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Activation of leukocyte rolling by the cysteine-rich domain and the hyper-variable region of HF3, a snake venom hemorrhagic metalloproteinase

Milene C. Menezes[a], Adriana F. Paes Leme[a], Robson L. Melo[a], Carlos A. Silva[a], Maisa Della Casa[b], Fernanda M. Bruni[a], Carla Lima[a], Mônica Lopes-Ferreira[a], Antonio C.M. Camargo[a], Jay W. Fox[c], Solange M.T. Serrano[a,*]

[a] *Laboratório Especial de Toxinologia Aplicada-CAT/CEPID, Instituto Butantan, Av. Vital Brasil 1500, 05503-900 São Paulo, Brazil*
[b] *Laboratório de Imunopatologia, Instituto Butantan, Av. Vital Brasil 1500, 05503-900 São Paulo, Brazil*
[c] *Department of Microbiology, University of Virginia, Charlottesville, VA 22908, USA*

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Abstract **The functionality of the disintegrin-like/cysteine-rich domains of snake venom metalloproteinases (SVMPs) has been shown to reside in the cysteine-rich region, which can interact with VWA-containing proteins. Recently, the hyper-variable region (HVR) of the cysteine-rich domain was suggested to constitute a potential protein–protein adhesive interface. Here we show that recombinant proteins of HF3, a hemorrhagic P-III SVMP, containing the cysteine-rich domain (disintegrin-like/cysteine-rich and cysteine-rich proteins) but not the disintegrin-like protein were able to significantly increase leukocyte rolling in the microcirculation. Peptides from the HVR also promoted leukocyte rolling and this activity was inhibited by anti-alpha$_M$/beta$_2$ antibodies. These results show, for the first time, that the cysteine-rich domain and its HVR play a role in triggering pro-inflammatory effects mediated by integrins.**

Keywords: Disintegrin-like/cysteine-rich domains; Hyper-variable region; Intravital microscopy; Leukocyte rolling; Snake venom metalloproteinase

1. Introduction

Local tissue destruction due to snake envenomation involves activation of the immune response in which the cascade of events is usually initiated by tissue macrophages and blood monocytes [1,2]. SVMPs are found mainly in viper venoms, and are primarily responsible for the observed hemorrhage due to the disruption of blood vessels as well as for inhibition of platelet-aggregation [3]. These enzymes have also been implicated in the local pro-inflammatory pathogenesis [2,4]. They are members of the Reprolysin subfamily of metalloproteinases, which also includes two groups of mammalian homologous proteins, A Disintegrin And Metalloproteinase (ADAM) and A Disintegrin And Metalloproteinase with Thrombospondin Motifs (ADAMTS) [4]. SVMPs are divided into three main classes depending on their domain organization [5]. The P-III class of SVMPs and the ADAMs and ADAMTSs share homologous metalloproteinase, disintegrin-like and cysteine-rich domains [5]. The disintegrin-like/cysteine-rich domains of SVMPs have been shown to play a role in the inhibition of collagen-stimulated platelet-aggregation promoted by these enzymes [6,7]. Members of the ADAMs group have been implicated in the control of membrane fusion, cytokine and growth factor shedding, and cell migration [8]. Accumulated evidence in the literature suggests that the disintegrin-like/cysteine-rich domains of the SVMPs as well as those of the ADAMs and ADAMTSs are involved in their interactions with specific ligands [5,9,10]. Various studies have shown that the functionality attributed to the disintegrin-like/cysteine-rich domains of SVMPs resides in the cysteine-rich domain region [11–13]. The biological activities attributed to this domain result from its ability to interact with other proteins, such as FACIT collagens, von Willebrand factor (VWF) and integrins [11–13]. Recently, the determination of the tridimensional structure of two P-III SVMPs revealed that the cysteine-rich domain of proteins with the ADAMs' metalloproteinase/disintegrin-like/cysteine-rich architecture show a hyper-variable region (HVR), which has a novel fold stabilized by conserved disulfide bonds, and constitutes a potential protein–protein adhesive interface [14,15].

Previous studies have shown that TNF-α antibodies reduced the size of venom-induced necrotic lesions, suggesting that endogenous mechanisms of the inflammatory response can be activated by SVMPs [16]. Moreover, a study on the inflammatory action of jararhagin, a hemorrhagic metalloproteinase from the venom of *Bothrops jararaca*, showed that it is a potent pro-inflammatory agent dependent on in situ activation of macrophages [17]. The activation of macrophages by jararhagin involves induction of TNF-α, IL1β and IL-6 expression, which is dependent on its proteolytic activity [18]. On the other hand, it was recently shown that the disintegrin-like/cysteine-rich domains of jararhagin are sufficient to locally activate the early events of an acute inflammatory response as leukocyte rolling and pro-inflammatory cytokine release [19].

HF3, a P-III SVMP of 62 kDa, is the most potent hemorrhagic toxin isolated from *B. jararaca* venom [20]. It causes hemorrhage on rabbit skin with a minimum hemorrhagic dose of 15 ng. Previously, we determined the complete amino acid sequence of HF3 by molecular cloning and showed that a recombinant protein composed of its disintegrin-like/cysteine-rich domains (DC-HF3) inhibits collagen-induced

*Corresponding author. Fax: +55 11 3726 1024.
E-mail address: solangeserrano@butantan.gov.br (S.M.T. Serrano).

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platelet-aggregation [21]. Moreover, we showed that native HF3 and DC-HF3 activate $\alpha_M\beta_2$-mediated phagocytosis of opsonized-zymosan particles by macrophages and this activity is inhibited by anti-α_M and anti-β_2 antibodies. The catalytic activity of HF3 did not contribute to its ability to stimulate phagocytosis by macrophages suggesting a role for the disintegrin-like/cysteine-rich domains of HF3 in the activation of phagocytosis mediated by $\alpha_M\beta_2$ integrin [21].

In order to further investigate the effect of HF3 and of its individual disintegrin-like (D) and cysteine-rich (C) domains on the pro-inflammatory response we tested for the first time in parallel the ability of recombinant proteins obtained in fusion with glutathione S-transferase (GST), composed of the domains together (GST-DC), and individually (GST-D and GST-C), and showed that only proteins containing the cysteine-rich domain, GST-DC and GST-C, have the ability to promote leukocyte rolling in the microcirculation as examined by intravital microscopy. We also showed that peptides corresponding to the HVR of the cysteine-rich domain of HF3 and berythractivase, a P-III non-hemorrhagic, pro-coagulant SVMP from *B. erythromelas* venom [22] can activate leukocyte rolling. Leukocyte rolling induced by the cysteine-rich domain and the HVR of HF3 was inhibited by anti-α_M/β_2 antibodies, suggesting a role for this region in triggering the $\alpha_M\beta_2$-mediated pro-inflammatory effects of HF3.

2. Materials and methods

2.1. Animals

Male Swiss mice (18–20 g) were housed in temperature controlled rooms and received water and food ad libitum. These studies were approved by the Experimental Animals Committee of Butantan Institute according to the procedures laid down by the Universities Federation for Animal Welfare.

2.2. Expression of the non-catalytic domains of HF3 in E. coli

The disintegrin-like/cysteine-rich domains of HF3 (GST-DC) was obtained in *E. coli* DH5α, as described earlier [21]. For the expression of the disintegrin-like domain the constructs pGEX-4T2-D16 (containing cDNA encoding amino acid residues 396–493 of HF3; GenBank AAG48931) and pGEX-4T2-D18 (containing cDNA encoding amino acid residues 396–505) were used; the construct to express the cysteine-rich domain (pGEX-4T2-C) contained amino acid residues 494-606. The following primers were used to amplify the sequences from the cDNA encoding the HF3 precursor by PCR:

Disintegrin-like (D16) – sense: 5′CGGAATTCCATTGAGAACA GATATT 3′, with a EcoRI restriction site; antisense: 3′ TTACCTGTTGGTACGACTGAGCTCGCC 5′, with a XhoI restriction site.

Disintegrin-like (D18) – sense: 5′CGGAATTCCATTGAGAACA GATATT 3′, with a EcoRI restriction site; antisense: 3′ ATGT TACCCTTAACGACTGAGCTCGCC 5′, with a XhoI restriction site.

Cysteine-rich (C) – sense: 5′-AGGAATTCTACTACATAACTACG GTTAC-3′, with a EcoRI restriction site; antisense: 3′-CGACTC GAGCGGCCGCATGCTGA-5′ with a NotI restriction site.

To generate fusion proteins with GST the PCR products were directly subcloned into the pGEX-4T2 vector (GE Healthcare). Plasmids were sequenced on both strands to ensure that the coding sequences were correct. The constructed plasmids were transformed into *E. coli* DH5α, and grown in 50 ml Luria-Bertani medium containing 100 μg ml^{-1} ampicillin at 37 °C to a cell density of A_{600} = 0.6–0.8. Protein expression was induced by adding 0.5 mM isopropyl-thio-β-D-galactopyranoside (IPTG) and incubation was continued for 3 h at 30 °C. Cells were collected by centrifugation at 4000 × g for 5 min and suspended in 1 ml lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100). After lysis by sonication the cell lysate was centrifuged and 80 μl of Glutathione-Sepharose (GE Healthcare) suspension were added to supernatant and incubated 16 h at 4 °C. The resin was washed successively with lysis buffer to eliminate unbound proteins and incubated with thrombin cleavage buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$) and 4U thrombin (Sigma) for 2 h at 37 °C. Recombinant proteins obtained in the supernatant (GST-D16, GST-D18 and GST-C) were analyzed by SDS–PAGE, Western-blot and N-terminal amino acid sequencing.

2.3. Intravital microscopy assays

Mice were injected with a muscle relaxant drug (0.4% Xilazin) (Coopazine®, Schering-Plough) and then anaesthetized with 2.5% chloral hydrate and the cremaster muscle was exposed for microscopic examination in situ as described by Lomonte et al. [23]. The number of rolling leukocytes was quantified as described by Shigematsu et al. [24]. Animals were maintained on a board thermostatically controlled at 37 °C, which included a transparent platform on which the tissue to be transilluminated was placed. After stabilization of the microcirculatory network, the alterations were visualized in post-capillary venules for 30 min after topical application of 20 μl solution of recombinant proteins at 5 μM or 15 μM, or synthetic peptides at 50 μM in PBS. Leukocyte rolling velocity was measured for the first five leukocytes entering the field of view at the time of recording and calculated from the average time required for a leukocyte to roll along a 500-μm length of venule. To evaluate the role of $\alpha_M\beta_2$ integrin on cysteine-rich domain- and peptide-induced leukocyte rolling, mice were injected in the ophthalmic plexus with 50 μg or 25 μg, respectively, of each monoclonal antibody in 100 μl PBS (anti-α_M, clone 5C6, Serotec; anti-β_2, clone YTS213.1, Serotec), and after 30 min GST-C or the peptide HF3-HVR1 was topically applied. Values are average of three independent experiments. The analysis of the microvascular system was performed with an optical microscope (Image.A1 Carl-Zeiss) coupled to a photographic camera (AxioCam ICc1) using an objective 40×/03.

2.4. Peptide synthesis

Peptides based on the HVR of HF3 and berythractivase were synthesized using an automatic synthesizer PSSM8 (Shimadzu) by the solid phase peptide synthesis FMOC strategy [25]. Peptides were purified by preparative reversed-phase chromatography (RP-HPLC), and the purity and identity of the peptides were confirmed by MALDI-TOF mass spectrometry on a Ettan Maldi-Tof/Pro instrument (Amersham Biosciences), and by analytical RP-HPLC in two different solvent systems. Oxidation of cysteinyl residues was carried out using oxygen in diluted aqueous solution of peptide in basic pH. Following oxidation, the peptide was lyophilized and purified by RP-HPLC.

2.5. Analytical procedures

Protein concentrations were determined by the Bio-Rad protein assay kit using bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to Laemmli [26]. Western-blot analysis was performed as described by Burnette [27]. For N-terminal sequencing, proteins were transferred to 0.2-μm pore PVDF membrane (Applied Biosystems) using the Transphor apparatus (GE Healthcare). Protein bands stained with Coomassie blue were sequenced in an Applied Biosystems 494 Procise sequencer using the manufacturer's pulsed liquid PVDF cycles.

3. Results and discussion

3.1. Expression of the individual non-catalytic domains of HF3

For the analysis of the ability of the non-catalytic domains of HF3 to activate leukocyte rolling we obtained the disintegrin-like and cysteine-rich domains together (GST-DC), and individually (GST-D and GST-C). The disintegrin-like domain was expressed in two versions considering the disulfide bonds observed in the crystal structure of P-III SVMPs VAP1 [14] and catrocollastatin/VAP2B [15]: D16 contains 16 Cys residues and D18 contains 18 Cys residues, including the disulfide bridge (Cys468–Cys499) which connects the disintegrin-like and cysteine-rich domains and stabilizes the continuous structure of the disintegrin-like domain (Fig. 1A). The cDNA

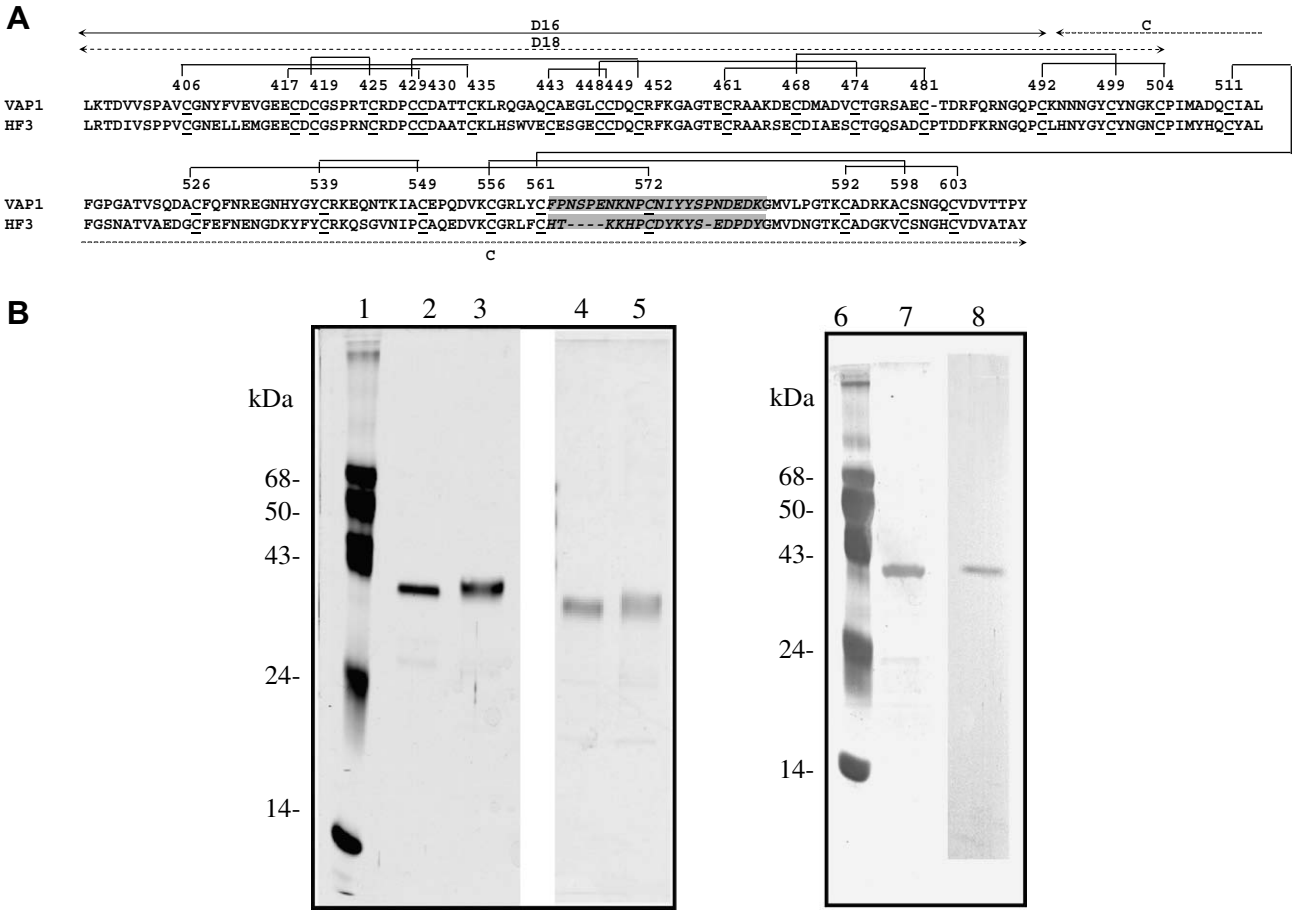

Fig. 1. Expression of HF3 non-catalytic domains. (A) Comparison of HF3 and VAP1 disintegrin-like/cysteine-rich domain sequences. Disulfide bonds of VAP1 are depicted [14]. Cysteine residues are underlined. Double-headed arrows indicate the sequence of HF3 recombinant proteins. The HVR is shaded in grey. (B) SDS–PAGE of molecular mass markers (lanes 1 and 6), GST-D16 (lane 2), GST-D18 (lane 3), GST-C (lane 7) purified by glutathione-Sepharose 4B chromatography, and Western-blot of purified GST-D16 (lane 4), GST-D18 (lane 5) and GST-C (lane 8) identified with anti-HF3 antibody.

sequences coding for the disintegrin-like and cysteine-rich domains of HF3 were subcloned in the expression vector pGEX-4T2, and the resultant plasmids were used to transform *E. coli* DH5α cells, and protein expression was induced with 0.5 mM IPTG for 3 h. The recombinant fusion proteins GST-D16, GST-D18 and GST-C were detected by SDS–PAGE and by Western-blot using anti-HF3 antibodies [20] as ~40 kDa proteins (Fig. 1B), which shifted to ~12 kDa after release of GST by thrombin (not shown) and showed the N-terminal amino acid sequences *G-S-P-G-I-P*-L-R-T-D-I-V-S-P (D16 and D18) and *G-S-P-G-I-P*-L-H-N (C) where the italicized letters correspond to amino acid residues encoded by the vector. The yield of the GST-D16 and GST-D18 proteins was estimated as 2 mg/l culture medium while GST-C yielded 1 mg/l.

3.2. Effect of the non-catalytic domains of HF3 on the rolling of leukocytes in the microcirculation

In a previous study we showed the ability of HF3 to activate macrophages for phagocytosis through the integrin $\alpha_M\beta_2$ and suggested that the disintegrin-like/cysteine-rich domains are important for this effect since a recombinant protein corresponding to these domains recapitulated the effects of native HF3 [20]. Moreover, orthophenanthroline-inactivated HF3 was also able to activate macrophages for phagocytosis, indicating that the proteolytic activity of HF3 was not important for the interaction with integrin $\alpha_M\beta_2$. Recent studies have indicated that it is the cysteine-rich domain of P-III SVMPs that functions as a targeting exosite for interaction with their substrates. We showed that the recombinant cysteine-rich domain of P-III SVMP atrolysin A was able to bind VWF [11], FACIT collagens and matrilins [12]. Synthetic peptides from the cysteine-rich domain of atrolysin A and of jararhagin were shown to block collagen-stimulated platelet-aggregation, presumably by binding integrin $\alpha_2\beta_1$ [7]. Similar peptides were recently shown to bind to VWF and to block the binding of the cysteine-rich domain to VWF [28]. These data clearly identify the cysteine-rich domain as the key substrate targeting domain in P-III SVMPs.

In this work we investigated the effect of HF3 and its disintegrin-like/cysteine-rich domains expressed together (GST-DC) or individually (GST-D16, GST-D18 and GST-C) in the pro-inflammatory response by analyzing the alterations on the microcirculatory network using intravital microscopy by transillumination of mice cremaster muscle. The first observation from these experiments is that none of these proteins interfered with microvasculature haemodynamic parameters,

such as vessel diameter, erythrocyte speed or blood flow rate (not shown). HF3 induced leukocyte rolling at 1.6 nM (Fig. 2), however, at higher doses it also caused hemorrhage (not shown). The proteins GST, GST-D16 and GST-D18 at 15 μM did not affect the number of rolling leukocytes, which remained at the basal level of ±20 leukocytes per min (Fig. 2); on the other hand, GST-DC and GST-C proteins at 5 μM increased the number of rolling leukocytes to 100 or higher per min in post-capillary venules between 15 and 30 min after topical application, suggesting that only the proteins containing the cysteine-rich domain (GST-DC and GST-C) were able to affect leukocyte rolling (Fig. 2). Furthermore, GST-DC and GST-C promoted adhesion of part of the leukocytes to the endothelial wall with transmigration being observed in some instances (Fig. 2B and D). These data corroborate the role of the cysteine-rich domain in the interaction of P-III SVMPs with their targets in vivo. Moreover, here we confirm the role of integrin $\alpha_M\beta_2$ in the pro-inflammatory effects of HF3 as monoclonal antibodies anti-α_M and anti-β_2, injected in mice prior to the application of GST-C, abolished leukocyte rolling (Fig. 2).

3.3. Effect of synthetic peptides from the HVR on the rolling of leukocytes in the microcirculation

The recently determined crystal structures of two P-III SVMPs, VAP1 and catrocollastatin/VAP2B showed that in both structures the disintegrin-loop, thought to interact with integrins, is packed by the cysteine-rich domain and inaccessible for protein binding [14,15]. Moreover, a region of the cysteine-rich domain, named the (HVR), was pointed as most divergent and variable in length (16–55 aa) among the ADAM sequences and was suggested to constitute a potential protein–protein adhesive interface (Fig. 1A). In VAP1 and catrocollastatin/VAP2B, the HVR is located at the distal end of the arm constituted by disintegrin-like domain and faces toward the catalytic site [14,15]. Here we investigated the effect of synthetic peptides corresponding to the HVR of HF3 and berythractivase, a pro-coagulant SVMP from *B. erythromelas* [22] in leukocyte rolling in the endothelium (Table 1). As most P-III SVMPs, the HVR of HF3 contains one conserved Cys residue (Cys572, VAP1 numbering) while berythractivase contains an extra residue, besides Cys572, located two positions afterwards (see Fig. 3 of [5]). By comparison of the primary structure of HF3 with that of VAP1 it is possible to infer that all Cys residues of the non-catalytic domains of HF3 form disulfide bonds in a similar fashion to VAP1, however it is not possible to predict what the state of the extra Cys of berythractivase HVR is, therefore, it was replaced by an Ala residue. Our synthetic peptides included another Cys residue (Cys561, VAP1 numbering) at the N-terminus which, although is not considered as part of HVR, served to test the role of a disulfide bond in the HVR-induced pro-inflammatory effects. As shown in

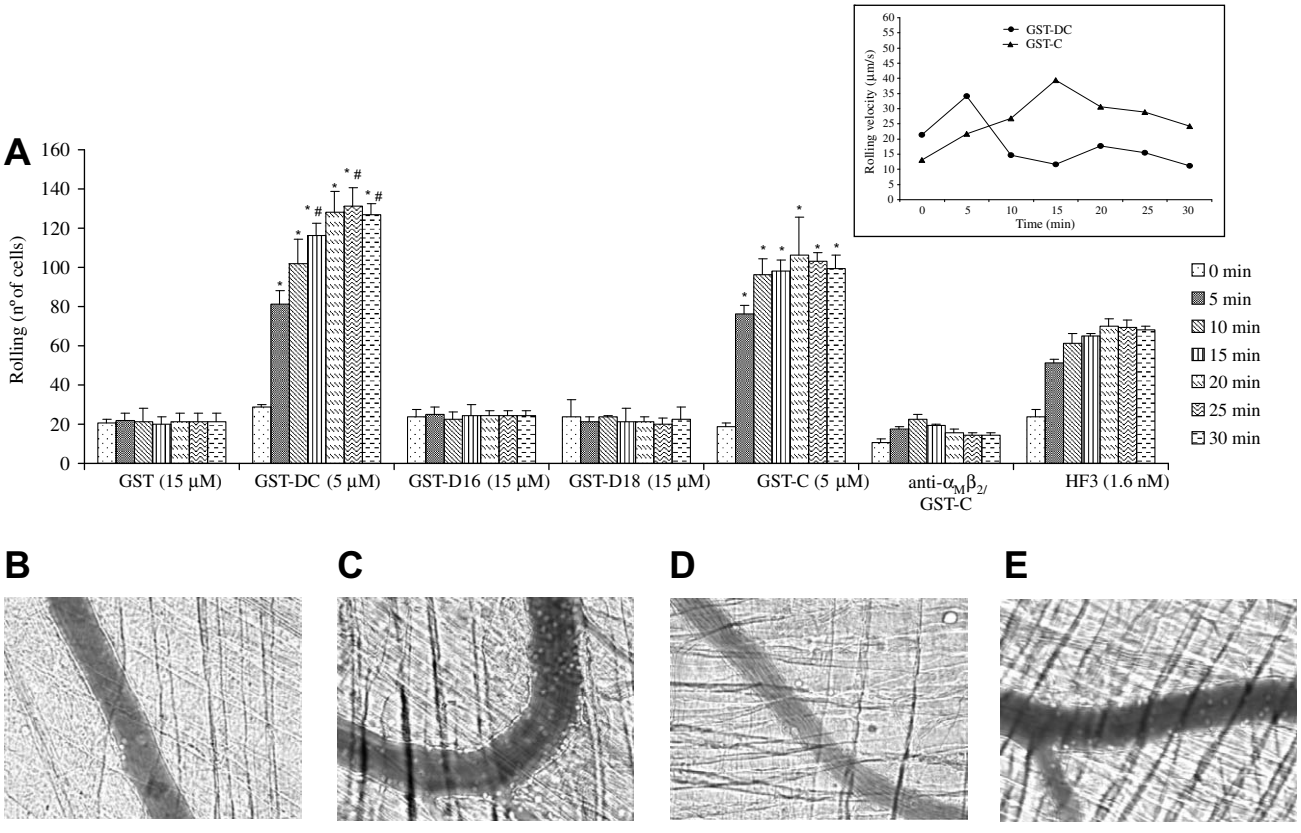

Fig. 2. Number of leukocytes rolling on endothelial wall at different times after the topic treatment with recombinant proteins (GST-DC and GST-C: 5 μM; GST-D16 and GST-D18: 15 μM). Inset: Leukocyte rolling velocity after treatment with GST-DC and GST-C (A). Intravital micrographs of cremaster muscle after topical application of GST (B), GST-DC (C), GST-D16 (D), and GST-C (E). The bars represent means ± S.E.M. of at least three experiments. $^{*}P < 0.05$ compared with GST; $^{*\#}P < 0.05$ compared with GST-C.

Table 1
Amino acid sequences of cysteine-rich domain synthetic peptides. Sequences are numbered according to the VAP1 sequence [14]; disulfide bonds are indicated.

HF3-HVR1	^{561}CHTKKHPCDYKYSEDPDY^{583}
HF3-HVR2	^{562}HTKKHPADYKYSEDPDY^{583}
HF3-HVR3	^{561}CHTKKHPCDYKYSEDPDY^{583}
HF3-CR1	^{526}CFEFNENGDKYFYC^{539}
HF3-CR2	^{526}CFEFNENGDKYFYC^{539}
HF3-CR3	^{548}PCAQEDVKCG^{557}
HF3-CR4	^{554}VKCGRLFCHTKKHPA^{572}
HF3-CR5	^{584}GMVDNGTKCADGKVC^{598}
HF3-CR6	^{591}KCADGKVASNGHCV^{604}
Berythractivase-HVR1	^{561}CNDNSPGQNNPCKAIYFPRNEDR^{583}
Berythractivase-HVR2	^{562}NDNSPGQNNPAKAIYFPRNEDR^{583}

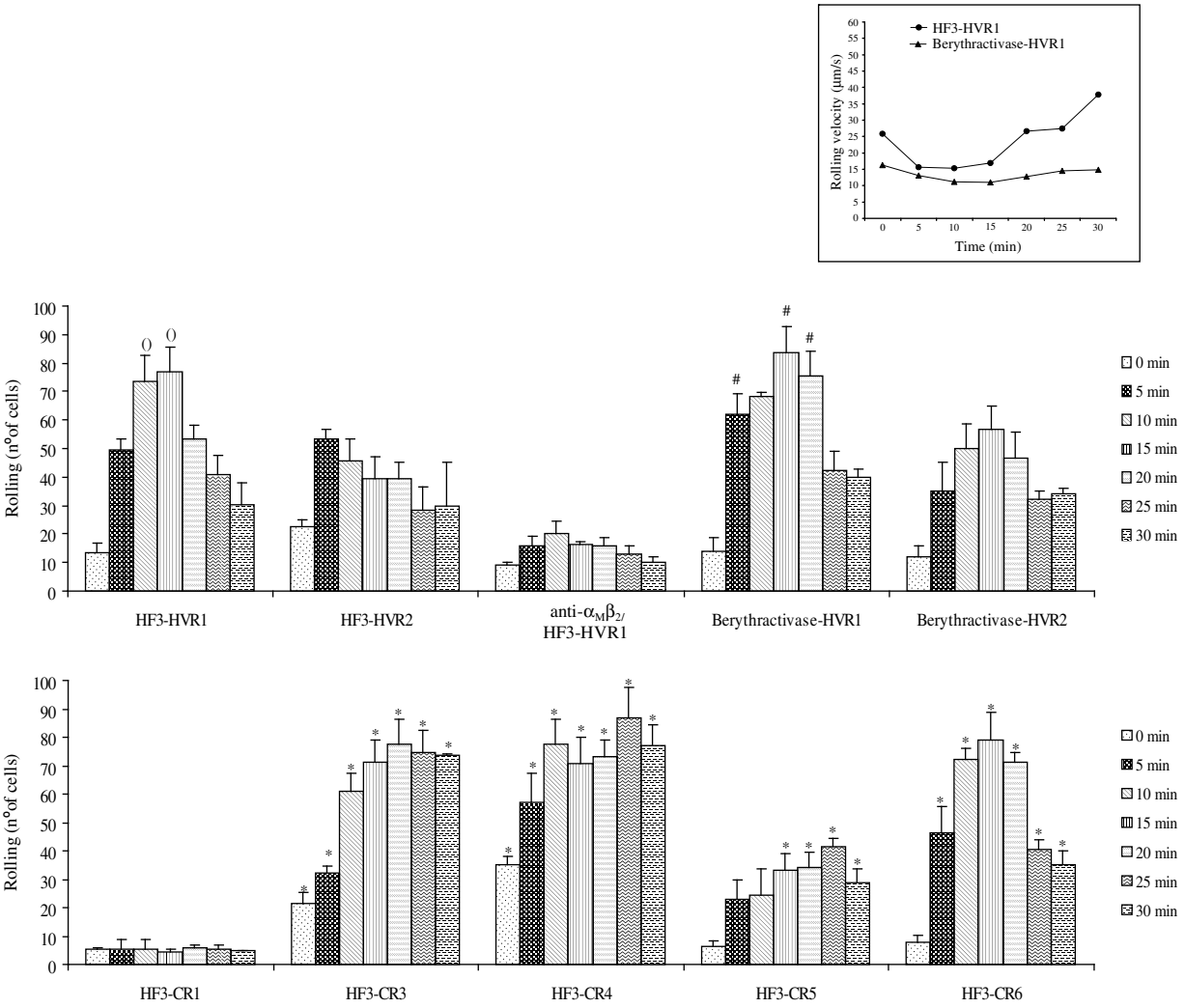

Fig. 3. Number of leukocytes rolling on endothelial wall at different times after the topic treatment with 50 μM peptides. Inset: Leukocyte rolling velocity after treatment with HF3-HVR1 and berythractivse-HVR1. The bars represent means ± S.E.M. of at least three experiments. $^{O}P < 0.05$ compared with HF3-HVR2; $^{\#}P < 0.05$ compared with berythractivase-HVR2; $^{*}P < 0.05$ compared with HF3-CR1.

Fig. 3, despite the notable divergence in the HVR sequences of HF3 and berythractivase, the peptides HF3-HVR1 and berythractivase-HVR1 at 50 μM significantly increased the rolling of leukocytes suggesting that this region of both hemorrhagic and non-hemorrhagic SVMPs may be involved in triggering pro-inflammatory effects. Both HF3-HVR1 and berythractivase-HVR1 peptides contained a disulfide bond, which was not present in peptides HF3-HVR2 and berythractivase-

HVR2, in which the cysteinyl residues were substituted by alanyl residues. The lack of Cys residues in these peptides caused a moderate but significant effect of decreasing the activation of leukocyte rolling (Fig. 3), indicating that the conformational constraint imposed by the disulfide bond may be important for the expression of the peptide pro-inflammatory activity. The significance of Cys residues in the HVR of HF3 was confirmed by testing a linear peptide (HF3-HVR3) that lacked the disulfide bond. The effect of this peptide on the microcirculatory network was rather unexpected as it caused such blood flow lowering in post-capillary venules that made it impossible to evaluate leukocyte rolling (not shown). Nevertheless, it is clear that the cysteinyl residue in the HVR of HF3 plays a role in HF3 effects in the microcirculatory network. As observed with GST-C, anti-αM/β2 antibodies significantly prevented leukocyte rolling induced by peptide HF3-HVR1 (Fig. 3).

We also tested peptides derived from sequences adjacent to the HVR of HF3 (Table 1). Interestingly, peptides corresponding to sequences flanking the N-terminal region of the HVR (HF3-CR3 and HF3-CR4) and the C-terminal region of HVR (HF3-CR5 and HF3-CR6) also induced leukocyte rolling (Fig. 3), suggesting that the C-terminal part of the cysteine-rich domain and not only the HVR may constitute a potential interacting interface. Finally, we tested the corresponding sequence of HF3 (peptides HF3-CR1 and HF3-CR2; Table 1) where three P-III class SVMPs (HR1B, BjussuMP_I, and kaouthiagin) have the integrin-binding motifs KGD or RGD in their cysteine-rich domains [5], while HF3 has a NGD motif. Peptide HF3-CR1, which contains a disulfide bond, showed no effect on leukocyte activation for rolling (Fig. 3), while the corresponding linear peptide (HF3-CR2) caused a striking blood flow lowering in post-capillary venules as was observed with HF3-HVR3 (not shown). Taken together, these data suggest that this portion of the cysteine-rich domain is not involved in the effects of HF3 on leukocytes.

It has been suggested that the HVR of ADAMs and SVMPs, but not the disintegrin domain should be the focus of searches for physiological targets of these enzymes [14]. This is the first report on the evaluation of the pro-inflammatory effects of recombinant individual non-catalytic domains of a P-III SVMP that clearly shows the role of the cysteine-rich domain and of its HVR in the activation of leukocyte rolling in the endothelium. An increasing body of evidence has emerged showing that proteins of the ADAM family critically act as regulators of cell migration in inflammation and invasion [29]. Integrins participate in rolling and mediate leukocyte adhesion. They are activatable receptors since intracellular signaling through cell-surface molecules is required to increase their ligand-binding capability [30]. The $\alpha_M\beta_2$ integrin can be activated by inside-out or outside-in signaling mechanisms to generate high-affinity binding sites on the integrin [31,32]. It is one of the key players of both phagocytic processes by macrophages, and leukocyte rolling and migration. Our previous work showing the activation of macrophage phagocytosis via $\alpha_M\beta_2$ integrin by the disintegrin-like/cysteine-rich domains of HF3 is in agreement with the findings of the present work that suggest that the cysteine-rich domain and its HVR may activate $\alpha_M\beta_2$ integrin for leukocyte rolling. Although there is only limited structural homology shared by the cysteine-rich domains of the P-III SVMPs and the ADAMs and ADAMTSs, our results suggest an analogous role for the cysteine-rich domains in certain members of the ADAM and ADAMTS families of proteins in inflammatory processes.

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