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Patagonfibrase modifies protein expression of tissue factor and protein disulfide isomerase in rat skin

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ABSTRACT

Patagonfibrase is a hemorrhagic metalloproteinase isolated from the venom of the South American rear-fanged snake *Philodryas patagoniensis*, and is an important contributor to local lesions inflicted by this species. The tissue factor (TF)-factor VIIa complex, besides triggering the coagulation cascade, has been demonstrated to be involved in inflammatory events. Our aim was to determine whether patagonfibrase affects the expression of TF and protein disulfide isomerase (PDI), an enzyme that controls TF biological activity, at the site of patagonfibrase injection, and thus if they may play a role in hemostatic and inflammatory events induced by snake venoms. Patagonfibrase (60 μg/kg) was administered s.c. to rats, and after 3 h blood was collected to evaluate hemostasis parameters, and skin fragments close to the site of injection were taken to assess TF and PDI expression. Patagonfibrase did not alter blood cell counts, plasma fibrinogen levels, or levels of TF activity in plasma. However, by semiquantitative Western blotting, patagonfibrase increased TF expression by 2-fold, and decreased PDI expression by 3-fold in skin samples. In agreement, by immunohistochemical analyses, prominent TF expression was observed in the subcutaneous tissue. Thus, patagonfibrase affects the local expression of TF and PDI without inducing any systemic hemostatic disturbance, although that they may be involved in the local inflammatory events induced by hemorrhagic metalloproteinases. Once antivenom therapy is not totally effective to treat the local injury induced by snake venoms, modulation of the activity and expression of TF and/or PDI might become a strategy for treating snake envenomation.

1. Introduction

Tissue factor (TF) is an essential component for initiating blood coagulation *in vivo*. The binding of TF to factor VIIa, in the presence of membrane phospholipids, cleaves factor X and IX, thus initiating blood coagulation. TF is a single-chain integral membrane protein constitutively expressed in vascular smooth muscle cells, adventitial fibroblasts and pericytes, but its expression can be induced on the surface of mononuclear cells, platelets and endothelial cells (van der Poll et al., 2011). Several inflammatory mediators have been shown to promote protein expression and enhanced biological activity of TF (Breitenstein et al., 2010). In addition to express TF molecules on their surfaces, cells have been demonstrated to regulate TF procoagulant activity by means of protein disulfide isomerase (PDI), which regulates the formation of the Cys_{186}-Cys_{209} disulfide bond in the extracellular domain of TF. The oxidation of this disulfide bond renders TF decrypted and with higher procoagulant activity (Kothari et al., 2013; Lysov et al., 2014).

Snake venoms are complex mixtures of proteins, peptides and small organic molecules with a variety of potent enzymatic and ligand-based biological activities (Mackessy and Mackessy, 2009). Among the enzyme-based toxins, an important class includes the snake venom metalloproteinases (SVMP) which act synergistically with many other toxins to induce a complex series of local and systemic pathophysiological effects upon envenomation (Gutiérrez et al., 2009). In a recent publication (Yamashita et al., 2014), increased TF activity in plasma, and increased protein expression of TF in lungs and at the site of inoculation were noticed in rats injected with *Bothrops jararaca* venom. When the crude venom was inhibited by EDTA, TF activity in plasma was drastically reduced, indicating that SVMP were crucial to this increase. Various PIII-

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SVMP (Fox and Serrano, 2008), such as jararhagin from *B. jararaca* venom (Moura-da-Silva and Baldo, 2012), by accumulating on the basement membrane of capillaries and venules, lead to hemorrhage, edema and necrosis, and thus play an important role in local tissue damage. Other PIII-SVMP, such as berythractivase from *Bothrops erythromelas* venom, preferentially exhibit a systemic procoagulant action (prothrombin activation) and are non-hemorrhagic (Baldo et al., 2010; Moura-da-Silva et al., 2008; Silva et al., 2003). Incubation of berythractivase with endothelial cells increases gene and protein expression of TF, whilst jararhagin does not (Pereira et al., 2006). Recently, an activator of factor X and prothrombin isolated from *Bothrops moojeni* (Sartim et al., 2015), moojenactivase, also induced increased procoagulant activity of TF in peripheral blood mononuclear cells.

Patagonfibrase (Pf) is a P-III class metalloproteinase isolated from the venom of the South American rear-fanged snake *Philodryas patagoniensis* (Peichoto et al., 2007, 2010). Local reactions – such as pain, ecchymosis, erythema and edema – are conspicuous signs of snakebites inflicted by this species, but no hemostatic systemic signs, such as hemorrhage or blood incoagulability, are noticed in patients bitten by this snake (de Medeiros et al., 2010). Taking into consideration previous studies (Peichoto et al., 2011) that demonstrated that Pf is an important contributor to local inflammation and local hemorrhage elicited by *P. patagoniensis* envenomation, this study aimed to understand whether altered TF expression induced by hemorrhagic SVMP in dermis could induce *per se* augmented levels of TF in plasma.

2. Materials and methods

2.1. Animals

Male Wistar rats (220–250 g) were obtained from the Animal House of Butantan Institute, and were supplied with free access to food and water. All procedures involving the use of rats were approved by the Animal Ethical Committee of Butantan Institute (protocol 883/12), and were in accordance with the Guide for Care and Use of Laboratory Animals (2011), the International Guiding Principles for Biomedical Research Involving Animals (2012), the Brazilian guidelines from the Conselho Nacional de Controle de Experimentação Animal (CONCEA) (2015), and ARRIVE guidelines. Rats were anesthetized by intraperitoneal administration of xylazine (10 mg/kg b.w.) and ketamine hydrochloride (100 mg/kg b.w.).

2.2. Philodryas patagoniensis venom (PpV) and purification of patagonfibrase (Pf)

A pool of PpV was obtained from wild specimens captured in northeastern Argentina and maintained at the serpentarium of the local Zoo, Corrientes, Argentina. Venom was extracted according to a procedure described previously (Ferlan et al., 1983). Pf was purified from PpV as previously described (Peichoto et al., 2007). Protein concentrations were determined (Smith et al., 1985) using bovine serum albumin (Sigma, USA) as a protein standard.

2.3. Envenomation protocol and sample collection

Pf (60 μg/kg) was administered s.c. to rats; this dose reproduced a characteristic hemorrhagic lesion. Rats injected with saline were used as negative controls. Three hours after Pf injection (period of time considered representative of the acute phase reaction of *Philodryas* envenomation), rats were anesthetized, and blood was collected by puncture of the abdominal aorta. For complete blood counts (CBC), blood (500 μL) was collected into plastic bottles containing 5 μL of 269 mM Na_2-EDTA, and samples were counted in an automated cell counter BC-2800 Vet (Mindray, China). To obtain plasma samples, blood (4.3 mL) was collected into plastic bottles containing 700 μL of CTAD anticoagulant (75 mM trisodium citrate, 42 mM citric acid, 139 mM dextrose, 15 mM theophylline, 3.7 mM adenosine, 0.2 mM dipyridamole, and 2 mM imipramine) (Santoro and Sano-Martins, 2004), and centrifuged at 2500 *g* for 15 min at 4 °C.

One circular 4-cm-diameter skin fragment, whose center was the point of Pf inoculation, was also removed from each animal and used to evaluate protein expression of TF and PDI by Western blotting (WB) (Yamashita et al., 2014), and immunohistochemistry (IH) (Santoro and Sano-Martins, 2004).

2.4. Assays in blood samples

Plasma fibrinogen was assayed as described elsewhere (Ratnoff and Menzie, 1951). Prothrombin time was assayed by incubating plasma samples (80 μL) with rat thromboplastin ((Yamashita et al., 2014), 40 μL) for 1 min at 37 °C, and then 50 mM $CaCl_2$ (40 μL) was added, and clotting times were measured on a Start4 coagulometer (Diagnostica Stago, France). TF activity was evaluated in plasma samples with Actichrome TF kit (American Diagnostica, USA), according to manufacturer's instructions.

2.5. Assays with skin samples

2.5.1. Western blotting

TF and PDI protein expression in rat skin lysate supernatants was evaluated as described previously (Yamashita et al., 2014), except that membranes were incubated at room temperature for 2 h with either 1:500 mouse monoclonal anti-TF antibody (TF9-10H10, Calbiochem, USA) or 1:5000 rabbit polyclonal anti-PDI antibody (Sigma P7372). Expression of β-actin, used as a loading control, was evaluated using 1:5000 mouse monoclonal anti-β-actin antibody (Sigma A5316), and 1:10000 peroxidase-conjugated anti-mouse IgG (Sigma A4416). Membranes were developed as reported elsewhere (Antunes et al., 2010), and densitometric analyses were carried out identically as described (Yamashita et al., 2014).

2.5.2. Histological analysis and immunohistochemistry

Histological sections were stained with hematoxylin-eosin or toluidine blue. TF protein expression in rat skin sections was evaluated as described (Santoro and Sano-Martins, 2004), except that slides were incubated with 1:50 mouse monoclonal anti-TF antibody (TF9-10H10, Calbiochem, USA); negative controls were performed without the use of primary antibody. Specimens were incubated later with 1:100 peroxidase-conjugated anti-mouse IgG (Sigma A4416), and the detection of primary antibody was performed with DAB (3,3′-diaminobenzidine tetrahydrochloride hydrate, Sigma D5637) or AEC (3-amino-9-ethylcarbazole, Sigma A5754) – according to manufacturer's instructions -, and counterstained with 1% neutral red or Mayer's hematoxylin counterstain, respectively.

2.6. Statistical analyses

One-way ANOVA, followed by the Tukey test, was used to compare quantitative results. Statistical analyses were performed using the software SigmaStat (version 3.5, USA). Differences with $p < 0.05$ were considered statistically significant. Data were expressed as mean ± standard deviation (SD).