Altered Metastatic Behavior of Human Breast Cancer Cells after Experimental Manipulation of Matrix Metalloproteinase 8 Gene Expression

Valerie Montel,1 Jeanine Kleeman,1 Dianne Agarwal,1 Dominic Spinella,2 Kanji Kawai,1 and David Tarin1

1Department of Pathology, University of California, San Diego Cancer Center, La Jolla, California, and 2Chugai Pharma USA LLC, San Diego, California

ABSTRACT

Previous work in our laboratory led to the cloning, from the same parent tumor cell line (MDA-MB-435), of two human breast cancer cell lines (M-4A4 and NM-2C5) with opposite metastatic phenotypes. Additional investigations revealed that the nonmetastatic cell line NM-2C5 overexpressed the neutrophil collagenase, matrix metalloproteinase (MMP)-8, relative to its partner. Because other studies have implicated the MMP family in promoting tumor metastasis, we investigated the apparently paradoxical expression of MMP-8 in these cell lines. By genetic engineering, we inverted its relative levels of expression in the two partners and studied the effects on the behavior of the tumors that they generated in athymic mice. Knock-down of expression in NM-2C5 cells by transduction with a sequence encoding a specific ribozyme and overexpression of MMP-8 in M-4A4 cells by retroviral transduction both strikingly changed metastatic performance in opposite directions, indicating that this gene plays a role in the regulation of tumor metastasis.

INTRODUCTION

Emergence of metastases in organs distant from the primary tumor is the culmination of a complicated sequential process that is systematic in its execution but defies basic rules governing the maintenance of multicellular organization and threatens the survival of the host. It occurs insidiously and is only recognized when secondary tumor deposits are already established. To understand and arrest the process, there is a need to identify the molecular mechanisms involved and to find markers that signal that the process is imminent or already in progress. To this end, we have developed a new experimental system that enables comparative molecular screening and functional evaluation of candidate metastasis-related genes in an isogenic background (1, 2). A large panel of monoclonal tumor cell lines was derived by limiting dilution cloning from the polyclonal breast carcinoma cell line MDA-MB-435 and systematically tested for metastatic behavior in athymic mice. The clone M-4A4 was selected for its highly metastatic capability, whereas a separate clone, NM-2C5, was found to be virtually nonmetastatic in such mice. Representative difference analysis of cDNA obtained from these two clones revealed a 20-fold greater expression of the neutrophil collagenase, matrix metalloproteinase (MMP)-8, in the nonmetastatic cell line relative to its metastatic counterpart. Other metalloproteases measured (MMP-9 and MMP-2) were equally expressed in the two lines (3). MMP-8 is a member of the large family of MMPs (4). More specifically, it is a Zn2+ metalloendopeptidase (5) predominantly expressed by neutrophil precursors (6, 7) but also expressed by fibroblasts (8, 9), endothelial cells (10), keratinocytes (11, 12), chondrocytes (13, 14), bronchial epithelial cells (15), macrophages (15, 16), and plasma cells (17). MMP-8 is expressed initially as a proenzyme and stored in specific granules of neutrophils (18). It is the most active collagenase against type I collagen but is also capable of cleaving collagens type II and III (19), cartilage aggrecan (20), the plasma serine proteinase inhibitor α1 antitrypsin (21), the tachykinin substance P (22), the angiotensin (22), and fibrinogen (23). The enzyme appears to play a major role in the turnover of connective tissue occurring in inflammatory processes, including periodontitis (24), osteoarthritis (25), and bronchiectasis (15).

Unlike other members of the metalloproteinase family, MMP-8 has not, to date, been clearly associated with tumorigenesis or metastasis (see Refs. 26 and 27 for reviews). Our observation of significant down-regulation of a MMP in the metastatic cell line or, conversely, of an up-regulation in the nonmetastatic line raised the hypothesis that it has an inhibitory role in regulating tumor metastasis. Therefore, we have investigated this possibility by generating reversed phenotypes of MMP-8 expression in both cell lines by transducing them with appropriate genetic constructs. The altered expression of the gene was evaluated in vitro, and specific clones showing marked up- or down-regulation of this collagenase in M-4A4 and NM-2C5 lineages, respectively, were selected for additional study. These were injected orthotopically into athymic mice to evaluate whether or not the inversion in MMP-8 expression could induce a change in the metastatic properties of the parental cell lines.

This investigation showed decreased metastatic performance of M-4A4 cells genetically engineered to overexpress MMP-8 and a corresponding increase in metastatic competence of NM-2C5 cells engineered to underexpress MMP-8, using ribozyme knock-down technology. Ribozymes are small RNA molecules that possess catalytic RNA cleavage activity (28, 29). They bind, by complementary base pairing, to a target RNA and enzymatically cleave it at that specific site. In this work, we combined this highly specific technology with sensitive and indelible enhanced green fluorescent protein (eGFP) tumor cell labeling to investigate whether the differential expression of MMP-8 in these cells is causally related to their differing metastatic phenotypes. The evidence suggests that this protein does have a role in regulating the metastatic process.

MATERIALS AND METHODS

Cell Lines. The NM-2C5 and M-4A4 cell lines were isolated in our laboratory from the MDA-MB-435 breast cancer cell line as described by Bao et al. (1). These monoclonal cell lines were routinely cultured in RPMI 1640 supplemented with 10% newborn calf serum (Life Technologies, Inc., Gaithersburg, MD), penicillin, and streptomycin at 37°C in a humidified atmosphere of 5% CO2-95% air.

Isolation of Total RNA. Fresh cell pellets or frozen solid primary tumors were homogenized in TRIzol reagent (Life Technologies, Inc.) for RNA extraction. The RNA pellets were resuspended in RNAase-free water, and the contaminating DNA was removed from the preparations with DNase I using the DNA-free kit (Ambion, Austin, TX). The yield of total RNA was measured using a spectrophotometer (Eppendorf, Westbury, NY), and the quality was assessed by running the samples in a 1% agarose gel.

Quantitative PCR. mRNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase and oligodeoxynucleotidic acid (oligo(dT)) from the Retroscrip cDNA synthesis system (Ambion). PCR primers were designed, based on the human MMP-8 cDNA sequence (GenBank accession number NM_002424), to specifically amplify human mRNA (MMP-8 sense primer 5'-ACCAATACTGGGCTCTGAGTGGCTAT and antisense primer

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Requests for reprints: David Tarin, University of California at San Diego Cancer Center, 9500 Gilman Drive, MC0912, La Jolla, California 92093-0912. Phone: (858) 822-2081; Fax: (858) 822-2084; E-mail: dtarin@ucsd.edu.
temperature, and the binding of the secondary antibody was detected by Agarwal Tris-buffered saline-Tween four times, blots were incubated with secondary were blocked in Tris-buffered saline-Tween buffer containing 5% dried milk according to the manufacturer’s instructions. Blots (Pierce, Rockford, IL). Denatured and reduced protein samples were separated concentration was determined with Coomassie Plus Protein Assay Reagent (Bio-Rad) using Biomax Ultrafree Centrifugal Filters (Millipore, Bedford, MA). Protein MMP-8 in a sample was determined by interpolation from a standard curve.

**MMP-8 Activation.** Procollagenase activation with the organomercurial compound p-aminophenylmercuric acetate was performed by incubating the recombinant MMP-8 (Chemicon International Inc., Temecula, CA) in activa- tion buffer [50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 10 mM CaCl2] containing 1 mM p-aminophenylmercuric acetate for 16 h at 37°C. The reaction was stopped by the addition of boiling denatured buffer for subsequent electro- phoretic analysis.

**Western Blotting.** To prepare serum-free conditioned medium, cells were washed six times with serum-free medium and resupplied with fresh serum- free medium. After 24 h, the culture supernatant was harvested, spun at 1,500 g to remove cellular debris, and concentrated approximately 200×

**Zymography.** The analyses were performed as described previously by Agarwal et al. (3). Briefly, 5 µg of protein samples from concentrated serum-free conditioned medium were loaded on 10% Tris-glycine polyacryl- amide gel with 0.1% gelatin incorporated as a substrate (Novex, San Diego, CA). After a refrigerated migration under nonreducing conditions, the gel was incubated (2 × 15 min at room temperature) in the renaturing buffer (Novex), equilibrated for 30 min at room temperature in the developing buffer (Novex), and incubated overnight at 37°C in fresh developing buffer. The gel was stained with 0.5% Coomassie Brilliant Blue R in 50% methanol/10% acetic acid for 30 min and destained in 7.5% acetic acid/5% methanol. The clear bands represent gelatinase activity.

**ELISA.** Quantitative determination of human MMP-8 in tissue culture supernatants and tissue homogenates was performed using a commercially available ELISA kit from Amersham Biosciences. The concentration of MMP-8 in a sample was determined by interpolation from a standard curve.

**DNA Constructs and Retroviral Infection.** Two strategies were used to knock-down MMP-8 expression: (a) transduction with an antisense sequence; and (b) transduction with specifically targeted hairpin ribozymes. There are several types of naturally occurring ribozymes, but the hairpin ribozyme was chosen because this RNA enzyme works optimally under physiological con- ditions, and its structure may be intrinsically more stable intracellularly than other ribozyme types. Hairpin ribozymes require a GUC in the substrate RNA, and cleavage occurs just 5’ of this GUC. The ribozyme-containing constructs were produced as follows: hairpin ribozyme cleavage sites were designed to select specific sequences within the target gene according to target recognition sequence requirements (XXXNNGTCXxxxxxx; X represents bases comple- mentary to helices 1 and 2). The target sequences in MMP-8 mRNA were recognized and cleaved at nucleotide positions 1612, 1549, and 1191 by the three chosen ribozymes as were: (a) 5’-GAGCAGTCAAGCAAT; (b) 5’-ATTGTTGCTCGTCTTAT; and (c) 5’-TACCTGTCCCTCCGTGA. These se-quences were used to construct a multiribozyme cassette in the shuttle vector T71.1, in which the expression of each of the ribozymes was driven by the human valine tRNA promoter. The first-phase ribozyme construct contained a single ribozyme that cleaves MMP-8 mRNA at nucleotide position 1612. The second-phase construct contained three ribozymes that cleave the mRNA at positions 1191, 1549, and 1612. Double-stranded ribozyme DNA inserts were generated by PCR using a target sequence-specific primer and a ribozyme sequence-specific primer, and the incorporated ribozyme sequence was con- firmed by DNA sequence analysis. The single ribozyme was ligated into retroviral vector pLNCX. The multiribozyme cassette was ligated into the Xba1 site in the 3’ long terminal repeat of the pLNCX2 retroviral vector from Clontech (Palo Alto, CA). Additionally, the eGFP sequence, under the control of cytomegalovirus promoter, was inserted into the multicloning site of the pLNCX2 vector to aid visualization of metastases made by cells transduced with the construct. The control construct for the ribozyme experiment consisted of the empty vector pLNCX2-eGFP alone. The packaging cell line PT67 was transfected with the retroviral vector containing the ribozyme sequences or the empty vector, and the transfectants were cultured in DMEM supplemented with 1 mM sodium pyruvate and Geneticin (Life Technologies, Inc., Carlsbad, CA) at 800 µg/ml for selection. After removal of the dead cells by changing the medium every day for 1 week, the culture supernatant containing the viral particles was collected and filtered through a 0.45-µm syringe filter. The filtered medium was supplemented with Polybrene to a final concentration of 8 µg/ml and used to infect the NC-2M5 cells with the retroviral vectors containing the ribozyme or the control construct by two 12-h incubations at 37°C. The transduced cells (Chu3-MuRzB containing the first- or second-phase ribozyme constructs and 2C5pLNCX2-eGFP containing the control vector) were then selected by culturing them in the presence of Geneticin (400 µg/ml) for 3 weeks before inoculation in mice.

**Orthotopic Inoculation and Metastasis Assays.** One million NM-2C5 or M-4A4 cells in 20 µl of a mixture of RPMI 1640 and extracellular matrix gel (Sigma Chemical Co., St. Louis, MO) were inoculated into the mammary fat pad of anesthetized mice. The rate of primary tumor growth was determined by plotting the diameters of the tumors,
measured at 7-day intervals. Animals were euthanized and autopsied at 3–7 months postinoculation when the primary tumors reached 20 mm in diameter. Metastasis formation was assessed by macroscopic observation of all major organs for secondary tumors and confirmed by histological examination. In experiments using green fluorescent protein (GFP)-labeled cells (i.e., ribozyme-transduced cells and corresponding controls), metastasis was also confirmed by looking for fluorescence of incorporated GFP under blue light (λ = 490 nm). Tissues from primary tumors and metastases were snap-frozen and stored at −80°C until used for RNA or protein extraction.

Statistical Analysis. The results were evaluated using Fisher’s exact t test by the Biostatistics Shared Resource of the University of California at San Diego Cancer Center.

RESULTS

Expression of MMP-8 in the Unmodified Breast Cancer Cell Lines and Their Transduced Counterparts in Vitro. It was shown previously by semiquantitative methods that MMP-8 gene transcription in the NM-2C5 cell line in culture is approximately 20-fold higher than that in the M-4A4 cell line (3). Western blots on serum-free conditioned medium confirmed the elevated expression of the corresponding protein. In the present study, the transcription and expression of this gene were precisely quantified by real-time PCR and ELISA, respectively, in the original cell lines, NM-2C5 and M-4A4, and in the derived cell lines transduced with different retroviral constructs designed to modulate its expression. Fig. 1A shows that the MMP-8 mRNA expression profiles for the wild-type cell lines are in accordance with our previous results: NM-2C5 showed 38 times higher transcription than M-4AA (left bars). The MMP-8 mRNA level was decreased in the clonal cell line NM2C5-ASM8 transduced with the antisense construct for this gene to 5.5% of that in the NM-2C5 cell line, from which it was derived. Conversely, the overtranscription of incorporated MMP-8 sequence driven by the cytomegalovirus promoter in the clonal cell line M4A4-M8 derived from M-4A4 was >300% in unmodified NM-2C5 cultures according to quantitative PCR measurements (Fig. 1A). These results were in close accordance with ELISA measurements of MMP-8 protein levels in the serum-free conditioned medium by the various cultured cell lines, as shown in Fig. 1B. Western blots also performed on the serum-free conditioned medium corroborated the identity of the secreted protein quantified in the ELISA assay (Fig. 2A). These blots showed a single band recognized by the monoclonal anti-MMP-8 antibody around Mr 75,000–80,000 corresponding to the molecular weight of the latent form (30, 31) as indicated by comparison with the p-aminophenylmercuric acetate activated form of human recombinant MMP-8 in Fig. 2C (left lane). In addition, this experiment provided confirmation that the electrophoretic pattern of the product synthesized from the exogenous sense sequence inserted in M4A4-M8 was similar to the endogenous MMP-8. The enzymatic activity of the collagenase was also demonstrated by zymography (Fig. 2B). The gelatin hydrolysis visible in the M 70,000 range in the right lane of the zymogram indicated that the induced MMP-8 in the M4A4-M8 cell line was not only secreted into the conditioned medium but was also as functional on the gelatin substrate as the endogenous enzyme (left lane).

Similar characterization of MMP-8 expression was performed on the ribosome-expression cell line Chu3MuR2ZB and compared with the parental cell line and the empty vector-transduced control. The real-time PCR analysis in Fig. 3A showed a 50% knock-down of the MMP-8 mRNA transcription attributable to the ribosome expression in this cell line, whereas the control cells (2C5PLNCX2-eGFP) were not significantly down-regulated compared with the parental line. The protein levels corroborated the reduced pattern of expression in ribosome-transduced cells, as shown by the Western blot in Fig. 3B. Collectively, these in vitro characterizations confirmed the successful
modulation of the neutrophil collagenase gene in the different transduced cell lines in vitro.

Expression of MMP-8 in the Tumors Originating from Breast Cancer Cell Lines NM-2C5 and M-4A4 and Their Transduced Counterparts in Nude Mice. Total RNA extracted from the frozen tissues was used as a template for quantitative PCR quantification of MMP-8 mRNA levels. The NM-2C5 tumors overtranscribed the MMP-8 gene by 25-fold compared with M-4A4 tumors, as displayed in Fig. 4A. Although the relative difference between these tumor cell types seen in vivo, with regard to the expression of this gene, was comparable with the relative difference seen in vitro, it was noticeable that the scale of transcription of this gene in the tumor was decreased by a factor of 100 relative to that seen in the corresponding cell line when normalized to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression. The values are the average of three measurements and the error bars are the SDs.

In the tumors made by M4A4-M8 cells, the RNA and protein levels of human MMP-8 were so up-regulated that the data did not fit the scales used for other tumor types in Fig. 4 and were elevated at least 1000-fold over the levels seen in the nonmetastatic tumors, but this may not be matched by a corresponding degree of increased activity. The induced overexpression of MMP-8 was therefore stable over several cell generations in vitro. Unexpectedly, the tumors made by NM2C5-ASM8 cells did not show suppression of human MMP-8 expression, and the levels observed varied between individual tumors (Fig. 4). Genomic DNA PCR confirmed that the construct was no longer present in the cells composing the NM2C5-ASM8 tumors, although it could still be detected in DNA from the same cell line growing in vitro (data not shown).

Conversely, the down-regulation of MMP-8 achieved using the ribozyme technology proved to be highly successful and sustained over time in vivo. With primers specifically designed to amplify only human but not mouse MMP-8 RNA, we confirmed that the ribozymes had reduced the transcripts of this gene in the cells composing the tumor. In addition, using primers specific for mouse but not human MMP-8, we confirmed that the levels of mouse MMP-8 expression remained low relative to those of their human counterpart in NM-2C5 and M-4A4 tumors (data not shown). A 70% decrease of MMP-8 protein expression was observed by ELISA in the Chum-MuRZB tumors compared with the parental cell line tumors (Fig. 4B), and similar reductions of MMP-8 levels were seen in the blood of the mice bearing tumors made by ribozyme-modified cells, relative to mice carrying control vector-only transduced tumors (data not shown).

Tumorigenicity and Metastatic Behavior of NM-2C5 and M-4A4 in Vivo. Although the tumorigenic and metastatic properties of the NM-2C5 and M-4A4 breast cancer cell lines have already been repeatedly assessed (2), we conducted new experiments to confirm that their concurrent baseline metastatic behavior corresponded to that
observed previously. Autopsy coupled with histological examination clearly established the presence of metastatic deposits in the lungs of 100% (12 of 12) of M-4A4 tumor-bearing animals and lymph node metastases in 40% of the same batch of mice, whereas only one instance of lung metastasis (but no lymph node metastases) was observed in a group of 18 mice that received an injection with NM-2C5 cells labeled with eGFP in a pLEIN vector to increase sensitivity of detection (Table 2).

NM2C5-ASM8 tumors did not form any metastases in the lungs or lymph nodes (Table 2), indicating that this transduced cell line, which had eliminated the construct during growth in vivo, behaved like its original parent. Conversely, we found that only 58% of the M-4A4-M8 tumor-bearing mice developed some secondary deposits in the lungs, which was a substantial ($P < 0.037$) decrease of metastatic capability compared with the M-4A4 cell line from which it was derived, and metastasis to the lymph nodes was also decreased (1 of 12).

The most striking pathological observation in the whole investigation was that 15 of 20 (75%) mice inoculated with cells transduced with ribozymes directed against the MMP-8 transcript formed metastases in the mediastinal, para-aortic, and pelvic lymph nodes, and 6 of them (30%) also had deposits in the lungs, all confirmed by histology (Figs. 5 and 6). In the first group of animals inoculated with NM-2C5 cells containing a single ribozyme, 3 of 12 (25%) tumor bearers had lung metastases, and 9 of 12 (75%) had lymph node metastases. In the second group (animals inoculated with Chu3MuRzB cells containing the triple ribozyme construct), three of eight (37.5%) animals had lung metastases, and six of eight (75%) animals had lymph node metastases. Because the results in the two groups were almost identical, the data are pooled in Table 2.

In contrast, tumor-bearing animals inoculated with the control cells, which were transduced with the 2CPspLNCX2-eGFP vector alone, showed no metastases in the lungs of any of the 10 tumor-bearing animals, and only 2 animals had tumor deposits in the local draining lymph nodes (Table 2). We deliberately chose to evaluate metastasis on a binary (present or absent) scale in these animals, rather than attempting to approximately assess metastatic load, because it is a more stringent analysis, and from a clinical perspective, the primary issue is whether a tumor can metastasize or not. The incidence of metastasis to the lungs and lymph nodes in animals inoculated with Chu3-MuRzB cells, therefore, represents a substantial, significant, and novel departure from the behavior of the parent NM-2C5-eGFP ($P < 0.00001$) cells from which they were derived and from the vector-transduced controls ($P < 0.007$).

**DISCUSSION**

This study focused on testing the possible role of a neutrophil collagenase, MMP-8, in determining the differential metastatic behav-

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**Table 2. Metastasis by human breast cancer cells orthotopically implanted in athymic mice**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of mice</th>
<th>Median days postinoculation (range)</th>
<th>Median size of primary tumor (mm) (range)</th>
<th>No. of animals with lung metastasis</th>
<th>No. of animals with LN metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM-2C5-eGFP</td>
<td>18*</td>
<td>150 (112–271)</td>
<td>17 (15–19)</td>
<td>1/18</td>
<td>0/18</td>
</tr>
<tr>
<td>M-4A4</td>
<td>12</td>
<td>95 (65–134)</td>
<td>18 (17–19)</td>
<td>12/12</td>
<td>5/12</td>
</tr>
<tr>
<td>NM2C5-ASM8</td>
<td>9</td>
<td>112 (84–168)</td>
<td>18 (18–19)</td>
<td>0/9</td>
<td>0/9</td>
</tr>
<tr>
<td>M4A4-M8</td>
<td>12</td>
<td>112 (77–168)</td>
<td>18 (15–19)</td>
<td>7/12</td>
<td>1/12</td>
</tr>
<tr>
<td>Chu3MuRzB</td>
<td>20</td>
<td>103 (79–185)</td>
<td>18 (12–20)</td>
<td>6/20</td>
<td>15/20</td>
</tr>
<tr>
<td>2CspLNCX2-eGFP</td>
<td>10</td>
<td>118 (66–225)</td>
<td>12 (6–20)</td>
<td>0/10</td>
<td>2/10</td>
</tr>
</tbody>
</table>

* Nine of these animals underwent tumor resection at approximately 15–20 mm diameter, which allowed them to survive longer and provided more stringent confirmation of their nonmetastatic status.

b LN, lymph node.
ior of two isogenic breast cancer cell lines (one is metastatic, and the other is nonmetastatic) after orthotopic implantation in athymic mice and found an inverse relationship between transcription of this gene and metastatic spread of the tumor. Specifically, genetic manipulation of the metastatic cell line to up-regulate the activity of this gene resulted in decreased metastatic spread; conversely, its down-regulation by incorporation of a targeted ribozyme in the nonmetastatic line resulted in the cells becoming impressively metastatic compared with control cells transduced with an empty GFP-labeled vector and untransduced NM-2C5 cells. Ribozyme technology has been used previously to identify cellular genes involved in hepatitis C translation (32) and, most recently, an upstream regulator in the expression of BRCA1, the familial breast cancer susceptibility gene (33), but this is the first example to our knowledge of their use to alter spontaneous metastatic behavior of orthotopic tumors. The combination of this technology with GFP labeling permitted a highly sensitive and specific analysis of the role of this gene in the regulation of metastasis in these cells.

Collectively, these findings suggest a regulatory role for MMP-8 in the metastatic process, as proposed in the hypothesis described in the “Introduction.” The effects on lymph node metastasis were unexpected and suggest that malfunction of this enzyme predisposes the affected cells to travel, survive, and grow better in the lymphatic system than by the hematogenous route to the lungs. This may indicate a mechanism for the predominant organ-specific metastasis to lymph nodes frequently observed in breast cancer patients.

The matrix metalloproteinase family enzymes have, because of their ability to digest fibrillar collagen and other connective tissue elements, long been considered to be candidates for facilitating tumor invasion and metastasis, and a large amount of data has been published supporting this concept (see Refs. 26 and 27 for reviews). Studies using knockout mice for distinct MMPs provided direct evidence for the role of MMPs in tumor growth and invasion, and numerous investigations in different types of human cancer have demonstrated correlations between MMP expression and clinicopathological findings in patients. Two aspects related to cancer progression have been considered in these numerous studies: (a) the association of MMP expression with tumor grade (or aggressiveness); and (b) the correlation with recurrence and metastasis. Indeed, used as markers, some MMPs can predict the risk of metastasis. For instance, MMP-2 and MMP-9 have been reported to be powerful predictors of metastasis in breast cancer (34, 35). The enhanced production of MMP-7 by human gastric carcinoma has also been reported as implicated in metastasis by this type of tumor (36).

Although MMP-8 is also highly effective in cleaving collagen I, the major component of formed collagen fibers in many tissues, its potential role in tumor invasion and metastasis has not been extensively studied. Importantly, MMP-8, like other MMP enzymes, is secreted as a proenzyme, which can subsequently be activated by a number of other enzymes including MMP-3 (37) and serine proteases, which themselves can be inactivated by specific tissue inhibitors (38). The interplay between these potential activators of MMPs and their inhibitors plays a significant role in the function of these enzymes.

Therefore, in addition to the differential expression of MMP-8 in these tumor cell lines, activation of the procollagenase could be an important regulatory step in its inhibitory effect on metastasis. With the exception of two publications reporting the collagenase as a potential tumor marker in patients with head and neck cancer (39, 40) and one showing its expression in several melanoma cell lines (14), most of the papers describing MMP expression in different types of cancers failed to observe any correlation between MMP-8 and invasion or metastasis (41–46). In breast cancer, Duffy et al. (47) did not find any relationship, but we have described previously that down-regulation of the enzyme in NM2C5-ASM8 breast cancer cells in vitro by gene transduction experiments significantly increased their ability to invade through Matrigel-coated membranes (3). These findings in vitro are consistent with the present results, but there are no other detailed studies of this topic.

The clear-cut differences in metastatic behavior seen in cells transduced with constructs that alter MMP-8 gene activity therefore contrast starkly with previous data on the putative roles of MMP-2 and MMP-9 in facilitating invasion and metastasis (34, 35), and the current study suggests that the observed inhibitory effects of MMP-8 are being mediated either by a fragment generated by the activity of MMP-8 or by a completely different pathway than the known catalytic effect of this enzyme on its main collagen substrate. There are some precedents for considering these possibilities. First, several investigators have described antiangiogenic and tumor-inhibitory activities of other MMP enzymes, including MMP-7, MMP-9 (48), and MMP-12 (49), resulting from the generation of angiostatin. Second, the work of Heiissig et al. (50) demonstrated that transgenic MMP-9 knockout animals became anemic because a previously unknown function of this metalloproteinase in cleaving the c-Kit cell surface receptor was abrogated, and it could no longer bind its ligand. Reasoning by analogy with such findings, it seems likely that the effects of manipulation of MMP-8 gene function that we have reported above also act by an as yet unknown route, and the data provide a starting point for investigation of the role that MMP-8 plays in regulating tumor metastasis. These results emphasize the need to design highly specific MMP inhibitors, if they are to be used in anticancer strategies.

It seems unlikely that the synthesis and release of an extracellular protease alone could cause the tumor cells to be unable to execute the entire sequential process of metastasis. However, it is possible that inappropriate inactivation or lowered expression of the protease could
sufficiently alter the balance of expression of a network of other genes in a cell line (NM-2C5), which we know from other experiments using GFP labeling to be already capable of disseminating widely without colonizing distant organs (51), and thus render it fully metastatic. The failure of the antisense methodology to cause the same effect as the ribozyme is explained by the finding that the antisense construct had been ejected by the cells that formed the tumor, although it had been effective in achieving down-regulation of MMP-8 in vitro. In our experience, the ribozyme technology proved much more stable and effective in vivo. However, the comparison with the antisense approach was useful to observe how such engineered cells can still revert to their former status, and the absence of metastasis in this group of animals acted as a further control, indicating that the changed phenotype was not simply a nonspecific byproduct of the engineering procedure itself.

In summary, these data provide novel evidence to conclude that intervention to alter MMP-8 production caused a clear change in the metastatic phenotype of both the metastatic and the nonmetastatic human breast cancer cells used in this study. Additional investigation of the mechanism involved may provide insights into how tumors become malignant and prone to forming secondary tumors in distant organs, whereas normal cells remain sedentary. It is hoped that such information will lead to clinically reliable prognostic markers for metastatic and nonmetastatic malignancy and therapeutic targets to inhibit cancer progression.

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