Specific conformational changes of plasminogen induced by chloride ions, 6-aminohexanoic acid and benzamidine, but not the overall openness of plasminogen regulate, production of biologically active angiostatins

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The overall conformation of plasminogen depends upon the presence of anions and molecules such as AHA (6-aminohexanoic acid) and BZ (benzamidine). The purpose of the present study was to determine the effect of conformation on the initial and secondary cleavages of plasminogen to generate active angiostatins. Plasminogen was digested with the physiologically relevant neutrophil elastase in one of the four Tris/acetate buffers: buffer alone or buffer plus NaCl, AHA or BZ. The initial cleavage of Glu¹-plasminogen was much slower in the tight NaCl-induced α -conformation, fastest in the intermediate BZ-induced β -conformation and intermediate both in the control and in the AHA-induced open γ -conformation. Although the buffer system determined the relative amounts of the initial cleavage products, the same four cleavage sites were utilized under all conditions. A fifth major initial cleavage within the protease domain was observed in the presence of BZ. N-terminal peptide cleavage

required for angiostatin formation occurred as either the initial or the secondary cleavage. Angiostatins were generated fastest in the presence of BZ and slowest in the presence of NaCl. Both the initial and secondary cleavages were affected by the modifying agents, indicating that they influence the conformation of both Glu-plasminogen and the initial cleavage products. The angiostatins produced under the different conditions inhibited proliferation of human umbilical-vein endothelial cells. These results suggest that plasminogen conversion into active angiostatins is dependent more on the specific conformation changes induced by the various modifying reagents rather than on the overall openness of the molecule.

Key words: 6-aminohexanoic acid, angiostatin, conformation, neutrophil elastase, N-terminal peptide, plasminogen.

INTRODUCTION

Plasminogen, the inactive precursor of the serine protease plasmin, circulates in the bloodstream as a 92 kDa glycoprotein consisting of seven domains. The NTP (N-terminal peptide) domain of 83 amino acids is susceptible to cleavage by plasmin and other proteases. Five internal kringle domains, each comprising approx. 80 amino acids, are double-looped disulphide structures that contain the LBSs (lysine-binding sites) responsible for plasminogen binding to extracellular matrix molecules [1,2], cell receptors [3,4] and inhibitors [5]. These kringles are connected by linker peptides of differing lengths. A C-terminal protease domain is cleaved at the Arg⁵⁶⁰–Val⁵⁶¹ peptide bond by specific plasminogen activators to convert plasminogen into plasmin. Plasmin principally functions as an effector of fibrinolysis in wound healing [6], but when bound to specific cell-surface and extracellular matrix receptors, plasmin participates in degradation of extracellular matrices during cell migration, tissue remodelling, tumour cell invasion and inflammation [7,8].

Plasminogen is also the precursor for a group of anti-angiogenic molecules, the angiostatins. These molecules, consisting of kringle domains K1–3 (kringles 1–3), K1–4 and K1–5, as well as the single kringle domains (other than K4), have anti-etherangiogenic activity [9–11]. Angiostatin molecules are usually generated by cleavage of plasminogen in the linker regions between kringle

domains; however, several active molecules are generated by cleavage and reduction of disulphide bonds within K5 [12].

Regulation between angiogenesis and anti-angiogenesis is a delicate balance controlled by complex mechanisms. Tumour cells and inflammatory cells synthesize plasminogen activators for the activation of plasminogen to plasmin, proteases that are capable of inducing angiogenesis, as well as forming angiostatin fragments from plasminogen [12–16]. Neutrophils present at sites of injury and inflammation release proteases including neutrophil elastase. This is the major protease produced in neutrophils that converts plasminogen into K1–3 angiostatin molecules with biological activity [17]. The mechanism of formation of angiostatin by neutrophil elastase has not been elucidated.

Plasminogen with the NTP intact, Glu¹-plasminogen, can assume at least three different conformations [18]. At physiological concentrations of NaCl, Glu¹-plasminogen exists in the tight α -conformation with the NTP bound to the LBS on K5 and a radius of gyration of 3.07 nm. In the presence of BZ (benzamidine), plasminogen is in the intermediate β -conformation with a radius of gyration of 4.21 nm. In the presence of AHA (6-aminohexanoic acid), plasminogen undergoes a dramatic change in conformation to assume the extended, flexible γ -conformation with a radius of gyration of 5.03 nm. This change occurs when the low-affinity LBSs on Glu¹-plasminogen are occupied by AHA, a molecule that competes with lysine residues on the protein for

Abbreviations used: AHA, 6-aminohexanoic acid; BZ, benzamidine; ECGS, endothelial cell growth supplement; FBS, foetal bovine serum; FGF-2, fibroblast growth factor-2; HRP, horseradish peroxidase; HUVEC, human umbilical-vein endothelial cells; K1–3, kringles 1–3; K1–3L, K1–3 with most of the linker region between K3 and K4; K1–3S, K1–3 with little of the linker region between K3 and K4; K4–5-Prot, K4–5 plus the protease domain; K4–5-ProtL, K4–5-protease domain with a long sequence from the linker region between K3 and K4; K4–5-ProtS, K4–5-protease domain with a short sequence from the linker region between K3 and K4; K5-Prot, K5 plus the protease domain; LBS, lysine-binding site; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NTP, N-terminal peptide; NK1–3, NTP plus K1–3; NK1–3L, NK1–3 with most of the linker region between K3 and K4; NK1–3S, NK1–3 with little of the linker region between K3 and K4.

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the LBS on the kringles. The openness of plasminogen is also affected by the presence of acetate ions. Plasminogen has an intermediate sedimentation coefficient in the presence of acetate ions ($s_{20,w}^0 = 5.4$ S) between that in the presence of Cl^- ($s_{20,w}^0 = 5.8$ S) and AHA ($s_{20,w}^0 = 4.7$ S) [19]. Addition of AHA to plasminogen in the presence of Cl^- or acetate ions reduces the sedimentation coefficient to $s_{20,w}^0 = 4.7$ S.

Conformation of the plasminogen molecule plays an important role in regulating the activation of plasminogen and thus the fibrinolytic activity of plasmin. Under physiological Cl^- concentrations, the α -conformation is favoured [20]. This likely prevents or limits activation to plasmin in the bloodstream, where the concentration of plasminogen is high, but the need for plasmin activity is low unless a fibrin clot has formed. In contrast, occupation of the LBS on the kringles by lysine residues of cell receptors or fibrin forces plasminogen into the easily activatable form (γ -conformation) [5]. Upon activation of plasminogen to plasmin, the active enzyme will degrade the fibrin clot during wound healing, or will degrade the extracellular matrix aiding cell migration.

Although the conversion of plasminogen into angiostatin molecules has been studied using a number of proteases [13,16,21], the role of plasminogen conformation in the formation of these kingle fragments has not been addressed. It would be expected that the conformation of plasminogen controls the generation of angiostatins. In blood or other solutions with physiological levels of NaCl, the need for anti-angiogenic activity is low. As a result, the α -tight conformation present under these conditions may prevent the conversion of plasminogen into angiostatins. Interaction of the LBSs of plasminogen with lysine residues or Arg-Gly-Asp sequences in cell-surface or extracellular matrix proteins leads to a conformational change of plasminogen [22], probably exposing cleavage sites for generation of angiostatins.

The purpose of the present studies was to examine the effect of plasminogen conformation induced by Cl^- , AHA (mimic of protein lysine residues [18]) and BZ (mimic of Arg-Gly-Asp sequences [23]) on the generation of active angiostatins by a physiologically relevant protease, human neutrophil elastase, that is released during inflammation. Because of the sensitivity of proteolysis for probing local conformational changes, use of known modifiers of overall plasminogen structure offers the opportunity to explore both local and known overall changes in conformation of plasminogen.

EXPERIMENTAL

Reagents

Human Glu¹- and Lys⁷⁸-plasminogens were purchased from Haematological Technologies (Essex Junction, VT, U.S.A.). Human neutrophil elastase was from Athens Research and Technology (Athens, GA, U.S.A.). Monoclonal antibodies raised against human plasminogen were obtained from American Diagnostica (Greenwich, CT, U.S.A.). The plasminogen antibodies used were directed against the NTP (#3641), K1-3 (#3642), K5-Prot (K5 plus the protease domain; #3644) and K4 (#3647). The HRP (horseradish peroxidase)-labelled anti-mouse IgG was from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Silver staining was performed using a kit from Pierce Endogen (Rockford, IL, U.S.A.). Casein, AHA and BZ were purchased from Sigma (St. Louis, MO, U.S.A.).

Casein digestion by human neutrophil elastase

Casein (300 $\mu\text{g}/\text{ml}$ final concentration) in each Tris-based buffer was digested with 150 ng/ml human neutrophil elastase for 60–

240 min. The products were separated by SDS/PAGE and the gel was stained with Coomassie Brilliant Blue.

Plasminogen digestion by human neutrophil elastase

Glu¹- or Lys⁷⁸-plasminogen was diluted in buffer at 200 $\mu\text{g}/\text{ml}$ and incubated in a shaking 37 °C water bath for 5 min. Control buffer (Con) consisted of 100 mM Tris acetate/100 mM sodium acetate (pH 7.8). Chloride buffer (Cl^-) consisted of 100 mM Tris/HCl/100 mM NaCl (pH 7.8). AHA buffer consisted of 100 mM Tris acetate/100 mM sodium acetate/100 mM AHA (pH 7.8). BZ buffer consisted of 100 mM Tris acetate/100 mM sodium acetate/50 mM BZ (pH 7.8). Buffers were filtered through a 0.22 μm filter (Pall Corp., Ann Arbor, MI, U.S.A.) before use to avoid any bacterial contamination. Human neutrophil elastase or buffer only was added at a final concentration of 2 $\mu\text{g}/\text{ml}$ and the mixtures were incubated in a shaking water bath. Samples were taken at timed intervals from 30 min to 4 h, added to SDS sample buffer (50 mM Tris/HCl, pH 6.8, 2%, w/v, SDS, 0.1% Bromophenol Blue and 15% glycerol) and frozen until used. Each digestion was repeated multiple times.

SDS/PAGE and Western-blot analyses

The digested samples were separated by SDS/PAGE (10% polyacrylamide) under non-reducing conditions and the proteins were transferred on to nitrocellulose membranes. Non-specific binding was blocked by incubating the membranes in 10% (w/v) non-fat dry milk (Nestle USA, Solon, OH, U.S.A.) in 20 mM TBS (Tris-buffered saline) containing 0.1% Tween 20 (pH 7.6) (TBS-T) overnight. After washing, the membranes were incubated with a primary monoclonal antibody directed to the NTP, K1-3, K4 or K5-Prot in 1% BSA in TBS-T. The membranes were washed and incubated with the HRP-labelled goat anti-mouse IgG, washed again and developed with ECL[®] (Amersham Biosciences, Piscataway, NJ, U.S.A.). The bands were visualized by exposure to an X-ray film. The bands observed on some blots were digitized using an α -imager 2000 (Packard Instrument, Meriden, CT, U.S.A.). The linear range was determined relative to a standard curve. Only blots in this linear range were digitized. Representative blots are given in Figures 1–3 and 5–8.

Electroelution

Single Coomassie Blue-stained bands from non-reducing SDS/PAGE were cut from the original gel and eluted using an electroeluter (Bio-Rad Laboratories) according to the manufacturer's instructions. The products were re-electrophoresed using SDS/PAGE in the presence of 5% (w/v) 2-mercaptoethanol and the proteins were silver-stained using a kit (Pierce Endogen).

N-terminal sequencing

The plasminogen degradation products generated in the Con, AHA and BZ buffers after 15 and 120 min were separated by non-reducing SDS/PAGE, transblotted on to PVDF membranes and stained with Amido Black. The blots were then submitted for N-terminal sequencing of the bands by Edman degradation at the Protein and Nucleic Acid Core Facility at the Medical College of Wisconsin.

Isolation of angiostatin molecules from digests of plasminogen

Digests of plasminogen by neutrophil elastase in Con, AHA and BZ buffers were inhibited after 4 h using 100 μM PMSF and used for isolation of angiostatin fragments. AHA digest was dialysed

extensively against 50 mM PBS (pH 7.4) before the next step. The digests, which contained only plasminogen degradation products, were passed over a 1 ml lysine–Sepharose column (Amersham Biosciences, Uppsala, Sweden). The column was washed sequentially with 10 ml of 50 mM phosphate buffer (pH 7.4), 5 ml of the same buffer plus 50 mM NaCl, 5 ml of the same buffer plus 500 mM NaCl and 5 ml of the initial buffer. The specifically bound products were eluted with 3 ml of 200 mM AHA. AHA was removed by dialysis versus water. Samples were concentrated to 1.5 ml using a spin concentrator with a 10 kDa molecular-mass cut-off (Millipore, Bedford, MA, U.S.A.). Samples were then dried using a spin vacuum system and resuspended in PBS (Invitrogen, Carlsbad, CA, U.S.A.). The amount of angiostatin material was measured using dot-blots with an angiostatin standard and a K1–3 antibody. Western-blot analyses were performed on the final samples to confirm the presence of angiostatin molecules and the absence of plasminogen or degradation products greater than 60 kDa.

Vascular endothelial cell proliferation assay

Human umbilical vascular endothelial cells were obtained from the A.T.C.C. (Manassas, VA, U.S.A.). The cells were grown in F12-K nutrient mixture Kaighn's modification (Invitrogen) containing 10% (v/v) FBS (foetal bovine serum; Hyclone, Logan, UT, U.S.A.), 0.03 mg/ml ECGS (endothelial cell growth supplement), 0.1 mg/ml heparin (Sigma) and 10 µg/ml ciprofloxacin (Serologicals Proteins, Kankakee, IL, U.S.A.). Cells were trypsinized and re-plated at 10^3 cells/well of a 96-well plate. At 24 h, the initial medium was exchanged for a medium containing 2.5% FBS and heparin, but without ECGS. Cells were cultured in the presence of 0.25–4 µg of angiostatin products/100 µl of medium and 10 ng/ml FGF-2 (fibroblast growth factor-2; Sigma). A K1–4 angiostatin standard (Haematologic Technologies) was included in each assay. The cells were cultured for 72 h and the number of cells was quantified using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay kit (Roche Applied Science, Indianapolis, IN, U.S.A.) according to the manufacturer's instructions. Formazan produced in this assay is the result of mitochondrial activity, and the amount of formazan formed is directly related to the number of live cells. The results were confirmed in an independent experiment. Statistical significance was determined using a one-way ANOVA for overall differences and the Tukey test for individual comparisons with the program SigmaStat (SPSS, Chicago, IL, U.S.A.).

RESULTS AND DISCUSSION

The susceptibility of Glu¹-plasminogen to initial neutrophil elastase cleavage is dependent on the presence of Cl[−], BZ and AHA, but not on the overall openness of the molecule

To study the effects of conformation on the ability of neutrophil elastase to convert plasminogen into angiostatin, four buffer conditions were chosen based on the known conformational information of plasminogen in these buffers. The Con buffer used was 100 mM Tris/acetate buffer (pH 7.8), which induces a conformation that is quite open but can further be opened by LBS-binding ligands [19]. The Con buffer plus 100 mM NaCl (Cl[−], tight) and 100 mM AHA (open) were used because they induce the two extremes in plasminogen conformation [18]. The Con buffer plus 50 mM BZ was used because it induces an intermediate β -conformation between the tight α and open γ forms [18].

Human neutrophil elastase was chosen for these studies because it is the major enzyme released by neutrophils at the site of injury or infection and mediates conversion of plasminogen into angiostatin [17]. To confirm that the activity of neutrophil elastase was not altered by the various conditions, casein was incubated with this enzyme in the presence of buffer only or buffer plus Cl[−], AHA or BZ for 0–240 min. No differences were noted among the SDS/PAGE-separated proteolytic products of casein or their levels generated over time in the different buffers (results not shown).

The loss of intact plasminogen at 83 kDa was used as a measure of initial cleavage of Glu¹-plasminogen by neutrophil elastase (Figure 1A). Human plasminogen was incubated with human neutrophil elastase in the four buffer systems, and samples were taken at various times and analysed by Western blots using the plasminogen NTP antibody. As would be predicted, full-length Glu¹-plasminogen was degraded slowest in the presence of Cl[−] over the 4 h period (Figures 1A and 1B). The susceptibility of plasminogen to neutrophil elastase in the presence of the Con and AHA conditions was similar and, as expected, was greater than in the presence of Cl[−]. Unexpectedly, plasminogen was cleaved fastest in the presence of BZ, a reagent which places plasminogen in the intermediate β -conformation [18]. In contrast with the Con and AHA samples, plasminogen was mostly degraded by 2 h in the presence of BZ rather than by 4 h. These results would suggest that the availability of peptide bonds in plasminogen susceptible to neutrophil elastase cleavage is not directly related to overall openness of plasminogen, but depends on the specific conformational changes induced by the four conditions.

The susceptibility of Lys⁷⁸-plasminogen to neutrophil elastase cleavage is altered relative to the control by BZ, but not by Cl[−] or AHA

The NTP is important in the assumption of the tight α -conformation and for the decreased rate of activation of plasminogen to plasmin in the presence of Cl[−] relative to that in the presence of AHA [20,24]. To determine whether the NTP is also required for the Cl[−] effect on initial cleavage by neutrophil elastase, the same experiment as given in Figure 1(A) was carried out using Lys⁷⁸-plasminogen that is missing the first 77 N-terminal amino acids. Neutrophil elastase cleaved Lys⁷⁸-plasminogen in the presence of Cl[−] at a similar rate to that of the Con and the AHA-containing buffer (Figure 1B), indicating that the NTP is important in determining the availability of peptide bonds in full-length Glu¹-plasminogen for cleavage by neutrophil elastase in the presence of Cl[−]. In the presence of BZ, Lys⁷⁸-plasminogen, like Glu¹-plasminogen, was more susceptible to cleavage than in the presence of the other three buffer conditions. Therefore the NTP is not involved in the increase in cleavage rate of Glu¹-plasminogen in the presence of BZ relative to Con and AHA.

Glu¹-plasminogen is initially cleaved at four independent cleavage sites by neutrophil elastase under control conditions

In the presence of the Con buffer, 100 mM Tris/acetate buffer (pH 7.8), plasminogen assumes a conformation intermediate between the open γ -conformation present in AHA and the tight α -conformation found in Cl[−] [18]. Plasminogen degradation products were generated with time in the presence of neutrophil elastase under Con conditions (Figures 2 and 3). Plasminogen incubated alone was not degraded over the 4 h time period (results not shown).

Within 1 min, initial cleavage of Glu¹-plasminogen by neutrophil elastase yielded two stable cleavage products and one

Figure 1 Effect of chloride, AHA and BZ on the initial cleavage of plasminogen by neutrophil elastase

Glu¹-plasminogen (**A**) or Lys⁷⁸-plasminogen (**B**) were digested in the presence of buffer alone (Con) or buffer with the addition of 100 mM NaCl (Cl), 100 mM AHA (AHA) and 50 mM BZ. Samples were removed at 1, 15, 30, 60, 120 and 240 min. The products were separated by SDS/PAGE under non-reducing conditions, transblotted and then probed with monoclonal antibodies directed to the NTP of plasminogen (**A**) or K1–3 (**B**). The densities of the bands were plotted. Pg, plasminogen.

Figure 2 Effect of chloride, AHA and BZ on the initial cleavage products of plasminogen by neutrophil elastase

Glu¹-plasminogen was digested in the presence of buffer alone (Con) or with the addition of 100 mM NaCl (Cl), 100 mM AHA or 50 mM BZ. Samples were removed at 1, 15, 30 and 60 min. The products were separated by SDS/PAGE under non-reducing conditions, transblotted and then probed with monoclonal antibodies directed to K5-Prot (**A**) or to K1–3 (**B**).

transient product (Figures 2A and 2B, Con, 1 min). A 38 kDa product was recognized by the K5-Prot antibody but not by the K1–3 antibody (Figure 2A versus Figure 2B, Con, 1 min), and a 45 kDa product was recognized by the K1–3 antibody but not by

Figure 3 Identification of the neutrophil elastase cleavage products of plasminogen produced under control conditions in 100 mM Tris/acetate buffer (pH 7.8) using monoclonal antibodies

Glu¹-plasminogen was incubated in the presence of neutrophil elastase for 240 min. The products were separated by SDS/PAGE under non-reducing conditions, transblotted and then probed with monoclonal antibodies directed to the NTP, K1–3, K4 or K5-Prot.

the K5-Prot antibody (Figure 2B versus Figure 2A, Con, 1 min), indicating that these are the two halves of Glu¹-plasminogen. The 38 kDa product reacted with the K5-Prot antibody but not with the K4 antibody (Figure 3), and had an N-terminal sequence of

Figure 4 Summary of the major neutrophil elastase cleavage sites that generate angiostatin from plasminogen

(A) Five major initial cleavage sites. (B) Initial products formed by the five initial cleavages. (C) Major secondary cleavage site. (D) Major secondary cleavage products. CHO, carbohydrate; Plgn, plasminogen.

Ala-Pro-Pro-Pro-Val-Val, showing that cleavage occurred between K4 and K5 at the Val⁴⁴³-Ala⁴⁴⁴ bond. Thus the 38 kDa band represents K5-Prot (Figures 4A and 4B4, cleavage 4). The 45 kDa band reacted with antibodies directed to the NTP, K1-3 and K4 antibody (Figure 3) and had an N-terminal sequence of Glu-Pro-Leu-Asp-Asp-Val. Hence the 45 kDa band represents the NK1-4 (NTP plus K1-4).

By 15 min, a 78 kDa cleavage product was observed (Figures 2A and 2B, Con, 15 min), which reacts with antibodies directed to K5-Prot, K1-3 and K4 but not with the NTP antibody (Figure 3). An N-terminal sequence of Leu-Phe-Glu-Lys-Lys-Val-Tyr confirmed the 78 kDa band as a plasminogen cleavage product missing the first 73 amino acids. Based on its apparent mass and the N-terminal amino acid sequence, this 78 kDa band

probably represents Leu⁷⁴-plasminogen (Figures 4A and 4B, cleavage 1).

In addition to the bands observed at 1 min, four additional products appeared at 15 min, two that reacted with the K5-Prot antibody at 46 and 49 kDa (Figure 2A, Con, 15 min) and two that reacted with the K1-3 antibody at 34 and 37 kDa (Figure 2B, Con, 15 min). The apparent masses of these bands suggest that these products represent two sets of cleavage pairs. In addition to the anti-K5-Prot antibody, the 46 and 49 kDa products also reacted with the K4 antibody (Figures 2A and 3). N-terminal analysis of the 49 kDa band revealed a sequence of Ser-Thr-Glu-Xaa-Leu-Ala-Phe. Cleavage at the Val³³⁸-Ser³³⁹ bond generated a product composed of a long segment of the K4-5-ProtL [K4-5-Prot (K4-5 plus the protease domain) with a long sequence from the linker

region between K3 and K4] (Figures 4A and 4B2, cleavage 2). N-terminal analysis of the 46 kDa band gave a sequence of Val-Gln-Asp-Xaa-Tyr-His-Gly, indicative of cleavage at the Val³⁵⁴-Val³⁵⁵ bond to generate a product composed of a short segment of the K4-5-ProtS (K4-5-Prot with a short sequence from the linker region between K3 and K4) (Figures 4A and 4B2, cleavage 3). The relative amounts of the 49 kDa versus the 46 kDa anti-K5-Prot reactive products (Figure 2A) imply that the Val³⁵⁴-Val³⁵⁵ bond is more available for cleavage compared with the Val³³⁸-Ser³⁹⁹ bond. The difference in apparent mass between these two cleavage products reflects not only a difference of 16 amino acids but also the presence of an O-linked carbohydrate chain attached to Thr³⁴⁶ [25]. This difference in apparent mass is also reflected in the corresponding anti-K1-3 reactive bands at 34 and 37 kDa (Figure 2B, Con). These bands react as well with the NTP antibody (Figure 3). Therefore we surmise that the 34 kDa band represents the NK1-3S [NK1-3 (NTP plus K1-3) with little of the linker region between K3 and K4] and the 37 kDa band represents the NK1-3L (NK1-3 with most of the linker region between K3 and K4) (Figures 4B3 and 4B4, cleavage 3). N-terminal sequencing confirmed that both of these bands have Glu¹ as the N-terminal amino acid.

These results suggest that there are four main early neutrophil elastase cleavage sites in plasminogen under control conditions (Figures 4A and 4B). Two of these four sites differ from those reported for pancreatic elastase [26]. Pancreatic elastase cleaves the Val⁷⁹-Tyr⁸⁰ and the Ala⁴⁴⁰-Ser⁴⁴¹ bonds rather than at the Val⁷³-Leu⁷⁴ and Val⁴⁴³-Ala⁴⁴⁴ bonds observed for neutrophil elastase. Both enzymes cleaved the Val³³⁸-Ser³³⁹ and the Val³⁵⁴-Val³⁵⁵ bonds. These results probably reflect the differences in specificity for the two enzymes. Pancreatic elastase cleaves on the C-terminal side of alanine, glycine, serine and valine, but has a preference for alanine in both the P1 and P2 sites, while neutrophil elastase cleaves on the C-terminal side of alanine, cysteine, serine and valine residue, with a preference for valine [27,28]. For this latter enzyme, the P2 site usually is an alanine, proline or valine.

The use of multiple initial cleavage sites for angiostatin production differs from that observed for efficient activation of plasminogen on the cell surface, where the NTP is initially cleaved, followed by the activation peptide in the protease domain [29]. Under control conditions, not only is the Val⁷³-Leu⁷⁴ bond in the NTP exposed, but sites in the K3-4 linker and the K4-5 linker are also available for cleavage.

Under control conditions, the initial NTP-containing products are secondarily cleaved to generate Leu⁷⁴-angiostatins

By 30 min, an additional band at 40 kDa was observed that reacts with anti-K1-3 (Figure 2B, Con, 30 min) and K4 antibodies, but not the N-terminal antibody (Figure 3). Sequencing of this band revealed an N-terminal sequence of Leu-Phe-Glu-Lys-Lys-Tyr, so this product appears to represent the angiostatin form of K1-4 beginning with Leu⁷⁴ (K1-4). At least two cleavage reactions were required for the generation of this angiostatin form, one at the Val⁷³-Leu⁷⁴ bond and the other probably at the Val⁴⁴³-Ala⁴⁴⁴ bond in the linker region between K4 and K5 (Figure 4, cleavages 1 and 4).

By 60 min, an additional smaller K1-3-containing 32 kDa product was observed (Figure 2B, Con) that does not react with anti-NTP (Figure 5A). By 240 min, a band at 29 kDa that does not react with the NTP antibody was observed (Figure 3). The N-terminal amino acid sequence for both products was Leu-Phe-Glu-Lys-Lys-Tyr, suggesting that these are K1-3 angiostatin products formed from the 37 and 34 kDa NK1-3 products and differ at the C-terminal end by the presence and absence of part of the linker

Figure 5 Comparison of the neutrophil elastase secondary cleavage products of plasminogen at 120 and 240 min in the presence and absence of 100 mM NaCl

Glu¹-plasminogen was digested in the presence of buffer alone (Con) (A) or buffer with 100 mM NaCl (Cl⁻) (B). Samples were removed at 120 and 240 min. The products were separated by SDS/PAGE under non-reducing conditions, transblotted and then probed with monoclonal antibodies directed to the NTP or to K1-3.

region between K3 and K4 [K1-3S (K1-3 with little of the linker region between K3 and K4) and K1-3L (K1-3 with most of the linker region between K3 and K4)] (Figure 4D, products 1 and 2).

By 120–240 min, additional bands were observed that react with anti-NTP and anti-K1-3 (Figures 3 and 5A). A 42 kDa band reacted with anti-NTP but not with anti-K1-3, suggesting cleavage within the kringle region that destroyed the epitope recognized by the anti-K1-3 antibody. Additional anti-K1-3 reactive bands were observed, i.e. 33 and 36 kDa, but these were not further studied because our focus was on the initial major cleavages leading to angiostatin formation.

These results support the hypothesis that the major secondary cleavage site that generates angiostatin molecules occurred at the Val⁷³-Leu⁷⁴ bond to generate K1-4 and K1-3 angiostatin molecules (Figure 4C). This is the same site that was cleaved to generate the 78 kDa initial cleavage product (Figures 4A and 4B). This 78 kDa Leu⁷⁴-plasminogen product is probably also further cleaved within the linker regions between K3 and K4 and between K4 and K5 to generate the observed angiostatin molecules at 29, 32 and 40 kDa. These results imply that cleavage of the NTP can occur as the first or second step.

In presence of Cl⁻, Glu¹-plasminogen in the tight α -conformation is less susceptible to cleavage by neutrophil elastase and less angiostatin is produced

Physiological concentrations of Cl⁻ maintain plasminogen in the tight α -conformation, in contrast with the more open conformation in the Con buffer [18]. In the presence of Cl⁻, very little plasminogen was degraded even at 240 min (Figure 1A); however, some degradation products were generated (Figures 2A and 2B, Cl, and Figure 5B). At 1 min, the same 38 kDa (K5-Prot) and 45 kDa (NK1-4) fragments were observed as in the control (Figure 2, Cl, 1 min). By 15 min, the 49 kDa protease-containing fragment (K4-5-ProtL) (Figure 2A, Cl, 15 min) and the 34 kDa K1-3-containing fragment (NK1-3S) (Figure 2B, Cl, 15 min) were observed at a concentration similar to that of the control (Figures 2A and 2B, Con, 15 min). In contrast, the levels of the 49 kDa protease-containing peptide (K4-5-ProtS) and the 37 kDa N-K1-3-containing-peptide (NK1-3L) were much lower in the presence of Cl⁻ than in the control (Figures 2A and 2B, Con versus Cl, 15–60 min). This probably reflects a difference in the availability of the Val³⁵⁴-Val³⁵⁵ cleavage site in the linker between

Figure 6 Effect of chloride and AHA on the generation of cleavage products of Lys-plasminogen by neutrophil elastase

Lys⁷⁸-plasminogen was digested in the presence of buffer alone (Con) or buffer with 100 mM NaCl (Cl) or 100 mM AHA for 240 min. The products were separated by SDS/PAGE under non-reducing conditions, transblotted and then probed with monoclonal antibodies directed to K1–3 (A) and K5-Prot (B).

K3 and K4 in the presence of Cl[−] from that in the Con buffer. Very little, if any, of the 78 kDa N-terminal cleavage product was generated in the presence of Cl[−], which would suggest that the frequency of the initial cleavage in the NTP is lower than in the kringle region.

The 40 kDa K1–4 angiostatin product was observed by 15 min (Figure 2B, Cl), and by 120–240 min, a small amount of the 32 kDa K1–3 angiostatin was formed (Figure 5B). Between 15 and 60 min, the levels of the initial products formed were not significantly increased (Figures 2A and 2B, Cl). By 120 and 240 min, the concentration of the products formed in the presence of Cl[−] was much less than that observed in the control (Figure 5B versus Figure 5A). It appears that in the tight α -conformation, the neutrophil-elastase-susceptible peptide bands in Glu-plasminogen are less exposed than in the more open form under the control conditions.

NTP is required for the effect of Cl[−] on generation of angiostatins by neutrophil elastase

To determine whether the effect of Cl[−] on the generation of angiostatin molecules was dependent on the presence of the NTP, Lys⁷⁸-plasminogen was incubated with neutrophil elastase in the presence and absence of Cl[−]. No differences were observed in the apparent mass or the amounts of products formed in the presence and absence of Cl[−] at 240 min (Figure 6, Con versus Cl) or at earlier times (results not shown). The anti-K5-Prot reactive bands at 38 kDa (K5-Prot), 46 kDa (K4–5-ProtS) and 49 kDa (K4–5-ProtL) were observed at similar levels to that for the control (Figure 6A). A band at 37 kDa was also noted, which probably represents cleavage at a second site within the K4–5 linker. The expected anti-K1–3 reactive angiostatin bands, the 40 kDa K1–4 band, the 32 kDa K1–3L band and the 29 kDa K1–3S band were observed at similar levels to those in the control (Figure 6B, Con versus Cl).

These results suggest that, in the presence of Cl[−], the NTP is required for the formation of a conformation in which the neutrophil-elastase-susceptible bonds are less exposed than in the presence of the Con alone. The resistance of neutrophil-elastase-susceptible bonds to cleavage in the presence of Cl[−] is similar to that for plasminogen activation [20]. The major difference is that the initial cleavage of plasminogen by neutrophil elastase occurs

Figure 7 Effect of AHA on the generation of secondary cleavage products of Glu-plasminogen by neutrophil elastase

Glu¹-plasminogen was digested in the presence of 100 mM AHA for 120 and 240 min. The products were separated by SDS/PAGE under non-reducing conditions, transblotted and then probed with monoclonal antibodies directed to the NTP, K1–3, K5-Prot and K4.

mainly within the kringle linkers rather than in the NTP observed during activation [29].

The initial neutrophil elastase cleavage of plasminogen in the AHA-induced open γ -conformation is similar to that of the control

Upon binding AHA to the LBSs of K1, K2, K4 and K5, plasminogen adopts a very open, flexible γ -conformation [18]. The initial cleavage products generated from plasminogen by neutrophil elastase in the presence of AHA were identical with that for the control (Figure 2, Con versus AHA). By 15 min, the 78 kDa Leu⁷⁴-plasminogen, the 49 kDa Val³⁵⁵ K4–5-ProtS, the 46 kDa Ser³³⁹ K4–5-ProtL and the 38 kDa Ala⁴⁴⁴-K5-Prot bands that react with the K5-Prot antibody were observed (Figure 2A, AHA). The anti-K1–3 reactive cleavage partners, the 34 kDa NK1–3S, 37 kDa NK1–3L and 45 kDa NK1–4, were visualized in the same time frame (Figure 2B, AHA). These initial cleavage products had the expected N-terminal sequences that were identical with those observed for the control.

The presence of AHA alters the rate of the secondary neutrophil elastase cleavage to form K1–3 and K1–4 angiostatins

Unlike under the other conditions, the cleavage products containing the NTP, the 45 kDa NK1–4, the 37 kDa NK1–3L and 34 kDa NK1–3S products were very stable, with the appearance of only low levels of other products even at 240 min (Figure 2B, AHA, and Figure 7). Since cleavage of the NTP to yield Leu⁷⁴-plasminogen was similar to that of the control (Figure 2A and 2B, AHA versus Con), the resistance to cleavage of the products containing the NTPs appears to be directly related to the presence of AHA. In the presence of AHA bound to the LBS, the NTP apparently alters the conformation of the NK1–4 and NK1–3 products in a manner that buries the neutrophil-elastase-susceptible bond, Val⁷³–Leu⁷⁴.

AHA does not alter the generation of angiostatins from neutrophil elastase cleavage of Lys⁷⁸-plasminogen

To determine whether AHA affects the cleavage of plasminogen in the absence of the NTP, Lys-plasminogen was incubated in the presence of 100 mM AHA and the products were analysed. The products formed that reacted with anti-K5-Prot, anti-K4 and anti-K1–3 and their amounts were similar to those for the control

Figure 8 Characterization of the high-molecular-mass neutrophil elastase cleavage products of Glu¹- and Lys⁷⁸-plasminogen in the presence of BZ

(A) Time course of generation of the high-molecular-mass Glu¹-plasminogen degradation products. Glu¹-plasminogen was digested in the presence of 50 mM BZ and samples were taken at 1, 15, 30, 60 and 240 min. The products were separated by SDS/PAGE under non-reducing conditions, transblotted and the membrane was probed with a monoclonal antibody directed to K1–3. (B) Identification of the Glu¹-plasminogen cleavage products. Blots from samples taken at 15 min in (A) were probed with antibodies directed to the NTP, K1–3, K5-Prot and K4. (C) Formation of a high-molecular-mass cleavage product in the presence of BZ. Lys⁷⁸-plasminogen was digested with neutrophil elastase for 15 min and the products were separated by SDS/PAGE, transblotted and the membrane probed with antibodies directed to K1–3, K4 and K5-Prot. (D) Identification of the molecular masses of the high-molecular-mass bands on reducing PAGE. Glu¹-plasminogen was digested with neutrophil elastase for 15 min. The samples were separated by SDS/PAGE under non-reducing conditions, the bands excised from the gel and then electroeluted. These bands were then re-electrophoresed under reducing conditions and the gel was stained with silver.

at 240 min (Figure 6). This was also true at the earlier time points (results not shown). These results suggest that, in the presence of AHA, the NTP does not affect cleavage within the kringle or protease regions.

In the β -conformation induced by BZ, additional high-molecular-mass products are generated from Glu¹-plasminogen by neutrophil elastase

In the presence of BZ, plasminogen assumes a conformation that is considered to be intermediate between the open γ -conformation and the closed α -conformation [18]. Incubation of plasminogen with neutrophil elastase in the presence of BZ resulted in the appearance of products at 69 and 64 kDa in addition to those observed under the other conditions used for the present study. At 1 min, a 69 kDa band was observed that reacted with anti-K1–3 but not anti-K5-Prot (Figure 2B versus Figure 2A). A product of this apparent mass was also observed transiently at lower levels in the Con and in the presence of AHA (Figure 2B, Con and AHA, 1 min). In the presence of BZ, this band decreased with time, with little observed by 120 min (Figures 2B and 8A). This cleavage product reacted with antibodies directed to the NTP, K1–3 and K4, but not with the antibody directed to K5-Prot (Figure 8B). N-terminal amino acid sequencing confirmed the presence of the glutamic residue as the N-terminal amino acid. Electroelution of this band from non-reducing gels followed by electrophoresis under reducing conditions confirmed that this product represents an intact polypeptide of 89 kDa (Figure 8D). The apparent mass of this product and the fact that it does not react with the K5-Prot antibody indicates that the cleavage site for the

generation of this product lies within the protease domain. There is one characteristic neutrophil elastase cleavage site found in the linker region between the two subdomains of the protease portion of plasminogen, Val⁶⁷³–Val⁶⁷⁴–Ala⁶⁷⁵, with a potential cleavage between Val⁶⁷⁴ and Ala⁶⁷⁵. Cleavage at this point would remove 117 amino acids or approx. 13 kDa from plasminogen. The estimated molecular mass of this product on reducing SDS/PAGE was 89 kDa and that for plasminogen was 102 kDa, suggesting that a 13 kDa polypeptide is removed. Sufficient amounts of the C-terminal peptide were not obtained for N-terminal analysis.

By 15 min, a unique band was observed at 64 kDa that reacted with anti-K1–3 but not anti-NTP (Figure 2B, BZ, and Figures 8A and 8B). N-terminal sequencing confirmed Leu⁷⁴ as the N-terminal amino acid. Although a band at 64 kDa was not observed using anti-K4 (Figure 8B), this product must include K4, because the electroeluted band when rerun under reducing conditions was observed at 82 kDa (Figure 8D). This band most likely represents K1–5 plus a part of the protease domain (K1–5-part of Prot) and probably is the N-terminal cleavage product of the 89 kDa (reducing) product.

In addition to these bands, the same initial cleavage bands were noted as those observed under other conditions: the 78, 49, 46 and 38 kDa anti-K5-Prot reactive bands (Figure 2A) and the 78, 45, 37 and 34 kDa anti-K1–3 reactive bands (Figure 2B). The concentration of the anti-K5-Prot reactive bands was less than that observed for the control, due to diversion of a population of the molecules to form the high-molecular-mass products (Figure 2A). N-terminal sequencing of these bands confirmed the same cleavage sites as those observed under the other conditions.

In the presence of BZ, Glu¹-plasminogen was initially cleaved by neutrophil elastase not only at the same four initial sites observed under the other conditions but also at an additional site in the protease domain. Three molecules of BZ bind to plasminogen, the LBS of K5, the active site of the protease domain and an unknown site in the protease domain [30]. Cleavage in the protease domain, implies that BZ altered the conformation of the protease domain exposing the linker region between the two protease domain subunits in addition to interrupting the interaction between the NTP and the K5.

Angiostatins are produced more rapidly in the presence of BZ than under the control condition

The K1–3 and K1–4 angiostatins were observed in the presence of BZ earlier than under control conditions (Figure 2B, BZ versus Con). By 15 min, the 40 kDa K1–4 and the 32 kDa K1–3 long forms were observed, and by 60 min, the short 29 kDa K1–3 form was detected. N-terminal sequencing confirmed Leu⁷⁴ as the N-terminal amino acid for these products. The early appearance of the K1–3 angiostatin forms indicates that BZ alters the initial plasminogen cleavage products, exposing the neutrophil elastase cleavage sites to a greater extent than under the other conditions.

In the presence of BZ, Lys⁷⁸-plasminogen is cleaved by neutrophil elastase to generate the 64 kDa protease domain cleaved product

Lys⁷⁸-plasminogen is considered to be in the β -conformation similar to that of Glu¹-plasminogen in the presence of BZ [18]. This would suggest that BZ would not alter the conformation of Lys-plasminogen. To determine whether BZ alters the conformation of Lys⁷⁸-plasminogen and whether cleavage within the protease domain of plasminogen is dependent upon the presence of the NTP, Lys⁷⁸-plasminogen was digested by neutrophil elastase in the presence of BZ. A band was observed at 64 kDa, which corresponds to the K1–5-Prot cleaved product observed upon digestion of Glu¹-plasminogen in the presence of BZ (Figure 8C

Table 1 Inhibition of HUVEC proliferation by angiostatins generated from plasminogen under Con, AHA and BZ conditions

Plasminogen was incubated in the Con, AHA and BZ buffers for 4 h and the angiostatin molecules were isolated by lysine–Sepharose chromatography. The angiostatin concentrations were determined using a dot-blot assay. FGF-2, purified angiostatins and control angiostatin (Std) were added to HUVEC cultures for 72 h and the cell numbers were determined using the MTT assay.

Angiostatin added	Angiostatin ($\mu\text{g}/100\ \mu\text{l}$)	FGF-2 (10 ng/ml)	% FGF-2 cells
—	0	+	100 $\pm$ 3.6
—	0	—	85.8 $\pm$ 1.6*
Std	80	+	71.3 $\pm$ 2.3*
Con-generated	0.5	+	86.0 $\pm$ 3.6*
Con-generated	1.0	+	84.0 $\pm$ 4.1*
AHA-generated	0.5	+	77.2 $\pm$ 2.5*
AHA-generated	1.0	+	75.5 $\pm$ 3.3*
AHA-generated	4	+	28.1 $\pm$ 0.2*
BZ-generated	0.25	+	70.3 $\pm$ 1.9*
BZ-generated	4	+	58.3 $\pm$ 1.1*

* A significant difference ($P < 0.5$) relative to FGF-2 plus HUVEC cells without angiostatin.

versus Figure 8A). The identity of the minor band at 68 kDa was not pursued, but may represent a more highly glycosylated form of cleaved plasminogen. It appears that BZ alters the conformation of the protease domain in both Glu¹- and Lys⁷⁸-plasminogens, resulting in cleavage within the protease domain.

The angiostatins generated by neutrophil elastase in the presence of Con, AHA and BZ inhibit HUVEC (human umbilical-vein endothelial cells) proliferation

The angiostatins produced by neutrophil elastase from plasminogen under Con, AHA and BZ conditions were isolated by lysine–Sepharose chromatography, dialysed and tested for inhibition of FGF-2-stimulated HUVEC proliferation. Because of the small amount of angiostatins generated in the presence of Cl[−] and their similarity to those produced under Con conditions, these products were not purified and tested. At all concentrations, the angiostatins significantly inhibited HUVEC proliferation (Table 1). Although the angiostatins generated under the various conditions differed, all conditions generated active angiostatins.

Conclusions and implications

The mechanism of neutrophil elastase cleavage of plasminogen to form biologically active angiostatin molecules depended upon the specific conformational changes induced by a given modifying molecule and not on the openness of plasminogen. The minimum number of cleavages required to convert plasminogen into an angiostatin molecule was two: one removed the NTP and the other removed either K4–5-Prot to form the K1–3 angiostatin or K5 plus the protease domain to form the K1–4 angiostatin. These two cleavages occurred sequentially, but the order was not fixed. There were two cleavage sites in the K3–4 linker, producing Ser³³⁹-K4–5-Prot and Val³⁵⁵-K4–5-Prot, and one in the K4–5 linker, producing Ala⁴⁴⁴-K5-Prot that produced angiostatins. In the NaCl-induced tight α -conformation, very little plasminogen was cleaved; however, low levels of angiostatins were formed. In the BZ-induced β -conformation, neutrophil elastase cleaved not only at the four sites observed under the other conditions, but also within the protease domain. Also, the generation of K1–3 angiostatin was faster than under other conditions. In the AHA-induced open γ -conformation, the initial cleavage of plasminogen

was similar to that of the Con buffer; however, secondary cleavage of the NTP to form angiostatin was very slow.

In a solution under normal physiological conditions, plasminogen conversion into angiostatin probably is controlled by its conformation to prevent the consumption of plasminogen when angiogenesis is low. In the presence of physiological Cl[−] concentrations, conversion of plasminogen into angiostatin most likely is very slow, based on its tight conformation with the unavailability of susceptible bonds. However, when plasminogen is bound to a binding protein through the LBS of the kringles, the present results obtained in the presence of AHA would predict that initial cleavages within the linkages between kringles would be relatively fast; however, cleavage of the NTP would be slow. This may be a secondary means of regulating angiostatin production. The conformation induced by BZ may predict the interaction of plasminogen with proteins with RGD sequences, because BZ has been considered as a mimic of this peptide [23].

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