTGG(A/G)GT(A/T)ACC-3'] and primer 2 (5'-ATCTGGA-GCCACAGTTGCACCTT-3') were designed and synthesized in accordance with the conserved KMCVGT sequence in the prodomain and the XATVXXD sequence stretch in the cysteine-rich domain of SVMP respectively. Amplification was performed by using *Taq* DNA polymerase (Ab peptides, Inc., St Louis, MO, U.S.A.) and primers 1 and 2 with a hot start at 94 °C for 10 min and 30 cycles of denaturation (1 min at 94 °C), annealing (1 min at 42 °C), and extension (2 min at 72 °C). The PCR products were analysed by 1% (w/v) agarose-gel electrophoresis and purified by electroelution. The purified DNA fragments were ligated to pCRII-TOPO vector with a TA cloning kit (Invitrogen, Carlsbad, CA, U.S.A.) and sequence analysis was performed. On the basis of the information, sequences near primer 2 but within the cysteine-rich domain and with the least similarity among SVMPs were selected to design specific oligonucleotides for performing an amplification towards the 3' end of the precursor cDNA of SVMP. In cloning of precursor cDNA of graminelysin I, the amplification was performed with primer 3 (5'-AATTG-CCCCATCATGTTTTAC-3', corresponding to the deduced amino acid sequence NCPIMFY) and M13 forward primer under the same conditions as the first amplification except that annealing was performed at 55 °C. The specificity of the second amplification was confirmed from the overlapping sequences that were distinguishable from the other SVMPs. Sequences were assembled with the GCG program (Wisconsin Package version 10.1, Genetics Computer Group).

MTT assay

HUVECs were seeded at 1.5×10^5 cells per well in 24-well plates (Costar, Cambridge, MA, U.S.A.). After 1 day the culture medium was replaced with medium containing 2% or 20% (v/v) FBS with or without the addition of graminelysin I. After incubation for 24 h, the culture cells were washed twice with PBS and cell viability was determined as described previously [20] with modification. In brief, the cells were incubated with 0.5 mg/ml MTT for 4 h at 37 °C. Formazan crystals resulting from MTT reduction were dissolved by the addition of 200 μ l of DMSO and gentle agitation for 30 min. The A_{550} of the supernatant was then measured spectrophotometrically in an ELISA reader.

DNA content analysis

For flow cytometric analysis, HUVECs with or without treatment with graminelysin I were collected by centrifugation and adjusted to 3×10^6 cells/ml; prechilled methanol was added to 0.5 ml of the cells and incubated at 4 °C overnight. Methanol was then removed by centrifugation and the DNA of the cells was stained with propidium iodide staining solution [100 μ g/ml propidium iodide/0.1% (v/v) Triton-X/1 mM EDTA in PBS] in the presence of an equal volume of DNase-free RNase (200 μ g/ml) and analysed immediately by FACScalibur (Becton-Dickinson, San Jose, CA, U.S.A.).

DNA fragmentation assay

A selective procedure for DNA extraction from apoptotic cells has been described previously [21]. In brief, graminelysin I or PBS-treated HUVECs were collected by centrifugation, resuspended in 1 ml of Hanks buffered salt solution and fixed by adding 10 ml of prechilled 70% (v/v) ethanol and maintaining them at -20 °C for 24 h. After centrifugation at 800 *g* for 5 min, the cell pellets (2×10^6 cells) were resuspended in 40 μ l of phosphate/citrate buffer, pH 7.8, consisting of 0.2 M

$\text{Na}_2\text{HPO}_4/0.1$ M citric acid (24:1, v/v), then incubated at room temperature for 30 min. After centrifugation at 1000 *g* for 5 min, the supernatant was concentrated by vacuum in a SpeedVac for 15 min. A 3 μ l portion of 0.25% NP-40 (Sigma) and 3 μ l of RNase A (1 mg/ml) were added. After incubation for 30 min at 37 °C, 3 μ l of proteinase K (1 mg/ml) was added and the incubation was continued for a further 30 min. After the addition of loading dye, the reaction mixture was subjected to 1% (w/v) agarose-gel electrophoresis. DNA ladders stained by ethidium bromide were detected under UV radiation.

Fibrinogenolytic assay

Fibrinogenolytic assay was performed by incubating human fibrinogen dissolved in PBS (20 mg/ml) with various concentrations of graminelysin I for different durations. After the addition of 5 $\times$ sample buffer containing 1.8 M 2-mercaptoethanol, the reaction mixtures were subjected to SDS/PAGE [7.5% (w/v) gel] followed by staining with by Coomassie Blue.

Collagenolytic assay

Collagenolytic activity of graminelysin I was measured by type I collagenase [matrix metalloproteinase 1 (MMP-1)] activity assay kit (Chemicon, Temecula, CA, U.S.A.) in accordance with the protocol provided by manufacturer. In brief, sample buffer was added to the reactions containing various concentrations of graminelysin I to make a volume of 150 μ l. FITC-labelled type I collagen solution (50 μ l) was added at 4 °C, mixed well and incubated for 1.5 h at 37 °C. The reactions were stopped by the addition of 200 μ l of stop solution, vortex-mixed thoroughly and kept on ice for 5 min. The samples were then stored at room temperature for 30 min. After centrifugation at 3000 *g* for 15 min, the supernatant was removed for measuring fluorescence intensity. Fluorescence intensity was measured at 485 nm (excitation)/530 nm (emission) with a CytoFluor fluorescence plate reader (Millipore). One unit of collagenase activity is defined as the capacity of the enzyme to degrade 1 μ g of collagen per min under the condition used. The collagenolytic activity of graminelysin I was calculated from a standard curve of MMP-1 and is represented as units/mg of protein.

Adhesion assay

Plates with 96 wells (Costar) were coated overnight with 50 μ l of fibrinogen (50 μ g/ml), 100 μ l of type I collagen (10 μ g/ml) or 1% (w/v) BSA in PBS at 4 °C. HUVECs collected from a culture near confluence were resuspended in Medium 199, and labelled with BCECF/AM (10 μ g/ml) for 30 min at 37 °C. The labelled cells were washed and resuspended in Medium 199 to a density of 5×10^5 cells/ml. A 90 μ l sample of the resuspended cells was incubated with 10 μ l of PBS or various concentrations of graminelysin I for 30 min at 37 °C. Control or the pretreated HUVECs were applied to the fibrinogen- or collagen-precoated 96-well plates and incubated for 90 min at 37 °C to allow adhesion. After being washed twice with PBS, the non-adherent cells were removed by aspiration and the 96-well plates were read with a CytoFluor 2300 fluorescence plate reader (Millipore).

Cell detachment assay

Plates with 96 wells (Costar) were coated with fibrinogen (50 μ g/ml), collagen (10 μ g/ml) or 1% (w/v) BSA in PBS at 4 °C overnight. HUVECs were collected and labelled with BCECF/AM as for the adhesion assay. A 100 μ l sample of the labelled cells (5×10^5 cells/ml) was seeded on each well of the precoated 96-well plates and incubated at 37 °C for 3 h in Medium 199

containing 2% (v/v) FBS to allow adhesion. After cell adhesion, the FBS-containing medium was replaced by Medium 199 with or without the addition of graminelysin I. Where indicated, 2.5 mM EDTA was preincubated with graminelysin I for 15 min before adding into Medium 199. After incubation for 1.5 h at 37 °C, detached cells were removed by two washes with PBS; the remaining BCECF-labelled cells were detected with a CytoFluor 2300 fluorescence plate reader.

RESULTS

Purification and characterization of graminelysin I

We first used DEAE-ion-exchange chromatography to purify graminelysin I from the crude venom of *T. gramineus* (Figure 1A). Fraction III, which possessed azocaseinolytic activity, was

Figure 1 Purification of graminelysin I

(A) Freeze-dried *T. gramineus* venom (0.6 g) was applied to a DEAE ion-exchange column; elution was performed as described in the Experimental section. The active fraction (fraction III) was pooled. Fraction XI, containing a disintegrin-like/cysteine-rich domain of programinelysin I, was pooled for direct N-terminal sequencing. (B) Fraction III was subsequently applied to a Sephadex G-100 chromatography column and eluted with a buffer containing 0.02 M ammonium bicarbonate, pH 7.8. The major fraction (II) was collected. (C) The major fraction was loaded on a FPLC Superdex 75 column. Elution was performed with 0.02 M ammonium bicarbonate containing 0.15 M NaCl buffer, pH 7.8, at a flow rate of 0.75 ml/min. The active fractions containing graminelysin I (indicated by an asterisk) were pooled. Inset, SDS/PAGE [12% (w/v) gel] analysis of graminelysin I. The gel was stained with Coomassie Blue. Lane 1, standard proteins (molecular masses indicated at the left); lane 2, purified graminelysin I (7 µg) under reduced conditions; lane 3, graminelysin I (7 µg) under non-reduced conditions.

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1  AAAATGTGTGGAGTTACCCAGAAATGGGAATCATATGAGTCCACCAAAAAGGCTCTCAG 60
   K E C S V T Q W E S V R S T K K A S Q
61  TTAATATCTACTCTGACAAACAAATGATGACGATGATGATGATGATGATGATGATGAT 120
   L N L T P E Q Q R F P Q R Y I K L G I F

       |--- putative N-terminal
122 GTGGACCAACGGAATGTACACAAATACAGTGGCAATCTGAAAGGATACAAAAGGGTA 180
   V D H G M Y T K Y S G N S E R I T K R V
181 CATCAATGATCAACAAATATAATATGATGTCAGAGCTCTGAAATATGTTACAACTG 240
   H Q M I N N I N M C R A L N I V T T L
241 AGTCTCTAGAAATTTGGTCCGAAAAGATTTGATTACGGTGACGGCATCAGCGCTACT 300
   S L L L E I W S E K D L T T V Q A S A F T
302 ACTTGAACCTTATTTGGAGCTTGGAGAGACAGTCTTGTGATGATGTCAGAGCTGAT 360
   T L T L F G A W R E T V L L M R T S H D
361 CATGCTCAGTACTACGGCCACTATCTTCATGGAAGCTTATAGGAAGGGCTCCCGTG 420
   H A Q L L T A T I F N G N V I G R A P V
421 GCGGCTATGTGACCCGAAGGCTCTGTAGCAATTTGTCGGATCATAACGCAATAGTT 480
   T G M C D P K R S V A I V R D H N A I V
481 TTGTGGTGTGAGTTACAATGACCATGAGATGAGTGGTCAATCTGGGCTGATCATGAT 540
   F V V A V T M Y H E M G H N L G M H H D

       catalytic site
541 GAAGATAAATGTAATTGTAAACACATGATTATGTAAAGTGTAAAGCGCAACTTCC 600
   E D K C N C N T C I M S K V L S R Q P S
601 AAATATTTTCAGCAATGTAGTAAAGGATTTATCAGACATTTCTTAACTATCAAAACCA 660
   K Y F S E C S K D Y Y Q T F L T N H N P
661 CAATGATTTCTCAATGACCTTGAGAACAGATACTGTTCACTCACTTTCTGGAAT 720
   Q C I L N A P L R T D T V S T P V S G N

       putative C-terminal |--- spacer region
721 GAATCTTTGGAGCGGAGAGATGTGACGTGGCTCTCTGCAATTCGCTGCTGAT 780
   E L L L A G E E C D C P F A N P C C D
       |---disintegrin-like/cysteine-rich domain
781 GGTGCAACTTGTAACTGACACAGGGGCGCAAGTGTGAGAGGAGCTGTTGTGACAG 840
   A A T C K L R P G A Q C G E L C C D Q
841 TGCAGATTTTACAGTTCAGGAACAGATGACGGGACGAGGAGTGAATGTGACATTCCT 900
   C R F T S A G T E C R A A R S E C D I A
901 GAAAGCTGGCTGGCCATCTGCTGACTGTCCACGAGTACTTCATGAGGAATGGACAA 960
   E S C A G Q S A D C P T D D F H R N G Q
961 CCATGCTTAAACCAACCGGTTACTGCTACAAATGGGAATGCCCATCATGTTTACCAA 1020
   F C L N N H G Y C Y N G N C F I M E Y Q
1021 TGATTTGCTCTGTGCTCAATGCTGCTGAGTGTGCTGAGTGTGTTGATGATGAT 1080
   C I A L F G S N A T V G Q D G C F D A N
1081 GACATAGGACATAAATATTTCCACTGACAAAGGATAATGAGAAATACATTCATGTGCA 1140
   D I G H K Y F H C R K D N E K Y I P C A
1141 CCACAGGATGTAAATGTGGCAGGTTATCTGTACTATATTTATGATATGATCTCTGC 1200
   P Q D V K C G R L F C T Y I Y D I D L C
1201 CGTTACGATGATTCAGCTAATGGAATGGTGTCTCAAGGACAAJAAATGTGAGATGGAAG 1260
   R Y D E S A N G M V A Q S T K A C A D G K
1261 GTCTGCAACAGCAACAGGCAAGTGTGCTGATGTGATACAGCTACTAATCAACCTCTGGC 1320
   V C N S N R Q C A D V N T A Y
1321 TTCTCTCAGATTTGATTTTGGAGATCTCTCTTCAGAAAGGTTTCACTTCCCTCTGGTCCA 1380
1381 AAGAGACCCATCTGCTGCTGATCTCTACTAGTAAATCAACCTTAGCTTCAGATGGCATCTA 1440
1441 AATTTCTGCAATATTTCTTCTCCACATTTAATCTGTATTCTTTTGTGTAATCAAACTT 1500
1501 TTTCCACCAACAAAGCTCCATGGGCTGTACACACCAAGAGCTTATTTGCTGTCAAGAA 1560
1561 AAAAAATGGCAATTTTACCGTTTGGCAATGCAAGTACATTTAATGCAACAGGTTCTGC 1620
1621 CTTTAGAGCTGTGATTTGGAAGTCAATGCTTCTCTCCCAAAATTTCACTGCTGCTTTC 1680
1681 CAAGATGTAGCTGCTTCCATGAATAACTCATTTTCAATTCACAAAAAAGAAAAA 1740
1741 AAA

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Figure 2 cDNA sequence and deduced amino acid sequence of the putative protein precursor of graminelysin I

The nucleotide sequence shown begins with the KMGVT conserved amino acid sequence of SVMs from which primer 1 used in PCR was designed. The translation stop codon is indicated by an asterisk. The amino acid sequences of three fragments of CNBr-digested reduced alkylated graminelysin I, determined by Edman degradation, are underlined. The putative N- and C-terminus and the conserved zinc-chelating sequence (catalytic site) of graminelysin I are indicated. Graminelysin I is followed by a spacer region and a disintegrin-like/cysteine-rich domain. The N-terminal amino acid sequence of an approx. 30 kDa protein from fraction XI (see the legend to Figure 1) is shaded; SECD sequence in the disintegrin-like/cysteine-rich domain is shown in bold.

further purified with a Sephadex G-100 column (Figure 1B). The major fraction obtained was then applied to a Superdex 75 FPLC column (Figure 1C). Graminelysin I, which eluted as the major peak, was characterized. Graminelysin I migrated with a mobility of approx. 30 kDa under both reduced and non-reduced conditions as examined by SDS/PAGE (Figure 1C, inset). The molecular mass of graminelysin I was further determined as 27020 Da by matrix-assisted laser desorption/ionization-time-of-flight MS (results not shown). Graminelysin I possesses azocaseinolytic activity; this activity was inhibited in the presence of 5 mM EDTA (results not shown). The N-terminus of graminelysin I is likely to be blocked because an attempt to sequence S-pyridylethylated graminelysin I failed. S-pyridylethylated graminelysin I was therefore digested with CNBr; the cleaved fragments were further purified by SDS/PAGE and HPLC for sequence determination. Three partial sequences were obtained (underlined in Figure 2). These partial sequences have high similarity to members of the SVMs family (Figure 3). Taken together, these results indicate that graminelysin I is a single-chain metalloproteinase of small molecular size.

graminellysin I	QQRFF--PQRY	IKGLGIFFVDHG	MVTRKYSGN	SE	RITKRHHQMI	NNINNMCRAL	NIVTT	53
protrigramin	QQRFF--PQRY	IKGLGIFFVDHG	MVTRKYSGN	SE	RITKRHHQMI	NNINNMCRAL	NIVTT	53
jararhagin	QQRFF--PQRY	IKGLGIFFVDHG	MVTRKYSGN	SE	RITKRHHQMI	NNINNMCRAL	NIVTT	54
vap1	QQRFF--PQRY	IKGLGIFFVDHG	MVTRKYSGN	SE	RITKRHHQMI	NNINNMCRAL	NIVTT	55
graminellysin I	LSLLEIWSERK	DLTVQASAP	TLLTLLFGAWR	ETVLLNATSH	DHAQLLTATH	PNGNV	108	
protrigramin	LSLLEIWSERK	DLTVQASAP	TLLTLLFGAWR	ETVLLNATSH	DHAQLLTATH	PNGNV	108	
jararhagin	LSLLEIWSERK	DLTVQASAP	TLLTLLFGAWR	ETVLLNATSH	DHAQLLTATH	PNGNV	109	
vap1	LSLLEIWSERK	DLTVQASAP	TLLTLLFGAWR	ETVLLNATSH	DHAQLLTATH	PNGNV	110	
graminellysin I	LGRAPVVGGM	DKRSVAIVR	DHNAIVFVVA	VTMTTHENGHN	LGMHDEDDKC	NCNT	162	
protrigramin	LGRAPVVGGM	DKRSVAIVR	DHNAIVFVVA	VTMTTHENGHN	LGMHDEDDKC	NCNT	162	
jararhagin	LGRAPVVGGM	DKRSVAIVR	DHNAIVFVVA	VTMTTHENGHN	LGMHDEDDKC	NCNT	164	
vap1	LGRAPVVGGM	DKRSVAIVR	DHNAIVFVVA	VTMTTHENGHN	LGMHDEDDKC	NCNT	165	
graminellysin I	JCIMS KVLRS	QPSKYFSECS	KDYQTFLLN	HNQCCLNAP	LRTDVSFPV	SGNEL	216	
protrigramin	JCIMS KVLRS	QPSKYFSECS	KDYQTFLLN	HNQCCLNAP	LRTDVSFPV	SGNEL	216	
jararhagin	JCIMS KVLRS	QPSKYFSECS	KDYQTFLLN	HNQCCLNAP	LRTDVSFPV	SGNEL	219	
vap1	JCIMS KVLRS	QPSKYFSECS	KDYQTFLLN	HNQCCLNAP	LRTDVSFPV	SGNEL	220	
graminellysin I	LEAGEDCDG	SP...ANPCC	DAATCKLRPG	AQCGEGLCCD	QCRTSAGTR	CRAAR	268	
protrigramin	LEAGEDCDG	SP...ANPCC	DAATCKLRPG	AQCGEGLCCD	QCRTSAGTR	CRAAR	268	
jararhagin	LEAGEDCDG	SP...ANPCC	DAATCKLRPG	AQCGEGLCCD	QCRTSAGTR	CRAAR	274	
vap1	LEAGEDCDG	SP...ANPCC	DAATCKLRPG	AQCGEGLCCD	QCRTSAGTR	CRAAR	275	
graminellysin I	SECDIAESCA	GSADCPD	FHENGQPCLN	NHGYCYNGNC	PIHFYQCIAS	PGSNA	323	
protrigramin	SECDIAESCA	GSADCPD	FHENGQPCLN	NHGYCYNGNC	PIHFYQCIAS	PGSNA	290	
jararhagin	SECDIAESCA	GSADCPD	FHENGQPCLN	NHGYCYNGNC	PIHFYQCIAS	PGSNA	329	
vap1	SECDIAESCA	GSADCPD	FHENGQPCLN	NHGYCYNGNC	PIHFYQCIAS	PGSNA	329	
graminellysin I	CVGQGGCFDA	NOIGHKVFHC	RKNNEKYVPC	APQDVKCGRL	FC...TYIYDI	DLCRY	376	
protrigramin	CVGQGGCFDA	NOIGHKVFHC	RKNNEKYVPC	APQDVKCGRL	FC...TYIYDI	DLCRY	290	
jararhagin	CVGQGGCFDA	NOIGHKVFHC	RKNNEKYVPC	APQDVKCGRL	FC...TYIYDI	DLCRY	384	
vap1	CVGQGGCFDA	NOIGHKVFHC	RKNNEKYVPC	APQDVKCGRL	FC...TYIYDI	DLCRY	384	
graminellysin I	...DSSANG	MVAQGTTCAD	GKVCNENRAC	ADVNTAY			409	
protrigramin	...DSSANG	MVAQGTTCAD	GKVCNENRAC	ADVNTAY			290	
jararhagin	...DSSANG	MVAQGTTCAD	GKVCNENRAC	ADVNTAY			420	
vap1	...DSSANG	MVAQGTTCAD	GKVCNENRAC	ADVNTAY			420	

Figure 3 Comparison of the predicted amino acid sequence of the putative protein precursor of graminelysin I with members of the snake-venom zinc metalloproteinase and disintegrin family

Amino acid sequences of programinelsin I, protrigramin, jararhagin and vascular apoptosis-inducing protein1 (vap1) were translated from their corresponding cDNA sequences by the GCG program PILEUP. Amino acid residues proposed to be involved in the processing of the disintegrin(-like)/cysteine-rich domain are indicated by asterisks. This alignment was generated with the GCG program PILEUP. Amino acid residues identical with and similar to those of programinelsin I are shown with black shading and grey shading respectively.

Figure 4 Effect of graminelysin I on cell viability

HUVECs were seeded at 1.5×10^5 cells in 24-well plates. When cells had reached confluence the culture medium was replaced with Medium 199 supplemented with 2% (open columns) or 20% (filled columns) FBS in the presence of PBS (control) or the indicated concentrations of graminelysin I for 24 h. Cell viability was measured by MTT reduction. Results are expressed as percentages of control and are means $\pm$ S.E.M. All *P* values are for comparisons between control and graminelysin I-treated cells. **P* < 0.05, ***P* < 0.001; *n* = 3.

Cloning and sequence determination of the cDNA of graminelysin I precursor protein

Previous studies have suggested that SVMPs are processed post-translationally from their precursor proteins [22]. To obtain the cDNA sequence of graminelysin I precursor, the oligonucleotides were synthesized in accordance with the conserved KMCGVT sequence in the prodomain of SVMP and a XATVXXD sequence

stretch in the cysteine-rich domain. The oligonucleotides were used as primers to perform PCR with a cDNA library of *T. gramineus* venom. After the first amplification and cloning, several recombinant clones were obtained, one of which contained a cDNA fragment of approx. 1060 bp, representing the graminelysin I precursor from the KMCGVT sequence to the beginning of the cysteine-rich domain. By using an oligonucleotide primer that possessed a unique sequence at the 3' end specific for graminelysin I precursor, amplification of the cDNA fragments was extended to the 3' untranslated region. Figure 2 shows the assembled cDNA sequence and the deduced amino acid sequence of the graminelysin I precursor, which indicates that graminelysin I precursor, designated programinelsin I, consists of a prodomain, a metalloproteinase domain, a disintegrin-like domain and cysteine-rich domain. The metalloproteinase and disintegrin-like/cysteine-rich domains consist of 201 amino acid residues from a putative start site of the mature protein and 192 residues respectively and are linked by a spacer region of 16 residues. Although only a partial sequence of the prodomain was obtained, the sequence was very similar to the prodomain sequence of protrigramin [10]. The sequences of the three CNBr-cleaved fragments of graminelysin I were found in the predicted amino acid sequence of programinelsin I with 100% identity (Figure 2, underlined sequences). The characteristic zinc-chelating sequence, HEXHXXGXXH, is similar to those of other metalloproteinases, whereas the disintegrin-like domain contains a SECD sequence instead of the RGD sequence found in most disintegrins (Figure 2 sequence in bold).

To investigate whether programinelsin I was processed, producing graminelysin I and a disintegrin-like/cysteine-rich domain, we screened the corresponding disintegrin-like/

Figure 5 Histograms of flow cytometric cell cycle and patterns of electrophoretic DNA fragmentation in graminelysin I-treated HUVECs

(A) HUVECs treated with control PBS (panels a and e) or various concentrations of graminelysin I (panels b–d and f) in the absence (panels a–d) or presence of EDTA (panels e and f) for 24 h were permeabilized and fixed, then stained with propidium iodide containing RNase, and analysed by flow cytometry. Panel (a) shows the cell-cycle stage of control HUVECs. The sub- G_1 region represents cells undergoing apoptosis-associated DNA degradation and is expressed as a percentage (A_0) with respect to the entire cell cycle. (B) HUVECs cultured in Medium 199 supplemented with 2% (lanes 2 and 3) or 20% (lanes 4 and 5) FBS were treated with PBS (lanes 2 and 4) or graminelysin I (40 μ g/ml) (lanes 3 and 5) for 24 h. DNA was isolated and analysed on a 1% (w/v) agarose gel. Lane 1 was loaded with a 100 bp DNA ladder marker (PROtech, Taipei, Taiwan). The photograph shows a representative result from three independent experiments.

cysteine-rich domain by comparing the pI of this domain with other proteins that have been isolated and characterized from the venom of *T. gramineus* in our laboratory. The N-terminus of an approx. 30 kDa protein in the fraction XI from DEAE-ion-exchange chromatography of *T. gramineus* venom (Figure 1A) was sequenced. The N-terminal sequence (EAGEDCDCGS) is identical with the disintegrin-like/cysteine-rich domain of programinlysin I (Figure 2, shaded sequence).

The predicted amino acid sequences of programinlysin I were aligned with other SVMP family members, including protrigramin, jararhagin and VAP1 (Figure 3). Programinlysin I has a proteinase domain with an amino acid sequence completely identical with that of protrigramin except for Leu⁵⁶. A Val $\rightarrow$ Leu replacement also occurred at the fourth position upstream of the putative start site of programinlysin I (Figure 2) [10]. Interestingly, residues Thr²⁰⁶, Thr²⁰⁹, Ser²¹² and Ala²¹⁹, which were proposed to be conserved among processed metalloproteinases, were also conserved in programinlysin I and protrigramin. Those residues are replaced by Ile²⁰⁶, Pro²⁰⁹, Cys²¹² and Val²¹⁹ respectively in the unprocessed, disintegrin-like/cysteine-rich domain-containing metalloproteinases such as jararhagin [22]. These indicate that graminelysin I might be a gene product processed post-translationally. Graminelysin I contains only a proteinase domain and belongs to the P-I class of repolysin subfamily of the metalloproteinase [23].

A prediction by the program GCG indicated that graminelysin I is a basic proteinase with an isoelectric point of 8.83, whereas the pI of the processed disintegrin-like/cysteine-rich domain is estimated to be 5.6.

Vascular cell apoptosis induced by graminelysin I

A typical pattern of apoptosis includes internucleosomal DNA degradation, DNA fragmentation and morphological changes. HUVECs that were cultured in the medium supplemented with 2% or 20% FBS were treated with various concentrations of graminelysin I at 37 °C for 24 h, the cell viability was decreased in a concentration-dependent manner as measured by performing

MTT assay (Figure 4). As shown in Figure 4, HUVECs cultured in 2% FBS-containing medium with graminelysin I were susceptible to death. Furthermore, graminelysin I induced morphological changes in HUVECs in a dose-dependent manner as examined by phase-contrast microscopy (results not shown). The effect of graminelysin I on HUVECs was not due to cytotoxicity because lactate dehydrogenase released to the medium was not affected compared with control (containing PBS only) (results not shown). In addition, graminelysin I (20 μ g/ml) exhibited a weaker effect on human smooth-muscle cells under the same conditions (results not shown), indicating that the effect of graminelysin I is relatively specific to vascular endothelial cells. To determine the mechanism of graminelysin I in decreasing the cell viability of HUVECs, the DNA contents of graminelysin I-treated HUVECs were examined by flow cytometry. As shown in Figure 5(A), control HUVECs (panel a) showed a typical cell cycle stage, consisting of a major diploid peak (G_0/G_1), a small hyperdiploid region (S) and a minor tetraploid peak (G_2/M). However, exposure of HUVECs to graminelysin I (20 and 40 μ g/ml) resulted in a significant decrease in the proportion of G_2/M -phase cells, concomitant with the appearance of cells with a fractional DNA content (sub- G_1). The increase in the fractional DNA content occurred in a dose-dependent manner. The increase detected in the hypodiploid cells (designated as A_0) (panels b–d) reflected that cells had undergone apoptosis-associated DNA degradation. The effect of graminelysin I in inducing apoptosis of HUVECs was confirmed by DNA fragmentation analysis. As shown in Figure 5(B), DNA isolated from PBS-treated HUVECs cultured in medium containing 20% FBS migrated as a single band with high molecular mass (lane 4). A little DNA fragmentation was observed with DNA isolated from HUVECs cultured in medium containing 2% FBS (Figure 5B, lane 2). Graminelysin I (40 μ g/ml) induced an apparent DNA fragmentation (Figure 5B, lanes 3 and 5). The internucleosomal DNA degradation and DNA fragmentation were inhibited when graminelysin I pretreated with 2 mM EDTA was added to the HUVECs and continuously cultured in the presence of 2 mM EDTA (Figure 5A, panel f, and results not shown).

Figure 6 Effect of graminelysin I on HUVECs adhesion and detachment

(A) Adhesion of HUVECs to immobilized fibrinogen and collagen. HUVECs collected as described in the Experimental section were incubated with PBS or various concentrations of graminelysin I pretreated with PBS or 2.5 mM EDTA, as indicated, at 37 °C for 30 min. Adhesion was performed by adding cells to 96-well plates precoated with fibrinogen (panel a) or collagen (panel b) for 1.5 h at 37 °C and measured with a CytoFluor fluorescence plate reader. (B) Detachment of HUVECs from immobilized fibrinogen. HUVECs were collected and allowed to attach to fibrinogen-precoated plates for 3 h at 37 °C. Detachment assay was performed in the absence (control) or presence of graminelysin I pretreated with PBS or 2.5 mM EDTA as indicated. After incubation for 1 h, non-adherent cells were removed and adherent cells were measured with a CytoFluor fluorescence plate reader. Results are presented as percentages of control (non-detached cells) and are means $\pm$ S.E.M. ($n = 4$).

Graminelysin I inhibits HUVEC adhesion to immobilized fibrinogen and induces HUVEC detachment from immobilized fibrinogen

To investigate the possible mechanism of action of graminelysin I on endothelial cells, the effect of graminelysin I on the adhesion of HUVECs to immobilized fibrinogen was examined. Graminelysin I inhibited the adhesion of HUVECs to immobilized fibrinogen in a concentration-dependent manner (Figure 6A, panel a). In contrast, graminelysin I showed little inhibition of the adhesion of HUVECs to collagen even at high concentration (80 μg/ml) (Figure 6A, panel b). Graminelysin I also detached the adherent HUVECs from immobilized fibrinogen (Figure 6B). However, pretreatment of graminelysin I with 2.5 mM EDTA for 15 min almost abolished the effects of graminelysin I (Figures 6A and 6B).

Effect of graminelysin I on cleaving fibrinogen and collagen

To examine whether graminelysin I possesses catalytic activity

Figure 7 Graminelysin I cleaves fibrinogen in a concentration- and time-dependent manner

Graminelysin I was added to human fibrinogen (20 mg/ml, 15 μl) to a final concentration of 10 μg/ml (A), 20 μg/ml (B) or 40 μg/ml (C) at 37 °C. At the indicated time points, a 5 μl aliquot of each reaction mixture was removed and the reactions were immediately stopped by adding 5 × sample buffer containing 2-mercaptoethanol (1.8 M). The reaction products were analysed by SDS/PAGE [7.5% (w/v) gel] followed by staining with Coomassie Blue.

Table 1 Comparison of collagenolytic activity of graminelysin I with those of collagenases from other sources

FITC-labelled collagen was incubated with various concentrations of graminelysin I for 1.5 h as described in the Experimental section. The collagenolytic activity of graminelysin I was calculated from the standard curve of standard type I collagenase and is expressed as mean $\pm$ S.E.M. The collagenolytic activities of vertebrate collagenases adapted from [24] are indicated by asterisks.

Source of proteinase	Activity (units/mg)
Graminelysin I	5 $\pm$ 0.9 ($n = 3$)
Standard collagenase (MMP-1)	119
Human rheumatoid synovium	312*
Rabbit tumour V2 ascites cell in muscle	3583*
Human skin	958*
Mouse bone	2850*
Pig synovium	26300*
Tadpole back skin	8400*

towards matrix proteins, human fibrinogen was incubated with graminelysin I at 37 °C for various periods and the reaction products were analysed by SDS/PAGE. Graminelysin I cleaved the α chain of fibrinogen preferentially and the β chain when the incubation time or the concentration was increased, but had little effect on the γ chain of fibrinogen (Figure 7). In addition, the collagenolytic activity of graminelysin I was examined with the use of a type I collagenase assay kit. As shown in Table 1, the collagenolytic activity of graminelysin I (5 $\pm$ 0.9 units/mg) was weaker than those of the standard collagenase (119 units/mg)

Figure 8 Effect of graminelysin I on HUVECs cultured on different matrices

HUVECs were cultured on 24-well plates precoated with fibrinogen (100 µg/ml), type I collagen (100 µg/ml) or PBS (control). When the culture cells had reached confluence, the culture medium was replaced with Medium 199 supplemented with 20% (v/v) FBS containing 0–40 µg/ml of graminelysin I as indicated, then incubated at 37 °C for a further 24 h. Cell viability was measured by inhibition of MTT reduction; the results are expressed as percentages of control and are means $\pm$ S.E.M. * P < 0.05, ** P < 0.001 compared with control (n = 3).

and other collagenases (312–26300 units/mg) purified from various sources [24].

HUVECs cultured on collagen are less sensitive to graminelysin I-induced cell death

Cultured endothelial cells secrete stromal components in the culture medium *in vitro* and in the ECM *in vivo* [25]. On the basis of the results shown above, it was possible that graminelysin I degraded ECM secreted by HUVECs, resulting in the detachment of HUVECs. To test this hypothesis, HUVECs were cultured on plates precoated with fibrinogen, collagen or PBS control and incubated with graminelysin I for 24 h. The MTT assay demonstrated that HUVECs cultured on fibrinogen and polystyrene (PBS control) were susceptible to death caused by graminelysin I treatment, whereas HUVECs cultured on collagen-precoated plates were less sensitive to graminelysin I-induced cell death (Figure 8).

DISCUSSION

SVMPs have been shown to induce haemorrhage, to inhibit platelet aggregation and to degrade ECMs. The classification for the SVMPs was based on the size of the cDNA/mRNA precursor [Nucleotide-based (N) class] or the size of the mature protein as isolated from the venom [protein-based (P) class]. The P class can be divided into four groups, P-I, P-II, P-III and P-IV, which differ from one another primarily by the presence of additional domains on the carboxy side of the metalloproteinase domain [8]. On the basis of the molecular mass and cDNA sequence, graminelysin I contains only a proteinase domain and belongs to the P-I class. In addition, graminelysin I had little inhibitory effect (less than 10% inhibition) on thrombin- and collagen-induced platelet aggregation even when preincubated with platelets for 20 min (results not shown). This might be due to the absence of an RGD- or KGD-like-containing disintegrin domain. Partial amino acid sequencing of the three fragments of the CNBr-digested alkylated graminelysin I and the matched corresponding nucleotide sequence of cDNA clones clearly suggests that graminelysin I is a metalloproteinase possessing only a proteinase domain.

Processing of P-III SVMPs generates disintegrin-like/cysteine-rich peptides, such as jararhagin-C and catrocollastatin-C [26,27]. However, only a few reports have indicated that P-I SVMP was post-translationally processed from P-II or P-III SVMP. The protease domain of atrolysin E (formerly known as Ht-e), a P-I SVMP from *C. atrox*, has been suggested to be processed from its P-II precursor [28]. The disintegrin domain of atrolysin E has been isolated from *C. atrox* [29]; in addition, recombinant proatrolysin E has been shown to be proteolytically processed by atrolysin A [30]. However, there is evidence that the disintegrin-like or disintegrin-like/cysteine-rich domain is autoproteolytically released from SVMPs *in vitro* [31,32]; the mechanisms of post-translational processing of P-II or P-III SVMPs are still unclear in snake venom. In our study, the corresponding cDNA sequence of graminelysin I precursor (N-III SVMP) was obtained from two overlapping cDNA clones. Although the amino acid sequence of the proteinase domain of programinelysin I is very similar to that of protrigramin, the cDNA clones of programinelysin I that we obtained are unlikely to be artifacts that occurred during the cloning procedures. First, the first cDNA clone ended in the cysteine-rich domain but not in the proteinase domain. The second cDNA clone was extended from a unique internal sequence of the first clone. The overlapping nucleotide sequences of the two cDNA clones are fully identical and distinguishable from other SVMPs (Figure 3). Secondly, direct sequencing of the N-terminus of an approx. 30 kDa protein in fraction XI of DEAE-ion-exchange chromatography revealed that the corresponding disintegrin-like/cysteine-rich domain of the programinelysin I might exist in *T. gramineus* venom (Figures 1A and 2).

Adhesion of cells to ECM has been shown to serve as a survival mechanism in several anchorage-dependent cell types. Primary cells of epithelial and endothelial origin rapidly undergo apoptosis when denied proper substrate adhesion [33]. Here we show that graminelysin I, a natural metalloproteinase, induced apoptosis of vascular endothelial cells when it was incubated with HUVECs for 24 h. The biochemical characteristics of apoptosis in HUVECs were further examined by electrophoresis and flow cytometry (Figure 5). Several lines of evidence indicate that the catalytic activity of graminelysin I towards matrix proteins is responsible for its apoptotic activity. First, graminelysin I cleaved the α and β chains of fibrinogen (Figure 7). Secondly, graminelysin I inhibited the adhesion of HUVECs to immobilized fibrinogen and detached adherent HUVECs (Figure 6), and these effects were abolished when graminelysin I was pretreated with EDTA. Thirdly, in the presence of EDTA, apoptosis of HUVECs induced by graminelysin I was blocked (Figure 6A). Fourthly, after pretreatment with graminelysin I (20 µg/ml), HUVECs probed with monoclonal antibodies raised against α_2 , β_1 , α_3 or $\alpha_v\beta_3$ integrins did not show a significant decrease in fluorescence intensity as examined by flow cytometry (results not shown). Cultured HUVECs are known to secrete matrix components and to attach to substratum. Therefore one of the action mechanisms of graminelysin I seems to be to degrade fibrinogen or other components in the matrix, detaching HUVECs from the matrix and inducing apoptosis. One further piece of evidence to support this hypothesis is that HUVECs cultured on fibrinogen matrix were more susceptible to death than those cultured on collagen matrix (Figure 8). These results are correlated with the weakest MMP-1 (collagenase) activity of graminelysin I (5 units/mg) in comparison with the standard MMP-1 (119 units/mg) used and other collagenases purified from various sources (more than 312 units/mg).

It has been shown that fibrinogen can induce endothelial cell attachment, spreading and migration [34,35]. Human fibrinogen

contains three potential integrin-binding sites, two RGD sequences within the A α chain [RGDS (residues α 572–575) and RGDF (residues α 95–98)] and a non-RGD sequence in the γ chain (C-terminal γ 400–411). Several distinct receptors have been implicated in mediating fibrinogen binding to endothelial cells; these include $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin receptors [36]. It remains to be determined whether the cleavage of the α and β chains of fibrinogen might affect the interaction between cells and matrix. In addition, it has been reported that the fibrinogen degradation product, fragment D, induces endothelial cell detachment by the activation of cell-mediated fibrinolysis [37]. Endothelial cells secrete stromal and ECM components, including collagen I, II, thrombospondin, fibronectin, elastin and proteoglycan. Nevertheless, there are few reports mentioning the possibility of fibrinogen secretion by cultured endothelial cells. It has been reported that four haemorrhagic metalloproteinases (Ht-a, Ht-c, Ht-d and Ht-e) are capable of cleaving fibronectin, laminin, type IV collagen, nidogen and gelatin [38]. Besides, it is known that forward-angle light scatter, which relates to cell diameter, is decreased during the initial stages of apoptosis [1]. We found that the forward-angle light scatter of HUVECs was decreased after treatment with FITC-labelled graminelysin I as examined by flow cytometry (results not shown). It therefore remains to be elucidated whether graminelysin I affects other steps such as the direct proteolytic effect on other ECM components (such as laminin and fibronectin) or whether the fragments from graminelysin-I-cleaved matrix components affect the viability of HUVECs directly or indirectly.

Comparisons of the structural organization of graminelysin I and VAP1 apoptosis-inducing protein reveal that graminelysin I has only a putative metalloproteinase domain but VAP1 possesses an additional disintegrin-like and a cysteine-rich domain. The apoptosis-inducing capability of graminelysin I seems to be weaker than those of VAP1 and VAP2. Besides, graminelysin I (1.48 μ M) induced apoptosis of human smooth-muscle cells to a smaller extent than did VAP1 and VAP2. The disintegrin (-like) and cysteine-rich domains are important for metalloproteinase function. Jia et al. [39] have suggested that ESECD in the disintegrin-like domain of atrolisin A is important in inhibiting platelet aggregation induced by ADP and collagen. Besides, a recombinant cysteine-rich domain of atrolisin A interacted with MG-63 osteosarcoma cells to inhibit cell adhesion to type I collagen [40]. It is unknown whether the presence of additional domains on the carboxy side of the metalloproteinase domain affects the specificity and potency of induction of apoptosis. The differences in apoptosis-inducing capability between graminelysin I and VAP metalloproteinase might be due to differences in their structural composition or in experimental conditions. The relationships between its molecular structure and the apoptosis-inducing capability of these metalloproteinases need to be further clarified.

In conclusion, we have determined the amino acid sequences of graminelysin I isolated from the venom of *T. gramineus*. Graminelysin I induced apoptosis in human endothelial cells, probably owing to the degradation of matrix proteins leading to cell detachment. The findings and concepts disclosed here provide a sound and important basis for a further mutagenesis experiment towards understanding the structure–activity relationship. In addition, graminelysin I might be used as a tool for studying the mechanism of vascular endothelial cell apoptosis.

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