Macrophage Elastase (Matrix Metalloproteinase-12) Suppresses Growth of Lung Metastases

A. McGarry Houghton,1 Jay L. Grisolano,2 Mary L. Baumann,2 Dale K. Kobayashi,3 R. Dean Hautamaki,1 Leslie C. Nehring,3 Lynn A. Cornelius,1 and Steven D. Shapiro1

Departments of 1Pediatrics, 2Medicine, and 3Dermatology, Washington University School of Medicine, St. Louis, Missouri

Abstract

Matrix metalloproteinases (MMP) have been implicated in virtually all aspects of tumor progression. However, the recent failure of clinical trials employing synthetic MMP inhibitors in cancer chemotherapy has led us to hypothesize that some MMPs may actually serve the host in its defense against tumor progression. Here we show that mice deficient in macrophage elastase (MMP-12) develop significantly more gross Lewis lung carcinoma pulmonary metastases than their wild-type counterparts both in spontaneous and experimental metastasis models. The numbers of micrometastases between the two groups are equivalent; thus, it seems that MMP-12 affects lung tumor growth, and not metastasis formation, per se. MMP-12 is solely macrophage derived in this model, being expressed by tumor-associated macrophages and not by tumor or stromal cells. The presence of MMP-12 is associated with decreased tumor-associated microvessel density in vivo and generates an angiostatic-angiogenic tumor microenvironment that retards lung tumor growth independent of the production of angiostatin. These data define a role for MMP-12 in suppressing the growth of lung metastases and suggest that inhibitors designed to specifically target tumor-promoting MMPs may yet prove effective as cancer therapeutics. (Cancer Res 2006; 66(12): 6149-55)

Introduction

Metastasis of tumors to distant sites represents a significant portion of the morbidity and mortality attributable to malignant disease. Metastasis formation is a complex process that requires the orderly and successful completion of a number of distinct steps to generate a tumor at a distant site (1). Many of these biological processes, such as vascular invasion by the primary tumor, extravasation of tumor cells into the target organ, and appropriate angiogenesis at the new site of tumor growth, are thought to require the presence of members of the matrix metalloproteinase (MMP) family (2, 3). There are 24 members of the MMP family that share the ability to degrade extracellular matrix proteins and possess common structural domains, such as an NH2-terminal proenzyme domain and a zinc-containing catalytic domain. Most have a hemopexin-like COOH-terminal domain and others may have additional domains such as a transmembrane domain (4).

MMPs are traditionally regarded as path clearers, destructive proteinases that inflammatory, tumor, and/or stromal cells release to degrade extracellular matrix components or enable tumors to invade vascular and other structures (5, 6). However, recent studies support more intricate roles for MMPs including the generation of chemotactic gradients, release of growth factors stored within the extracellular matrix, and the ligation of integrins, thus directly influencing cell behavior (7–9). Taken together, members of the MMP family (MMPs 2, 3, 7, 9, and 13) have been shown to promote tumor growth at virtually all possible stages of tumor progression (4, 9–14). Several of these studies have shown important roles for MMPs in tumor-associated angiogenesis. Gelatinase A (MMP-2) promotes tumor growth by interacting with the αvβ3 integrin on vascular endothelial cells and directly promoting endothelial cell migration (9, 15). Gelatinase B (MMP-9) has also been shown to promote tumor growth (16) by influencing the "angiogenic switch."

The implication of MMPs in tumor progression has made them attractive targets for therapeutic intervention (17), a concept seemingly validated by the successful inhibition of tumor growth by the administration of MMP inhibitors in murine models (18, 19). These studies have made the negative results of several recently reported clinical trials employing MMP inhibitors all the more perplexing (20, 21). Although it is possible that the failure of these trials stems from a lack of adequate MMP inhibition, another plausible explanation is that the nonspecific nature of the agents employed would theoretically eliminate the function of all MMPs, including those that serve the host in its defense against tumors. Literature to support roles for MMPs in the host defense against tumors is admittedly sparse; however, there is evidence that macrophage elastase (MMP-12), among other MMPs, can generate angiostatic peptides, such as angiostatin, a cleavage product of plasminogen, and thus retard tumor growth (22). This concept was confirmed in vivo when melanoma cells, genetically engineered to overexpress MMP-12, displayed reduced tumor growth rates in mice (23).

The exact role of MMP-12 in human cancers remains controversial. Whereas the expression of MMP-12 has been associated with angiostatin production and favorable outcomes in hepatocellular and colon carcinoma (24, 25), the reverse has been reported in dermal squamous cell carcinoma and non–small-cell lung carcinoma (26, 27). To better understand the role of MMP-12 in metastasis and lung tumor growth, we subjected MMP-12 deficient (MMP-12−/−) mice to the Lewis lung carcinoma (LLC) model of lung metastasis. Here we show a pro-host role for macrophage-derived MMP-12 by suppressing lung metastasis growth via tumor-associated angiogenesis inhibition.

Materials and Methods

Mice. Mice deficient in MMP-12 (MMP-12−/−) and wild-type (WT) littermates were used for all experiments. The MMP-12−/− mice were previously generated in our laboratory and have been described elsewhere.
(28). Briefly, intron 1 and exon 2 were disrupted by the insertion of the phosphoglycerol kinase promoter and neomycin resistance genes (PGK-neo). Initially, experiments were done in mice on a mixed C57/129 SvJ background but were repeated after 10 generations of backcrossing the MMP-12−/− mice to obtain a pure C57BL/6 background. Age- and sex-matched WT littermates were used as controls. The Harvard Standing Committee for Animal Research at Harvard University School of Public Health approved all animal experiments.

Cells. LLC cells and B16-F10 melanoma cells were used for the in vivo experiments. These cells were grown in DMEM (with t-glutamine, glucose, and 1 mmol/L sodium pyruvate), 10% FCS, 1× NEAA, 1× penicillin/streptomycin, and 1× vitamin supplement. Of note, both LLC and B16-F10 cells were derived from C57BL/6 mice on the same background as the MMP-12−/− and WT control mice.

Human dermal microvascular endothelial cells (Clonetics, San Diego, CA) were used for the in vitro endothelial cell proliferation assays. These cells were cultured in endothelial cell basal medium (Clonetics). All cells were maintained at 37°C with 5% CO2.

Tumor models. To study the role of MMP-12 in lung metastasis formation, we subjected MMP-12−/− and WT littermate control mice to two models of lung metastasis formation: (a) a spontaneous model in which metastases arise from a s.c. primary tumor and (b) an experimental model in which the malignant cells are directly injected i.v. such that lung metastases develop in the absence of a primary tumor. In the spontaneous model, LLC cells were trypsinized and resuspended at a concentration of 1 × 107/mL in PBS before injecting 0.1 mL of the cells s.c. into the dorsal left flank of mice. Twenty-eight days after injection, the mice were sacrificed and the tissues were processed as described below. For the experimental model, the LLC cells were trypsinized and resuspended in PBS at a concentration of 2.5 × 107/mL. The mice received 400 μL of these cells via tail vein injection. Fourteen days later, the mice were sacrificed and the tissues processed as described below. All of the above experiments were done in mice initially on a mixed C57/129 SvJ background but were repeated on MMP-12−/− and WT control mice on a pure C57BL/6 background. Additionally, B16-F10 melanoma cells (0.1 mL of 1 × 107/mL suspension) were injected i.v. to assess the effect of MMP-12 on a different malignant cell type.

Tissue processing. The mice were sacrificed at the conclusion of each experiment. After the initial dissection, blood was removed by right ventricle puncture and stored at −80°C. Gross lung metastases (>1 mm) were quantified by the naked eye. The right ventricle was then flushed with 3–4 mL normal saline to remove blood from the lungs before inflating the lungs at 25 cm H2O pressure with 10% buffered formalin for 10 minutes via an intrathoracic catheter. The lungs were removed and fixed for 24 hours before embedding in paraffin. Serial mid sagittal sections of 5-μm thickness were obtained for histologic analysis. A subset of the lungs were homogenized in 0.05 mol/L Tris (pH 8.0)/0.01 mol/L CaCl2/0.15 mol/L NaCl buffer and stored at −80°C to allow for further analysis. The primary tumors were resected, weighed, and fixed in 10% buffered formalin. A total of 6 μg of serum proteins was loaded per well.

Bone marrow transplantation. To show that MMP-12 was macrophage derived and not stromal cell derived, we did bone marrow transplantation experiments in MMP-12−/− and WT mice, both in pure C57BL/6 backgrounds. Recipient mice were irradiated with two doses (450 rad each) of full-body radiation in a cesium-137 irradiator located within our animal facility. Donor mice were sacrificed by CO2 narcosis followed by exsanguination. After removal of the hind limbs, the ends of the femur and tibia were cut and the marrow cells flushed out using sterile PBS containing 2% FCS and penicillin/streptomycin. The cells were pelleted and resuspended in the PBS/FCS/penicillin-streptomycin solution. The donor marrow was then administered to the recipient mouse via tail vein injection of 2 × 108 bone marrow cells in 250 μL PBS. Following a 3-week recovery period, the mice were subjected to the experimental LLC tumor model as described above.

Statistics. The data are expressed as mean ± SD unless otherwise indicated. Statistical significance was determined using the Student's t test (two-tailed distribution with a two-sample equal variance). P < 0.05 was considered significant.

Results

Spontaneous gross LLC lung metastases are increased in MMP-12−/− mice. To investigate the role of MMP-12 in lung metastasis formation and tumor growth, we subjected MMP-12−/− mice and WT littermate controls, in a mixed C57/129 SvJ background, to the spontaneous model of LLC lung metastasis in which 1 × 106 LLC cells were injected into the dorsal left flank of mice. LLC cells are known to spontaneously metastasize to the lung from a primary flank tumor within 2 to 4 weeks. Four weeks after flank injection, there was no difference in the size or weight of the primary tumors between MMP-12−/− and MMP-12−/− mice (Fig. 1A). However, examination of the lungs from these mice...
showed the presence of numerous gross lung metastases in MMP-12−/− mice, such that the lungs were markedly increased in size when compared with WT control (Fig. 1B, inset). The number of gross lung metastases (defined as lung tumors >1 mm³) in MMP-12−/− mice was nearly 10-fold greater (P < 0.0001) than in MMP-12+/+ controls (Fig. 1B). Histologic analysis revealed that the number of micrometastases coming from the primary tumor to the lung was not statistically different in MMP-12−/− versus MMP-12+/+ mice, suggesting that MMP-12 affects lung tumor growth, and not metastasis formation, per se (data not shown).

To eliminate the possibility that minor histocompatibility differences between the mouse and the LLC cell line (pure C57BL/6) were contributing to the observed phenotype, we backcrossed the mixed MMP-12−/− mice into a pure C57BL/6 background to produce a syngeneic experimental system. There was no significant difference in primary tumor size between WT and MMP-12−/− mice 4 weeks after injection of LLC cells (Fig. 1A). Consistent with the above findings, MMP-12−/− mice in a pure C57BL/6 background developed significantly more gross lung metastases (Fig. 1B) than C57BL/6 controls (P < 0.001). Thus, it did not seem that minor histocompatibility differences were influencing the observed phenotype. Nonetheless, we used MMP-12+/+ and MMP-12−/− mice in a pure C57BL/6 background for the remainder of the experiments.

MMP-12 suppresses the growth of LLC lung metastases in the absence of a primary tumor. Previous studies with the spontaneous LLC model of lung metastasis have suggested that an angiostatic factor was generated by the primary tumor or neighboring stromal cells, such that lung metastases experience accelerated growth on removal of the primary tumor. This factor was termed angiostatin, a cleavage product of plasminogen that is accelerated growth on removal of the primary tumor. This factor

Inflammatory cell accumulation is not impaired in MMP-12−/− mice. Differences in inflammatory cell recruitment/accumulation between experimental groups are a potential explanation of the above results. We have previously shown that MMP-12 is not required for proper monocyte/macrophage chemotaxis or macrophage survival (32); however, in some models, MMP-12 is required for monocyte recruitment (33). In addition, alterations in the numbers of key mediators of tumor killing, such as CD8+ T lymphocytes and natural killer (NK) cells, could affect lung tumor growth. Therefore, we did total lung digestion followed by flow cytometry to determine the immune cell content for MMP-12+/+ and MMP-12−/− tumor-bearing mice and nontumor controls using antibodies to CD3, CD4, CD8, Mac-1, and Gr-1. Although there were increases in inflammatory cell content in the tumor-bearing mice as compared with nontumor controls, there were no significant differences in immune effector cell recruitment between WT and MMP-12−/− tumor-bearing mice (Table 1).

Lung macrophage-derived MMP-12 is responsible for the observed phenotype. Previous studies have shown that MMPs can have deleterious actions when expressed by tumor cells themselves or by neighboring stromal cells (34). Because MMP-12 serves the host in our model, we hypothesized that its expression was likely limited to macrophages (as is the case under normal physiologic conditions) and not expressed by tumor cells, which would serve as a marker for an aggressive, poorly differentiated cell type. Therefore, we did immunohistochemistry for MMP-12 using polyclonal antiserum raised against MMP-12, generated in our laboratory, on lung sections from MMP-12+/+ and MMP-12−/− tumor-bearing mice that were subjects of the experimental LLC model. The macrophage-specific Mac-3 stain was also employed (Fig. 2A), which showed the presence of tumor-associated
macrophages in similar quantity in MMP-12+/+ and MMP-12−/− tumor-bearing mice. These tumor-associated macrophages express MMP-12 in the MMP-12+/− group, as evidenced by MMP-12 immunostaining. There was no expression of MMP-12 by MMP-12−/− macrophages nor was there expression of MMP-12 by the tumor or stromal cells (Fig. 2B) in either genotype. Thus, it seems that MMP-12 elaborated by lung tumor-associated macrophages exerts an antitumor effect on lung metastasis growth.

To test this concept, we did bone marrow transplantation in a pure C57BL/6 background in which MMP-12−/− mice received WT C57BL/6 bone marrow. Following a 3-week recovery period posttransplant, these mice received LLC injections as per experimental protocol. There was a significant decrease in gross lung metastasis (P < 0.01) number for WT bone marrow recipient MMP-12−/− mice when compared with MMP-12−/− mice that did not undergo bone marrow transplantation (Fig. 2C).

**Increased tumor-associated vessel density in MMP-12−/− mice.** Because MMPs (and MMP-12 in particular) have been linked to the generation of angiostatic factors such as angiostatin and endostatin, we hypothesized that host tumor-associated macrophages and MMP-12 functioned to produce an angiostatic-angiogenic tumor microenvironment. Therefore, we measured the tumor-associated vessel density in vivo using a CD31 antibody to label endothelial cells. Quantifying these results within the lung is challenging, given the ubiquitous nature of endothelial cells residing in adjacent alveolar structures. Therefore, we have expressed our results as microvessel number using the methods of Weidner (30) within (a) tumor center, (b) tumor periphery, and (c) micrometastasis including a 20-μm band of surrounding lung tissue. Not surprisingly, LLC lung metastases displayed increased tumor-associated vessel density in MMP-12−/− mice as compared with MMP-12+/+ controls (Fig. 3).

**Increased endothelial cell proliferation in MMP-12−/− mice.** The increased tumor-associated vessel density observed above could be the result of a decrease in an angiostatic factor or an increase of an angiogenic factor in the absence of MMP-12. To study the angiostatic versus angiogenic properties of MMP-12+/+ versus MMP-12−/− mice in this model, we used serum and tumor homogenate samples from WT and MMP-12−/− tumor-bearing mice in an in vitro endothelial cell proliferation assay. Both the serum and tumor homogenate samples isolated from MMP-12+/+ tumor-bearing mice inhibited the proliferation of human dermal microvascular endothelial cells to a greater extent than their MMP-12−/− counterparts (Fig. 4A and B). Thus, it seems that the MMP-12−/− samples lack an angiostatic factor that is generated in the MMP-12+/+ specimens.

**Angiostatin production is not dependent on MMP-12.** Because MMP-12 has been shown to generate angiostatin and because our results are consistent with the production of an angiostatic factor, we analyzed serum from WT and MMP-12−/− tumor-bearing mice for the presence of angiostatin. Western blot analysis of serum samples using a plasminogen antibody showed that both the 38-kDa Kringle regions 1-4 fragment (angiostatin) and the 35-kDa Kringle regions 1-3 fragment were equally present in the serum of MMP-12+/+ and MMP-12−/− mice (Fig. 4C). In addition, we were unable to detect expression of endostatin
(35), another antiangiogenic proteolytic fragment of type XVIII collagen (data not shown).

**MMP-12 suppresses B16-F10 melanoma growth in vivo.** To show that the effects of MMP-12 on lung tumor growth are not limited to LLC cells, we subjected MMP-12−/− mice and WT littermate controls in a pure C57BL/6 background to the i.v. injection of B16-F10 melanoma cells, which are also of C57BL/6 lineage. Similar to the above findings with LLC cells, experimental gross lung metastases were observed in significantly greater number (P < 0.01) in MMP-12−/− mice as compared with MMP-12+/+ controls 2 weeks postinjection (Fig. 5).

**Discussion**

MMPs are traditionally thought of in terms of their capacity to cause tissue destruction and promote disease progression. This is particularly true for MMP-12, an elastolytic enzyme strongly implicated in the pathogenesis of emphysema but for which normal physiologic roles remain poorly understood (33). The advent of genetic engineering in the mouse has illuminated many novel functions for MMPs, including the generation and termination of chemotactic gradients that mediate cell movement (36), participation in proper embryogenesis and development (37), and host defense against microorganisms (38). Here, we show yet another novel function for MMP: host defense against tumor progression via generation of an angiostatic-angiogenic tumor microenvironment.

The LLC model of pulmonary metastasis was employed to show a pro-host role for MMP-12 in this regard. The experiments were carried out in two distinct genetic backgrounds, mixed C57/SvJ 129 and pure C57BL/6, to show that minor histocompatibility differences were not responsible for the observed phenotype. Flow

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Increased microvessel density in MMP-12−/− tumor-bearing mice. A, microvessel counts were tabulated on CD31-immunostained sections from MMP-12+/+ (n = 4) and MMP-12−/− (n = 5) tumor-bearing mice. Columns, microvessel number that was generated by counting five high-power fields per slide using the method of Weidner et al. (30); bars, SE. *, P < 0.0001. B, representative CD31 sections from central tumor for MMP-12+/+ and MMP-12−/− mice.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Decreased angiostatic activity in MMP-12−/− tumor-bearing mice. Microvascular endothelial cell proliferation assays were done to study the angiostatic capacity of lung homogenates (A) and serum (B) from MMP-12+/+ and MMP-12−/− tumor-bearing mice at a concentration of 2 μg/mL. All samples were stimulated with bFGF. Control samples were generated from microvascular endothelial cells stimulated with bFGF in the presence or absence of homogenation buffer (A) and from microvascular endothelial cells stimulated with bFGF in the presence or absence of normal mouse serum (B). Columns, mean (n = 8 for each group); bars, SE. Each sample was run in triplicate. *, P < 0.001. C, representative Western blot showing the presence of angiostatin in the serum of MMP-12+/+ and MMP-12−/− tumor-bearing mice. Polyclonal antiserum raised against intact plasminogen was employed to show reactive proteins in serum samples after passing through lysine-sepharose columns. Plg, plasminogen; Ang, 38-kDa Kringle 1-4 fragment and the more prominent 35-kDa Kringle 1-3 fragment.
Increased angiogenesis in vivo

MMP-12 elaborated from lung tumor-associated macrophages is responsible for the observed findings. The tumor-suppressing functions of MMP-12 presented here represent a relatively novel finding for an MMP. There have been two prior reports suggesting an antitumor role for an MMP (or MMP fragment). Brooks et al. (45) discovered that PEX, the COOH-terminal hemopexin-like domain of MMP-2, functioned as an angiostatic peptide and thus inhibited tumor growth. MMP-2 itself, however, is a potent angiogenic substance that induces endothelial cell migration via interaction with the αvβ3 integrin. When MMP-2 is removed entirely, as in MMP-2−/− mice, tumor growth and angiogenesis are inhibited (10). Therefore, it seems that although a fragment of MMP-2 is capable of inhibiting angiogenesis, the net effect of MMP-2 in vivo is to promote tumor-associated angiogenesis and assist tumor growth. Neutrophil collagenase (MMP-8) has also been reported to serve the host in its defense against cancer (46). MMP-8-deficient mice display increased tumor growth and neutrophil influx in a carcinogenic model of skin cancer. The effect of MMP-8 in this model seems to be indirect, however, influencing tumor growth by altering the chemokine gradients that mediate neutrophil recruitment to the site of tumorigenesis, and not related to any MMP-8/tumor cell interactions or specific MMP-8-generated factor. The increase in tumor-associated neutrophils in MMP-8−/− mice was postulated to promote angiogenesis and tumor growth via elaboration of reactive oxygen species and proteinases as has previously been reported (47, 48).

The findings presented here, that macrophage-derived MMP-12 serves the host by generating an angiostatic-angiogenic tumor microenvironment (in a tumor type that does not express MMP-12), offer a plausible explanation about why MMP-12 expression has been associated with both favorable and unfavorable outcomes in different human malignancies. In the event that MMP-12 originates from its normal physiologic host, the tissue macrophage, as described here, angiogenesis is inhibited and tumor growth halted. However, when neoplastic cells are sufficiently dedifferentiated that they express MMP-12 themselves, as seen in squamous cell carcinoma of the skin and non–small-cell lung carcinoma (27, 28), the prognosis is poor. This hypothesis has actually been confirmed within the same malignancy (i.e., vulvar carcinoma). Kerkel et al. (49) reported a series of 33 patients in which the expression of MMP-12 by macrophages was associated with favorable outcomes whereas its expression by tumor cells conferred a poor prognosis. Whether tumor cell–derived MMP-12 has some specific pathophysiologic role aiding tumor progression...
or simply serves as a marker for a poorly differentiated tumor remains to be elucidated.

Lastly, pro-host functions for MMPs provide data crucial to the proper interpretation of the now largely defunct movement to develop synthetic MMP inhibitors as cancer therapeutics. Although still unpublished, the results from trials employing broad-spectrum MMP inhibitors were not ineffective as widely believed, but rather beneficial in certain tumor types and detrimental in others (50). In light of the findings presented here, it is mandatory that we have a better understanding of the function(s) of specific MMPs in specified tumor types, and furthermore, that we develop specific inhibitors to eliminate carefully targeted aspects of MMP biology.

It is therefore plausible that MMP inhibitors still represent a viable therapeutic strategy for cancer chemotherapy, albeit in more carefully defined circumstances.

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References

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