Regulation of Angiostatin Production by Matrix Metalloproteinase-2 in a Model of Concomitant Resistance*

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We have previously reported the identification of the endogenous angiogenesis inhibitor angiostatin, a specific inhibitor of endothelial cell proliferation in vitro and angiogenesis in vivo. In our original studies, we demonstrated that a Lewis lung carcinoma (LLC-LM) primary tumor could suppress the growth of its metastases by generating angiostatin. Angiostatin, a 38-kDa internal fragment of plasminogen, was purified from the serum and urine of mice bearing LLC-LM, and its discovery provides the first proven mechanism for concomitant resistance (O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M. A., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. (1994) Cell 79, 315-328). Subsequently, we have shown that systemic administration of angiostatin can regress a wide variety of malignant tumors in vivo. However, at the time of our initial discovery of angiostatin, the source of the protein was unclear. We hypothesized that the tumor or stromal cells might produce an enzyme that could cleave plasminogen sequestered by the primary tumor into angiostatin. Alternatively, we speculated that the tumor cells might express angiostatin. By Northern analysis, however, we have found no evidence that the tumor cells express angiostatin or other fragments of plasminogen (data not shown). We now report that gelatinase A (matrix metalloproteinase-2), produced directly by the LLC-LM cells, is responsible for the production of angiostatin, which suppresses the growth of metastases in our original model.

Recently, in vitro studies of cancer cell lines (3-7) have shown that a variety of enzymes can cleave plasminogen into fragments of plasminogen with a similar sequence and activity to that of the angiostatin protein we first described (1). However, none of these reports have identified the specific enzyme involved in the production of the biologically active angiostatin in the in vivo Lewis lung model in which angiostatin was first discovered nor have they demonstrated the production of angiostatin with identical sequence, composition, or biological activity to that which we first described. Here we present the first report of the production of biologically active angiostatin by tumor cells that correlates to the inhibition of tumor growth by tumor mass.

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EXPERIMENTAL PROCEDURES

Cell Culture—LLC-LM lines were isolated from tumors growing in mice and were maintained in culture in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and 1% glutamine, penicillin, and streptomycin (1, 2). Serum-free CM was prepared by washing nearly confluent cells with phosphate-buffered saline (3 washes) and then allowing them to condition the media composed of Dulbecco's modified Eagle's medium and 1% glutamine, penicillin, and streptomycin for 24 h. Media were then collected, centrifuged, filtered (0.45 microns), and stored at 4 °C.

Substrate Gel Electrophoresis—Substrate gel electrophoresis was conducted according to a modification (9) of the method of Herron et al. (10).

Endothelial Cell Proliferation Assay—Bovine capillary EC were maintained and the inhibition assay was performed as described previously (1, 11). Briefly, cells were plated onto gelatinized 24-well culture plates (12,500 cells/well) and were incubated at 37 °C in 10% CO2 for 24 h. The medium was then replaced with 0.25 ml of Dulbecco's modified Eagle's medium, 5% bovine calf serum, 1% antibiotics, and the test samples were added. After 20 min of incubation, media and basic fibroblast growth factor (bFGF) were added to each well to obtain a final volume of 0.5 ml of Dulbecco's modified Eagle's medium, 5% bovine calf serum, 1% antibiotics, 1 ng/ml bFGF. After incubation for 72 h, media were aspirated, and the cells were removed by trypsinization, resuspended in Hematall, and counted electronically with a cell counter.

Processing and Purification of Angiostatin—Human plasminogen was purified from outdated human plasma and then incubated with 125 ml of LLC-CM obtained as above at 37 °C for 24 h with native MMP-2 (8). Following incubation, test samples or control samples (serum-free control media not conditioned by cells but identically treated otherwise) were applied to a lysine-Sepharose (Amersham Pharmacia Biotech) column that had been equilibrated with 50 mM phosphate buffer, pH 7.4. The column was reequilibrated with 50 mM phosphate buffer, pH 7.4, followed by phosphate-buffered saline until the baseline (A280) was stable. Samples were fractionated by elution with 0.2 M aminocaproic acid as described previously (2). All fractions eluted from the lysine affinity column, which contained protein as measured spectrophotometrically.

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§§ The abbreviations used are: MMP, matrix metalloproteinase; bFGF, basic fibroblast growth factor; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; EC, endothelial cells.
MMP-2 Processing of Bioactive Human Angiostatin

Fig. 1. A, Gelatin zymography of conditioned media of LLC-LM. Substrate gel electrophoresis was conducted on 30 μl of unconditioned media conditioned by LLC-LM cells or media alone (Control) treated identically. Gelatinase activity was detected as zones of clearance migrating with an apparent mass of approximately 64 kDa. The two right lanes represent the zymographic analysis of CM and media alone following incubation with 1 mm aminophenylmercuric acetate (APMA) as described under “Experimental Procedures.” B, immunoblot analysis of conditioned media of LLC-LM. Conditioned and control media were analyzed by SDS-PAGE and Western blot using a monoclonal anti-MMP-2 antibody.

Fig. 2. Reverse phase HPLC chromatography of purified angiostatin. Following incubation of LLC-LM CM with human plasminogen and subsequent affinity chromatography on a lysine-Sepharose affinity column as described under “Experimental Procedures,” the protein-containing samples were applied to a reverse phase HPLC column, and fractions were eluted using a gradient of 0.1% trifluoroacetic acid in H2O to trifluoroacetic acid in CH3CN. Representative chromatograms of CM incubated with plasminogen in the absence (A) and presence (B) of a neutralizing antibody to MMP-2 (anti-IVase). Angiostatin eluted at approximately 30–35% CH3CN and unprocessed plasminogen at approximately 40–45%.

RESULTS

Following our original method (1, 2), pure cell populations of Lewis lung carcinoma were obtained by repeated in vitro passage. We have previously found that the in vivo phenotype of these cells can be changed after in vitro passage. To confirm that these cells would still form tumors that could suppress their metastases in vivo, they were injected into mice (1, 2). The ability of these cells to form tumors that can suppress the growth of their metastases was assessed in vivo (data not shown) as previously reported by us. Zymographic analysis of conditioned media of these LLC-LM cells revealed a prominent zone of clearance, which migrated at an apparent molecular mass of approximately 64 kDa, consistent with it being gelatinase A or MMP-2 (Fig. 1A). Treatment with aminophenylmercuric acetate did not result in a decrease in molecular weight of this proteolytic activity (Fig. 1A), suggesting that the Lewis lung carcinoma cells produce an active MMP-2 species. Immunoblot analysis using MMP-2-specific antibodies (Oncogene Sciences, Cambridge, MA) verified the identification of this proteolytic species as being gelatinase A (Fig. 1B). A second immunoblot using the MMP-2-specific antibody, anti-IVase (8), verified this identification. Treatment with 1,10-phenanthroline resulted in a total abrogation of proteolytic activity demonstrating that the 64-kDa enzyme was a metal-dependent protease (10) (data not shown).

Having demonstrated that LLC-LM cells produce gelatinase A, we next tested the ability of the conditioned media to process human plasminogen to angiostatin. Human plasminogen was prepared from outdated human plasma as described previously (2). Optimal processing of plasminogen into angiostatin was obtained by combining 1 mg of plasminogen with 125 ml of conditioned media. This mixture was incubated on a shaker at 37 °C for 72 h. Following incubation, test samples as well as controls (serum-free control media not conditioned by cells but identically treated otherwise) were fractionated on a lysine-Sepharose column and eluted in a buffer containing 0.2 M aminocaproic acid (2). Bound material eluted as a single broad peak from the column. All fractions eluted from the lysine affinity column that contained protein as measured spectrophotometrically at A280 were pooled, concentrated in a spin concentrator (4000 molecular weight cut-off), and applied to a SynChropak RP-4 (100 × 4.6 mm) reverse phase HPLC column. Protein was eluted using a gradient of 0.1% trifluoroacetic acid in H2O to trifluoroacetic acid in CH3CN. Representative chromatograms of CM incubated with plasminogen in the absence (A) and presence (B) of a neutralizing antibody to MMP-2 (anti-IVase) (Fig. 2A) as described previously (1). Aliquots of each fraction were immediately concentrated by vacuum centrifugation, re-suspended in H2O, and tested for their ability to inhibit capillary EC proliferation stimulated by bFGF as described above. Fractions were also analyzed on SDS-PAGE gels followed by silver staining according to standard protocols.

MMP-2 Neutralization Experiments—LLC-CM was preincubated with a polyclonal antibody raised against gelatinase A (anti-IVase), which has been shown to specifically block the proteolytic activity of MMP-2 (8), in a ratio of 1:50 (antibody:CM) at 37 °C for 10 min. This mixture was then incubated with whole plasminogen as described above. Controls included a sample of LLC-CM incubated with human plasminogen under the same conditions but in the absence of the neutralizing antibody. Following the incubation with human plasminogen, samples from each of the treatment groups were fractionated over a lysine affinity column, followed by reverse phase HPLC chromatography as described above.

Protein Microsequencing—The 38-kDa protein obtained from LLC-LM CM processing was purified to homogeneity as described above, resolved by SDS-PAGE, electroblotted onto polyvinylidene difluoride (Bio-Rad), detected by Ponceau S staining, and excised from the membrane. The N-terminal sequence was determined by William S. Lane, Harvard Microchemistry Facility (Cambridge, MA), using automated Edman degradation on a PE/ABD model 470A protein sequencer (Foster City, CA) operated with gas phase delivery of trifluoroacetic acid. Protein was eluted using a gradient of 0.1% trifluoroacetic acid in H2O to trifluoroacetic acid in CH3CN. Representative chromatograms of CM incubated with plasminogen in the absence (A) and presence (B) of a neutralizing antibody to MMP-2 (anti-IVase) as described under “Experimental Procedures.” B, immunoblot analysis of conditioned media of LLC-LM. Conditioned and control media were analyzed by SDS-PAGE and Western blot using a monoclonal anti-MMP-2 antibody.
(Fig. 4A). As with angiostatin derived from the urine of tumor-bearing mice, the band migrated at 28 kDa when run under nonreducing conditions (data not shown).

To determine if other enzymes in the conditioned media could account for the production of angiostatin, we conducted neutralization experiments using a blocking polyclonal antibody raised against gelatinase A (anti-IVase) (8). Representative reverse phase HPLC chromatograms of LLC-LM CM following incubation without (Fig. 2A) and with (Fig. 2B) gelatinase A-neutralizing antibodies are shown. SDS-PAGE analysis of these samples followed by silver staining verified the identification of a single band consistent with angiostatin (38 kDa, Fig. 4A) detected in the CM samples incubated in the absence of neutralizing antibody. In contrast, angiostatin is absent, and a band corresponding to intact plasminogen is prominent in the CM of samples, which were incubated in the presence of the neutralizing gelatinase A antibody (Fig. 4A).

Gelatinase A digestion of plasminogen generated angiostatin as a single 38-kDa peptide identical to that seen after incubation of plasminogen with the conditioned media of Lewis lung carcinoma. This is in contrast to previous reports of multiple bands with angiostatin activity seen with the digestion of plasminogen (3–7).

Upon assay of the fractions containing protein from both of these columns for their ability to inhibit capillary EC proliferation, we found that only the fractions containing angiostatin inhibited EC proliferation. We did not detect any significant antiproliferative activity in any of the other fractions tested. Protein microsequencing of the inhibitory fraction identified this protein as human angiostatin (Fig. 4B). Interestingly, this microsequencing revealed an N terminus of residues 98–99, in comparison to that reported in the original angiostatin study (amino acid residues 97–98) (1). One explanation for this difference may be that the angiostatin reported in the original study was derived from murine plasminogen, whereas in the current report the angiostatin was derived using human plasminogen as a substrate. This shift in the N-terminal residue has been consistently observed in our studies of mouse and human angiostatin. C-terminal sequencing did not yield unequivocal C-terminal identity.

Given that the “net” proteolytic activity is typically represented by a balance between enzyme and inhibitor activity, we have also tested the LLC-LM CM for tissue inhibitors of metalloproteinases bioactivity using a solid radiolabeled collagen film assay (11). We were unable to detect significant inhibition of MMP activity in this assay using unconcentrated samples. Therefore, it would appear that, at the level of the LLC-LM tumor cell CM, the proteolytic balance is shifted in favor of the production of MMPs, in particular gelatinase A.

DISCUSSION

These studies demonstrate that gelatinase A, in the same in vivo system in which angiostatin was first discovered, can process plasminogen into bioactive angiostatin. Although several other enzymes have been shown to process plasminogen to angiostatin, MMP-2 is the first enzyme that can produce angiostatin identical to that which we first described. Recent reports have documented that metalloproteinases can process precursor or parent proteins into their bioactive form. Metalloproteinases have now been implicated in the processing of the tumor necrosis factor-α precursor protein (12, 13), transforming growth factor-α (14), the β-amyloid precursor protein (14), the lymphocyte L-selectin adhesion molecule (15–17), the interleukin-6 receptor ectodomain (18), the human thyrotropin receptor ectodomain (19), and the fibroblast growth factor receptor type 1 ectodomain (20). More recently, we have demonstrated that MMP-3 (stromelysin) can release soluble and active heparin-binding epidermal growth factor-like growth factor from its membrane-anchored precursor (21). In light of the fact that MMPs have recently been shown to play a role in the processing of nontraditional MMP substrates, we focused on the role that MMPs might be playing in the processing of plasminogen into mature angiostatin.

The production of angiostatin by gelatinase A from the tumor cells helps to resolve the question as to why a primary tumor might be producing angiostatin. By increased production of gelatinase A, the tumor may become more locally invasive.
However, our findings clearly demonstrate that gelatinase A can also mobilize angiostatin. Therefore, the current study helps to explain the growth of the primary tumor, although the metastases are suppressed. In addition, the increased permeability of the tumor vessels may lead to the sequestration of circulating plasminogen into the neostroma of the tumor (22). This plasminogen could then be cleaved into active angiostatin that then suppresses the growth of the metastases. Thus, the tumor microenvironment may localize the enzyme to the substrate resulting in the generation of angiostatin.

Further evidence to support the role of enzymes in regulating angiogenesis comes from the emerging theme that angiogenesis inhibitors are fragments of much larger proteins with distinct functions (23). For example, an internal 16-kDa fragment of prolactin inhibits angiogenesis, whereas the parent molecule, intact prolactin, does not (24). Other inhibitors of EC proliferation that are fragments of larger molecules include fragments of platelet factor 4 (25), thrombospondin (26), epidermal growth factor (27), laminin (28), and endostatin (29). We suggest that angiogenesis may depend not only upon the balance of endothelial stimulators and inhibitors but also upon the balance of matrix-degrading proteases and their endogenous inhibitors. The regulation of angiogenesis by MMPs suggests that clinical strategies that decrease the enzymatic activity of a tumor may also decrease the release of angiogenesis inhibitors. This possibility suggests that, with respect to the therapeutic use of MMP inhibitors, it will be important to define the precise role(s) of MMPs in the modulation of angiogenesis before utilizing therapeutic strategies that inhibit their activity.

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