Matrix Metalloproteinase-8 Functions as a Metastasis Suppressor through Modulation of Tumor Cell Adhesion and Invasion

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Abstract

Collagenase-2 (matrix metalloproteinase-8, MMP-8) is an MMP mainly produced by neutrophils and associated with many inflammatory conditions. We have previously described that MMP-8 plays a protective role in cancer through its ability to regulate the inflammatory response induced by carcinogens. Moreover, it has been reported that experimental manipulation of the expression levels of this enzyme alters the metastatic behavior of human breast cancer cells. In this work, we have used mutant mice deficient in MMP-8 and syngenic melanoma and lung carcinoma tumor cells lines overexpressing this enzyme to further explore the putative antimetastatic potential of MMP-8. We report herein that MMP-8 prevents metastasis formation through the modulation of tumor cell adhesion and invasion. Thus, tumor cells overexpressing MMP-8 have an increased adhesion to extracellular matrix proteins, whereas their invasive ability through Matrigel is substantially reduced when compared with control cells. Analysis of MMP-8 in breast cancer patients revealed that the expression of this metalloproteinase by breast tumors correlates with a lower incidence of lymph node metastasis and confers good prognosis to these patients. On this basis, we propose that MMP-8 is a tumor protective factor, which also has the ability to reduce the metastatic potential of malignant cells in both mice and human.

Introduction

The matrix metalloproteinases (MMP) are a group of structurally related endopeptidases that have the ability to collectively degrade the different protein components of the extracellular matrix and basement membranes (1). On the basis of these degrading activities, MMPs have been traditionally considered as key enzymes in the invasive and metastatic properties of tumor cells. However, evidence that MMPs are not exclusively implicated in the proteolytic breakdown of tissue barriers for metastatic spread is accumulating. Thus, these enzymes may target nonmatrix substrates and influence other critical events in tumor evolution, such as cell proliferation, differentiation, angiogenesis, or apoptosis (2, 3). These findings, together with clinical and experimental data associating MMPs with tumor progression, stimulated the search for inhibitors to block the unwanted activities of these enzymes in cancer. However, most clinical trials with MMP inhibitors have not provided appreciable benefits to patients with advanced cancer (4). These negative results might be partly due to the fact that the broad range of inhibitors originally used in these trials could have also reduced the putative host protective antitumor properties of certain MMPs. This possibility has made necessary a reconsideration of previous MMP inhibition strategies, including the detailed analysis of the specific role of each individual MMP in the multiple stages of tumor evolution (5, 6). This is the case for collagenase-2 (MMP-8), a member of the MMP family which has recently emerged as a candidate to play a protective role during tumor progression (7). MMP-8 is mainly produced by neutrophils, and it has been implicated in a variety of tissue remodeling processes associated with inflammatory conditions (8–16). MMP-8 is very efficient in the degradation of fibrillar collagens (17), but may also target additional extracellular matrix proteins, as well as other proteases, cell adhesion proteins, protease inhibitors, growth factors, and chemokines (18, 19).

The unexpected finding that MMP-8 might play tumor-defying functions first derived from studies of cancer susceptibility in a murine model of MMP-8 deficiency (7). Thus, the absence of MMP-8 strongly increased the incidence of skin tumors in male Mmp8−/− mice. Bone marrow transplantation studies provided additional evidence that neutrophil-derived MMP-8 is sufficient to restore the antitumor protection mediated by this metalloproteinase (7). The relevance of MMP-8 as a protective factor in cancer has been further extended by the finding that experimental manipulation of the expression levels of this enzyme alters the metastatic behavior of human breast cancer cells (20). However, to date, no information is available about the molecular mechanisms underlying the putative role of MMP-8 in the regulation of the metastatic process. In this work, we have used mutant mice deficient in MMP-8 and syngenic melanoma and lung carcinoma tumor cells lines overexpressing this enzyme to further explore the possibility that MMP-8 may influence the in vivo metastatic potential of different mouse cancer cells.

Materials and Methods

Animals. The generation of Mmp8−/− mice has been previously described (7). These animals were back-crossed to C57BL/6 background for at least eight generations, and littermates were used as controls. All experiments were performed with 8-wk-old to 12-wk-old male mice and...
were conducted in accordance with the guidelines of the Committee on Animal Experimentation of Universidad de Oviedo.

**Cell lines and transfectants.** The murine melanoma B16F10 and the Lewis lung carcinoma (LLC) cell lines were obtained from the American Type Culture Collection and grown in standard conditions. For the generation of stable transfectants, B16F10 cells were transfected with pcDNA3 or with the same plasmid containing the full-length murine MMP-8 cDNA (9). Transfected clones were selected with G418 and analyzed by Western blot. A catalytically inactive MMP-8, in which the glutamic acid residue at position 218 was replaced by glutamine, was used to generate the following oligonucleotides: mMmp8E218Q-For 5' GGCGTTGCTCATCAAATTTGGAC and mMmp8E218Q-Rev 5'-TGCCCAATTGATGACGCCG. The resulting plasmid was used to generate stable transfectants, which were selected as above.

**Western blot analysis and enzymatic assays.** Cells stably transfected with MMP-8 cDNA or with an empty vector were incubated in serum-free media for 36 h, and secretion of MMP-8 was analyzed by Western blot using an anti-MMP-8 rabbit polyclonal antibody (7). To confirm that the MMP-8 probe was, however, the MMP-8 E218Q mutant, was auto-processed to its active form, the conditioned medium was incubated with 4-aminophenylmercuric acetate (APMA) at 37°C for 1 h and analyzed by Western blot. Enzymatic assays were performed with 50 μL of the above media using the quenched fluorescent substrate Mca-Pro-Leu-Gly-Leu-Dpa-Afa-Arg-NH₂ as described (21).

**Cell proliferation assays.** To quantitate cell proliferation, a CellTiter96 AQ nonradioactive cell proliferation kit was used (Promega Corp.). A hundred B16F10 cells were seeded in triplicate in 96-well plates and incubated at 37°C, 5% CO₂ for 4 d. Cell proliferation was quantified by measuring the conversion of 3-(4,5-dimethyl thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) into a water-soluble formazan by dehydrogenase enzyme in living cells, and the absorbance was measured at 490 nm using a microplate reader. All data are the mean of three independent experiments.

**Invasion and transendothelial migration assays.** The *in vitro* invasion potential was evaluated using Matrigel-coated invasion chambers with an 8-μm pore size (BD Biosciences). Briefly, 1 × 10⁵ cells per 100 μL were allowed to migrate for 8 h through the Matrigel-coated membranes using 5% fetal bovine serum (FBS) as chemoattractant for melanoma cells or 1 × 10⁶ cells/100 μL for 4 h using FBS as chemoattractant for bone marrow cells. Cells that reached the lower surface of the membrane were stained and counted in 20 random fields per section of the case in the case of B16F10 cells (magnification, 400×), or for the experiments using bone marrow cells, the total number of cells in the lower chamber was determined. For experiments involving the use of recombinant MMP-8, the protein was produced and purified as previously described (21). Data are the mean of three independent experiments. Transendothelialmigration assays were performed as described (22). Briefly, human umbilical vascular endothelial cell (HUVEC; 150,000) were grown to confluence (48 h) on top of an 8-μm pore size collagen-coated membrane of a 6.5-mm migration chamber (Transwell Costar). Subconfluent B16F10 cells, either expressing MMP-8 or not, were labeled for 45 min with cell tracker green Bodipy (Invitrogen). Afterwards, cells were trypsinized and added in migration buffer [DMEM, 10 mM/L HEPES (pH 7.4), 0.5% bovine serum albumin (BSA)] to the HUVEC monolayer, previously washed with the same buffer. Migration buffer containing 10% FCS was used as chemoattractant on the lower compartment. After 20 h, cells on the upper face of the membrane were scraped and the number of tumor cells that migrated to the lower face of the filter was counted using a fluorescence microscope.

**Cell adhesion assays.** For cell adhesion assays, non–tissue culture-treated plates were coated either with laminin-1 (5 μg/mL), type I collagen (10 μg/mL) or fibronectin (5 μg/mL) by overnight adsorption at 4°C and blocked with 5% BSA. Then, 1 × 10⁵ cells were plated and allowed to adhere for 1 h. Plates were washed, bound cells were fixed with 1% paraformaldehyde in PBS and stained with 0.1% crystal violet, and color was read at 570 nm.

**In vivo tumorigenicity.** Subconfluent B16F10 cells stably transfected with MMP-8 cDNA or with an empty vector (5 × 10⁴/100 μL) were injected s.c. at one flank into 8-wk-old C57BL/6 male mice. Two weeks later, mice were sacrificed, and tumors were harvested and weighed. In these experiments, 12 mice were used per condition and all assays were done in triplicate.

**Metastatic models.** We performed two models of lung metastasis formation: (a) a spontaneous model in which metastases arise from an s.c. primary tumor and (b) an experimental model in which the cells are directly injected i.v. in mice. In the spontaneous model, LLC cells (5 × 10⁶ cells) were injected s.c. in the dorsal right flank of mice (n = 12). Three weeks after injection, mice were sacrificed and the tissues were collected for histologic studies. For the experimental model, B16F10 cells (5 × 10⁵ cells) were injected in the lateral tail vein (n = 12). Three weeks later, mice were sacrificed and lungs were collected for histologic analysis. Serial sections of the lung (10 sections spaced 50 μm) were H&E stained, and metastatic foci were counted. Metastases were classified in small (<10 cells), medium (between 10 and 50 cells), and large (>50 cells). Graphs show the total number of metastatic foci per lung.

**Immunofluorescence.** To visualize actin filaments, B16F10 cells stably transfected with MMP-8 or control vector were allowed to attach to glass coverslips coated with laminin-1. One hour later, cells were fixed in paraformaldehyde, permeabilized with Triton X-100, and blocked with 10% FBS in PBS for 1 h. Coverslips were incubated with TRITC-phalloidin (Sigma) and mounted using Vectashield with DAPI (Vector Laboratories). Images were taken using a confocal fluorescent microscope (Leica), and differences in spreading between control B16F10 cells and those expressing MMP-8 were calculated by measuring the surface area occupied by the cells 1 h after plating using the MetaVue Imaging System (Molecular Devices). To detect host-derived MMP-8, LLC tumors grown on Mmps−/− mice or in wild-type littermates were collected, embedded in optimum cutting temperature, and frozen in liquid nitrogen. Sections (7-μm thick) were blocked as above and incubated with a rabbit anti–MMP-8 antibody or with a rat antibody against the neutrophil-specific antigen Ly6G (BD Pharmingen). Sections were washed with PBS and incubated with Alexa488-labeled goat anti-rat or Alexa594-labeled goat anti-rabbit secondary antibodies (Molecular Probes). Coverslips were mounted using Vectashield, and images were taken using a confocal fluorescent microscope (Leica).

**Immunohistochemistry and real-time PCR analysis of human tissues.** The current study involving human subjects was approved by the institutional ethical committee of the Radboud University Nijmegen Medical Center and the Norfolk Research Ethics Committee. The description of patients, their treatments, tissue processing, and RNA extraction have been previously reported (23). Quantitative real-time TaqMan reverse transcription–PCR (RT-PCR) analysis was carried out as described (24). The isolation and characterization of cell populations from normal human mammary gland obtained from reduction mammoplasty specimens has been described in detail elsewhere (24, 25). Immunohistochemistry was performed on 4-μm formalin-fixed, paraaffin-embedded sections of breast tissue using a standard avidin-biotin complex (ABC) technique. Briefly, slides were dewaxed and hydrated by passing through xylene and an alcohol gradient. After blocking with avidin and biotin blocking solutions (Vector Laboratories) and 20% normal goat serum (Sigma), sections were incubated with a rabbit anti-human MMP-8 polyclonal antibody (1/1,000, AbCam) overnight at 4°C followed by a goat anti-rabbit biotinylated secondary antibody (30 min, room temperature, Dako) and a streptavidin ABC complex/horseradish peroxidase tertiary antibody (30 min, room temperature, Dako). The staining was visualized using a 3,3′-diaminobenzidine substrate kit for peroxidase (Vector Laboratories), counterstained with Mayers hematoxylin, dehydrated, and mounted with DPX (BDH Chemicals Ltd.).

**Statistical analyses.** Statistical analyses were carried out using SPSS 12.0.1 software (SPSS Benelux BV). Normality of distribution was tested by the method of Kolmogorov-Smirnov. Differences in the proportion of tumors with or without detectable MMP-8 expression from patients categorized by clinicopathologic characteristics, used as grouping variables, were assessed with Pearson χ² test or Spearman correlation if more than two ordinal categories were involved. After excluding the samples without detectable MMP-8 mRNA, differences in absolute levels of log transformation
Collagenase-2 Reduces Tumor Metastasis and Cell Invasion

MMP-8 reduces lung metastasis of B16F10 mouse melanoma cells. MMP-8 is mainly produced by infiltrating neutrophils, although its presence has also been detected in some types of cancer (20, 26, 27). Likewise, previous studies have shown that murine MMP-8 has a protective role against tumor development (7). To explore the possibility that this metalloproteinase could also influence the metastatic potential of mouse tumor cells, we first used murine B16F10 melanoma cells which are highly metastatic, do not express MMP-8, and are syngentic with the C57BL/6 mice strain to which Mmp8+/− mice have been backcrossed, thus allowing the use of animals with an intact immune system. To evaluate the role of MMP-8 in this cell line, we transfected the murine MMP-8 cDNA in B16F10 cells and isolated several stably transfected clones with ability to produce this protein. Three of these clones (8, 11, and 21) were used for subsequent studies. Western blot analysis with a specific antibody against rodent MMP-8 revealed that these cells mainly produced the inactive zymogen of this protease, proMMP-8 (Fig. 1A). We also confirmed that this proenzyme was autoprocessed to its active form after treatment of culture media with APMA (Fig. 1B). By contrast, an MMP-8 mutant containing a point mutation in the zinc binding region (E218Q) failed to become activated after APMA treatment (Fig. 1B) and did not show any enzymatic activity against an MMP substrate (Fig. 1C). As expected, MMP-8 could not be detected in cells transfected with an empty vector (Fig. 1A).

To examine the effect of MMP-8 on the metastatic ability of melanoma cells, B16F10 cells, either transfected with MMP-8 cDNA or with an empty vector, were injected via the lateral tail vein of C57BL/6 mice, and the number of lung metastases was determined. Injection of B16F10 control cells resulted in the formation of numerous lung metastases (182 ± 51), whereas expression of MMP-8 reduced the number of metastases by >70% (Fig. 2A). This protective effect of MMP-8 could be observed in three independent clones expressing MMP-8 (data not shown), reinforcing the idea that the observed effect was solely dependent on the expression of this metalloproteinase. Furthermore, differences between cells expressing MMP-8 and control cells were not limited to the number of lung metastasis, as histochemical analysis also revealed differences in the histologic grade. Thus, metastasis classified as large (>50 cells per section) were more frequently found in mice injected with control cells than in those with cells expressing MMP-8 (68% versus 26%). In contrast, half of the metastatic foci formed by MMP-8–expressing cells were mainly classified as small (<10 cells per section). On the basis of these results, we conclude that MMP-8 expression is sufficient to reduce the metastatic ability of B16F10 cells.

To determine whether the proteolytic activity of MMP-8 was necessary for this protective effect, we established B16F10 transfectants expressing a mutant form of this protein, in which the active site glutamic acid was replaced by a glutamine residue. This E218Q mutant form of MMP-8 lacked any apparent proteolytic activity, as it was not activated by APMA (Fig. 1B), nor had any activity against different MMP substrates (Fig. 1C). Analysis of the metastatic potential of cells expressing E218Q-MMP-8 in C57BL/6 mice showed that the number of lung metastases produced by them was much higher than those generated by cells expressing similar amounts of wild-type MMP-8 and similar to those obtained with control cells, indicating that the proteolytic activity of MMP-8 is necessary for its antimetastatic activity (Fig. 2B).

MMP-8 expression does not affect melanoma growth. The formation of tumor metastasis is a complex process in which numerous events are implicated, including degradation of the extracellular matrix, cell migration, extravasation, and growth at
the site of metastasis (28, 29). Because of the characteristic features of the B16F10 cell model, which is based on the direct injection of tumor cells in the bloodstream, it is tempting to speculate that MMP-8 could influence either cell migration during extravasation or tumor growth. To determine which of these processes was affected by MMP-8 expression, we first carried out cell proliferation assays using the melanoma cell lines producing this metalloproteinase. We observed that the proliferation rate of B16F10 cells expressing MMP-8 or a control vector was similar (Fig. 2C) with a slightly lower proliferation rate for those cells expressing MMP-8, suggesting that this protease does not influence cell proliferation in vitro or might have a minor effect. However, as cell proliferation might be affected by numerous factors, we also analyzed the in vivo ability of these MMP-8–producing melanoma cells to form tumors. To this purpose, we injected s.c. 5 × 10⁴ cells in C57BL/6 mice, and after 2 weeks, tumors were harvested and weighed. All animals injected with control B16F10 cells developed tumors, as well as those injected with cells expressing MMP-8. However, we did not find differences in tumor weight between tumors expressing MMP-8 and control tumors (Fig. 2D). Together, these data indicate that MMP-8 is an antimetastatic protease, but does not directly affect melanoma growth in vitro or in vivo.

Melanoma cells expressing MMP-8 have reduced invasive capacity. The finding that MMP-8 does not affect cell proliferation in vitro or tumor growth in vivo prompted us to investigate whether the antimetastatic activity of this metalloproteinase could be due to changes in cell invasion that might affect extravasation. For this aim, we performed cell invasion assays using Matrigel as extracellular matrix. As can be seen in Fig. 3A, B16F10 control cells easily invaded through this reconstituted basal membrane in <8 h. Interestingly, expression of MMP-8, an enzyme with a potent collagenolytic activity, resulted in >80% reduction of invading cells (292 ± 41 versus 49 ± 10, P < 0.05; Fig. 3A). This effect of MMP-8 in cell invasion could be observed in three independent clones expressing this enzyme, suggesting that it is a general effect of MMP-8 on this melanoma cell line. The effect of MMP-8 on cell invasion could be further confirmed by incubating B16F10 control cells for 24 h in the presence or absence of recombinant murine MMP-8. After this treatment, Matrigel invasion experiments were performed, which showed that addition of recombinant MMP-8 was sufficient to reduce by >60% the number of invading cells (207 ± 10 versus 71 ± 20, P < 0.05; Fig. 3B). According to these results, we can conclude that MMP-8 is able to reduce the invasive capacity of a tumor cell line, in contrast with the activity of most MMPs that have been reported to promote invasion of tumor cells (2, 30).

To examine whether the observed effect of MMP-8 on cell migration was specific to the B16F10 cell line or a more general mechanism, we used animals deficient in MMP-8 and performed a comparative analysis of the invasive capacity of cells from mutant and control mice. As MMP-8 expression is mainly limited to neutrophils, we isolated bone marrow cells from Mmp8−/− and wild-type mice and performed Matrigel invasion assays using fMLP as chemoattractant. In agreement with the results obtained with B16F10 cells, we observed that bone marrow cells from Mmp8−/− animals were almost twice more invasive than bone marrow cells from wild-type mice (274,000 versus 150,000, P < 0.05; Fig. 3C). On the basis of these data, we conclude that MMP-8 has the ability to reduce the invasive properties of cells from different types.

Additionally, we investigated whether the effect of MMP-8 on cell invasion could contribute to diminish the capacity of tumor cells to extravasate and form metastasis. To this aim, we performed transendothelial migration assays and we observed that B16F10 cells expressing MMP-8 showed a reduction in their ability to transmigrate through a HUVEC monolayer when compared with B16F10 control cells (Fig. 3D). Together, these data suggest that the
antimetastatic activity of MMP-8 might be mediated, at least in part, by its ability to reduce the capacity of tumor cells to invade and extravasate at the sites of metastasis.

Expression of MMP-8 increases cell adhesion. To further explore the precise mechanism by which MMP-8 is able to regulate cell invasion, we investigated whether migration or cell adhesion to different extracellular matrix components were altered in cells expressing this metalloproteinase. By using wound-healing assays in plate, we did not find differences in cell migration between B16F10 expressing MMP-8 or control cells (data not shown), suggesting that this mechanism is not affected by the presence or absence of this protease. However, analysis of cell adhesion to components of the extracellular matrix revealed that MMP-8–expressing cells had an increased adhesion to type I collagen and laminin-1 when compared with control cells (Fig. 4A). In contrast, adhesion to fibronectin was very low in both cell types, and no significant differences were found. This increased adhesion to collagen and laminin-1 was detected in three independent clones with ability to produce MMP-8, suggesting that the observed effect is caused by the expression of this metalloproteinase. To further evaluate the activity of MMP-8 in the regulation of cell adhesion processes, we treated B16F10 cells with recombinant murine MMP-8 for 24 h and then cell adhesion assays were performed. As can be seen in Fig. 4B, addition of recombinant MMP-8 increased the adhesion of B16F10 cells to collagen type I and laminin-1 by six and three times, respectively. The effect of MMP-8 on cell adhesion could also be observed when cell spreading and cytoskeletal organization of filamentous actin (F-actin) were examined microscopically. Thus, when cells were allowed to attach to laminin-1 for 1 h, those expressing MMP-8 showed a rapid spreading and the appearance of actin stress fibers distributed throughout the entire cell (Fig. 4C and D). This contrasted with B16F10 control cells, which at the same time point showed less spreading, and the distribution of F-actin was more limited to the cell periphery. On the basis of these results, we can conclude that MMP-8 potentiates cell adhesion, and this might be a candidate mechanism by which this protease reduces cell invasion.

Increased metastasis in mutant mice deficient in MMP-8. MMP-8 has been previously shown to protect against skin tumor development (7), and we have shown herein that this protective effect can be extended to the formation of tumor metastasis in mice. However, the relevance of host-derived MMP-8 in this process is still unknown. We reasoned that if MMP-8 has an antimetastatic activity, mice deficient in this protease might be more susceptible to develop metastasis independently of the MMP-8 status of tumor cells. To test this hypothesis, we used two different models of tumor metastasis. First, we injected B16F10 cells that do not express MMP-8 in wild-type or Mmp8<sup>-/-</sup> mice via the tail vein, and the formation of lung metastases was examined. As expected, 3 weeks after injection of B16F10 cells, lung metastases developed in mice of both genotypes. However, the number of lung metastases in Mmp8<sup>-/-</sup> mice was significantly higher than in wild-type animals (71 ± 4 versus 30 ± 6, <i>P</i> < 0.05; Fig. 5A). This finding indicates that host-derived MMP-8 also has a protective effect against the formation of metastases caused by B16F10 melanoma cells, thereby extending our previous results, showing that tumor-derived MMP-8 diminished the ability of tumor cells to develop metastasis.

Although injection of tumor cells in the bloodstream is a widely used model to investigate tumor metastasis, it does not reflect the complexity of this process, in which interactions between tumor and inflammatory or stromal cells regulate tumor growth and dissemination (28, 31). To study the activity of MMP-8 in a model that could be closer to the in vivo situation, we used lung carcinoma LLC cells, as they have the ability to form primary tumors that later produce lung metastasis. We injected s.c. 1 × 10<sup>5</sup> LLC cells in wild-type or Mmp8<sup>-/-</sup> mice, and 3 weeks later, primary tumors and lungs were removed to compare tumor growth and to analyze the number of metastasis. We observed that the growth of LLC s.c. tumors was similar between wild-type and Mmp8<sup>-/-</sup> mice (Fig. 5B). However, mice deficient in MMP-8 developed more lung metastases than wild-type animals (7 versus 2, <i>P</i> < 0.05; Fig. 5C). As these metastatic cells originated from the primary subcutaneous tumor and LLCs do not express MMP-8, we reasoned that host-derived MMP-8 in wild-type animals was contributing to reduce the metastatic potential of LLC cells. In line with these results, immunofluorescence analysis of LLC tumors revealed that infiltrating neutrophils were the main source of host-derived MMP-8 (Fig. 5D). Therefore, it seems that secretion of MMP-8 by
tumor-infiltrating neutrophils might contribute to reduce tumor dissemination.

MMP-8 expression in human breast cancer is associated with lower incidence of synchronous lymph node metastases. To investigate whether the protective effect of MMP-8 against mouse tumor metastasis formation could also be observed in humans, we first analyzed the expression of this gene in normal human breast tissue, as well as in benign and malignant breast tumors. MMP-8 was detected in all normal and benign breast tissue samples with expression being localized almost exclusively to the myoepithelial–basement membrane interface (Supplementary Fig. S1). Staining was strongest in ducts but was also maintained in glandular acini. In those samples of ductal carcinoma in situ (DCIS) in which MMP-8 was expressed, diffused cytoplasmic staining of the neoplastic cells filling the ducts was evident in both low-grade and high-grade diseases (Supplementary Fig. S1). Interestingly, the well-defined staining for MMP-8 at the myoepithelial–basement membrane interface was no longer evident in DCIS. Invasive breast carcinomas exhibited a variable pattern of staining, some tumors lacking expression of MMP-8, whereas others displayed strong cytoplasmic expression in the majority of tumor cells (Supplementary Fig. S1). A consistent finding was expression of MMP-8 in vascular smooth muscle cells (Supplementary Fig. S1).

To try to extend these findings, we next analyzed the expression of MMP-8 by quantitative RT-PCR in a large panel of breast cancer cases. Of 250 patients with invasive breast cancer, 169 (68%) had no detectable MMP-8 mRNA in their breast cancer tumor tissue. The distribution of the MMP-8 expression levels in the remaining 81 (32%) MMP-8–positive tumors was normal after a log transformation (not shown). Patients with MMP-8–negative tumors did not differ from those with MMP-8–positive tumors with regard to age, menopausal status, type of surgery, histologic grading, or tumor size (not shown). MMP-8–negative tumors, however, were more likely to be found in patients with axillary lymph node involvement \((P = 0.006)\), or with ER–positive \((P = 0.008)\) or PgR–positive \((P = 0.007)\) tumors. Of the 103 patients that presented without involved axillary lymph nodes, 61 (51%) had a MMP-8–negative primary breast tumor (Fig. 6A). When one to three nodes were found to contain tumor cells, as seen in 74 patients, 54 (73%) had no detectable MMP-8 mRNA in their corresponding primary tumor. In the primary tumors of 45 patients who had four or more involved axillary lymph nodes, no MMP-8 mRNA was found in 36 (80%, Fig. 6A). The association of MMP-8 expression with the number of
involved axillary lymph nodes was not only detectable when patients were divided by the presence or absence of MMP-8 mRNA \((P = 0.006)\); when excluding the samples without detectable MMP-8 mRNA, there was a significant difference in the absolute level of MMP-8 depending on the nodal category (ANOVA \(P = 0.028\), Fig. 6A). Even more, the expression level of MMP-8 was negatively correlated with the number of involved axillary lymph nodes \((R = -0.328, P = 0.005)\) in the group of MMP-8–positive tumors.

Finally, we determined whether the prognostic value of MMP-8 expression differed by the number of involved lymph nodes that were found after primary surgery. Only in the patients that had no involved axillary lymph nodes could a difference in relapse-free survival be found \((P = 0.01, \log-rank; \text{Fig. 6B})\). Kaplan-Meier analysis survival data of patients with MMP-8–negative or MMP-8–positive tumors that were not treated with adjuvant therapy revealed a highly significant difference in RFS time \((P = 0.009; \text{Fig. 6C})\).

**Discussion**

In this work, we provide evidence that MMP-8, a member of the MMP family of metalloproteinases, prevents metastasis formation through modulation of tumor cell adhesion and invasion. We also report that the expression of this enzyme is associated with less axillary lymph node metastases and confers good prognosis to breast cancer patients whose tumors have yet to metastasize. These findings extend previous results showing that MMP-8 plays antitumor roles through its ability to regulate inflammatory responses to carcinogens (7), and show that this enzyme has also the capacity to influence the metastatic behavior of both human and mouse malignant cells.

The experimental approach followed to identify the antimetastatic role of mouse MMP-8 was based on the establishment of murine melanoma and lung carcinoma cell lines overexpressing this protease, as well as on the utilization of Mmp8–null mice previously generated in our laboratory (7). Analysis of the metastatic ability of different B16F10 melanoma cell clones stably transfected with MMP-8 cDNA revealed that the expression of this metalloproteinase was sufficient to reduce the number of lung metastases by \(>70\%\). Furthermore, the size of the metastases derived from melanoma cells producing MMP-8 was significantly lower than that of control cells. Interestingly, these antimetastatic effects of MMP-8 depend on its proteolytic activity because melanoma cells producing a catalytically inactive form of MMP-8 exhibit higher metastatic potential than those expressing the fully active protease.

In this work, we have also tried to elucidate the molecular mechanisms underlying the observed antimetastatic effects mediated by mouse MMP-8. By performing Matrigel-based invasion experiments and transendothelial migration assays, we have observed that B16F10 melanoma cells producing this metalloproteinase exhibit a strikingly reduced invasive ability through Matrigel or through a HUVEC monolayer. Likewise, incubation of melanoma cells with recombinant MMP-8 significantly reduced their invasive properties. These effects were not exclusive of melanoma cells because similar analysis performed with bone marrow cells, from either wild-type or Mmp8–/– mice, revealed that loss of this metalloproteinase results in a significant increase in the invasive ability of these nontransformed cells. On the basis of these results, we can conclude that the antimetastatic role of MMP-8 derives, at least in part, from the reduction of invasive properties and extravasation capacity of both malignant and nonmalignant cells conferred by the sole expression of this enzyme. Further analysis of this negative regulation of cell invasiveness mediated by MMP-8 revealed that it is associated with an increased

![Image of increased metastases in Mmp8–/– mice.](https://www.aacrjournals.org)
adhesion of cells expressing MMP-8 to different extracellular matrix components, such as type I collagen and laminin-1, a situation similar to that previously reported for PRL-1 phosphatase and hepatocyte growth factor in lung and prostate cancer cells, respectively (32, 33). These changes are accompanied by a rapid spreading and actin fiber reorganization, consistent with the increased adhesion of cells expressing MMP-8. After these studies based on the utilization of tumor cells overexpressing MMP-8, we evaluated the hypothesis that animals deficient in this enzyme might be more susceptible to develop metastases independently of the MMP-8 status of the analyzed tumor cells. Consistent with the above results, both B16F10 and LLC cells generated much more pulmonary metastases in Mmp8−/− mice than in wild-type animals, demonstrating that host-derived MMP-8 has also an antimetastatic effect. At present, we can only speculate about the precise molecular mechanisms mediating the observed effects of MMP-8 on cell adhesion and invasion. One possibility is that this protease may target specific cell adhesion proteins or selected integrins and contribute to change the adhesive and invasive properties of the corresponding cells. Nevertheless, we cannot rule out the possibility that MMP-8 may also cleave different extracellular matrix proteins and alter the cellular microenvironment or generate signaling molecules that activate intracellular pathways and finally contribute to modify adhesion and migration of metastatic tumor cells.

The identification of these antimetastatic properties of mouse MMP-8 prompted us to explore whether a similar situation could occur in human malignancies, with the finding that the expression of this protease in breast carcinomas confers a favorable prognosis to the corresponding patients. The fact that this prognostic value is restricted to those patients that as yet have no axillary lymph node metastases adds to the finding that MMP-8 is involved in the metastatic cascade. This observation agrees well with previous results, indicating that MMP-8 expression levels in tumor cell lines derived from a human breast carcinoma were negatively correlated with the metastatic potential of these cells (20). Also in this regard, it is noteworthy the recent finding that MMP-8 gene variation is associated with breast cancer prognosis (34). Interestingly, the high-expression T allele of the MMP-8 gene is associated with lower susceptibility to metastasis and better survival in breast cancer patients, which fully agrees with our present findings and emphasizes the putative relevance of MMP-8 in the context of novel metastasis suppressor genes.

The results presented in this work also provide additional evidence on the diversity of functional roles played by MMPs in cancer (2, 3, 35). For many years, it has been assumed that these enzymes are directly involved in tumor invasion and metastasis through their ability to degrade all major protein constituents of extracellular matrix and basement membranes. Accordingly, high MMP levels have been traditionally associated with invasive lesions and poor clinical prognosis in cancer patients (2). Furthermore, two MMP family members, MMP-1 and MMP-2, have been recently found to be important functional mediators in the generation of pulmonary metastases from breast carcinomas (36). Likewise, RNA interference inhibition of MMP-1 prevents melanoma metastasis by reducing tumor collagenase activity and angiogenesis (37). However, the observations reported herein for MMP-8, as well as recent findings for other MMP family members (38–42), clearly indicate that the cancer protective roles of MMPs may be much...
more relevant than anticipated. These findings will likely be extended to other protease families with multiple components whose expression is dysregulated in cancer (6, 24, 43–48) and emphasize the need to precisely define the complete set of proteases produced by each specific tumor before developing protease inhibitor-based approaches for cancer treatment (6). Finally, the recent development of global systems for profiling proteases produced by each specific tumor before developing more relevant than anticipated. These findings will likely be extended to other protease families with multiple components whose expression is dysregulated in cancer (6, 24, 43–48) and emphasize the need to precisely define the complete set of proteases produced by each specific tumor before developing protease inhibitor-based approaches for cancer treatment (6). Ultimately, the recent development of global systems for profiling proteases produced by each specific tumor before developing more relevant than anticipated. These findings will likely be extended to other protease families with multiple components whose expression is dysregulated in cancer (6, 24, 43–48) and emphasize the need to precisely define the complete set of proteases produced by each specific tumor before developing protease inhibitor-based approaches for cancer treatment (6). Finally, the recent development of global systems for profiling proteases produced by each specific tumor before developing more relevant than anticipated. These findings will likely be extended to other protease families with multiple components whose expression is dysregulated in cancer (6, 24, 43–48) and emphasize the need to precisely define the complete set of proteases produced by each specific tumor before developing protease inhibitor-based approaches for cancer treatment (6). Finally, the recent development of global systems for profiling proteases produced by each specific tumor before developing more relevant than anticipated. These findings will likely be extended to other protease families with multiple components whose expression is dysregulated in cancer (6, 24, 43–48) and emphasize the need to precisely define the complete set of proteases produced by each specific tumor before developing protease inhibitor-based approaches for cancer treatment (6). Finally, the recent development of global systems for profiling proteases produced by each specific tumor before developing more relevant than anticipated. These findings will likely be extended to other protease families with multiple components whose expression is dysregulated in cancer (6, 24, 43–48) and emphasize the need to precisely define the complete set of proteases produced by each specific tumor before developing protease inhibitor-based approaches for cancer treatment (6). Finally, the recent development of global systems for profiling proteases produced by each specific tumor before developing more relevant than anticipated. These findings will likely be extended to other protease families with multiple components whose expression is dysregulated in cancer (6, 24, 43–48) and emphasize the need to precisely define the complete set of proteases produced by each specific tumor before developing protease inhibitor-based approaches for cancer treatment (6).
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