Angiostatin-converting Enzyme Activities of Human Matrilysin (MMP-7) and Gelatinase B/Type IV Collagenase (MMP-9)*

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Angiostatin is one of the most potent inhibitors of angiogenesis. Reports have shown that metalloelastase, pancreas elastase, plasmin reductase, and plasmin convert plasminogen to angiostatin. However, the cleavage sites of plasminogen by these enzymes have not been determined. Here we demonstrate that two members of the human matrix metalloproteinase (MMP) family, matrilysin (MMP-7) and gelatinase B/type IV collagenase (MMP-9), hydrolyze human plasminogen to generate angiostatin fragments. The cleavage sites have been determined. The 58-kDa bands derived from plasminogen by MMP-7 and MMP-9 both have the N-terminal sequence KYVLYSEKKTG, which corresponds to that of angiostatin. This N terminus is identical to that of the starting plasminogen itself and corresponds to residues 97–106 of prepro-plasminogen. The 42- and 38-kDa bands generated by MMP-7 both have the N-terminal sequence VVLLPNVETP, which corresponds to the amino acid sequence 467–478 of prepro-plasminogen, between kringle domain 4 and 5. MMP-9 cleaves plasminogen to generate a 42-kDa fragment with the N-terminal sequence PVPVLLPNVE, 1 residue upstream of the MMP-7 cleavage site. These results indicate that MMP-7 and MMP-9 may regulate new blood vessel formation by cleaving plasminogen and generating angiostatin molecules.

Angiogenesis is a fundamental process by which new microblood vessels are formed (1, 2). Highly regulated and transient angiogenesis plays a pivotal role in development, morphogenesis, wound repair, and reproduction (1–9). Embryo implantation and placentation, embryo growth and morphogenesis, menstruation, and wound healing all require new blood vessel formation to supply oxygen and other nutrients. Neovascularization is also critical for patients with coronary artery disease; the newly formed collateral circulation allows blood flow to bypass the blocked coronary artery and supply blood to the heart in preventing heart attacks. Uncontrolled and persistent angiogenesis is involved in many pathological processes such as cancer growth and metastasis and vascular, rheumatoid, menstruation, and wound healing all require new blood vessel formation (1–3). Embryo implantation and placentation, embryo growth and morphogenesis, menstruation, and wound healing all require new blood vessel formation to supply oxygen and other nutrients. Neovascularization is also critical for patients with coronary artery disease; the newly formed collateral circulation allows blood flow to bypass the blocked coronary artery and supply blood to the heart in preventing heart attacks. Uncontrolled and persistent angiogenesis is involved in many pathological processes such as cancer growth and metastasis and vascular, rheumatoid, and other diseases (4–6). Because angiogenesis is essential for tumor growth and metastasis, anti-angiogenic therapy may become the most effective treatment for starving tumor cells and preventing the spread of cancer to other parts of the body (1, 6, 7).

Angiostatin is an internal fragment of plasminogen consisting of the first four kringle domains of plasminogen, which is a key proenzyme in the fibrinolytic system (8–10). Angiostatin is a specific inhibitor of endothelial cell proliferation and one of the most effective and specific natural inhibitors of angiogenesis. It mediates the suppression of metastasis in a Lewis lung carcinoma (11). Angiostatin represents a molecule that is generated by a primary tumor to inhibit both primary and secondary tumor growth. Recombinant angiostatin has been used successfully to suppress tumor growth and metastasis in animal model systems (12, 13).

Angiostatin, a fragment of plasminogen, and endostatin, a fragment of type XVIII collagen, are emerging examples of endogenous inhibitors of angiogenesis derived from larger proteins with distinct and varied functions (11, 14). The identification of the responsible enzymes that cleave the precursor proteins and the elucidation of the cleavage sites in these proteins can uncover new mechanisms of growth regulation in the vascular system. One of the enzymes responsible for the generation of angiostatin in Lewis lung carcinoma has been identified as macrophage-derived metalloelastase (matrix metalloproteinase-12, MMP-12)1 (15). In addition, plasmin and plasmin reductase are responsible for generating angiostatin from plasminogen (16, 17). However, none of the cleavage sites by these enzymes have been determined. We now show that human matrilysin (MMP-7) and human neutrophil gelatinase B/type IV collagenase (HNG, MMP-9) also convert human plasminogen to angiostatin fragments. Furthermore, we determine that the cleavage sites in plasminogen by MMP-7 and MMP-9 are located between kringle domains 4 and 5, which is in good agreement with the estimation that angiostatin consists of the first four kringle domains in plasminogen (11).

EXPERIMENTAL PROCEDURES

Materials—Human plasminogen was purchased from Sigma. Human plasminogen was purchased from Boehringer Mannheim. Plasminogen was diluted to a final concentration of 10 mg/ml. Active human neutrophil gelatinase B/type IV collagenase (HNG, MMP-9) was purified as described (18). Active human recombinant matrilysin (MMP-7) was a generous gift from Dr. Harold E. van Wart of Syntex Discovery Research, Palo Alto, CA (18, 19).

Digestion of Plasminogen by a Variety of Enzymes—Human MMP-7 and MMP-9 were tested for the ability to digest plasminogen in a 1× assay buffer (50 mM Tricine buffer, pH 7.5, containing 0.2 M NaCl, 10 mM CaCl2, 50 μM ZnSO4, 0.05% Brij-35 (polyoxyethylene lauryl ether), and 0.02% NaN3). Time course of digestion of plasminogen for each enzyme varied according to enzyme concentration and catalytic activity. All reactions were stopped with a half-volume of 3× denaturing buffer (0.15 M Tris, pH 6.7, 6% SDS, 20% glycerol, 0.1 M EDTA, 0.03% bromophenol blue, 8 μl urea) and run on 12% SDS-polyacrylamide gels under reducing conditions using a Bio-Rad mini-gel apparatus. Protein bands were visualized using a silver stain method (20).

Electrophoretic Transfer and Protein N-terminal Sequencing—The electrophoresis and electrophoretic transfer of proteins were carried out

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1 The abbreviations used are: MMP, matrix metalloproteinase; HNG, human 98-kDa neutrophil gelatinase B; MMP-2, 72-kDa gelatinase A/type IV collagenase; MMP-7, matrilysin; MMP-9, 92–98-kDa gelatinase B/type IV collagenase; MMP-12, metalloelastase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis.
Angiostatin-converting Enzymes

RESULTS

Plasminogen, at 15 μM, was incubated with 1 μM MMP-9 (human neutrophil gelatinase B, HNG) or 1 μM MMP-7 (matrilysin) in 1× assay buffer at 37 °C for 48 h. As shown in Fig. 1, MMP-9 digested plasminogen at a much faster rate than MMP-7. Plasminogen (about 108 kDa) was transformed into three major bands at about 58, 42, and 38 kDa by MMP-9 and a broad band of 38–45 kDa by MMP-7. To assure band formations were in fact enzyme products of plasminogen, all incubations performed contained controls of plasminogen and the enzymes by themselves for the allotted time.

A detailed time course study of digestion of plasminogen by MMP-9 and MMP-7 was carried out (Fig. 2). Plasminogen at 15 μM was incubated with 1 μM MMP-9 or 1 μM MMP-7 in 1× assay buffer at 37 °C for a total of 120 h for MMP-9 and 24 h for MMP-7. Samples were taken at 0, 24, 48, 72, 96, and 120 h for MMP-9 (Fig. 2A) and 0, 2, 4, 8, 16, and 24 h for MMP-7 (Fig. 2B). Digestion was stopped by addition of a half-volume of 3× denaturing buffer with reduction and heating. As shown in Fig. 2, both MMP-9 and MMP-7 converted the 108-kDa plasminogen into three major bands at an estimated molecular mass of 58, 42, and 38 kDa. Cleavage of plasminogen by MMP-9 is very slow (Fig. 2A), whereas cleavage of plasminogen by MMP-7 is fast and almost complete within 24 h (Fig. 2B). Attempts to quantitatively analyze the digestion products produced over time using a densitometric scanning method were unsuccessful.

The 58-kDa bands generated from plasminogen by MMP-9 and MMP-7 both have the N-terminal sequence KVVL-SEXKTG (Fig. 3), which is the same as the N-terminal sequence of the angiostatin generated by elastase (11). The N terminus is also identical to that of the starting 108-kDa plasminogen band sequenced and corresponds to residues 97–106 of the pro-pre-plasminogen. The 42- and 38-kDa bands generated from plasminogen by MMP-7 both have the N-terminal sequence VVLLPNVETP (Fig. 3), which corresponds to residues 467–476 of the pro-pre-plasminogen, at the junction between kringle domain 4 and kringle domain 5 of the plasminogen molecule. The 42-kDa band generated from plasminogen, cleaved from MMP-9, has the N-terminal sequence PVVLLPNVE (Fig. 3), which corresponds to residues 1–19, signal peptide; 20–96, activation peptide; 20–810, plasminogen; 97–580, plasmin chain A; 581–810, plasmin chain B; 97–440, an estimated angiostatin sequence by O'Reilly et al. (11). The 38-kDa band corresponds to residues 467–476 of the pro-pre-plasminogen.

DISCUSSION

These experiments show that matrilysin (MMP-7) and gelatinase B/type IV collagenase (MMP-9) have angiostatin-converting enzyme activities. Both cleave plasminogen at the sites between kringle domain four and five to generate angiostatin molecules consisting of the first four kringle domains of plasminogen.
minogen. These two enzymes show slightly different cleavage site specificities, MMP-7 cleaving the Pro^{466}-Val^{467} peptide bond, and MMP-9, the Pro^{465}-Pro^{466} peptide bond in the pre-pro-plasminogen sequence. All reported angiostatin species that consist of the first four kringle domains of human and murine plasminogen, generated both in vitro and in vivo, or made as recombinant proteins exhibit the biological activities of angiostatin isolated from plasma (e.g. inhibition of endothelial proliferation, angiogenesis, and tumor growth and metastasis) (11–13, 21). Therefore, the angiostatin fragments consisting of the first four kringle domains of plasminogen generated by MMP-7 and MMP-9 should also have these biological activities.

The physiological relevance and significance of the generation of angiostatin by MMP-7 and MMP-9 in vivo remain to be investigated. Other reports have not demonstrated any direct evidence to exclude the possibility that MMP-7 and MMP-9 are the responsible enzymes for generating angiostatin. Serine proteinase activities, but not metalloproteinase activities, have been reported to be responsible for the generation of bioactive angiostatin from purified human plasminogen or plasmin in three human prostate carcinoma cell lines (PC-3, DU-145, and LN-Cap) (22). Recently, Soff and co-workers (16) have determined that plasmin itself in the presence of reduced glutathione is the enzyme responsible for the conversion of plasmin to angiostatin. To investigate why the metalloproteinase activities were not responsible for converting plasminogen to angiostatin in those three prostate cancer cell lines, we have examined if those cell lines produce active MMP-7 and MMP-9. None of the three cell lines produce any MMP-7. PC-3 and DU145 cells produce pro-MMP-9, and LN-Cap expresses some pro-gelatinase A (pro-MMP-2). None of those cell lines expresses any active forms of MMP-9. Thus, it is not possible for MMP-7 and MMP-9 to be the responsible enzymes for generating angiostatin because the active forms of these two enzymes are not available in that particular prostate cancer model system. In summary, it is possible that matrix metalloproteinases can act as angiostatin-converting enzymes in other model systems.

One member of the MMP family, macrophage metalloelastase (MMP-12), has been shown to generate angiostatin and inhibit tumor growth and metastasis in vivo (15). Some correlative results in that study have suggested that MMP-9 produced by macrophages in tumors is not likely to be responsible for angiostatin-converting enzyme activity. However, those data did not specify if the MMP-9 activity detected was due to pro-MMP-9 or activated MMP-9, because the gelatin zymogram assay can detect both latent and active forms of MMP-9. Pro-MMP-9 does not cleave plasminogen, so it cannot generate angiostatin. No direct biochemical evidence has been provided to confirm the conclusion that MMP-9 is not the responsible enzyme to generate angiostatin (15). Furthermore, the paper alternatively suggested, the levels of MMP-9 in their system may not have been high enough to produce the amount of angiostatin that can be detected in the bioassay. The activity of MMP-9 toward plasminogen may be lower than MMP-12. In summary, it is still possible that active MMP-9 can be partially responsible for the angiostatin-converting enzyme activity. This possibility is confirmed by our biochemical studies. Our biochemical data have demonstrated that MMP-9 cleaves plasminogen to generate an angiostatin fragment, although the rate of catalysis is slower than that of MMP-7. Moreover, it is well documented that MMP-7 and MMP-9 are expressed in many types of tissues and cells, for example tumor tissues (23, 24), fibroblasts (25), and developing mononuclear phagocytes (26), supporting the hypothesis that these two enzymes may also be physiologically relevant angiostatin-converting enzymes in vivo.

It would be interesting to find out if any other members of the MMP family can also convert plasminogen to angiostatin molecules. The specificity, efficiency, detailed cleavage sites, biological activity, and physiological relevance of angiostatin fragments generated by different enzymes remain to be determined and compared. We are currently undertaking such studies. The angiostatin species generated by an enzyme with an identical C terminus as angiostatin isolated from human plasma will be the most physiologically relevant, and this enzyme will be identified as the angiostatin-converting enzyme. The most potent angiostatin species may be selected for clinical trial for the inhibition of tumor growth and metastasis.

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