The serine protease granzyme A does not induce platelet aggregation but inhibits responses triggered by thrombin*

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Granzyme A is a serine protease stored in cytoplasmic granules of cytotoxic and helper T lymphocytes. This protease seems to elicit thrombin receptor-mediated responses in neural cells, thereby triggering neurite retraction and reversal of astrocyte stellation. Here we report that granzyme A does not cause platelet aggregation even at concentrations that are more than two orders of magnitude higher than the EC_{50} for granzyme A in causing morphological changes in neural cells. However, granzyme A blocks thrombin-induced platelet aggregation in a dose-dependent manner without affecting the response to either ADP or to the peptide agonist of the thrombin receptor SFLLRN that corresponds in sequence to the tethered ligand domain. The inability of granzyme A to cause aggregation and its inhibition of thrombin-induced aggregation were seen in platelets from man, rat and mouse. Granzyme A does not affect the catalytic activity of thrombin in cleaving a chromogenic substrate or the macromolecular substrate fibrinogen. However, granzyme A does seem to cleave the thrombin receptor on platelets to produce a weak Ca^{2+} signal and reduce the response to subsequent challenge with thrombin, but does not induce a signal in thrombin-stimulated platelets. It is proposed that granzyme A interacts with the thrombin receptor found on platelets in a manner that is insufficient to cause aggregation, but sufficient to compete with thrombin for the receptor. These results suggest that granzyme A cleaves the thrombin receptor at a rate that is insufficient to cause platelet aggregation but is sufficient to cause morphological changes in neural cells. Furthermore, these observations demonstrate that granzyme A release occurring during immune responses within blood vessels would not directly cause platelet aggregation.

INTRODUCTION

Granzymes are a family of serine proteases found in granules of cytotoxic and helper T lymphocytes [1]. These proteases are released from their cytoplasmic stores when the T-cell interacts with its target. The role played by the granzymes in cytolytic and other immune responses remains incompletely understood. The best characterized proteases of this family are granzymes A and B. When purified granzymes A and B are added to target cells together with the pore-forming protein perforin, cells die rapidly by apoptosis [2] and the degradation of nuclear DNA is greatly impaired in target cells lysed by T lymphocytes devoid of granzyme B [3]. Similarly to thrombin, granzyme A hydrolyses synthetic substrates after basic amino acids, with preference for arginine and lysine, whereas granzyme B cleaves substrates after aspartate [4,5]. Evidence was recently presented that the thrombin receptor in neural cells can be a target for granzyme A [6].

The thrombin receptor that is activated by cleavage, abbreviated here ThR, is a G-protein-coupled receptor with seven transmembrane domains [7,8]. Thrombin is thought to bind through its anion-binding exosite to an acidic region within the extracellular domain of the receptor, followed by cleavage between Arg^{41} and Ser^{42} of the human, hamster and murine receptors [7–9] or Arg^{45} and Ser^{46} of the rat receptor [10]. The newly created N-terminus then acts as a tethered ligand that causes receptor activation through interaction with the proximal N-terminal exodomain and the second extracellular loop [11]. Exogenously added synthetic peptides corresponding in sequence to the newly created N-terminus induce a thrombin-like response in *Xenopus* oocytes microinjected with ThR mRNA, in platelets, in neural cells and in other systems [7,12–17]. Neural cells, *in vivo* or *in vitro*, express the ThR mRNA that is found in platelets [12,18–20]. In cultured neural cells, granzyme A causes neurite retraction and reversal of astrocyte stellation in a manner indistinguishable from that induced by thrombin [6]. These effects were dependent on the proteolytic activity of granzyme A. Furthermore, a synthetic peptide spanning the cleavage site of the ThR is cleaved by granzyme A and antibodies raised against domains within the newly created N-terminus of the ThR block the effects induced by either thrombin or granzyme A in neural cells [6]. As brain-infiltrating $CD4^+$ lymphocytes are the effector cells in experimental allergic encephalomyelitis, we proposed that an aberrant activation of the ThR by granzyme A might contribute to the aetiology of autoimmune disorders in the nervous system.

The observation that granzyme A activates the ThR (or a homologous receptor with a similar activation sequence) on neural cells raised the possibility that this protease also cleaves the receptor in other structures, such as platelets. This seemed important in the context of modulation of platelet function by granzyme A in blood, in response to an immune attack occurring in the vessels. We therefore examined whether granzyme A causes platelet aggregation.

Here we report that granzyme A not only fails to induce platelet aggregation but also inhibits the aggregation caused by thrombin. Granzyme A is shown to induce a weak Ca^{2+} signal and to inhibit Ca^{2+} signalling by thrombin. We suggest that, through a weak interaction with the ThR found on platelets,

Abbreviations used: GPIb, glycoprotein Ib; ThR, thrombin receptor.

* We dedicate this work to Carlo Tapparelli, who died in May 1995. Carlo generously opened his laboratory to us, thereby providing the opportunity to begin this investigation. His good spirit will be dearly missed.

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granzyme A does not cause aggregation but reduces the thrombin response via the receptor. The functional significance of this apparent selectivity in inducing ThR-mediated responses in one system but not in another is discussed.

MATERIALS AND METHODS

Materials

Granules of the murine B6.1 cytolytic T lymphocyte cell line were the source of granzyme A preparations; these were only slightly contaminated by granzyme C [21]. Human α-thrombin previously characterized and prepared as described [22] was a gift from Dr. Stuart Stone, University of Cambridge, Cambridge, U.K. Recombinant rat protease nexin-1 was used [23]. Glycoprotein Ib (GPIb) and glycocalicin were prepared as described [24]. ADP was purchased from Boehringer Mannheim and the chromogenic substrate S-2288 from Chromogenix, Mölndal. ThR peptide SFLLRN was synthesized (9050 PepSynthesizer, Milligen), purified by HPLC, its molecular mass ascertained by mass spectroscopy and its composition verified by amino acid analysis, or it was purchased from Bachem.

Platelet aggregation assay *in vitro*

Washed platelets from man, rat and mouse were prepared as previously described [25]. Human platelet-rich plasma was obtained from the Kantonsspital, Basel. The blood of 2–3-month-old rats (Sprague–Dawley genetic background) and mice (BALB/c) was collected on citrate from anaesthetized animals by direct cardiac puncture. The animals were supplied by the Ciba Animal Facilities, Sisseln. The assay buffer (pH 7.4) included 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 10 mM Hepes, 0.35% BSA and 0.1 unit/ml apyrase. The washed platelet count was adjusted to 3.3×10^8/ml and the starting volume of the suspension was between 270 and 480 μl. Assays were performed in a Payton aggregometer (Merz and Dade) in which the platelets were stirred at 1100 rev./min at 37 °C.

Measurement of platelet-free Ca^{2+} concentrations with fura-2

Human platelets were isolated from citrate-treated buffy coats from the Central Laboratory of the Transfusion Service of the Swiss Red Cross, approx. 15 h after blood collection, and diluted with citrate buffer (30 mM sodium citrate, 100 mM NaCl, 3 mM KCl and 9.6 mM glucose, pH 6.5), then centrifuged to yield platelet-rich plasma. The platelets were pelleted by centrifugation, 0.5 ng/ml Iloprost (Schering) was added, washed twice (137 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 5.6 mM glucose and 5 mM Hepes, pH 6.5) and resuspended in the same buffer to a count of 10^9/ml. A platelet suspension (4.4×10^8 platelets, enough for four experiments) was incubated for 20 min at 37 °C with 1 μM fura 2 AM (Fluka; 0.23 nmol of fura 2 AM per 10^9 platelets) dispensed from a 1 mM stock solution in DMSO. Further loadings were carried out at 10 min intervals to allow an uninterrupted supply. Fura 2-loaded platelets (10^8/ml) were washed in test buffer and then stimulated while being magnetically stirred in 1 ml polystyrene cuvettes at 37 °C. Agents were added with a microsyringe. A 100 W mercury lamp was used for excitation through a bandpass filter 10 nm wide with maximum transmission at 334 nm. Broadband fluorescence above 486 nm was separated from the exciting light by a cut-off filter and was detected by an EMI 9817B photomultiplier. The fluorescence of fully saturated fura 2 (F_{max}) was obtained by adding 1 μM ionomycin. The fluorescence was fully quenched with 1 mM $MnCl_2$. Data were converted into $[Ca^{2+}]$ by using a K_d value of 135 nM for fura 2 and Ca^{2+} [26].

Preparation of fibrinogen gels

Fibrinogen gels were prepared by mixing two solutions as follows: (1) nine volumes of a 45 °C-heated solution of 1% (w/v) agarose (DNA grade, Bio-Rad), 10 mM Hepes, 144 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$ and 165 μM $MgSO_4$, pH 7.4, and (2) one volume of a 37 °C-heated solution of 20 mg/ml fibrinogen (from bovine plasma, Serva) in the above buffer that also included 1% (w/v) glucose and 0.35% BSA. The mixture was poured without delay on to a 37 °C-heated glass plate. Gels were 80 cm^2 in surface area and 6 mm in thickness. Plastic rings of approx. 6 mm inner diameter were siliconized on one rim and placed on the gel. Proteases with or without inhibitors were prepared in a solution that contained buffer, salts, glucose and BSA at the above-indicated final concentrations.

RESULTS

Granzyme A does not cause platelet aggregation and inhibits the response to thrombin

Figure 1A shows that the murine T lymphocyte-derived granzyme A at concentrations between 30 and 100 nM did not cause aggregation of washed human platelets. Moreover, granzyme A inhibited thrombin-induced platelet aggregation in a dose-dependent manner, without affecting the ability of platelets to aggregate in response to ADP. At 100 nM granzyme A completely blocked the aggregation caused by 1 nM thrombin, but at 30 nM it had no apparent effect. The rate of aggregation caused by 1 nM thrombin declined when granzyme A was present at 50 nM. The inhibitory effect of 100 nM granzyme A was overcome by thrombin at concentrations above 1 nM (results not

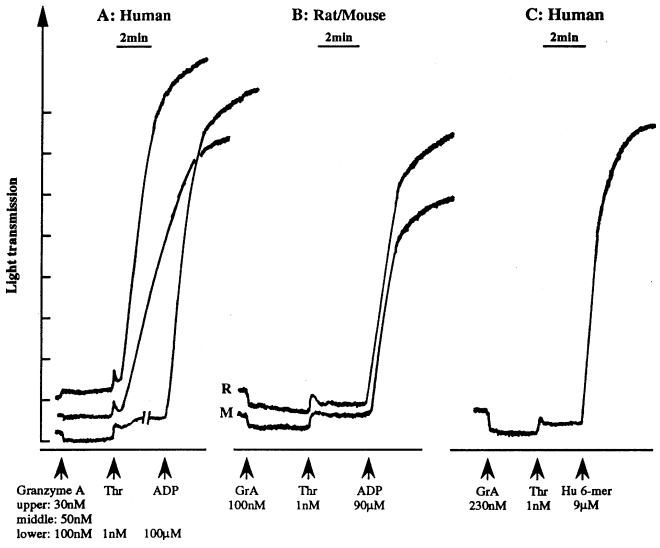

Figure 1 Granzyme A does not cause platelet aggregation

Human (**A** and **C**) and rat (R) and mouse (M) (**B**) platelets were isolated and assayed for aggregation as described in the Materials and methods section. The concentrations were as indicated at the time of the particular addition but decreased by 5–10% owing to the dilution effect caused by the next addition. In (**A**), thrombin-induced aggregation in the presence of 30 nM granzyme A was indistinguishable from that seen with thrombin alone (results not shown). The interruption in the lower trace of (**A**) was for 6 min. Abbreviations: Thr (thrombin), GrA (granzyme A) and Hu 6-mer (human ThR peptide SFLLRN).

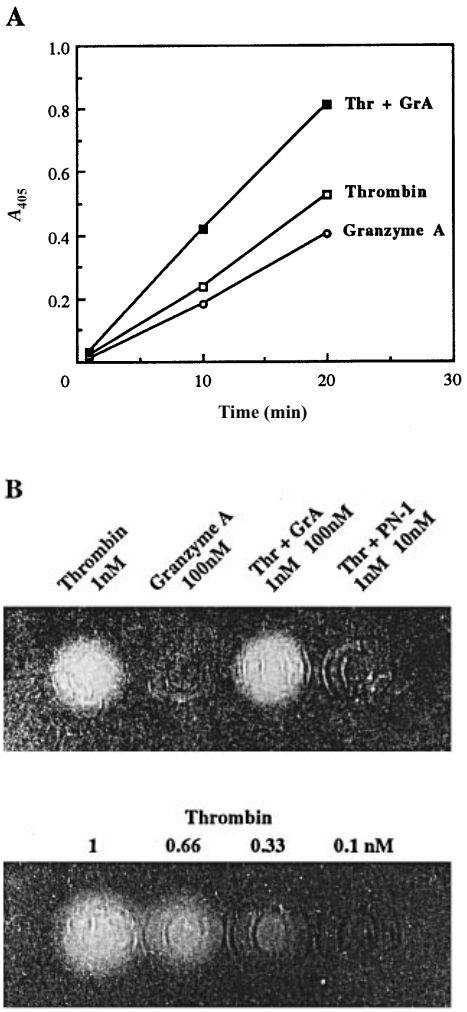

Figure 2 Granzyme A does not inhibit the proteolytic activity of thrombin

(**A**) Chromogenic assay. The assay was carried out in the presence of the chromogenic substrate S-2288 (*H*-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide dihydrochloride; 1.3 mM) in 100 μl of buffer containing 133 mM NaCl, 67 mM Tris/HCl, pH 8.0, and 0.13% polyethylene glycol 6000 in a 96-well microtitre plate under humid conditions at 37 °C. Thrombin (Thr) and granzyme A (GrA) were at 1 and 100 nM respectively. Additions at 15 s intervals were in the order: (1) buffer + thrombin, (2) granzyme A, and (3) substrate. The reaction was stopped at the times indicated by adding 35 μl of a 50% (v/v) aqueous solution of acetic acid. Absorbance at 405 nm was monitored in a microplate reader (Molecular Devices). (**B**) Fibrinogen cleavage test. Fibrinogen/agarose gels were prepared as described in the Materials and methods section. Thrombin, granzyme A and protease nexin-1 (PN-1) were diluted in 144 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$ and 165 μM $MgSO_4$, 10 mM Hepes, 1% (w/v) glucose and 0.35% BSA, pH 7.4. Solutions containing the indicated agents were preincubated for 5 min at 37 °C; 10 μl samples were added to wells created by placing siliconized plastic rings on the gel surface. The gel was then incubated under humidified conditions for 19 h at 37 °C, after which the rings were removed and photographs taken. Opacity indicates the polymerization of fibrin after fibrinogen cleavage.

shown). Granzyme A failed to cause platelet aggregation at concentrations between 1 and 230 nM. Furthermore, no aggregation could be detected in response to 100 nM granzyme A even in the presence of 0.1 mg/ml human fibrinogen or when platelets were initially sensitized by submaximal concentrations of ADP with or without fibrinogen (results not shown).

To determine whether the inability of the murine granzyme A to cause aggregation of human platelets was due to weak reactivity across species, the responsiveness of mouse and rat platelets to this protease was examined. Figure 1B shows that 100 nM granzyme A did not cause aggregation of rodent platelets and prevented thrombin-induced aggregation, whereas the ability to respond to ADP remained. As with human platelets, concentrations of up to 230 nM were ineffective in causing aggregation (results not shown). Thus the inability of the murine granzyme A to cause aggregation and its inhibition of the thrombin effect were not due to poor receptor recognition resulting from species differences.

The inability of granzyme A to cause platelet aggregation was unexpected because it had previously been shown that this protease activates the ThR expressed by rodent neural cells with an EC_{50} value of approx. 1 nM [6]. These results raised the possibility that there is a connection between the inability of granzyme A to cause aggregation and its inhibition of the thrombin-induced effect. In such a case, a possible simple explanation is that granzyme A is unable to cleave the ThR found on platelets at a rate that would trigger an aggregation response. Accordingly, granzyme A and thrombin could compete for binding to the same molecule, resulting in an inhibition of thrombin-induced platelet aggregation. Evidence supporting this hypothesis is provided below.

Granzyme A does not inactivate the ThR

Granzyme A could interact with the ThR on platelets in a manner that would cause receptor inactivation. Data presented in Figure 1C strongly argue against this possibility: granzyme A-treated human platelets that did not aggregate in response to thrombin did so on addition of the ThR-activating peptide SFLLRN.

The possibility that granzyme A cleaves the acidic region of the ThR (human sequence, Lys^{51}–Glu^{60} [7,8]) and thereby prevents the binding of thrombin through its anion-binding exosite is also unlikely. First, there is no putative granzyme A cleavage site within this stretch of receptor sequence. Secondly, previous experiments showed that the human ThR peptide Pro^{29}–Glu^{60} spanning the cleavage and the binding sites is hydrolysed by either thrombin or granzyme A only at the putative cleavage site ProArg↓Ser^{42} [6].

Granzyme A does not affect the proteolytic activity of thrombin

Thrombin is cleaved by autolysis or by a number of serine proteases, such as trypsin, α-chymotrypsin, elastase and a mast-cell-derived protease [27–32]. It was therefore possible that granzyme A inhibited thrombin-induced aggregation by directly or indirectly affecting the proteolytic activity or the binding ability of thrombin. To test these possibilities we examined first whether granzyme A alters the catalytic activity of thrombin in cleaving a small substrate. The chromogenic substrate S-2288 was used because it is cleaved at a higher rate by thrombin than by granzyme A. Figure 2A shows that the rate of cleavage of this substrate by 1 nM thrombin was not inhibited in the presence of 100 nM granzyme A. When present together, no sign of any cross-inhibition was seen: the rate of substrate cleavage by the two proteases was nearly additive. Additivity in the rate of cleavage was also seen when the chromogenic substrate was used at a concentration close to the K_m for thrombin (used at 10 μM; K_m for thrombin is 3 μM; results not shown). Secondly, we examined whether the ability of thrombin to bind to macromolecular substrates through its anion-binding exosite was affected by granzyme A. To test this possibility, fibrinogen was chosen as a substrate because it is not cleaved by granzyme A (J. Tschopp, unpublished work) and because the thrombin anion-binding exosite is crucial for binding to fibrinogen [33–35] as it is

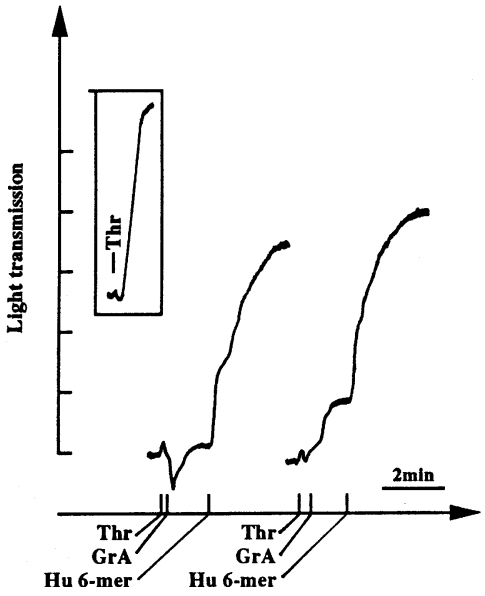

Figure 3 Granzyme A blocks platelet aggregation initiated by thrombin

Final concentrations of thrombin (Thr), granzyme A (GrA) and the human ThR peptide SFLLRN (Hu 6-mer) were 1 nM, 100 nM and 100 μM respectively after addition to human platelets. Granzyme A was added either 0.25 or 0.5 min after thrombin (left and right traces respectively). The insert (scale reduced by 30%) shows the response to thrombin without granzyme A.

for binding to the ThR [36] and to other substrates such as thrombomodulin [35,37], heparin cofactor II [38], hirudin [37,39] and single-stranded DNA aptamers [40]. Figure 2B shows that 100 nM granzyme A did not cause the formation of detectable fibrin polymers or alter the extent of polymerization induced by 1 nM thrombin. However, the potent thrombin inhibitor protease nexin-1 [41,42] completely abolished the formation of opacity induced by thrombin (Figure 2B, upper row). The sensitivity of this assay was sufficient to detect relatively small changes in the ability of 1 nM thrombin to cleave fibrinogen (Figure 2B, lower row). It therefore seems that neither the catalytic activity of thrombin nor the activity that is dependent on the anion-binding exosite is affected by granzyme A.

Another possibility was that granzyme A indirectly affected the proteolytic activity of thrombin by inducing platelets to release an inhibitor. A form of protease nexin-1, for example, is released from platelets on activation [43,44]. However, no difference in thrombin inhibitory activity (as measured in a chromogenic assay) was detected between the supernatants of untreated and of granzyme A-treated platelets (results not shown).

Taken together, these results indicate that the inability of thrombin to cause aggregation of granzyme A-treated platelets is not due to an alteration of thrombin properties as a proteolytic agonist of the ThR.

Granzyme A can interrupt an aggregation response triggered by thrombin

If granzyme A and thrombin compete for interaction with the same receptor, it is predicted that the addition of granzyme A to platelets that are being stimulated by thrombin will attenuate the aggregation response. Figure 3 shows that when granzyme A was added to human platelets 0.25 or 0.5 min after stimulation by thrombin, the aggregation response was indeed muted. However, aggregation was resumed after a challenge with the ThR peptide agonist SFLLRN. These observations are most readily explained by competition between thrombin and granzyme A for the same receptor.

Granzyme A cleaves GPIb inefficiently, primarily at a site not directly involved in thrombin binding

Because the effects of granzyme A on the platelet response to thrombin can be overcome by raising the thrombin concentration, it was possible that cleavage of GPIb was involved. GPIb contains a thrombin-binding site and modulates the response of platelets to this protease. Numerous proteases that affect the platelets' response to thrombin have been shown to cleave GPIb efficiently. These include calpain, elastase, cathepsin G, trypsin and chymotrypsin [45–48]. Therefore to examine whether cleavage of GPIb is involved, platelets (5×10^8/ml) or

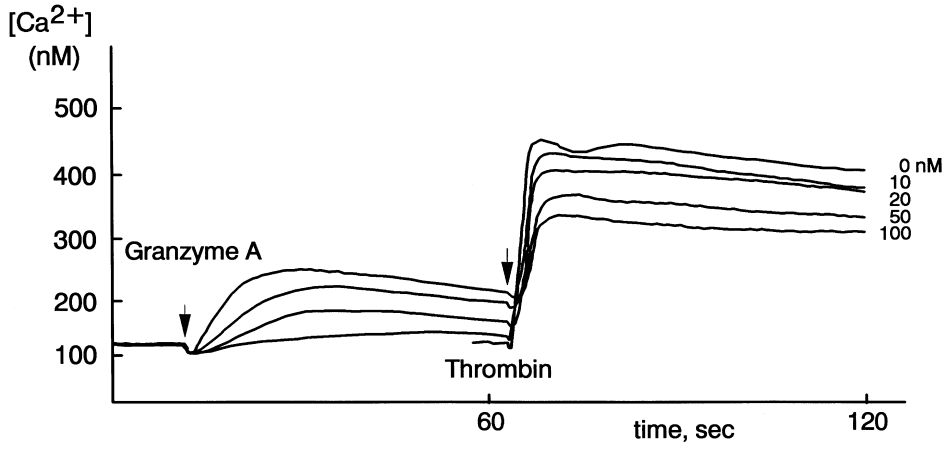

Figure 4 Granzyme A induces Ca^{2+} transients in platelets and inhibits subsequent thrombin-induced increase in Ca^{2+}

Washed human platelets were loaded with fura 2 (see the Materials and methods section) and treated with 10, 20, 50 or 100 nM granzyme A, followed after 50 s by 1 nM thrombin. The response to thrombin alone (0 nM granzyme A) is shown for comparison.

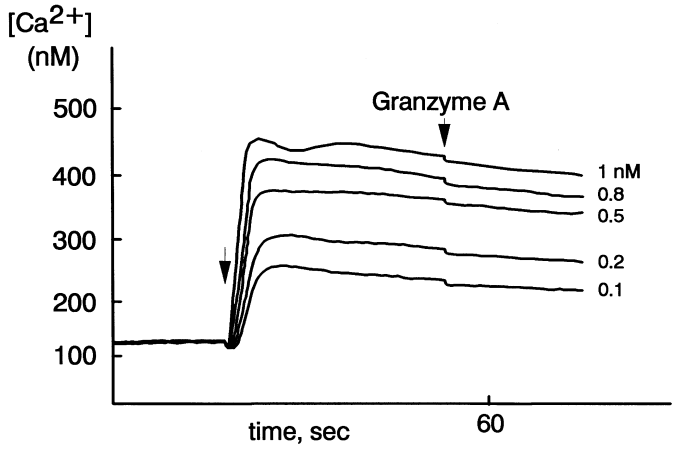

Figure 5 Thrombin prevents granzyme A-induced Ca^{2+} transients in platelets

Washed human platelets were loaded with fura 2 and treated with 0.1, 0.2, 0.5, 0.8 or 1 nM thrombin, followed after 35 s by 100 nM granzyme A.

purified GPIb (1 mg/ml) or the extracellular domain of GPIb, glycocalicin (1 mg/ml), were incubated for various times with 200 nM granzyme A and analysed by gel electrophoresis and Western blotting (in the case of platelets, the supernatant was also analysed). No appreciable GPIb cleavage occurred when platelets were subjected to granzyme A. Purified GPIb was slowly cleaved to a glycocalicin-like fragment, requiring about 30 min for total cleavage. After 10 min, only approx. 30% of GPIb was cleaved. Glycocalicin was only cleaved to a very minor extent between the 45 kDa N-terminal and the macroglycopeptide domains after prolonged (0.5–1 h) incubations with granzyme A (results not shown). Thus these results indicate that although granzyme A cleaves GPIb inefficiently, yielding a glycocalicin-like fragment, the cleavage site involved seems to be inaccessible on intact platelets. It is therefore unlikely that the effects of granzyme A in inhibiting the action of thrombin are due to cleavage of GPIb.

Granzyme A induces Ca^{2+} signalling in platelets and affects a subsequent thrombin challenge

When platelets loaded with fura 2 were treated with granzyme A, they showed a weak Ca^{2+} transient as response (Figure 4). At least 20 nM granzyme A was needed to detect an appreciable Ca^{2+} response. This concentration-dependent response was considerably slower than that measured with thrombin and rapidly returned to baseline values. When such platelets were then challenged with thrombin, the resulting Ca^{2+} transient was smaller than that seen in platelets that were not pretreated with granzyme A. The higher the granzyme A concentration, the smaller was the subsequent thrombin response. If granzyme A was added after thrombin, the signal showed only a slight drop and the slow decline in Ca^{2+} levels was not affected (Figure 5). This was also seen at lower thrombin concentrations.

Although the thrombin-induced Ca^{2+} response was attenuated in platelets pretreated with granzyme A, the response to the ThR-activating peptide SFLLRN was not affected (Figure 6). When SFLLRN was added first, granzyme A induced a weak response in some experiments at low concentrations of the peptide (results not shown).

These results support the notion that granzyme A binds to, but cleaves inefficiently, the ThR on platelets. This leads to a slow and weak Ca^{2+} transient but not to aggregation. In addition, the results show that granzyme A reduces subsequent Ca^{2+} responses to thrombin. Thus the inhibition of thrombin-induced aggregation and Ca^{2+} responses can be most readily explained by both proteases competing for a common binding site on the platelet surface.

DISCUSSION

The present work has established that the T lymphocyte-derived protease granzyme A, which activates the ThR expressed by neural cells and thereby causes neurite retraction and reversal of astrocyte stellation, does not cause platelet aggregation. Furthermore, granzyme A inhibits thrombin-induced platelet aggregation in a dose-dependent manner. The effect of the murine granzyme A was seen in platelets from man, rat and mouse. We propose that granzyme A is unable to activate the ThR found on platelets to an extent that is sufficient to cause aggregation and

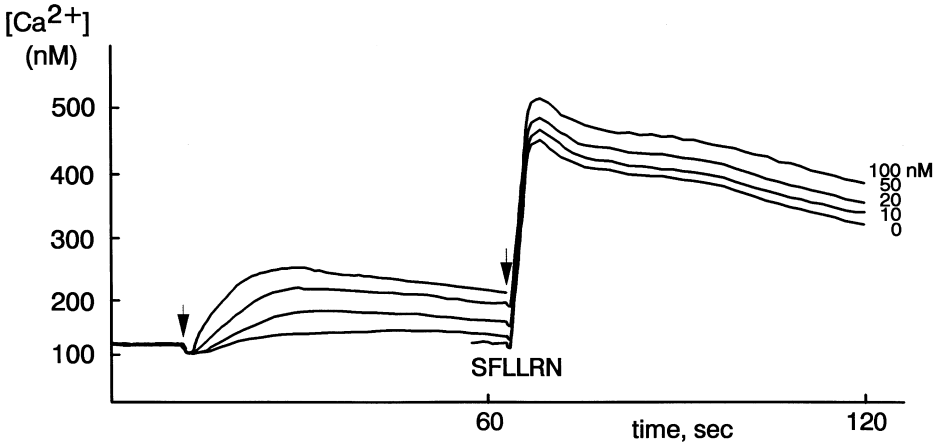

Figure 6 Granzyme A does not affect Ca^{2+} transients induced by SFLLRN

Washed human platelets were loaded with fura 2 and treated with 10, 20, 50 or 100 nM granzyme A, followed after 50 s by 1 nM SFLLRN. The response to SFLLRN alone (0 nM granzyme A) is shown for comparison.

that it competes with thrombin for this receptor. This proposition is supported by the following observations.

1. Granzyme A does not otherwise seem to alter platelet function or affect GPIIb–IIIa because in the presence of the protease, aggregation could still be induced by non-ThR agonists, such as ADP.

2. Granzyme A causes Ca^{2+} signalling in platelets, but the signalling is weak and slow compared with that induced by thrombin. Moreover, the Ca^{2+} signal triggered by thrombin is markedly decreased if the platelets are pretreated with granzyme A.

3. Granzyme A does not seem to inactivate the ThR on platelets, because platelets that do not respond to thrombin in the presence of granzyme A aggregate and give rise to a normal Ca^{2+} signal on addition of the peptide agonist SFLLRN. In this context it is of interest to note that the mutant ($Ser^{42} \rightarrow Tyr$) ThR peptide YFLLRNP inhibits thrombin- and SFLLRNP-induced platelet aggregation, probably owing to low-affinity binding to the ThR [49]. Contrasting this result with the inhibition by granzyme A of the thrombin-induced, but not the peptide-induced, platelet aggregation, highlights that the sites on the ThR that bind the tethered ligand remain available for activation in the presence of granzyme A.

4. Granzyme A is unlikely to affect the integrity of the acidic region of the ThR that binds thrombin, because no putative granzyme A cleavage sites are present within the ThR binding domain. In addition, granzyme A cleaves a synthetic peptide corresponding to a part of the ThR N-terminus only at the putative thrombin cleavage site and not in the binding region [6].

5. Granzyme A does not seem to desensitize the ThR efficiently because receptor activation can occur by decreasing the molar ratio of [granzyme A]/[thrombin], either by augmenting the concentration of thrombin or by decreasing that of granzyme A. Furthermore, granzyme A added a few seconds after thrombin can rapidly inhibit platelet aggregation. It should be mentioned that the responsiveness of platelets to the peptide agonist SFLLRN in the presence of granzyme A does not necessarily argue against the desensitization possibility. A desensitization of the response in some systems can occur to thrombin but not to the peptide [50,51]. However, our results are not compatible with such a selective desensitization mechanism.

6. GPIb on platelets is not cleaved by granzyme A, thus eliminating proteolysis of this thrombin-response modulator as an explanation. Although it seems most likely that granzyme A and thrombin compete for the ThR on platelets, it remains formally possible that granzyme A blocks the thrombin-binding site on GPIb.

7. Granzyme A does not directly or indirectly affect the catalytic activity of thrombin when tested in the chromogenic assay, nor does it seem to alter directly the ability of thrombin to bind through its anion-binding exosite and thereby cleave physiological substrates such as fibrinogen.

These results, taken together, clearly distinguish the effect of granzyme A from those of other proteases such as cathepsin G, chymotrypsin and plasmin, all of which were found to inhibit the effect of thrombin by catalysing an aberrant cleavage of either the ThR and/or GPIb (see [48,52,53] and references therein).

Why does granzyme A not cause platelet aggregation?

Granzyme A at concentrations of up to 230 nM did not cause platelet aggregation, whereas it caused neurite retraction in mouse neuroblastoma cells and reversed the stellation of rat astrocytes, apparently through activation of the ThR, with an EC_{50} of approx. 1 nM [6]. In comparison, the EC_{50} of thrombin in causing neurite retraction is approx. 2 pM [12] and that for causing platelet aggregation is generally accepted to be approx. 300 pM. If it is assumed that the ratio of the thrombin EC_{50} values for causing aggregation over retraction (300 pM/2 pM = 150) is equal to the ratio of the granzyme A EC_{50} values for inducing these two effects (x nM/1 nM = 150), the predicted EC_{50} of granzyme A for causing platelet aggregation would be approx. 150 nM. Thus the highest concentration of 230 nM granzyme A tested in this work would have been over this theoretical EC_{50}. Although such sub-millimolar concentrations are meaningless in a physiological context, it remains puzzling why no sign of platelet aggregation is seen with granzyme A.

The neural cells express the established ThR mRNA that is found in platelets ([12]; S. P. Niclou, unpublished work). Nevertheless it cannot be ruled out that more than one proteolytically activated receptor is involved in mediating the responses to the proteases, or that post-translational modifications produce different forms of the ThR in these systems. Discrepancies have been observed between thrombin and ThR peptides in eliciting particular responses. ThR peptides were found not to mimic the thrombin effect in causing aggregation of degranulated platelets [54], in stimulating platelet procoagulant activity [55], in activating p44 mitogen-activated protein kinase in fibroblasts [56], in inducing Ca^{2+} signals in osteoblast-like cells [57], in promoting expression of platelet-derived growth factor mRNA in endothelial cells [58] and in mediating contraction of isolated arteries [59]. In some species (e.g. rat, rabbit or Chinese hamster), moreover, ThR peptides do not elicit platelet aggregation [60,61]. Despite that, the hamster peptides are fully active in inducing platelet shape change and an increase in the proliferation of cultured fibroblasts [61]. In this context it is worth noting that mouse and rat ThR peptides, which fail to cause aggregation of mouse and rat platelets, are fully active in causing morphological changes in neural cells derived from these rodents (H. S. Suidan, unpublished work). On another level, thrombin and cathepsin G were suggested to induce platelet aggregation via two distinct receptors on the basis of a comparison of the sensitivity of the two responses to an anti-ThR antibody, elastase pretreatment or desensitization [62]. In a similar approach in liver cells, thrombin and trypsin were proposed to stimulate the production of prostaglandins via different receptors [63]. It is worth mentioning that a new member of the protease-activated receptor family has recently been cloned [64]. This receptor, however, seems irrelevant to the present study because it is activated by trypsin but not by thrombin.

Other explanations for the observations seen with granzyme A remain plausible. For example, cleavage of the ThR is sufficient to cause neurite retraction, whereas platelet aggregation requires additional interactions with other surface molecules such as integrins [65–68] and these may not occur efficiently with granzyme A. Another possibility is that the ThR is poorly cleaved by granzyme A in neural cells as in platelets (granzyme A induces a weak and slow Ca^{2+} response in platelets), but the intracellular signals leading to neurite retraction and reversal of astrocyte stellation can be generated with a relatively small number of clipped receptors.

Perspectives

The results presented in this study demonstrate that any release of granzyme A within blood vessels, for example during extravasation of T-cells or cell-mediated immune responses, would not directly cause platelet aggregation. Furthermore these observations highlight differences in responses mediated by proteolytically activated receptor(s) in different systems. The present

results, showing that granzyme A inhibits the effect of thrombin on platelets by competition, could serve as a basis for the design of novel antithrombotic agents that do not interfere with the clotting pathway.

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