Antihemostatic Activity of Human Granzyme B Mediated by Cleavage of von Willebrand Factor*

Marguerite S. Buzza*, Jennifer M. Dyson†, Hiawan Choi†, Elizabeth E. Gardiner†, Robert K. Andrews‡, Dion Kaiserman†, Christina A. Mitchell†, Michael C. Berndt†, Jing-Fei Dong‡, and Phillip I. Bird§

From the †Department of Biochemistry and Molecular Biology, Monash University, Clayton 3800, Victoria, Australia, §Thrombosis Research Section, Department of Medicine, Baylor College of Medicine, Houston, Texas 77030, and the ‡Department of Immunology, Monash University, Alfred Medical Research and Education Precinct, Melbourne 3004, Victoria, Australia

The cytotoxic lymphocyte protease granzyme B (GrB) is elevated in the plasma of individuals with diseases that elicit a cytotoxic lymphocyte-mediated immune response. Given the recently recognized ability of GrB to cleave extracellular matrix proteins, we examined the effect of GrB on the pro-hemostatic molecule von Willebrand factor (VWF). GrB delays ristocetin-induced platelet aggregation and inhibits platelet adhesion and spreading on immobilized VWF under static conditions. It efficiently cleaves VWF at two sites within the A1–3 domains that are essential for the VWF-platelet interaction. Like the VWF regulatory proteinase ADAMTS-13, GrB-mediated cleavage is dependent upon VWF conformation. In vitro, GrB cannot cleave the VWF conformer found in solution, but cleavage is induced when VWF is artificially unfolded or presented as a matrix. GrB cleaves VWF with comparable efficiency to ADAMTS-13 and rapidly processes ultra-large VWF multimers released from activated endothelial cells under physiological shear. GrB also cleaves the matrix form of fibrinogen at several sites. These studies suggest extracellular GrB may help control localized coagulation during inflammation.

Cytotoxic T lymphocytes and natural killer cells eradicate abnormal cells by releasing cytotoxins into the target cell cytoplasm. These cytotoxins include the serine proteinases granzyme A (GrA, a Trypt-ase) and granzyme B (GrB, an Asp-ase) (1–3). Elevated levels of circulating extracellular granzymes occur in viral and bacterial infections, rheumatoid arthritis, and certain allergic responses (4). GrB is also elevated in vascular diseases such as atherosclerosis, where levels correlate with disease severity (5, 6). GrB is found in all regions of the atherosclerotic plaque in advanced atherosclerosis, where it appears to be produced by foam cells and macrophages (6, 7). Plasma levels of GrB are also significantly elevated in atherosclerosis, with highest levels in patients with unstable plaques (8, 9).

Many extracellular substrates of granzymes are matrix components (4). Extracellular matrix targets of GrB include aggregan, fibronectin, vitronectin, and laminin. This matrix degrading activity of GrB disrupts cell adhesion, which in turn can induce cell death in susceptible cells (10, 11). Most of the GrB cleavage sites in matrix proteins have not been determined; however, one cleavage site in vitronectin has been identified as the integrin-binding motif, RGD, which explains the ability of GrB to modulate cell adhesion and adhesion-dependent processes such as cell spreading and migration (10).

Given the importance of integrins in platelet adhesion and aggregation, and the elevated levels of granzymes in vascular disease, we were interested to determine whether granzymes affect blood coagulation. Mouse GrA de-sensitizes platelets to thrombin-induced aggregation (12); however, the effect of GrB on coagulation is unknown. Here we show that GrB cleaves von Willebrand factor (VWF) at two sites important for platelet interactions, and not only inhibits VWF-mediated platelet aggregation and spreading on surface-bound VWF but also blocks platelet tethering and adhesion to ultra-large VWF multimers under shear flow conditions. Importantly, GrB does not cleave soluble VWF under static conditions, and in this respect mimics the physiological VWF regulator ADAMTS-13. GrB is therefore the first example of a serine protease that regulates the platelet-adhesive function of VWF in a strictly conformation-dependent manner, suggesting a unique role for extracellular GrB in controlling localized coagulation during inflammation.

EXPERIMENTAL PROCEDURES

Materials—Human VWF was prepared from human Factor VIII concentrate (13–15) or human cryoprecipitate (16) and did not contain ultra-large forms. Human fibrinogen was from Sigma. Ristocetin was from Helena Laboratories (Beaumont, TX), and botrocetin was prepared as described (13). Mouse anti-VWF (clone 6G1) recognizing the A1 domain Glu700–Asp709 has been described (17). The rabbit polyclonal anti-VWF antibody was from Dako. Production of glycolcalcin and the rabbit anti-GPlbα antibody are described in Ref. 18. Recombinant human GrA, GrB, and an active site mutant (GrBS183A) are described in Ref. 10. Recombinant ADAMTS-13 was produced as described (19).

Platelet Aggregation Assays—Platelet-rich plasma (PRP) was prepared from blood of healthy individuals who had not taken...
aspirin in the previous 2 weeks, and was used in ristocetin-induced aggregation assays (14).

Platelet Adhesion and Spreading on Immobilized VWF—Washed platelets were prepared from PRP as described previously (20). GrA or GrB was added to 0.5-ml platelet aliquots (in duplicate) at the indicated final concentration. Platelets were applied to VWF-coated glass coverslips, allowed to adhere and spread, and stained with Texas Red-conjugated phalloidin (Molecular Probes) (20). Images were captured using a confocal microscope (100× oil-immersion objective with a numerical aperture of 1.4 on a Leica TCS NT upright microscope running Leica T5 software; Monash University Micro Imaging Facility). Five fields per coverslip were analyzed. For platelet spreading, results are expressed as the percentage of platelets/field exhibiting lamellipodia. In one experiment, VWF-coated coverslips were pretreated with 100 nM GrA or GrB at 37 °C for 30 min. Treated and control treated coverslips were then washed five times in PBS prior to adding untreated washed platelets.

Assessment of GrB Cleavage of GPIbα—200 ng of glycocalcin was incubated with GrB for 45 min at 37 °C in TBS. Reactions were stopped by addition of reducing Laemmli sample buffer, resolved by 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with rabbit anti-GPIbα antibody (1:2000). To assess cleavage of full-length GPIbα on platelets, washed platelets in Tyrode’s buffer were treated with 170 nM GrB for 1 h at 37 °C. GPIbα was assessed by flow cytometry using anti-GPIbα monoclonal antibodies W/M23 and AK2, followed by fluorescein isothiocyanate-conjugated anti-mouse IgG. Binding of VWF was also assessed by mixing untreated and GrB-treated platelets with Alexa488-conjugated VWF (gift of Dr. Sue Cranmer, Monash University) in the presence or absence of botrocetin (10 μg/ml).

VWF Cleavage Assays—Wells of a 24-well tray were coated with 500 ng of VWF in 200 μl of PBS overnight at 4 °C, washed with PBS, and then treated with GrB or GrBS183A in 200 μl of TBS for 1 h at 37 °C. Supernatants were removed, and matrix was solubilized in 40 μl of reducing Matrix Lysis buffer (21). VWF cleavage in solution was performed with the indicated concentration of VWF and GrB in either TBS or 5 mM Tris-HCl, pH 7.5. In one experiment urea was also included. To stop the reaction reducing Laemmli sample buffer was added. For comparison of GrB with ADAMTS-13, purified VWF was incubated with ADAMTS-13 or GrB in 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, for 10 min at 37 °C. 150 μl were transferred onto a hydrophilic filter membrane (VSWP, 25 mm diameter; Millipore, Bedford, MA) floating on 50 ml of dialysis buffer (1.5 M urea; 5 mM Tris-HCl, pH 8.1) and incubated at 37 °C. At 3, 5, and 16 h, 10-μl aliquots were mixed with 1 μl of 0.2 M EDTA, pH 7.4, and then analyzed by reducing SDS-PAGE. For fibrinogen cleavage, 2 μg of fibrinogen in TBS or 5 μg of fibrinogen coated onto wells was incubated with GrB for 1 h at 37 °C. Samples were separated by SDS-PAGE and either transferred to nitrocellulose and probed with anti-VWF antibodies or stained using Rapid Ag Stain (ICN Biomedicals) or Coomassie Blue. Secondary antibodies were used at 1:4000 and developed by enhanced chemiluminescence (Western Lightning, PerkinElmer Life Sciences). For determination of GrB cleavage sites, samples were transferred to polyvinylidene difluoride membrane (Bio-Rad), and N-terminal sequencing was performed by the Monash Biomedical Proteomics Facility (Monash University).

Cleavage of UL-VWF strings was measured as described (22). Washed platelets were perfused over histamine-stimulated HUVECs at a shear stress of 2.5 dynes/cm² for 3 min. The number of platelet-decorated UL-VWF strings was counted in 10 continuous view fields (×200). GrB was added either to the perfusion buffer alone or was included during HUVEC stimulation.

RESULTS

GrB Delays Ristocetin-induced Platelet Aggregation—VWF initiates platelet adhesion in arterioles or diseased/narrowed vessels via interaction with the platelet membrane protein GPIbα (23, 24). In vitro, VWF-mediated platelet aggregation can be triggered by the antibiotic ristocetin, which binds plasma VWF, inducing a conformation recognized by GPIbα (14). Initially we examined whether GrB influences platelet aggregation in PRP, using ristocetin as the aggregation stimulus. Using protease concentrations consistent with previous investigations of leukocyte protease-platelet interactions (12, 25), we assessed platelet aggregation in PRP from eight donors after a 30-min pretreatment in the presence or absence of GrB. GrB significantly delayed platelet aggregation in 4/8 donors (Fig. 1 shows examples of two positive and one negative donor). We noted that the aggregation curves in untreated samples in response to ristocetin varied from donor to donor, as did the response to GrB. For example, in donor 1 PRP, GrB significantly delayed the initiation of aggregation, whereas in donor 2 PRP, which produced a more biphasic aggregation response, it showed a greater delay in the second phase of aggregation. This phase is thought to represent the release of platelet granules and activation of integrin-mediated binding to plasma VWF and fibrino-
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GrB Inhibits Platelet Adhesion and Spreading on Immobilized VWF—The inhibition of ristocetin-induced platelet aggregation by GrB suggested that it acts either on VWF or on GPIbα. We assessed the effect of GrB on binding of washed platelets to immobilized VWF. Here coverslips coated with VWF mimic its conformation in the sub-endothelial matrix; thus unstimulated platelets bind via GPIbα, which induces the activation of integrin αIIbβ3 to mediate stable adhesion and spreading (28). Untreated washed platelets adhere and spread well on the VWF matrix, as visualized by actin staining (Fig. 2A). By contrast, if GrB was added to the platelet suspension prior to plating, platelet adhesion and spreading were reduced by 70–80% (Fig. 2B). These results suggest that GrB disrupts GPIbα or integrin αIIbβ3 interactions with VWF. This activity of GrB is specific to VWF, as GrB had no effect on platelet adhesion and spreading on immobilized collagen (data not shown). By contrast, GrA had little effect on platelet interactions with immobilized VWF under identical conditions, with only a slight reduction in spreading at 60 min noted (Fig. 2, A and B), suggesting that a specific site is targeted by GrB, which is not susceptible to adventitious proteolysis.

As platelet adhesion to VWF is mediated by GPIbα, we tested whether GrB can cleave this receptor. The extracellular domain of GPIbα, glycocalcin (18, 29), was incubated with several concentrations of GrB for 45 min and assessed for cleavage by immunoblotting. GrB did not cleave glycocalcin (Fig. 3A). Full-length GPIbα on the platelet surface was not altered by pretreatment with GrB, as assessed by binding of the GPIbα-specific antibody AK2 (Fig. 3A). Furthermore, we showed that botrocetin-dependent Alexa488-VWF binding to platelets is also unaffected by pretreatment of the platelets with GrB, confirming that GPIbα remains intact and functional (Fig. 3B). These results show that GrB does not cleave the platelet VWF receptor and suggest that it must affect the VWF matrix itself. To confirm this, the VWF on coverslips was pretreated with GrB prior to applying platelets, which significantly inhibited both adhesion and spreading (Fig. 3C).

GrB Cleaves Immobilized VWF—Although the above experiments suggested that GrB cleaves VWF, no cleavage was detected when VWF in Tris-buffered saline (“soluble” VWF) was treated with GrB (Fig. 4A, left panel). Because GrB cleavage of vitronectin and fibronectin is conformationally dependent (10), and VWF adopts different conformations depending on its location and quaternary state, we coated VWF onto plastic to mimic its form in the platelet adhesion assays (immobilized VWF). Under these conditions GrB cleaved VWF at several sites (Fig. 4A, center and right).
panels), and no cleavage was detected using the GrB active site mutant.

GrB Cleaves VWF Conformers Susceptible to ADAMTS-13—In vivo, the plasma metalloproteinase ADAMTS-13 regulates VWF by cleaving large multimers into less hemostatically active forms (30, 31). ADAMTS-13 will only cleave a conformer of VWF that has exposed a specific cleavage site in the A2 domain (30, 32, 33). In vivo, this occurs under shear stress (22, 34). In vitro, this ADAMTS-13 susceptible VWF conformation can be induced by partially unfolding VWF in urea or guanidine–HCl (32, 33) or by removal of salt (32, 35).

We examined whether a GrB-susceptible VWF conformer of soluble VWF is induced under conditions similar to those that allow ADAMTS-13 cleavage. Urea in physiological concentrations of salt did not permit cleavage by GrB (Fig. 4B); however, the removal of salt facilitated complete cleavage of VWF without requiring urea (Fig. 4C). Adding back salt progressively inhibited GrB-mediated VWF cleavage (Fig. 4C). This resembles the effect of salt on ADAMTS-13 cleavage in vitro (32, 35, 36). Ristocetin binding also renders VWF susceptible to ADAMTS-13 cleavage (35). However, ristocetin did not increase GrB cleavage in the presence or absence of salt (data not shown).

Overall, five cleavage GrB products were apparent by silver staining or by immunoblotting with the anti-VWF monoclonal antibody (6G1) (Fig. 5). Four products ranging from 65 to 150 kDa were detected. The 65- and 90-kDa products were reminiscent of the products generated by GrB treatment of immobilized VWF (Fig. 4A). The antibody did not detect one larger product (>175 kDa) evident by silver staining, suggesting that this species has had the epitope removed.

GrB Cleaves the A1 and A3 Domains of VWF—Four of the five VWF GrB cleavage products (except the 62-kDa product) were analyzed by N-terminal sequencing. The sequence of product 1 showed that GrB cleaves after Asp709, within the sequence 706LPPD\^MAQV\_113. This site is in the VWF A1 domain, just C-terminal to the disulfide loop (Fig. 5C). Product 2 indicated cleavage after Glu\_910, within the sequence 907CSGE\_GLQI\_914. The Glu\_910 residue is the first residue of the A3 domain and is 65 residues downstream of the ADAMTS-13 cleavage site in the A2 domain (Fig. 5C). Products 3 and 4 contained the N terminus of mature VWF, and they probably represent the other portion of VWF generated by cleavage at sites 2 and 1, respectively. Time course assays showed that site 2 (Glu\_910) is cleaved more efficiently under these conditions (Fig. 5B).
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**GrB Cleaves VWF with Comparable Efficiency to ADAMTS-13—**We next directly compared GrB-mediated and ADAMTS-13 cleavage of VWF under standard ADAMTS-13 assay conditions (Fig. 6A). GrB cleaved VWF with similar efficiency to ADAMTS-13, and no degradation of VWF was noted in the absence of either protease (data not shown). Only two major VWF products are generated by GrB under these conditions. By comparison with products 2 and 3 in Fig. 5, it appears cleavage only occurs at GrB site 2 (Glu910), which is closer to the ADAMTS-13 cleavage site, and was the major site cleaved in the GrB time course assay (Fig. 5B).

**GrB Cleaves Ultra-large VWF Multimers under Flow—**Both platelets and endothelial cells store ultra-large multimers of VWF (UL-VWF), which are released upon stimulation (30). After release from endothelial cells, UL-VWF remains anchored to the cell surface, forming “string-like” structures that capture platelets under fluid shear stress (22). Although in vitro assays require nonphysiological denaturation of VWF to induce ADAMTS-13 cleavage, exposure of UL-VWF attached to endothelial cells to shear induces a conformation that allows cleavage by ADAMTS-13 (22, 34). We determined whether such VWF conformers are also sensitive to GrB (method according to Ref. 22). As shown in Fig. 6B, GrB rapidly cleaved UL-VWF strings released from activated endothelial cells under physiological flow, suggesting that it has antihemostatic capacity in vivo. As the duration of this assay is 3 min, it appears GrB is extremely efficient at recognizing and cleaving shear-induced VWF conformers.

**GrB Also Cleaves Fibrinogen in a Conformation-dependent Manner—**Fibrinogen bound to the extracellular matrix interacts with activated platelet integrin αIIbβ3 to mediate platelet adhesion, forms platelet-platelet bridges, and contributes to thrombus growth (37, 38). Fibrinogen molecules include two disulfide-linked sets of three polypeptide chains termed Aα, Bβ, and γ. Given that GrB cleaves matrix VWF, we assessed whether it has additional effects on hemostasis by cleaving fibrinogen (supplemental Fig. 1). GrB was unable to cleave soluble fibrinogen, similar to its inability to cleave soluble VWF, fibronectin, or vitronectin. However, when fibrinogen was coated onto plastic, GrB cleaved all three subunits. One cleavage site in the Bβ chain was identified by N-terminal sequencing after Asp230 within the sequence 227IQPD↓SSVK234, which represents a typical GrB cleavage site. Another fragment identified by sequencing was the γ-chain N terminus, which confirmed at least one GrB cleavage site in this chain. We also assessed whether GrB could dissolve thrombin-generated fibrin clots using a fibrin-agarose plates and fibrin turbidity assays (according to Ref. 39). Although plasmin was able to dissolve fibrin, GrB did not appear to have any effect under these conditions (data not shown).

**DISCUSSION**

The two new substrates of human GrB identified here, VWF and fibrinogen, are important components of blood coagulation pathways. We have demonstrated the effect of GrB cleavage on the biological function of these extracellular matrix molecules using assays for platelet adhesion, platelet aggregation, and cleavage of platelet strings under flow conditions. The following points argue for the physiological relevance of our findings. 1) The main cleavage point for GrB in VWF is very close to the site cleaved by the well characterized regulator, ADAMTS-13, suggesting that GrB has a similar anticoagulant effect on VWF. 2) GrB cleaves VWF from endothelial cells with similar efficiency to ADAMTS13 under experimental conditions that mimic shear forces in venous blood; and (3) cleavage of VWF by GrB is conformationally dependent such that only a specific procoagulant conformer of VWF is targeted by GrB. (In this respect, GrB is the first example of a protease other than ADAMTS13 cleaving within the same domain of conformationally altered VWF.) Although the biological impact of GrB-mediated fibrinogen cleavage was not determined in this study, it may have contributed to the delay in the second phase of ristocetin-induced aggregation that was observed in some donors.

VWF monomers are composed of multiples of four types of domains (A–D) (40, 41). The two GrB cleavage sites identified in VWF are within the A1 domain and at the boundary of the A2 domain.
and A3 domains. These domains play essential roles in the function of VWF. For example, the interaction of the A1 domain with GPIIb or is essential for platelet tethering under flow. This in turn stimulates intracellular signaling, activating platelet integrin by which then binds to VWF and fibrinogen to promote stable adhesion and clot formation (reviewed in Refs. 24, 42, 43). The A2 domain is the site of processing of VWF multimers by the physiological regulator ADAMTS-13, and the A3 domain is involved in collagen binding.

GrB cleavage of VWF would disrupt the initial platelet interaction with the A1 domain. It is also likely that GrB cleavage disrupts interactions of the A1 domain with molecules such as collagen, heparin, and sulfatides (40, 44, 45). Interestingly, the snake venom metalloproteinase kaouthiagin cleaves VWF at a single site adjacent to the GrB site 1, between and inhibits VWF-mediated platelet aggregation (46). However, is also the site of a single nucleotide polymorphism that alters residue 709 to His (47). Given that GrB cleaves after Asp or Glu (48), it is extremely unlikely that it would cleave VWF containing His at this site. Whether the VWF preparations employed in this study contained this polymorphism is unknown, but a mixture of both forms may potentially explain the donor variability and the (apparently) more efficient cleavage at site 2 (Glu).

Neither GPIb nor ADAMTS-13 interacts with circulating VWF multimers. However, both proteins bind VWF attached to collagen or other surfaces (49, 50). Ristocetin induces a conformational change in VWF that allows recognition by both GPIb and ADAMTS-13 (35, 51), and binding of GPIb to the A1 domain can also induce cleavage by ADAMTS-13 (52). As shown here, GrB also does not cleave VWF multimers but will cleave VWF on a surface. We suggest the binding of VWF to GPIb induces a conformation that allows GrB cleavage and the inhibition of ristocetin-induced platelet aggregation. In vivo, the biologically active conformation of VWF is induced under shear stress, which exposes GPIb and ADAMTS-13 (35, 51), and binding of GPIb to the A1 domain can stabilize a conformation that prevents ADAMTS-13 from binding collagen or other surfaces (40, 44, 45). Interestingly, the snake venom metalloproteinase kaouthiagin cleaves VWF at a single site adjacent to the GrB site 1, between and inhibits VWF-mediated platelet aggregation (46). However, is also the site of a single nucleotide polymorphism that alters residue 709 to His (47). Given that GrB cleaves after Asp or Glu (48), it is extremely unlikely that it would cleave VWF containing His at this site. Whether the VWF preparations employed in this study contained this polymorphism is unknown, but a mixture of both forms may potentially explain the donor variability and the (apparently) more efficient cleavage at site 2 (Glu).

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Circulating VWF multimers are held in an ADAMTS-13-resistant conformation by salt. A chloride-binding site in the A1 domain stabilizes a conformation that prevents ADAMTS-13 cleavage of the A2 domain. Interestingly a type 2 von Willebrand disease mutant (VWD R543W) has increased susceptibility to ADAMTS-13 and a lower affinity for chloride (36). There are also other examples of type A and B VWD mutations that increase the efficiency of ADAMTS-13 cleavage in physiological concentrations of salt (55). The lack of VWF cleavage by GrB in salt suggests chloride stabilization also renders soluble VWF resistant to GrB and that GrB will more efficiently cleave destabilized natural VWF mutants.

There is no direct evidence to suggest that GrB cleaves VWF in healthy individuals, as the sizes of VWF fragments in normal plasma are consistent with cleavage by ADAMTS-13 alone. However, locally elevated GrB has the potential to cleave VWF in a disease setting, and thus VWF fragments of a size different from those generated by ADAMTS-13 may be evident in conditions such as atherosclerosis. We propose that at sites of inflammation, where endothelial cells are activated and release UL-VWF forms, GrB produced by cytotoxic lymphocytes, mast cells (56, 57), or macrophages (7) may act with ADAMTS-13 to prevent or delay thrombosis. Coupled with previous findings that GrA prevents platelet aggregation by cleaving the thrombin receptor (12), this points to complementary roles for the two major granzymes in controlling coagulation and provides another example of the potential for cross-talk between the immune and coagulation systems during inflammation.

It should be noted that the concentrations of GrB used in our studies are higher than the sub-picomolar levels noted in the plasma of normal individuals or the picomolar levels in those with sepsis (58, 59). However GrB is stored within cells, and because of its propensity to strongly bind membranes and extracellular matrix (10, 60) would remain cell- or matrix-associated on release. Hence at an inflammatory site it would accumulate locally to levels much higher than those in the circulation. It is most likely that plasma GrB results from low level constitutive release from circulating lymphocytes (8) or arises from lymphocyte necrosis during inflammation.

Finally, direct evidence for regulation of VWF by GrB will require the study of an appropriate human or mouse mutant. Currently this is not possible because no human GrB deficiencies have been described, and there is no VWF patient with a mutation in the GrB cleavage site. Mouse knock-out models are problematic because of the following. 1) ADAMTS13-deficient mice do not develop thrombotic thrombocytopenic purpura, indicating interspecies differences in VWF regulation (61). 2) Mouse GrB has a different substrate specificity than human GrB (62). However, there are several recent reports of human GrB associated with inflamed sites in atherosclerosis and vascular disease (6–8). These pathologies involve endothelial and smooth muscle cell damage that would expose matrix VWF, leading to a hypercoagulable state favoring platelet aggregation. Thus the GrB observed in such settings is in a realistic position to cleave and control VWF.

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