Angiostatin Formation Involves Disulfide Bond Reduction and Proteolysis in Kringle 5 of Plasmin*

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Plasmin is processed in the conditioned medium of HT1080 fibrosarcoma cells producing fragments with the domain structures of the angiogenesis inhibitor, angiostatin, and microplasmin. Angiostatin consists of kringles 1–4 and part of kringle 5, while microplasmin consists of the remainder of kringle 5 and the serine proteinase domain. Our findings indicate that formation of angiostatin/microplasmin involves reduction of plasmin by a plasmin reductase followed by proteolysis of the reduced enzyme. We present evidence that the Cys461-Cys540 and Cys511-Cys535 disulfide bonds in kringle 5 of plasmin were reduced by plasmin reductase. Plasmin reductase activity was secreted by HT1080 and Chinese hamster ovary cells and the human mammary carcinoma cell lines MCF-7, MDA231, and BT20 but not by the monocyte/macrophage cell line THP-1. Neither primary foreskin fibroblasts, blood monocyte/macrophages, nor macrovascular or microvascular endothelial cells secreted detectable plasmin reductase. In contrast, cultured bovine and rat vascular smooth muscle cells secreted small but reproducible levels of plasmin reductase. Reduction of the kringle 5 disulfide bonds triggered cleavage at either Arg529-Lys530 or two other positions C-terminal of Cys461 in kringle 5 by a serine proteinase. Plasmin autoproteolysis could account for the cleavage, although another proteinase was mostly responsible in HT1080 conditioned medium. Three serine proteinases with apparent Mr of 70, 50, and 39 were purified from HT1080 conditioned medium, one or more of which could contribute to proteolysis of reduced plasmin.

The formation of new blood vessels from preexisting vessels is an important factor in a broad spectrum of diseases (1). New blood vessel growth by the process of angiogenesis is balanced by several protein activators and inhibitors. One such inhibitor is angiostatin, which accumulated in the murine circulation in the presence of a growing Lewis lung tumor and disappeared when the tumor was removed (2). The angiostatin produced by the primary tumor was found to inhibit the neovascularization and growth of its remote metastases. Angiostatin has been shown to inhibit the growth of a number of murine and human primary carcinomas in mice (3–5). The mechanism of action of angiostatin is not known but may relate to the induction of endothelial cell apoptosis (6).

Angiostatin is an internal fragment of plasminogen consisting of approximately the first four kringle domains. Both metalloproteinase and serine proteinase activity have been implicated in the formation of angiostatin. Angiostatin fragments are generated from plasminogen by metalloelastase (7), MMP-7 (8), MMP-9 (8), and MMP-3 (9). Dong et al. (7) proposed that angiostatin is produced by metalloelastase secreted by tumor-infiltrating macrophages. Serine proteinase activity was required for the generation of angiostatin from plasminogen or plasmin by cultured human prostate carcinoma cells (10), and generation of angiostatin from plasmin by Chinese hamster ovary (CHO) or HT1080 human fibrosarcoma cells (11).

Production of angiostatin by CHO or HT1080 cells involves reduction of one or more disulfide bonds in plasmin followed by proteolysis of the reduced enzyme by a serine proteinase (11). The plasmin disulfide bond(s) are reduced by a secreted reductase, which we have called plasmin reductase. Plasmin reductase requires a small cofactor for activity, and physiologically relevant concentrations of reduced glutathione or cysteine fulfill this role. Angiostatin can also be generated from plasmin with the reductants, thioredoxin (11), protein disulfide isomerase (11), or high concentrations of small thiols (12).

In this study, we present evidence that the Cys461-Cys540 and Cys511-Cys535 disulfide bonds in kringle 5 of plasmin were reduced by plasmin reductase. Plasmin reductase activity was secreted by the transformed cell lines, HT1080, CHO, MCF-7, MDA231, and BT20, but not by the monocyte/macrophage cell line, THP-1. Cultured bovine and rat vascular smooth muscle cells secreted small but reproducible levels of plasmin reductase, but neither primary foreskin fibroblasts, blood monocyte/macrophages, nor macrovascular or microvascular endothelial cells secreted detectable plasmin reductase. Reduction of the kringle 5 disulfide bonds triggered cleavage at either Arg529-Lys530 or two other positions C-terminal of Cys461 in kringle 5 by a serine proteinase. Three serine proteinases were purified from HT1080-conditioned medium (HT1080cm), one or more of which could account for proteolysis of reduced plasmin.

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1 Angiostatin has been loosely defined in the literature as an internal fragment of plasminogen consisting of kringle 1–4 or smaller fragments thereof. We define angiostatin as a protein consisting of kringle 1–4 and part of kringle 5 of plasmin.

2 The abbreviations used are: MMP, matrix metalloproteinase; CHO, Chinese hamster ovary K-1; HT1080cm, HT1080 conditioned medium; MMP, 3′-N-maleimidylpropionylbiocytin; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis; SBTI, soybean trypsin inhibitor; ELISA, enzyme-linked immunosorbent assay; MES, 4-morpholinethanesulfonic acid.
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EXPERIMENTAL PROCEDURES

Chemicals and Proteins—3-(N-maleimidylpropionyl)biocytin (MPB) was purchased from Molecular Probes (Eugene, OR), while soybean trypsin inhibitor (SBTI) and SBTI-agarose were from Sigma-Aldrich (Sydney, Australia). Plasminogen was purified from fresh frozen human plasma and separated into its two carbohydrate variants according to published procedures (13). Glu1-plasminogen was used in the experiment. Plasmin was produced by incubating plasminogen activator for 5 min from serum on 37°C. Results were corrected for control wells not incubated with MPB-labeled angiostatin.

Gelatin Zymography—Gelatin zymography was a modification of the technique originally described by Heussen and Dowdle (22). Bovine type B gelatin (Sigma-Aldrich) was incorporated into a 10% SDS-polyacrylamide gel at a final concentration of 1 mg/mL. After electrophoresis, gels were washed twice with 20 mM Hapes, 0.14 mM NaCl, pH 7.4 buffer containing 2.5% Triton X-100 for 30 min to remove the SDS. Gels were then incubated in 20 mM Hapes, 0.14 mM NaCl, pH 7.4 buffer for 20 min at room temperature with orbital shaking. Absorances were read at 405 nm using a Molecular Devices Thermomax Kinetic Microplate Reader (Molecular Devices Corp., Palo Alto, CA). Results were corrected for control wells not incubated with MPB-labeled angiostatin.

Plasmin amidolytic activity—Plasmin (10 μg) was incubated with Hapes (10 mM) at 37°C. At discrete time intervals, aliquots (20 μL) of the reactions were diluted 10-fold into 20 mM Hapes, 0.14 mM NaCl, 1 mg/mL PEG 6000, pH 7.4 buffer containing 200 mM H-Et-Val-Leu-Lys-7-benzo-2-oxa-1,3-diazole-4-sulfonic acid and resolved by reverse-phase high performance liquid chromatography as described previously (23). Plasmin amidolytic activity was determined using the Bio-Rad protein assay kit and plasminogen as the standard. All proteins were aliquoted and stored at −80°C until use.

Cell Culture—Human foreskin fibroblasts (15), human umbilical vein (16), human dermal microvascular (17), and human smooth muscle cells (20) were harvested and cultured as indicated. Bovine vascular smooth muscle cells were purchased from Cell Applications (San Diego, CA). HT1080, CHO-K1, MCF-7, BT20, MDA231, and THP-1 cells were purchased from American Type Culture Collection (Rockville, MD). All media components were from Life Technologies, Inc. Conditioned medium was collected by incubating cells at 80% confluence with Hanks’ balanced salt solution containing 25 mM Hepes at pH 7.4 for 6 h. The ratio of number of cells to volume of conditioned medium was 1.3 × 10^6 cells/mL. All conditioned medium was passed through a 0.22-μm filter prior to storage at −80°C.

Angiostatin Generation—Conditioned medium (1 mL) was incubated with plasmin (10 μg) for 2 h at 37°C. Angiostatin fragments were labeled with MPB (100 μM) for 30 min at room temperature, followed by quenching of the unreacted MPB with GSH (200 μM) for 10 min at room temperature. Unreacted GSH and other free sulfhydryls in the system were blocked with iodoacetamide (400 μM) for 10 min at room temperature. The plasmin kringle products were collected on 50 μL of packed lysine-Sepharose beads by incubation on a rotating wheel for 1 h at room temperature; washed three times with 20 mM Hapes, 0.14 mM NaCl, pH 7.4 buffer, and eluted with 50 mM e-aminoacrylic acid in the Hepes buffer.

Purification of Angiostatin from HT1080cm—Angiostatin was generated from 1 mg of plasmig in 100 mL of HT1080cm as described above. The conditioned medium was applied to a 1 × 20-cm column of lysine-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) as described previously (14). Miniplasmin was generated by incubating miniplasminogen (2 μM) with urokinase plasminogen activator (20 μM) for 2 h in 20 mM Hapes, 0.14 mM NaCl, pH 7.4 buffer at 37°C. Thioredoxin-derived angiostatin fragments were prepared as described previously (11). Protein concentrations were determined using the Bio-Rad protein assay kit and plasminogen as the standard. All proteins were aliquoted and stored at −80°C until use.

RESULTS

Angiostatin Fragments Produced in HT1080cm—Stathakis et al. (11) observed that three angiostatin fragments were made in CHO or HT1080cm with apparent Mr of 45, 41, and 38. These fragments were generated in HT1080cm, labeled with MPB, and purified by lysine-Sepharose affinity and gel filtration chromatography. Accordingly, three fragments were purified with apparent Mr of 45, 41, and 38 on nonreducing SDS-PAGE (Fig. 1A). The apparent Mr of the three fragments on reducing SDS-PAGE were 66, 60, and 57 (not shown). All three fragments were labeled with MPB, indicating that all fragments contained free thiols. The angiostatin fragments with similar Mr were visualized by reducing plasminogen with reduced thioredoxin as previously reported (Fig. 1B) (11). ELISA for MPB-labeled Angiostatin—To estimate secretion of plasmin reductase by cultured cells, an ELISA assay for MPB-labeled angiostatin was developed. This assay measured angiostatin generated by plasmin reductase and, therefore, was a relative measure of plasmin reductase activity. Briefly,
plasmin was incubated with conditioned medium, and the angiostatin fragments were labeled with the biotin-linked maleimide, MPB. The MPB-labeled angiostatin fragments were adsorbed to avidin-coated microtiter plate wells, and the bound angiostatin was detected using a murine kringle 1–3 monoclonal antibody and a secondary peroxidase-conjugated antibody.

To test the specificity of the ELISA, plasminogen, plasmin, or the plasminogen fragment kringle 1–3 (K1–3), K1–4, K4, or K5-serine proteinase (10 μg/ml) were incubated in either Hepes-buffered saline or HT1080cm for 2 h at 37 °C, and the angiostatin fragments were labeled with MPB and quantitated by ELISA. The plasminogen fragments were prepared by limited proteolytic digestion of plasminogen with porcine elastase and purified by a combination of lysine-Sepharose affinity and gel filtration chromatography (14). MPB-labeled angiostatin fragments were only produced in HT1080cm from plasmin or plasmin derived endogenously from plasminogen (11) (Fig. 2). As anticipated, no MPB-labeled angiostatin fragments derived from the plasminogen fragments in HT1080cm. Also, no MPB-labeled angiostatin fragments were produced from incubation of plasminogen, plasmin, or any of the plasminogen fragments in Hepes-buffered saline for 2 h at 37 °C. This result served as a negative control for plasmin reductase. The response of the ELISA was linear up to a plasmin kringle fragment concentration of ~200 ng/ml (Fig. 2B).

It is important to note that the ELISA assay was not an absolute measure of angiostatin formation. It is possible that one or more of the free thiols on angiostatin were refractive or inefficiently labeled by MPB due to steric factors or that two thiols on a proportion of the angiostatin molecules oxidized to form an intra- or interchain disulfide bond, which was not labeled with MPB. These considerations would have resulted in underestimation of the angiostatin generated. Nevertheless, the ELISA was a relative measure of angiostatin formation or plasmin reductase activity in serum-free conditioned medium. For example, generation of MPB-labeled angiostatin was a linear function of the concentration of HT1080cm in the reaction or of plasmin reductase concentration (r = 0.99) (Fig. 2C).

Plasmin Reductase Activity Secreted by Selected Primary and Transformed Cells—Conditioned medium from selected primary and transformed cells was collected by incubating cells at ~80% confluence with Hanks’ balanced salt solution containing 25 mM Hepes, pH 7.4, buffer for 6 h. The ratio of number of cells to volume of conditioned medium was between 1 and 3 × 10^6 cells/ml. Plasmin (10 μg/ml) was incubated with the conditioned medium, and MPB-labeled angiostatin fragments were quantitated by ELISA (see Fig. 2). ELISA results were corrected for background angiostatin formation in unconditioned medium, which was negligible.

Neither human foreskin fibroblasts, bovine aortic endothelial cells, human umbilical vein endothelial cells, nor human dermal microvascular endothelial cells secreted plasmin reductase activity (Fig. 3). However, cultured rat or bovine vascular smooth muscle cells secreted plasmin reductase and converted plasmin to angiostatin, although the activity was 6% of the reductase activity secreted by HT1080 cells. CHO and MCF-7 cells secreted approximately 60%, BT20 cells 27%, and MDA231 cells 20% of the plasmin reductase activity secreted by HT1080 cells. THP-1 cells did not secrete detectable levels of plasmin reductase.

Fig. 1. Angiostatin generated in HT1080cm or by thioredoxin. A, plasmin (10 μg/ml) was incubated in HT1080cm for 24 h at 37 °C, and the angiostatin fragments were labeled with MPB. Angiostatin fragments were purified from HT1080cm by lysine-Sepharose affinity and Sephacryl S-200 gel filtration chromatography, and 2 μg was resolved on nonreducing 10% SDS-PAGE and stained with Coomassie Blue (lane 1) or transferred to PVDF membrane and blotted with streptavidin peroxidase to detect the MPB label (lane 2). The positions of M markers are shown at the left. B, thioredoxin-derived angiostatin fragments (2 μg) resolved on nonreducing 10% SDS-PAGE and stained with Coomassie Blue (11). The positions of M markers are shown at the left.
activity on its own, while a concentration in HT1080cm, had no angiostatin-generating activity of undialyzed HT1080cm (Fig. 4). Dialysis of HT1080cm using a 12–14-kDa cut-off membrane. Plasmin (10 μg/ml) was incubated in the dialyzed conditioned medium (dCM) or Hepes buffer for 2 h in the absence or presence of 0, 1, 5, or 10 μM GSH, and angiostatin formation was quantitated by ELISA (see Fig. 2). The bars represent the mean and range of duplicate experiments.

Cofactor Requirements of Plasmin Reductase—The angiostatin-generating activity secreted by HT1080 cells was the highest of the cells examined and was used to further investigate the cofactor requirements of plasmin reductase. We previously reported that plasmin reductase secreted by HT1080 cells requires two components for activity, a protein component that can be heat-inactivated and a low M₆ cofactor that can be GSH (11). The low M₆ thiol compounds in HT1080cm were derivatized with the fluorescent compound 7-benzo-2-oxa-1,3-diazole-4-sulfonic acid and resolved by reverse-phase high performance liquid chromatography (24). The only low M₆ thiol detectable in HT1080cm was GSH (not shown). The concentrations of GSH and GSSG were determined as described by Vandeputte et al. (25). The concentration of GSH in the HT1080cm was 1.1 ± 0.12 μM. This corresponded to secretion of 0.27 ± 0.03 nmol of GSH/10⁶ cells/h. No GSSG was detected in HT1080cm.

To examine the GSH requirements of plasmin reductase, we measured the plasmin reductase activity of either Heps-buff ered saline or dialyzed HT1080cm supplemented with 0, 1, 5, or 10 μM GSH (Fig. 4). Dialysis of HT1080cm using a 12–14-kDa cut-off membrane reduced the angiostatin-generating activity to 12% of control. Supplementation of the dialyzed HT1080cm with 1 μM GSH doubled the angiostatin-generating activity, while 10 μM GSH restored the angiostatin-generating activity to that of undialyzed HT1080cm. This is in accordance with our previous findings (11). GSH at a concentration of 1 μM, the concentration in HT1080cm, had no angiostatin-generating activity on its own, while 10 μM GSH had 6% of the angiostatin-generating activity of undialyzed HT1080cm (Fig. 4).

The Kringle 5-Serine Proteinase Fragment of Plasminogen Is a Substrate for Plasmin Reductase—The plasminogen fragment K4, K1–3, K1–4, K5-serine proteinase was incubated with HT1080cm for 2 h and then labeled with MPB. The fragment was resolved on 15% SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin-peroxidase to detect the MPB label. Of the plasminogen fragments, only K5-serine proteinase incorporated MPB. The positions of M₆ markers are shown at left.

This observation suggested that the target disulfide bonds in plasmin for plasmin reductase reside in K5. Incubation of plasmin in pH 11 buffer causes reduction and isomerization of K5 disulfide bonds and results in formation of microplasmin (26, 27). Microplasmin has a Lys⁵³⁰ N terminus that is within K5. We compared the plasmin fragments generated by plasmin reductase with those generated by alkaline pH.

Comparison of Microplasmin Fragments Generated in either HT1080cm or in pH 11 Buffer—Plasmin was incubated in either 0.1 M glycine, pH 11 buffer or HT1080cm for 12 h at 37 °C. Samples were resolved and detected on gelatin zymography. Three major catalytically active plasmin fragments with apparent M₆ of 40, 30, and 29 were generated in either pH 11 buffer or HT1080cm (Fig. 6A). The M₆ 29 fragment corresponded to the M₆ 29 microplasmin fragment described by Wu et al. (26, 27).
Microplasmin hydrolyzes the tripeptidyl p-nitroanilide substrate, H-d-Val-Leu-Lys-p-nitroanilide with 1.4-fold higher efficiency than plasmin. This is a consequence of a 1.4-fold increase in the catalytic constant (26). Plasmin was incubated in HT1080cm, and the initial rate of hydrolysis of H-d-Val-Leu-Lys-p-nitroanilide was measured at discrete time intervals (Fig. 6B). Plasmin activity is reported as the fraction of control plasmin activity. The efficiency of hydrolysis of the chromogenic substrate increased with time of incubation and peaked at ~2-fold enhanced efficiency at ~8 h. The initial rate of hydrolysis returned to control levels after 24 h of incubation.

Serine Proteinase(s) Other than Plasmin Were Mostly Responsible for Proteolysis of Reduced Plasmin in HT1080cm—Angiostatin is generated from reduced plasmin by a serine proteinase in CHO or HT1080cm (11). Serine proteinase activity is also required for generation of angiostatin from plasminogen or plasmin by cultured human prostate carcinoma cells (10). Plasmin autoproteolysis can account for angiostatin formation in the presence of protein reductants (11) or small thiols (12). To examine whether autoproteolysis is the operative mechanism in HT1080cm, we compared formation of angiostatin from either active plasmin or plasmin inactivated with Val-Phe-Lys-chloromethyl ketone (VFK-plasmin).

Plasmin or VFK-plasmin was incubated with HT1080cm for 2 h, and the angiostatin fragments were labeled with MPB. The MPB-labeled fragments were either resolved on 15% SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin-peroxidase to detect MPB-labeled angiostatin. Note that MPB labeled both intact VFK-plasmin and angiostatin fragments derived from the inactivated plasmin. The positions of Mr markers are shown at the left. B, plasmin or VFK-plasmin (10 μg/ml) was incubated in HT1080cm in the absence or presence of SBTI (25 μg/ml) for 2 h, and angiostatin formation was quantitated by ELISA (see Fig. 2). C, HT1080cm (10 ml) was passed over either a Sepharose 4B or SBTI-Sepharose 4B column (2 ml). Plasmin (10 μg/ml) was incubated with the column eluate for 2 h, and angiostatin formation was quantitated by ELISA (see Fig. 2).
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proteinases in HT1080cm reduced the angiostatin-generating activity to 34% of control.

SBTI-inhibitable serine proteinases in HT1080cm were purified on a SBTI-agarose column, and the bound enzymes were eluted with benzamidine. Serine proteinases from the equivalent of 3 ml of HT1080cm were resolved and detected using gelatin zymography (Fig. 8). Proteinases with apparent $M_r$ of 70, 50, and 39 were evident. These enzymes did not correspond to plasmin or catalytically active plasmin fragments.

DISCUSSION

The formation of angiostatin from plasmin in the conditioned medium of transformed cells is a two-step process (11). First, one or more disulfide bonds in plasmin are reduced by a protein disulfide bond reductase, which we have called plasmin reductase, and a reductase cofactor, which can be a small thiol such as GSH. Second, reduced plasmin is cleaved by a serine protease producing angiostatin. Three angiostatin fragments are produced with apparent $M_r$ of 45, 41, and 38 on nonreducing SDS-PAGE, which have the same Lys530 N terminus but different C termini.

Plasmin reductase activity was secreted by the human fibrosarcoma cell line HT1080; the human mammary carcinoma cell lines, MCF-7, MDA231, and BT20; and CHO cells. In contrast, the monocyte/macrophage cell line THP-1 did not secrete significant levels of plasmin reductase before or after stimulation with phorbol ester (not shown). Neither primary foreskin fibroblasts, blood monocyte/macrophages, nor macrovascular or microvascular endothelial cells secreted detectable plasmin reductase. In contrast, cultured bovine and rat vascular smooth muscle cells secreted small but reproducible levels of plasmin reductase. In general terms, cellular transformation appeared to be associated with secretion of plasmin reductase and angiostatin formation. This result suggested that angiostatin formation is driven by tumor cells in vivo. Production of angiostatin by vascular smooth muscle cells is an interesting observation and suggested that angiostatin may function in the atherosclerotic vessel wall. The metalloproteinase inhibitor EDTA did not have any effect on angiostatin production by the cell lines used in this study.

Gately et al. (12) have shown that a sufficient concentration of small thiols alone can generate angiostatin from plasmin. The concentration of GSH in the HT1080cm was 1.1 ± 0.12 $\mu$m, and it was the only small thiol detected in the medium. This corresponded to secretion of 0.27 ± 0.03 nmol of GSH/10^6 cells/h, which is comparable with the level of secretion of GSH by other cultured cells (28, 29). To examine the contribution of the GSH to the plasmin reductase activity of HT1080cm, the medium was dialyzed, and the angiostatin-generating activity was determined. Dialysis of HT1080cm reduced the angiostatin-generating activity to 12% of control. Supplementation of the dialyzed HT1080cm with 1 $\mu$m GSH doubled the angiostatin-generating activity, while 10 $\mu$m GSH restored the angiostatin-generating activity to that of undialyzed HT1080cm. In contrast, 1 $\mu$m GSH, the concentration in HT1080cm, had no angiostatin-generating activity, while 10 $\mu$m GSH had 6% of the angiostatin generating activity of HT1080cm. These results support our previous findings (11) and the proposal that plasmin reductase requires a small thiol cofactor such as GSH to provide the hydrogens and electrons to reduce the plasmin disulfide bonds. We suggest that high concentrations (100 $\mu$m) of small thiols have enough reducing power to reduce plasmin (12); however, these concentrations are not achievable in cell culture. Considered together, these observations implied that plasmin reduction in HT1080cm was catalyzed by plasmin reductase using GSH as a cofactor and not by GSH directly.

One or more disulfide bonds in the K5-serine proteinase fragment of plasminogen was reduced in HT1080cm. In contrast, neither K4, K1–3, nor K1–4 were substrates for plasmin reductase. This finding suggested that the target disulfide bond(s) for plasmin reductase were in K5. Plasmin undergoes autoproteolysis in alkaline pH, producing a catalytically active microplasmin fragment with a Lys530 N terminus (26, 27). Microplasmin consists of the last 10 amino acids of K5, the remaining 21 amino acids of the A chain, and the serine proteinase domain. Wu et al. (26, 27) noticed that both the Cys561–Cys540 and Cys511–Cys535 disulfide bonds in K5 must have
been reduced to release microplasmin from K1–4. They proposed that the increased OH ion concentration at pH 11 was responsible for reducing the two disulfide bonds. We observed that the proteinase fragments produced from plasmin in pH 11 buffer were of identical Mr to the proteinase fragments generated from plasmin in HT1080cm. Fragments with apparent Mr of 40, 30, and 29 were generated. The Mr 29 fragment is the same size as microplasmin. We suggest that the mechanism of plasmin proteolysis at pH 11 is the same as the mechanism of proteolysis in HT1080cm. In other words, plasmin reductase achieves at neutral pH what is achieved by OH ion at pH 11.

Three major angiostatin fragments (Fig. 1A) and three major serine proteinase fragments (Fig. 6A) were produced in HT1080cm. Based on the close similarity in plasmin fragments produced at alkaline pH and in HT1080cm, we suggest that at least the Cys461–Cys482 and Cys511–Cys535 disulfide bonds in K5 were reduced by plasmin reductase. For instance, reduction of only the K5 Cys482–Cys523 and Cys511–Cys535 disulfide bonds would not have resulted in release of angiostatin from plasmin.

Similarly, angiostatin would have remained covalently linked to the remaining kringle if the Cys461–Cys482 and Cys511–Cys535 disulfide bonds were reduced, but not the disulfide bond at Cys511–Cys535. Reduction of the Cys461–Cys482 and Cys511–Cys535 disulfide bonds is consistent with all of the experimental data, although we cannot exclude reduction of the Cys482–Cys523 disulfide bond in K5 or other disulfide bonds in K1–4.

The largest angiostatin fragment (Mr 45) and the smallest catalytically active fragment (Mr 29; microplasmin) probably resulted from cleavage at the Arg529–Lys530 peptide bond. We hypothesize that cleavage can also occur at either the Arg472, Ala474 or Arg503, Ala504 peptide bond. Cleavage at these sites is favored by serine proteinases with plasmin-like specificity and would produce fragments of the size observed experimentally. Also, all three angiostatin fragments contained one or more cysteine residues (Fig. 1A). For the smallest angiostatin fragment (Mr 38) to contain a free thiol, proteolysis must have occurred C-terminal of Cys461. A model of K5 and the proposed reduction and proteolytic events in this kringle expose the motif.

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