Inhibition of Matrix Metalloproteinase 9 Expression by a Ribozyme Blocks Metastasis in a Rat Sarcoma Model System

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ABSTRACT

Matrix metalloproteinases (MMPs) have been implicated in tumor progression, but the exact roles that each member of this family may play in contributing to the behavior of malignant tumors are only beginning to be understood. MMP-9 (gelatinase B or the 92-kDa gelatinase/type IV collagenase) expression has been associated with metastasis in a variety of model systems including that of rat sarcomas generated by transformation of rat embryo cells with rasH and myc. To determine the effect that MMP-9 expression has on metastasis, we inhibited the expression of MMP-9 using a hammerhead ribozyme. Introduction of an expression vector for a ribozyme directed against the rat MMP-9 mRNA sequence into a metastatic rat embryo cell line transformed by rasH and myc (2.10.10) that constitutively secretes MMP-9 resulted in the absence of detectable MMP-9 mRNA and loss of released 92-kDa gelatinase activity. These cells were no longer metastatic in a lung colonization assay but retained tumorigenicity. Introduction of an expression vector for a control hammerhead ribozyme had no effect. These data document the requirement for MMP-9 expression in metastasis in this system.

INTRODUCTION

MMPs3 have been implicated in tumor progression in large part because of the repeated finding of overexpression of this class of enzymes in tumors compared with their expression in normal tissues (1—3). Although it has been suspected that these enzymes play a role in metastasis and invasion, the function of each member of this family in tumor progression is only beginning to be determined. There are at least 14 members of this family of enzymes that have been divided into four subgroups, the collagenases, the gelatinases, the stromelysins, and the membrane-type MMPs. With several exceptions (4), the MMPs are secreted as proenzymes and then activated by cleavage of an NH2-terminal peptide that is responsible for maintaining the latency of the enzyme. This cleavage occurs at a sequence that is highly conserved throughout the MMP family, yet the different MMPs are often activated via different mechanisms. The MMPs share a highly conserved catalytic domain that depends on Zn+2 for activity.

MMP-9, which has also been called the 92-kDa gelatinase/collagenase or gelatinase B, is one member of this family that shares some common features (1—3). MMP-9 is secreted as a 92-kDa molecule and is processed via an inactive 87-kDa intermediate to active 82- or 83-kDa forms. Active products in a range between 67 and 45 kDa can also be seen. Association with tissue inhibitor of metalloproteinases 1 (TIMP-1), a naturally occurring inhibitor of MMP-9 alters its processing in vitro. MMP-9 can be activated in vitro through exposure to 4-aminophenylmercuric acetate, stromelysin, MMP-2 or plasminogen activator, but its physiological activators are currently unknown (5—12). Since MMP-9 can be activated by electrophoresis procedures, its gelatinase activity will be revealed on zymography or gelatin substrate gel electrophoresis even in its 92-kDa form. Once active, it has a wide range of proteolytic activity with the capacity to degrade extracellular matrix components, including denatured collagen or gelatin, type IV and V collagens, and elastin. It cleaves gelatin on cell surfaces and also can process tumor necrosis factor in vitro; it is not known whether it serves these functions in vivo (13, 14). MMP-9 bears considerable homology to another member of the MMP family, MMP-2. These enzymes have similar substrate specificities when tested against synthetic peptides but show one major difference; MMP-9 is highly active against casein, whereas MMP-2 is not (15).

There are suggestive data that expression of MMP-9 plays a role in invasion and metastasis (16). The release of MMP-9 has been correlated with metastasis in several systems. Metastasis by rasH-transformed NIH 3T3 cells correlated with MMP-9 release (17). Cells derived from an osteosarcoma treated with tumor necrosis factor α or U937 cells treated with 12-O-tetradecanoylphorbol-13-acetate show increased production of MMP-9-associated with increased lung colonization or invasion in cell culture (18). Cells isolated from more advanced melanomas are induced to express MMP-9 after treatment with transforming growth factor β, whereas cells from less aggressive lesions are not (19). Sehgal et al. (20) have similar data in a rat prostatic carcinoma model system. Transfection of transformed rat embryo cells with the oncogene EIA resulted in a virtual shutoff of MMP-9 mRNA and released 92-kDa gelatinase (21). This correlated with inhibition of metastasis in a lung colonization assay, although decreased stromelysin 1 expression was also seen in this case (22—24). Transient transfection of an expression vector for MMP-9 into the tumorigenic but nonmetastatic rat embryo cells transformed with rasH plus E1A resulted in metastasis by these cells, suggesting that MMP-9 was the effector for metastasis in this system (25). This system proved problematic, however, in that stable transformants of these cells that expressed MMP-9 could only rarely be isolated.

To extend the evidence that MMP-9 is required for metastasis in the rat sarcoma model system, we have now inhibited MMP-9 expression in a metastatic, tumorigenic, transformed rat embryo cell line that expresses high levels of MMP-9. We turned to a ribozyme method to specifically inhibit MMP-9 expression. Like antisense approaches, this method depends on using homology of a complementary RNA to specifically target a mRNA. To make a ribozyme, catalytic RNA sequences are flanked by complementary sequences to the targeted mRNA. This brings the catalytic region into proximity with the targeted mRNA and leads to its cleavage (26—31). The advantage of this method over other types of antisense technology is that the catalytic capacity of the ribozyme means that one ribozyme molecule can result in the cleavage of many targeted mRNAs; therefore, levels of the sequence that might be insufficient to lead to inactivation as an antisense molecule may be sufficient in the form of a ribozyme. Thus, this method is particularly suitable for lowering the amount of abundant mRNAs. We found that a ribozyme directed against MMP-9 was effective in inhibiting MMP-9 expression, and that inhibition of MMP-9 expression resulted in inhibition of metastasis. These results clearly establish the role of MMP-9 in metastasis.

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3 The abbreviations used are: MMP, matrix metalloproteinase; CMV, cytomegalovirus.

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MATERIALS AND METHODS

Plasmids. The expression vectors for the MMP-9 ribozyme and the control hammerhead were constructed by subcloning DNA fragments that would code for the ribozymes shown in Fig. 1 into the expression vector pRC/CMV. The pRC/CMV plasmid contains the CMV promoter upstream from a multicloning site and an SV40 polyadenylation site downstream. It also contains a separate cassette for expression of neomycin resistance. The oligonucleotide 5'-GACCAGTTTCTCCTACGAGTCACTCGCTTCACGCTGCA-3' and the complementary oligonucleotide 5'-AGCTGGCCGCGC-GCTTAGTACTGCTCCGTGAGGCAAACTGCTTCCG-3' were used to generate the MMP-9 ribozyme, and oligonucleotides 5'-GACCAGTTTCTCCTACGAGTCACTCGCTTCACGCTGCA-3' and 5'-AGCTGGCCGCGC-GCTTAGTACTGCTCCGTGAGGCAAACTGCTTCCG-3' were used to generate the expression vector for the control hammerhead. Each oligonucleotide (synthesized from a multicloning site and an SV40 polyadenylation site downstream) was annealed to its complement by mixing equal molar amounts, heating to 80°C, and slowly cooling to 30°C. The annealed, double-stranded DNA was separated from single-stranded oligonucleotides by electrophoresis through a 12% nondenaturing polyacrylamide gel and then eluted in DNA buffer (0.5 MNH4OAc and 1 mM EDTA) at 37°C for 4 h. The double-stranded DNA was then subcloned into the multicloning site (at HindIII and NotI) of pRC/CMV. The sequence of the insert and the region flanking the insert was confirmed for both the MMP-9 ribozyme and the control ribozyme by DNA sequencing.

Cells and Cell Culture. Cell line 2.10.10 is a metastatic rat embryo fibroblast line that was transformed by rasH and v-myc and that stably expresses MMP-9, as described previously (21, 22). Cells were cultured at 37°C and 5% CO2 in DMEM supplemented with penicillin, streptomycin, and 5% heat-inactivated fetal bovine serum. The 2.10.10 cell line was transfected with the ribozyme expression vector pRC/CMV with the insert coding for the MMP-9 ribozyme; and subclones 7E2, 7E4, 40, and 4D were isolated after transfection of the ribozyme construct, which should hybridize to both the MMP-9 mRNA and the control hammerhead ribozyme.

Northern Blotting. Total cellular RNA was extracted from cells by the method of Chomczynski and Sacchi (33), and polyadenylated RNA was extracted from cells using the direct mRNA mini kit (Qiagen, Chatsworth, CA). Ten μg total RNA or 5 μg polyadenylated RNA were separated by electrophoresis through a formaldehyde/0.9% agarose gel and transferred to a Hybond-N+ membrane (Amersham, Arlington Heights, IL). The RNA blots were hybridized with P-labeled probes derived from the 146-bp Sad and ApaI insert fragment released from pRC/CMV for detection of rat MMP-9 mRNA (25) and from the 1.3-kb EcoR! insert fragment from cDNA p8P2a to detect rat MMP-9 mRNA (25) and from the ribosomal protein rPL32 as an internal standard (34, 35) or a probe derived from the 146-bp Sad and ApaI insert fragment of pRC/CMV coding for the MMP-9 ribozyme; and subclone C116 was isolated after transformation with pRC/CMV coding for the hammerhead control ribozyme.

RESULTS

Ribozyme to MMP-9. We constructed expression vectors that would lead to constitutive expression of a ribozyme directed against MMP-9 mRNA as well as a control ribozyme. An oligonucleotide that would code for the catalytic sequence of a hammerhead type ribozyme flanked by 15 (8 on the 5' side and 7 on the 3' side) bases complementary to rat MMP-9 was synthesized and subcloned into the multicloning site of pRC/CMV (Fig. 1). Because the hammerhead ribozyme cleaves 3' to GU, where X is C, A, or U, the sequence was designed to ensure that the hammerhead would lead to constitutive expression of a ribozyme directed against MMP-9 mRNA. The sequence labeled Ribozyme depicts the ribozyme sequence that the MMP-9 ribozyme expression vector was designed to generate. The flanking vector-generated sequences are not shown. Left, ribozyme that is antisense to MMP-9; right, control hammerhead.

Gelatin Substrate Gel Electrophoresis. Substrate PAGE or zymography was performed as described (22). The serum-free medium samples were collected as follows. Cells were plated at a density of 1 X 105 on a 24-well plate (Corning, Corning, NY) in DMEM supplemented with 5% heat-inactivated fetal bovine serum, 250 μg G418 sulfate penicillin, and streptomycin and incubated for 24 h. Cells were then washed with serum-free medium and resupplied with fresh serum-free DMEM. The serum-free DMEM supernatant was harvested after 24 h. SDS-PAGE gels were prepared in a 7% polyacrylamide gel containing 0.1% porcine gelatin (Sigma Chemical Co., St. Louis, MO). Gels were washed in 0.05 mM Tris (pH 7.4) and 2% Triton X-100 for 30 min, rinsed in 0.05 mM Tris (pH 7.4) for 15 min, and incubated at 37°C overnight in 0.05 mM Tris (pH 7.4), 5 mM CaCl2, 1% Triton X-100, 0.2 M NaCl, and 0.02% NaN3. Gels were stained in 0.2% Coomassie blue for 1 h and destained in 20% (v/v) methanol, and 10% (v/v) acetic acid. The clear bands indicate gelatinase activity.

Lung Colonization, Metastasis, and Tumorigenicity Assays. Four- to 6-week-old female NCR-nu/nu mice were obtained from Taconic Farms (Germantown, NY) and housed aseptically in the animal facilities of the Wistar Institute (Philadelphia, PA). Cells used for injection were grown to subconfluence, trypsinized, washed once, and resuspended in serum-free DMEM. The cell suspensions were examined microscopically to ensure that they were composed of single-cell suspensions. For metastasis studies, mice were injected in the tail vein with 5 X 104 single cells/0.1 ml. For tumorigenicity assays, mice were injected s.c. in the flank with 5 X 103 single cells/0.1 ml. Tumors were measured using calipers for the calculation of tumor size. Animals were sacrificed when exhibiting labored breathing or at 4 weeks. A few tumor nodules from lungs were cut out, minced, and plated in a 100-mm dish in selective medium for growth. Lungs were inflated and fixed in 10% formalin. A dissecting microscope was used to count the lung tumors for evidence of metastasis.
MMP-9 Ribozyme
7E2 7E4 4G 4D

Fig. 3. MMP-9 mRNA levels in 2.10.10 cells transfected with the MMP-9 ribozyme.
Ten μg total RNA from four independent 2.10.10 cells transfected with the expression vector for the MMP-9 ribozyme (7E2, 7E4, 4G, and 4D) and from three 2.10.10 clones transfected with pRC/CMV (D, C, and B) were probed sequentially with a probe for rat MMP-9 and for rPL32, a gene that codes for a ribosomal protein that was used as a loading control. MMP-9 mRNA in the rat typically yields a double band, probably due to alternate polyadenylation.

coding for the MMP-9 ribozyme into 2.10.10 cells. Clones were isolated after selection for G418 resistance and tested for release of 92-kDa gelatinase activity. Of eight clones isolated, all showed diminished levels of secreted 92-kDa gelatinase activity, and four had a complete loss of detectable gelatinase activity. The gelatinase activity released by these four clones is shown in Fig. 2 along with the activity from five independent control transfectants of 2.10.10 cells. Gelatinase activity at 92 kDa was not found in the conditioned medium from the clones bearing the MMP-9 ribozyme, whereas all five of the subclones of line 2.10.10 that had been transfected with pRC/CMV released 92-kDa gelatinolytic activity, as did the clones transfected with the control ribozyme. Thus, introduction of a ribozyme directed against MMP-9 specifically eliminated 92-kDa gelatinase expression, but subclones of the parental cells, including those with a construct for a control ribozyme, continued to release 92-kDa gelatinase activity.

To confirm that expression of the MMP-9 ribozyme had resulted in depletion of MMP-9 mRNA, we compared the levels of MMP-9 mRNA in the 2.10.10 subclones with that in the clones transfected with the expression vector for the MMP-9 ribozyme. With comparable amounts of RNA loaded, it is apparent that the clones transfected with the MMP-9 ribozyme have no detectable MMP-9 mRNA under conditions in which this RNA is easily seen in the controls (Fig. 3). We documented the expression of the RNA of the ribozyme in clones transfected with the expression vector for the ribozyme against MMP-9 or with expression vector for the control hammerhead ribozyme (Fig. 4). Thus, the mRNA for MMP-9 was down-regulated only in the MMP-9 ribozyme-expressing cells.

Effect of the MMP-9 Ribozyme on Metastasis. To determine the effect of down-regulation of MMP-9 by the ribozyme on metastatic potential, 2.10.10 cells, 2.10.10 cells transfected with the hammerhead control, and two independent clones containing the ribozyme directed against MMP-9 were tested for their lung-colonizing ability in nude mice. Both 2.10.10 lines and the control transfectant were highly metastatic, whereas the numbers of lung colonies seen in the mice injected with the MMP-9 ribozyme-bearing cells were markedly lower (Table 1). The lung nodules seen were of equivalent size (data not shown). Mice injected with 2.10.10 or control cells did not survive beyond 3 weeks, and metastases were counted in animals sacrificed at 2 weeks. The mice injected with the MMP-9 ribozyme-bearing cells survived for at least 4 weeks. We counted the number of colonies in these mice at both 2 and 4 weeks. At 4 weeks, there were 35 colonies after injection of clone 7E4 and 31 colonies after injection of clone 4G compared with 49 and 23 colonies, respectively, at 2 weeks. Thus, the number of nodules seen at 4 weeks was not significantly different from that at 2 weeks. In both cases, this was substantially lower than the number of pulmonary metastases (205 metastases) after injection of the parental cells counted at 2 weeks. Thus, specific inhibition of MMP-9 expression decreased metastasis in this rat sarcoma model system.

Individual nodules were isolated from mice injected with clone 4G.
The growth rates of the tumors were estimated by measuring the rate of growth of s.c. tumors. Both the cells bearing the ribozyme and the clones used in the metastasis assays readily gave rise to tumors. An understanding of the role of MMP-2 in metastasis is further complicated because of its membrane localization in invasion. MMP-7, also named matrilysin, was shown to influence tumorigenic-

### Table 1  Metastatic potential of 2.10.10 cells transfected with the expression vector for a ribozyme directed against MMP-9

The lung colonization assay was performed as described in "Materials and Methods." Animals were sacrificed at the indicated times, and lung nodules were counted. Four mice were used for each time point. The average number of lung colonies is shown with the SD. The median is in parentheses with the range (median, range). The difference between the numbers of lung nodules formed by 2.10.10 and CI16 cells compared with the numbers from 4G and 7E was significant at P = 0.001 in each experiment using a Mann-Whitney test.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MMP-9 ribozyme</th>
<th>4G</th>
<th>7E4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 wk</td>
<td>4 wk</td>
<td>2 wk</td>
</tr>
<tr>
<td>Parental 2.10.10, 2 wk</td>
<td>205 ± 34 (190, 170-250)</td>
<td>185 ± 31 (170, 150-220)</td>
<td>31 ± 14 (34, 11-42)</td>
</tr>
<tr>
<td>Control ribozyme CI16, 2 wk</td>
<td>165 ± 22 (155, 140-187)</td>
<td>126 ± 14 (116, 112-141)</td>
<td>19 ± 7 (20, 9-26)</td>
</tr>
</tbody>
</table>

![Fig. 5. Gelatinase activity in conditioned medium from isolated metastatic (mets) nodules. Three nodules from the lung colonization after injection with 4G, a clone of 2.10.10 cells that had been transfected with the MMP-9 ribozyme, and two from injection with CI16, a clone of 2.10.10 cells that had been injected with the control ribozyme, were isolated in selective medium, and conditioned medium was taken for gelatin substrate gel electrophoresis.](image)

Thus, MMP-9 appears to be critical for metastasis but not tumor growth in these cells. Thus, the functions that MMPs can perform in tumors are starting to be defined. We have shown here that MMP-9 has a role in metastasis but not tumor growth. MMP-2 is also likely to be involved in metastasis. Kawamata et al. (36) expressed MMP-2 in a bladder cell line and found an increased area of lung metastases coincident with the increased expression. An understanding of the role of MMP-2 is further complicated because of its membrane localization in invasodopha and association with MT1-MMP and integrin αVβ3 (37-39).

### DISCUSSION

In this report, we investigated the effect of down-regulation of MMP-9 on metastasis. Transfection of a ribozyme directed against MMP-9 mRNA and led to a loss of detectable secreted 92-kDa gelatinase. This approach allowed us to examine the in vivo behavior of cells in which expression of MMP-9 was drastically decreased.

Tumorigenicity and the rate of tumor growth was equivalent for tumors derived from parental cells expressing MMP-9, cells transfected with a control vector, and cells down-regulated in MMP-9 expression. In contrast, down-regulation of MMP-9 led to significantly decreased numbers of metastases. This was true even when the metastases from the ribozyme-transfected cells were allowed to grow for twice as long as the controls, indicating that differential growth rates did not explain the differences seen. Isolation of some of the lung nodules that formed after injection of the MMP-9 ribozyme-transfected cells also confirmed this conclusion, because these nodules, unlike the injected population, now expressed MMP-9, indicating that selection for metastasis resulted in selection for MMP-9 expression. Our previous data indicated that transfection of an expression vector for MMP-9 into transformed rat embryo cells enhanced their metastatic potential, a result complementary to that reported here (25). Thus, MMP-9 appears to be critical for metastasis but not tumor growth in these cells.

![Fig. 6. Growth rate of tumors derived from 2.10.10 cells transfected with expression vector for ribozymes. Dissociated cells (5 x 10⁶) were injected s.c. Five mice were injected with each cell type bilaterally, and each injection led to a tumor with each cell line. The volume of the tumors was monitored three times/week using calipers. The averaged volume for each cell type is plotted as indicated. Bars, SD.](image)
ity in a colon carcinoma system but affected invasion in a prostatic carcinoma model system. Witty et al. (40) manipulated matrilysin expression in a colon carcinoma system. In that case, increased matrilysin expression did not alter invasion but did enhance tumor take, whereas down-regulation decreased tumorigenicity. Transfection of a matrilysin expression vector into DU 145 prostatic carcinoma cells led to invasion of the diaphragm after i.p. injection, whereas the untransfected cells implanted on the peritoneal surface of the diaphragm but did not invade (41). Thus, expression of matrilysin appears to have different effects in different systems.

It is tantalizing to note that MMP-9 is highly expressed in a wide range of human malignancies, leading to the suggestion that MMP-9 may play a role in metastasis in these tumors. Tumor cells from squamous cell carcinomas of the head and neck, and lung, high-grade gliomas, pancreatic carcinomas, sarcomas, lymphomas, and many breast carcinomas have been found to express higher levels of MMP-9 than their corresponding normal tissue (42–53). In general, MMP-9 is only expressed at low levels in normal quiescent adult tissues, although it is found during embryogenesis in the invading trophoblast, in the brain at the stages when neuroblasts are dividing and sending out processes, and in the thymus, lung, thyroid, and bone at distinctive phases during development (54–57). Thus the presence of MMP-9 in many potentially invasive and metastatic human tumors along with the experimental evidence demonstrating the requirement for MMP-9 expression in metastasis leads to the conjecture that MMP-9 expression may play a role in metastasis in many human tumors.

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REFERENCES


DOWN-REGULATION OF MMP-9 INHIBITS METASTASIS


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