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Angiostatin-like molecules are generated by snake venom metalloproteinases

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Abstract

Angiostatin is a plasminogen-derived anti-angiogenic factor composed of its first four kringle structures. This molecule is generated by proteolytic cleavage of plasminogen by some proteolytic enzymes in vitro. Since venoms of viper snakes are a rich source of both serine- and metalloproteinase, we hypothesized that angiostatin-like polypeptides could be generated during the envenomation after snake bites and play a pathophysiological role in the local tissue damage and regeneration. Our results showed that crude venoms from several species of *Bothrops* snakes were able to generate angiostatin-like polypeptides and purified metalloproteinases but not serine proteinases from *Bothrops jararaca* and *Bothrops moojeni* venoms were responsible for their generation in vitro. The putative plasminogen cleavage sites by the crude venoms and purified proteinases were determined by N-terminal amino acid sequencing of the angiostatin-like molecules. Angiostatin-like peptides derived from human plasminogen digestion by jararhagin, a metalloproteinase isolated from *B. jararaca* venom, inhibited endothelial cell proliferation in vitro. These results indicate that angiostatin-like molecules can be generated upon snakebite envenomations and may account for the poor and incomplete regenerative response observed in the damaged tissue.

Keywords: Angiostatin; Metalloproteinases; Snake venom; Bothropic snakes; Plasminogen

Angiogenesis, the formation of new vessels from microvessels, is important in embryogenesis, development, wound healing, tumor growth, and other diseases. Several stimuli, including hypoxia, tumor drive, inflammatory or connective tissue cells, generate angiogenic molecules such as fibroblast growth factors (FGF), vascular endothelial growth factors (VEGF), transforming growth factor-beta (TGF-beta), platelet-derived growth factor (PDGF), and angiopoietin (Ang1). Angiogenesis inhibitory factors may also occur under physiological or pathological conditions. The balance between pro- and anti-angiogenic factors will control the generation of new vessels and therefore the blood supply in the new areas of recently formed tissue. Therefore, manipulation of the conditions necessary for vessels formation in angiogenesis has been of great value in processes such as wound healing, inflammatory diseases, ischemic heart and peripheral vascular diseases, and cancer, providing new therapeutic options and understanding of pathological mechanisms [1].

Angiostatin has been shown to be a very important anti-angiogenic factor. It is a circulating angiogenesis

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inhibitor that was first isolated from serum and urine of mice bearing a murine Lewis lung carcinoma. This polypeptide was characterized as a primary factor controlling the dormant state of secondary metastatic tumor cells by inhibition of angiogenesis, resulting in consequent decrease in blood supply and reduction of tumor growth. Angiostatin shows selective inhibition of endothelial cell growth and is composed of a 38 kDa internal plasminogen fragment, containing its first four kringle structures [2,3]. Functional angiostatin-like molecules can be generated from plasmin reduction and proteolysis [4], plasminogen digestion by pancreas elastase [5], urokinase-activated plasmin [6], prostate specific antigen [20], cathepsin D [21], and by several matrix metalloproteinases, including the macrophage metalloelastase or MMP-12 [6,10], human matrilysin or MMP-7, gelatinase B/type IV collagenase or MMP-9 [7,10], stromelysin or MMP-3 [8,10], gelatinase A or MMP-2 [9,10].

Since viper snake venoms are a rich source of proteinases, mainly zinc-metalloproteinases structurally related to matrix metalloproteinases (MMPs) and to the ADAMs (a disintegrin and metalloproteinases) [11,12], we hypothesized that the snake enzymes would be able to generate functional angiostatin-like molecules that would explain in part the impairment of wound healing and regeneration of the damaged tissues observed upon viperidae snake bite envenoming. In this study, we showed that active angiostatin-like molecules are generated by snake venom metalloproteinases in vitro, providing support to our hypothesis.

Materials and methods

Materials. All snake venoms were obtained from Instituto Butantan (São Paulo, Brazil). Plasminogen was purchased from Chromogenix (Sweden). Jararhagin, bothropasin, HF3, PA-BJ, and KN-BJ proteinases from *B. jararaca* and *Moojeni* protease A from *B. moojeni* were purified as described [13–18]. The purity of the enzymes was assessed by SDS–PAGE. All other reagents used were of analytical grade.

Sequencing of human plasminogen fragments. Human plasminogen (30 μg) was incubated with 5 μg of each crude venom or 1.5 μg of each purified proteinase in a 50 μl reaction volume containing 0.1 M Tris–HCl, pH 8.5, 0.01 M $CaCl_2$ for different time intervals at 37 °C. The reaction was stopped with the SDS–PAGE sample buffer, fractionated on a 12% SDS–polyacrylamide gel, and electroblotted onto a PVDF membrane as described [19]. The proteins were visualized with 0.1% Coomassie blue R250 in 50% methanol and destained and the bands of interest were cut out with a clean razor. The proteins in the PVDF membrane were sequenced on an Applied Biosystems model 477A sequenator.

Inhibitory activity of angiostatin-like molecules on the proliferation of endothelial cells. Human umbilical vein endothelial cells (HUVECs) were kept in RPMI medium containing 10% fetal calf serum and 10 ng/ml of recombinant fibroblast growth factor-2 (FGF-2 or bFGF) [39]. For inhibitory proliferation activity assay of angiostatin-like molecules, 1.2×10^4 cells were plated on each well (24-multiwell plate, Corning) in RPMI medium containing 10% fetal calf serum. In the next day, angiostatin-like molecules with or without FGF-2 were added to the cells. After 72 h, the cells were detached with trypsin and counted under a phase-contrast microscope.

Results and discussion

Bothrops snakes are important representatives of vipers. They are involved in approximately 90% of all human accidents in South America. Their venom induces a severe coagulopathy, followed by lesions at the site of the bite characterized by inflammatory reaction, hemorrhage, and necrosis of difficult regeneration, which are currently related to the direct action of the venom toxins and also to the hypoxia induced by intravascular coagulation and consequent reduction of blood supply. In this paper, we analyzed whether the generation of anti-angiogenic factors such as angiostatin occurs by the action of snake venom components thus contributing to the impairment of the regenerative response. Our first step was to evaluate whether angiostatin could be liberated by the action of venom proteinases. For this purpose, we incubated human plasminogen with *B. jararaca*, *B. pradoi*, *B. alternatus*, *B. moojeni*, *B. leucurus*, *B. pirajai*, *B. neuwiedi*, *B. insularis*, and *B. bilineata* venoms for 2 h and evaluated the degradation products by SDS–PAGE. Plasminogen is a 92 kDa protein of the human plasma composed of the N-terminal preactivation peptide, followed by five kringle structures and the C-terminal proteolytic domain. Its concentration in the human plasma is around 200 μg/ml (2 μM). Angiostatin is composed of an internal 38 kDa fragment of plasminogen containing only the first four kringle structures. As shown in Fig. 1, the plasminogen degradation products after incubation with several bothropic snake venoms included bands of molecular mass of approximately 38 kDa, compatible with the size expected for angiostatin, supporting the idea that angiostatin-like fragments can be generated upon envenoming by different species of *Bothrops* snakes. It is interesting to note that plasminogen was not hydrolyzed to small peptides but remained as large fragments around 36–38 kDa, despite the presence of large amounts of proteinases in bothropic venoms.

To evaluate the ability of snake venom proteinases to generate angiostatin-like polypeptides, we next incubated human plasminogen, for different time intervals, with purified proteinases isolated from *B. jararaca* or *B. moojeni* venoms. The serine proteinases KN-BJ and PA-BJ, isolated from *B. jararaca* venom, cleave preferentially peptide bonds containing arginine or lysine residues [16,17]. KN-BJ releases bradykinin from kininogen and converts fibrinogen to fibrin by splitting fibrinopeptide A. PA-BJ induces platelet aggregation by cleaving the protease-activated G protein-coupled receptors PAR1 and PAR4 [37]. Jararhagin [13], both-

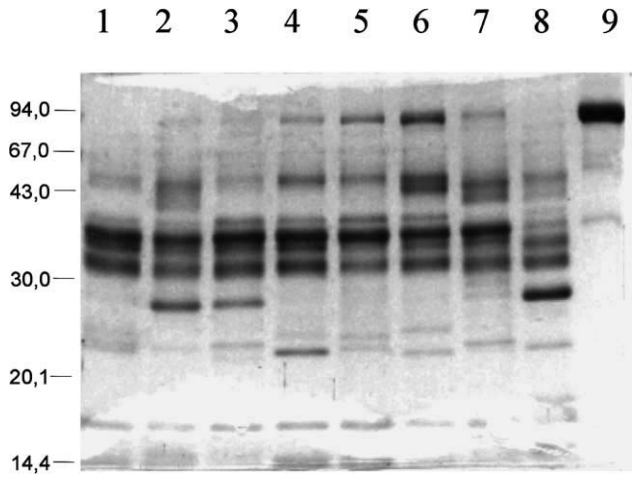

Fig. 1. Digestion of human plasminogen by crude snake venoms. Human plasminogen was incubated for 120 min with *B. pradoi* (lane 1), *B. alternatus* (lane 2), *B. moojeni* (lane 3), *B. leucurus* (lane 4), *B. pirajai* (lane 5), *B. neuwiedi* (lane 6), *B. insularis* (lane 7), and *B. lineata* (lane 8). Non-digested human plasminogen is in lane 9. The 12% SDS–PAGE was performed under reducing condition and further stained with Coomassie blue.

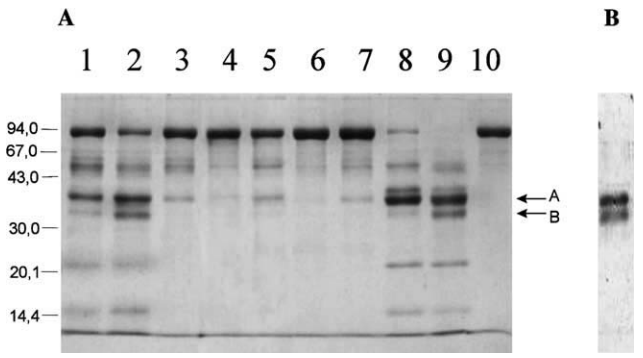

Fig. 2. SDS–PAGE analysis of human plasminogen digestion by snake venom metalloproteinases. (A) Human plasminogen was incubated by 30 min (lanes 1, 3, 4, 6, and 8) or 120 min (lanes 2, 5, 7 and 9) with *B. jararaca* crude venom (lanes 1, 2), purified jararhagin (lane 3), HF3 (lanes 4, 5), bothropasin (lanes 6, 7), and *moojeni* protease A (lanes 8, 9). Non-digested human plasminogen is in lane 10. The 12% polyacrylamide gel was stained with Coomassie blue. The corresponding protein bands A and B (indicated by the arrows) were submitted for N-terminal sequencing after electroblotting a replica gel to a PVDF membrane. (B) Enriched angiostatin-like peptides (bands A and B, Fig. 2) after digestion of human plasminogen with jararhagin. The 12% SDS–PAGE was performed under reducing condition and further stained with Coomassie blue.

ropasin [14], and HF3 [15] are long chain metalloproteinases of PIII class, with potent hemorrhagic activity, slightly differing in molecular size due to differences in the degree of glycosylation. *Moojeni* protease A (MPA) [18] is a PI class venom metalloproteinase isolated from *B. moojeni* venom, with minor hemorrhagic activity. Both KN-BJ and PA-BJ serine proteinases completely failed in hydrolysing human plasminogen (results not shown). However, all the purified metalloproteinases generated fragments around 38 kDa (Fig. 2), the same molecular mass reported for angiostatin. MPA was the most active metalloproteinase on human plasminogen, yielding several bands around 36–38 kDa. These bands were enriched upon prolonged incubation time and seemed to be very stable and resistant to further cleavage by the proteinase, even after 24 h of incubation (results not shown).

The identity of the 38 kDa (band A) and 36 kDa (band B) fragments generated by proteolysis was confirmed by N-terminal amino acid sequencing and further comparison with angiostatin N-terminal sequence (Fig. 3). A replica gel was run, electroblotted to a PVDF membrane and the corresponding bands indicated by the arrows in Fig. 2 were cut out from the membrane and sequenced. Angiostatin primary structure [2] shows plasminogen Val^{79} as the N-terminal amino acid residue and predicts Glu^{399}, Ala^{440} or Ser^{441} as C-terminal amino acid residue. The N-terminal sequences obtained from bands A and B are summarized in Fig. 3. All the A bands generated either by *B. jararaca* venom or by the isolated proteinases jararhagin, bothropasin or HF3 showed the N-terminal sequence VVAPPPV. In the case of MPA, the N-terminal sequence obtained for band A was SVVAPPPV. These results showed that the putative cleavage site was either between the Ser^{441}–Val^{442} or Ala^{440}–Ser^{441} bonds, respectively. In the case of the band B (Fig. 2), different N-terminal sequences were obtained, depending on the proteinase used in the human plasminogen digestion. Comparing to angiostatin N-terminus, most of these angiostatin-like peptides have their amino acid sequence shortly extended at N-terminus (Fig. 3, arrows 1–5) and are structurally very similar to angiostatin. Two N-terminal sequences resulted in a putative shorter angiostatin-like peptide than the reported angiostatin (Fig. 3, arrows 6 and 7). In all cases, the preactivation peptide was removed and almost all the resulting fragments contained the first four kringles as reported for angiostatin [2]. Only one N-terminal sequence, obtained by digestion with MPA (Fig. 3, arrow 7), indicated that the cleavage site was after the Cys^{84}, resulting in the loss of kringle 1 structure. However, kringles 2, 3, and 4 are expected to be conserved in this case. It is interesting to note that isolated kringles (K1, K2, K3, K4, and K5) as well as kringles in tandem (K1-3, K1-4, K1-5, and K2-3) are all active, differing in their potencies [5,6]. The most active anti-angiogenic plasminogen-derived polypeptide is displayed by the K1-5 polypeptide, with a half-maximal concentration of approximately 50 pM, a value 50-fold greater than that reported for K1-4 angiostatin [6].

The observed cleavage sites were consistent with the expected specificity of venom metalloproteinases, except N-termini indicated by arrows 1, 5, and 7, generated by MPA. On the insulin B-chain, this enzyme shows the typical metalloproteinase specificity for hydrophobic

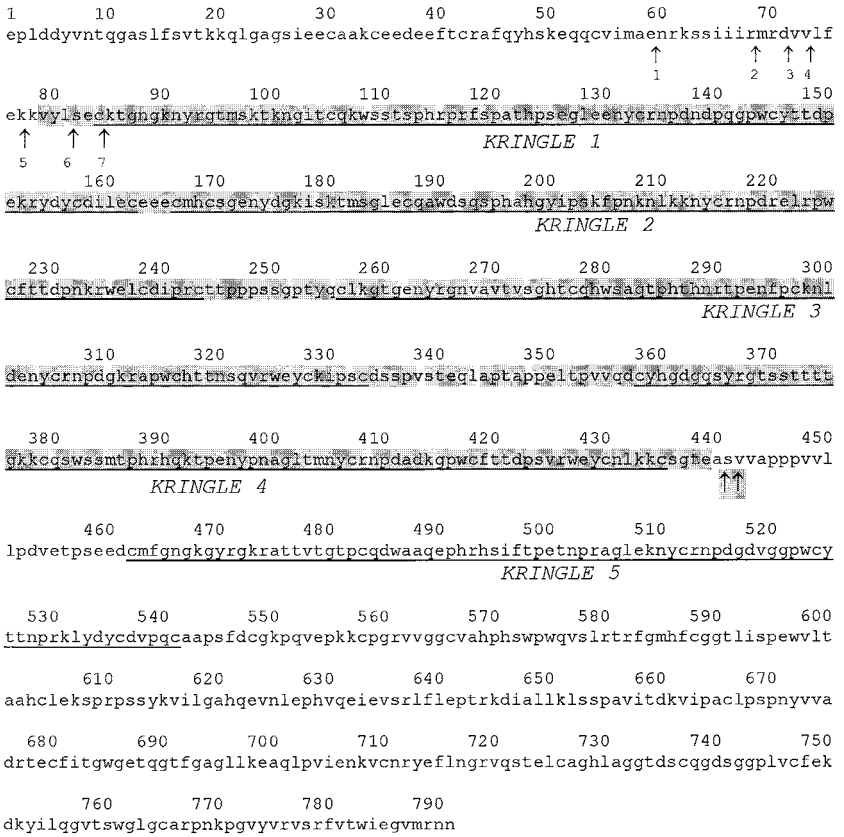

Fig. 3. Human plasminogen amino acid sequence and the internal angiostatin fragment. The five kringle structures of plasminogen are underlined and indicated. The reported (1) angiostatin amino acid sequence is shaded gray. The identified cleavage sites to generate angiostatin-like peptides by snake venom metalloproteinases digestion of human plasminogen are indicated by arrows. Gray shaded arrows indicate the cleavage sites identified by N-terminal sequencing of band A (Fig. 2). The non-shaded arrows show the putative cleavage sites obtained by N-terminal sequencing of band B (Fig. 2) after *B. moojeni* protease A (arrow 1), *B. jararaca* venom, *B. moojeni* protease A, and bothropasin (arrow 2), *B. jararaca* venom (arrow 3), *B. moojeni* protease A (arrow 4), *B. jararaca* venom, bothropasin, and *B. moojeni* protease A (arrow 5), jararhagin (arrow 6), and *B. moojeni* protease A (arrow 7) digestions of human plasminogen.

residues. However, upon longer incubation time, it can also cleave the Glu–Arg bond [14,40]. So, the peptide bonds 1, 5, and 7 may have been cleaved by extensive incubation with this enzyme, or they may be the result of a first cleavage on hydrophobic residues, which exposed the fragment to the action of the catalytic domain of plasminogen or of other minor contaminating enzymes. According to our data, MPA showed the highest proteolytic activity on plasminogen. MPA is a class I snake venom metalloproteinase (SVMP), comprising only the metalloproteinase domain, like some MMPs. In contrast, jararhagin, HF3, and bothropasin presented lower catalytic activity but higher specificity. These enzymes are class III SVMPs, which display additionally to the MMP-like metalloproteinase domain, the disintegrin and cysteine rich domains, similar to the ADAM protein family. The disintegrin domain is apparently involved in a closer contact of the toxin to the endothelium, thus, enhancing the hemorrhagic activity of the enzyme. Our data suggest that the disintegrin domain is not necessary for plasminogen cleavage and may explain the number of MMPs reported as processing enzymes for angiostatin generation. On the other hand, the presence of the disintegrin domain appears to impose a higher specificity to the metalloproteinase domain. All the PIII class enzymes tested presented only one cleavage site compatible with angiostatin N-terminus, whereas the PI class MPA presented several possible cleavage sites.

By these results, it is conceivable that during the envenoming process, a complex mixture of polypeptides and proteins is generated, including these angiostatin-like molecules. To determine if this angiostatin-like polypeptide mixture is active on endothelial cells, we carried out a purification protocol to enrich the 36–38 kDa fragments to avoid other cellular responses [12] that would be elicited by the presence of SVMPs after human plasminogen incubation with purified jararhagin (Fig. 2B). We have used a combination of FPLC gel filtration on Superose 12, hydrophobic chromatography on Phenyl-Superose, and C18 RP-HPLC (results not shown). The final preparation contained predominantly bands A and B (Fig. 2B) that are composed of the kringle 5 plus the catalytic domain of plasminogen and the kringles 1–4, respectively. This fraction was able to

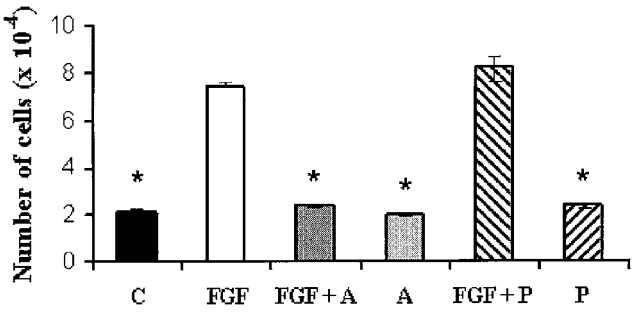

Fig. 4. Anti-proliferative activity of angiostatin-like peptides obtained by human plasminogen digestion with purified jararhagin. The HUVECs (1.2×10^4) were plated on a 24-multiwell plate in 10% fetal calf serum in RPMI/199 medium (1:1). On the next day, 2.5 μg/ml angiostatin-like peptides (A) and/or 10 ng/ml FGF-2 (F) were added to the wells and the cells were trypsinized and counted under microscope after additional 3 days in culture. As control, 5 μg/ml uncleaved plasminogen (P) with or without 10 ng/ml FGF-2 was added to the cells in the same conditions as described above. The cells kept in 5% fetal calf serum in RPMI/199 without any other additive throughout the assay are represented by (C). $*P < 0.003$ when compared to the value obtained from cells stimulated with FGF-2 (Student's t test).

inhibit the proliferation of HUVECs induced by FGF-2. In contrast, the proliferation of HUVECs incubated with FGF-2 was not inhibited by uncleaved human plasminogen (Fig. 4). The anti-proliferative activity towards endothelial cells could be attributed to band B, the angiostatin-like fragment present in our preparation. However, the activity of band A cannot be excluded, since it was recently reported that recombinant kringle 5 is also active on endothelial cells [22] as well as other angiostatin-like peptides with different kringle structures representing multiple forms of angiostatins [5,6,22,23].

Envenomation by viper snakes is characterized by severe local tissue damage. The local pathophysiological alterations develop very rapidly after the venom injection and often result in permanent sequelae. Several venom components are responsible for this effect, including metalloproteinases. Snake venom metalloendopeptidases are secreted as preproenzymes and contain additional regulatory modules, which are presumably responsible for interactions with the extracellular matrix and integrins [11,36]. Venom metalloproteinases are probably involved in local myonecrosis, skin damage, edema, and associated inflammation [12]. It is well documented that venom metalloproteinases impair muscle regeneration and drastically affect microvessels. Unlike metalloproteinases, venom myotoxic phospholipase A_2 induces myonecrosis without damaging and altering the capillar vessels. The injured skeletal muscle regenarates due to activation of satellite cells, which are myogenic cells located beneath the basal lamina of muscle fibers [33] and this process is dependent on adequate blood supply [12]. However, when the myotoxic PLA_2 is injected in the presence of the snake venom metalloproteinase BaH1 from *B. asper*, the regenerative response is poor, showing reduced density of capillary vessels [34,35]. So, it is conceivable that venom metalloproteinases impair local damage regeneration and one mechanism involved could be the production of angiogenesis inhibitors that account for bad blood supply to the damaged tissue and consequent poor regenerative response.

Recently, it has been proposed that in several cases, venom metalloproteinases are mimicking the roles of endogenous metalloproteinases. The normal conversion of pro-TNF-α into active TNF-α by the TNF-α converting enzyme (TACE) is mimicked by purified SVMPs that should account in part for the local tissue damage induced by venoms of viper snakes [24]. We anticipate that other cellular responses mediated by mammalian metalloproteinases or ADAMs can also be elicited by snake venom metalloproteinases, like shedding of ligand receptors [25] and generation of angiogenesis inhibitors. Angiostatin is an endogenous angiogenic inhibitor produced by digestion of plasminogen. Our data on the in vitro generation of angiostatin-like peptides from human plasminogen by venom metalloproteinases provide support to this hypothesis. Other endogenous inhibitors can also be generated like angiostatin, by cleavage and release of active anti-angiogenic protein domains, such as those derived after proteolytic cleavage of thrombospondin, platelet factor 4, kininogen, prothrombin, collagen XVIII, and antithrombin [26–32]. All these data provide strong evidence that the clotting and fibrinolytic pathways are directly involved in the regulation of angiogenesis probably by enzymatic actions on clotting and fibronolytic substrates in some cases [38]. Since viperidae venoms severely affect these pathways, it is possible that novel or some of these inhibitors can also be generated by snake venom proteinases during envenomation, in addition to angiostatin or angiostatin-like molecules as shown here. In addition, due to the relative facility to obtain purified amounts of snake venom metalloproteinases, the use of these enzymes can be of low cost and an alternative to other matrix metalloproteinases to produce angiostatin by digestion of plasminogen.

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