Inhibition of Invasion and Metastasis in Cells Transfected with an Inhibitor of Metalloproteinases

Yves A. DeClerck, Norma Perez, Hiroyuki Shimada, Thomas C. Boone, Keith E. Langley, and Shirley M. Taylor

ABSTRACT

The balance between levels of metalloproteinases and their corresponding inhibitors is a critical factor in tumor invasion and metastasis. Down-regulation of the activity of these proteases was achieved by transfection of invasive and metastatic rat cells with the complementary DNA for metalloproteinase inhibitor/tissue inhibitor of metalloproteinase 2 (MI/TIMP-2), a novel inhibitor of metalloproteinases recently described. (Y. A. DeClerck et al., J. Biol. Chem., 264: 17445-17453, 1989; W. G. Stetler-Stevenson et al., J. Biol. Chem., 264: 17374-17378, 1989). Secretion of functional MI/TIMP-2 protein in stably transfected cells resulted in a marked decrease in metalloproteinase activity. Partial suppression of the formation of lung colonies after i.v. injection in nude mice was observed in a transfected clone expressing high levels of MI/TIMP-2. Production of MI/TIMP-2 in four clones markedly reduced tumor growth rate in vivo after s.c. injection and completely suppressed local tissue invasion. Thus, down-regulation of metalloproteinase activity has a striking effect on local invasion and partially suppresses hematogenous metastasis.

INTRODUCTION

Metastasis, a major cause of mortality and morbidity in cancer patients, is a complex multistep process, during which tumor cells locally invade the surrounding tissues, penetrate blood or lymphatic vessels (intravasation), and exit vessels at distant sites (extravasation) to form secondary tumors (1). Proteolytic degradation of the ECM 

MMP are a family of Zn$^{2+}$-dependent endopeptidases with a broad spectrum of proteolytic activity for several components of the ECM (4). The family includes interstitial collagenase, type IV collagenases (M$, 72,000$ progelatinase, and M$, 92,000$ progelatinase) and stromelysin (rat transsin). These proteases are substrate specific. Interstitial collagenase (MMP-1) degrades collagen types I, II, and III; M$, 72,000$ progelatinase (MMP-2) and M$, 92,000$ progelatinase (MMP-9) degrade collagen types IV and V; and denatured collagen (gelatin) and stromelysin (MMP-3) degrades fibropectin, laminin, and proteoglycans.

The secretion of MMP in an inactive proenzyme form is an important feature that regulates their activity in the extracellular milieu. A second mechanism regulating the extracellular activity of these enzymes is provided by inhibitors of metalloproteinases. TIMP, a M$, 28,000$ ubiquitous glycoprotein, is considered to be a major regulator of metalloproteinase activity in tissues. This inhibitor is secreted by many cells in culture including fibroblasts (5), endothelial cells (6, 7), chondrocytes (8), and vascular smooth muscle cells (9) and is present in bone, cartilage, and amniotic fluid (10–13). TIMP inhibits MMP by forming an irreversible 1:1 stoichiometric complex with the activated enzyme (14). We have recently reported the purification (15) and cloning (16) of a MI related to but distinct from TIMP and other investigators have described an inhibitor that forms a complex with M$, 72,000$ progelatinase in human tumor cells (17, 18). This latter protein, designated TIMP-2, has a nucleotide sequence identical to the nucleotide sequence of MI (19).

Under normal conditions, the balance between MMP and their corresponding inhibitors is a critical factor that maintains the homeostasis of connective tissue proteins (4). We have therefore postulated that under pathological conditions associated with excessive degradation of the ECM such as tumor invasion and metastasis, there is an imbalance between metalloproteinases and metalloproteinase inhibitors. The observation of a direct correlation between the secretion of MMP by tumor cells and their invasive and metastatic potential suggests that such imbalance can be achieved by increased production of MMP by tumor cells (20–22). Alternatively, this imbalance could be created by a decreased production of inhibitors, as suggested by the observation of an inverse correlation between levels of TIMP production and the invasive potential of tumor cells (23, 24).

In this report, we have investigated whether down-regulation of the extracellular activity of metalloproteinases in tumorigenic, invasive, and metastatic cells could reestablish the balance between proteases and inhibitors and subsequently alter their phenotype. c-Ha-ras I expressing rat embryo cells [clone 4R (25)] were selected because of the large amount of metalloproteinases secreted (26) and the absence of detectable inhibitor. Down-regulation of their metalloproteinase activity was achieved by stable transfection and expression of the cDNA of MI/TIMP-2. Our previous studies have shown that recombinant MI/TIMP-2 can inhibit the degradation of ECM and the invasion of artificial tissue substrates by these rat embryo cells in vitro (27). Here we examine the effect of increased expression of MI/TIMP-2 in the same cells on their in vitro and in vivo properties.

MATERIALS AND METHODS

Cell Culture. c-Ha-ras-1 transfected rat embryo cells (4R) were obtained from Dr. L. A. Liotta (NIH, Bethesda, MD). Cells were cultured in Eagle's minimal essential medium (Grand Island Biological, Santa Clara, CA) containing 10% (v/v) fetal bovine serum (Irving Scientific, Irvine, CA), penicillin (100 units/ml), and streptomycin (100 µg/ml).

Construction of pcDNA.HMI Vector and Cell Transfection. The human MI/TIMP-2 cDNA was inserted into an expression vector containing the cytomegalovirus promoter and enhancer elements (pcDNA;
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Invitrogen, Inc., San Diego, CA). Human MI/TIMP-2 cDNA was isolated as a NcoI/StuI fragment from plasmid pUC.HMI (28), blunt ended, and ligated into plasmid pCDNA to form plasmid pCDNA.HMI (Fig. 1). Correct orientation was verified by restriction endonuclease digestion. Cotransfection using calcium phosphate precipitation (29) was carried out with plasmid pY3 which encodes the hygromycin B phosphotransferase gene as dominant selectable marker (30).

Metalloproteinase-Metalloproteinase Inhibitor Assays. Concentrated (50×) serum free conditioned media from cell cultures were tested for proteolytic activity for type I and type IV collagen and for inhibitory activity against rabbit fibroblast collagenase. Proteolytic activity in conditioned medium was measured after activation of metalloproteinases with 1 mM p-aminophenylmercuric acetate for 30 min at 37°C. Aliquots of activated conditioned medium were then incubated in 96-well microtiter plates coated with 14C-labeled type I or type IV collagen as described previously (9, 26). Inhibitory activity was measured by incubating various amounts of concentrated serum free conditioned medium in the presence of rabbit fibroblast collagenase (0.05 unit) for 15 min at 22°C followed by the determination of residual proteolytic activity. One unit is defined as the amount of inhibitor that inhibits the proteolytic activity of 2 units of collagenase by 50% (15). The method measures the level of inhibitory activity that is not already complexed with secreted enzymes.

Acrylamide Gel Electrophoresis and Reverse Zymogram. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (31) using 12.5% (w/v) acrylamide gels. Reverse zymograms were used to detect the presence of gelatinases and metalloproteinase inhibitors in culture media as reported previously (9, 15). In brief, gels (0.1%, w/v) was incorporated into the acrylamide mixture prior to polymerization. Samples were electrophoresed at 4°C without heat treatment. After electrophoresis, gels were incubated in Triton X-100 (2.5%, v/v) for 1 h with 2 changes to remove sodium dodecyl sulfate, followed by 3 h at 37°C in the presence of gelatinase to allow partial proteolysis of gelatin. After overnight incubation at 37°C in 50 mM Tris-200 mM NaCl-10 mM CaCl2, pH 7.5, the gels were stained with 0.25% (w/v) Coomassie brilliant blue and destained in methanol:acetic acid:water (50:10:40). A clear zone indicates gels were stained with 0.25% (w/v) Coomassie brilliant blue and destained in methanol:acetic acid:water (50:10:40). A clear zone indicates the presence of an inhibitor of gelatinase.

Results

Transfection of 4R Cells with a Full Length MI/TIMP-2 cDNA. We constructed an expression vector, pcDNA.HMI, containing human MI/TIMP-2 cDNA cloned from human heart tissue (16), and placed under the control of a cytomegalovirus promoter and enhancer (Fig. 1). This expression plasmid was introduced into highly invasive and metastatic 4R cells. Fifty-two clones cotransfected with pcDNA.HMI and pY3 plasmids (hygromycin resistance marker) were established in the presence of 0.4 mg/ml of hygromycin B and screened for the secretion of MI/TIMP-2 by immunodot-blot analysis. Untransfected cells and cells transfected with pY3 alone were negative for the production of recombinant human MI/TIMP-2 and were used as control. We selected 5 clones cotransfected with pcDNA.HMI and pY3 (2 strongly positive, 2 weakly positive, and 1 negative) and 2 clones transfected with pY3 plasmid alone for a more detailed analysis. Northern analysis demonstrated the presence of a 1.85-kilobase message in four clones positive for MI/TIMP-2 production by immunodot-blot analysis. Untransfected cells and cells transfected with pY3 alone were negative for the production of recombinant human MI/TIMP-2 and were used as control. We selected 5 clones cotransfected with pcDNA.HMI and pY3 (2 strongly positive, 2 weakly positive, and 1 negative) and 2 clones transfected with pY3 plasmid alone for a more detailed analysis. Northern analysis demonstrated the presence of a 1.85-kilobase message in four clones positive for MI/TIMP-2 production by immunodot-blot analysis (Fig. 2, A and B). A 1.3-kilobase message was also detected in parental cells and in all transfected clones indicating a low level of endogenous expression. The level of c-Ha-ras-1 transcript was generally unaffected by transfection and expression of MI/TIMP-2 (Fig. 2, C and D). Immuno blot analysis of serum free conditioned medium of these clones (Fig. 2E) indicated a correlation between the presence of the 1.85-kilobase transcript and the detection of a 21,500 immunologically reactive protein of expected size for MI/TIMP-2 (15, 17). By reverse zymogram analysis (Fig. 2F), we also demonstrated the 4R cells a local daily injection of 0.1 mg (in 0.1 ml of phosphate buffered saline) of sterile recombinant MI/TIMP-2 obtained from Chinese hamster ovary cells (28). This injection was done initially at the site of the injection of tumor cells and later at the posterior port of the tumor as it developed. The tumor on the left flank received a daily injection of 0.1 ml of sterile phosphate buffered saline (control). For experimental metastatic assays, 6-10-week-old athymic (nu/nu) mice were given injections in the lateral tail vein of 2×10⁵ cells suspended in 0.1 ml of sterile phosphate-buffered saline. Cell viability, determined by trypan blue exclusion prior to i.v. injection, was higher than 85%. After 2 weeks, animals were sacrificed and the number of colonies at the surface of the lungs was determined using a dissecting microscope after endotracheal injection of India ink (26).

Tumorigenicity and Metastatic Assays. For tumorigenic assay, cells were given injections in the lateral tail vein of 2×10⁴ cells suspended in 0.1 ml of serum free conditioned medium (control). For experimental metastatic assays, 6-10-week-old athymic (nu/nu) mice were given injections in the lateral tail vein of 2×10⁵ cells suspended in 0.1 ml of sterile phosphate-buffered saline. Cell viability, determined by trypan blue exclusion prior to i.v. injection, was higher than 85%. After 2 weeks, animals were sacrificed and the number of colonies at the surface of the lungs was determined using a dissecting microscope after endotracheal injection of India ink (26).
secretion of two type IV collagenases ($M$, 92,000 and $M$, 72,000 gelatinases) by all clones selected and the presence of functional MI/TIMP-2 in clones expressing the 1.85-kilobase transcript (clones 8.27, 8.39, 8.60, and 8.68). The small amount of MI/TIMP-2 detected by reverse zymogram in clones 4.17 and 8.71 (gelatinases) by all clones and the presence of functional secretion of two type IV collagenases (M, 92,000 and M, 72,000) in clones 8.27, 8.39, 8.60, and 8.68. The small amount of MI/TIMP-2 in clones expressing the 1.85-kilobase transcript cross-react with our anti-human MI/TIMP-2 antiserum. (Fig. 2F) and not detected by Western blot analysis (Fig. 2E) likely represents endogenous (rat) MI/TIMP-2 that does not cross react with our anti-human MI/TIMP-2 antiserum.

Phenotypic Analysis of Transfected Clones. Analysis of the effect of increased MI/TIMP-2 expression on proteolytic activity, growth rate in vitro, and tumorigenic and metastatic potential was performed in the same 7 clones described above (Table 1). The level of free inhibitor activity measured in the conditioned medium closely correlated with the presence of the 1.85-kilobase message in transfected clones and no free inhibitor activity was detected in parental cells or clones transfected with the pY3 plasmid alone. As anticipated, the level of free inhibitor activity detected in conditioned media of positive clones inversely correlated with the level of proteolytic activity for type I and type IV collagens. Clone 4.6 which was found to have the highest level of proteolytic activity for type IV collagenase (Table 1), secreted smaller amounts of $M$, 72,000 and $M$, 92,000 MI/TIMP-2 (Fig. 2).

Fig. 2. Analysis of production of functional MI/TIMP-2 in transfected 4R cells. A-D, Northern analysis of 4R cells and 4R cells transfected with pY3 and pcDNA.HMI. Samples of cytoplasmic RNA (20 µg; A and B) and polyadenylated RNA (5 µg; C and D) were electrophoresed on 1% formaldehyde-agarose gels and blotted to nylon membranes. Probes used were MI/TIMP-2 oligonucleotide probe 198.29 (16) which does not cross-hybridize with TIMP (A and C) and H-RAS protooncogene from American Tissue Culture Collection (B and D). Left, positions of standard RNA markers (kilobases). E, Immunoblot analysis of concentrated serum free conditioned media. Samples (unreduced) corresponding to $8.5 \times 10^6$ cells were loaded on sodium dodecyl sulfate-polyacrylamide gelatinase as described in material and methods. Right, positions of prestained molecular weight markers (in thousands). F, Reversed zymogram analysis of concentrated conditioned media. Samples (unreduced and not heated) corresponding to $4.2 \times 10^6$ cells were electrophoresed on gelatin-containing sodium dodecyl sulfate-polyacrylamide and treated with gelatinase as described in material and methods. Right, positions of $M$, 92,000 and $M$, 72,000 gelatinases (Gel.) and MI/TIMP-2. For all sections (A to F): Lane I, 4R parental cells; Lane 2, clone 4.6; Lane 3, clone 4.17; Lane 4, clone 8.27; Lane 5, clone 8.39; Lane 6, clone 8.60; Lane 7, clone 8.68; Lane 8, clone 8.71. Clones 4.6 and 4.17 were transfected with the pY3 plasmid alone; clones 8.27, 8.39, 8.60, 8.68, and 8.71 were cotransfected with pcDNA.HMI and pY3 plasmids.

<table>
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<tr>
<th>Clone</th>
<th>Secreted inhibitory activity* (milliunits/10⁶ cells)</th>
<th>Secreted collagenase activity+</th>
<th>Type I (µg/10⁶ cells)</th>
<th>Type IV (µg/10⁶ cells)</th>
<th>Doubling time* (h)</th>
<th>Tumor*</th>
<th>Metastatic potential*</th>
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<tr>
<td>Parent cells</td>
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<td>None</td>
<td>189 ± 1.6</td>
<td>158 ± 10.6</td>
<td>19.2</td>
<td>8/8</td>
<td>234 ± 11 (10)</td>
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<td>112.2 ± 9.5</td>
<td>597 ± 12.1</td>
<td>18.0</td>
<td>8/8</td>
<td>175 ± 16 (11)</td>
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<td>70.5 ± 22</td>
<td>123 ± 14.6</td>
<td>15.6</td>
<td>8/8</td>
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<td>8.27</td>
<td>1103</td>
<td>7.42 ± 52</td>
<td>19.5 ± 0</td>
<td>16.8</td>
<td>8/8</td>
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<td>518</td>
<td>2.16 ± 0.3</td>
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<td>3.54 ± 2.8</td>
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<td>6.12 ± 1.8</td>
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<td>19.2</td>
<td>8/8</td>
<td>351 ± 37 (10)</td>
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* Free inhibitory activity in serum free conditioned medium was calculated in the presence of rabbit fibroblast interstitial collagenase.
+ Values represent amount of collagen degraded over 24 h at 37°C. Data points represent mean ± SD of triplicate samples and were corrected for cell numbers.
Doubling time was calculated from growth curves obtained from cultures of cells plated at 10⁶ cells in 35-mm tissue culture dishes. Values represent the doubling time measured during the exponential phase of growth.
Tumor cells were injected at $5 \times 10^6$ (4 injections) and $1.2 \times 10^6$ (4 injections) s.c. in athymic (nude) mice and animals were observed for the formation of tumors. Data represent number of tumors formed per number of injections. In all animals a tumor was detected within 10 days.
Metastatic potential was calculated as the number of nodules per number of injections. Numbers in parentheses, total number of animals given injections during 2 separate experiments.

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gelatinases other than clones (Fig. 2F). The absence of production of endogenous MI/TIMP-2 in this clone (as detected by reverse zymogram; Fig. 2F, Lane 2) may partially explain this apparent discrepancy. It is also possible that this clone produces other proteases (such as plasminogen activators) that may be involved in the proteolytic degradation of type IV collagen (36).

Since it had previously been shown that down-regulation of TIMP in Swiss 3T3 cells can affect tumorigenicity (37), we also examined growth rate in vitro and tumorigenicity in vivo in all clones as well as parental 4R cells. Expression of MI/TIMP-2 had no effect on these parameters. We then used an experimental metastatic model to determine whether inhibition of metalloproteinase activity in transfected clones would affect their ability to form lung colonies after i.v. injection into nude mice. The number of lung colonies formed by these clones was in general not significantly altered by expression of MI/TIMP-2 with one exception. Clone 8.60 showed a 66% decrease in the number of metastatic nodules (Fig. 3). The level of inhibitory activity produced by this clone was 6–12-fold higher than the levels detected in the three other producing clones.

Expression of MI/TIMP-2 in Metastatic Tumor Nodules. The absence of complete suppression of metastasis raised the possibility that there was heterogeneous expression of MI/TIMP-2 in transfected clones and that these lung colonies may have originated from subpopulations of cells that did not express functional MI/TIMP-2. Northern analysis of RNA isolated from metastatic lung nodules derived from clone 8.60 cells and subcultured in vitro demonstrated that this was not the case, since the levels of MI/TIMP-2 and c-Ha-ras-1 transcripts in five cultured metastases were similar to the levels observed in clone 8.60 (Fig. 4, A and B). The same results were obtained when RNA was extracted directly from tumor nodules (Fig. 4C). Furthermore, cultured metastases secreted levels of immunologically reactive and functional MI/TIMP-2 similar to parental clone 8.60 (Fig. 4, D and E), eliminating the possibility that the transfected cDNA may have been mutated to a non-functional gene.

Effect of MI/TIMP-2 Expression on Tumor Growth and Local Invasion in Vivo. Since the experimental metastatic model used only allows for the investigation of a late step in metastasis (tumor cell extravasation), we also examined the effect of MI/TIMP-2 expression on an early step of tumor progression, i.e., local tumor growth and invasion. We injected transfected clones s.c. into the flank of nude mice and found a significant difference in the growth rate in vivo of tumors formed by clones that expressed recombinant MI/TIMP-2 in comparison with 4R parental cells and clones that did not express MI/TIMP-2 (Fig. 5). The latency period for the appearance of a small tumor nodule (0.1–0.2 cm³) was the same for all clones and 4R parental cells (8 days) but the average tumor size measured 14 days after injection was less than 1 cm³ in the 4 clones expressing MI/TIMP-2. In nonexpressing clones and parental cells, the average tumor size ranged between 1.66 and 5.12 cm³). Furthermore, these tumors showed a striking difference in their invasiveness in vivo. Histological analysis demonstrated that tumors derived from 4R cells and from clones not expressing MI/TIMP-2 had rapidly invaded the muscle of the abdominal wall, had penetrated the peritoneal cavity, and in one case had penetrated the kidney (Fig. 6). In contrast, tumors derived from clones expressing MI/TIMP-2 were entirely confined to the s.c. tissue layer by a capsule of dense connective tissue and had not invaded the muscle layer. The data therefore demonstrate that elevated MI/TIMP-2 expression in 4R cells has a major effect on the process of local growth and invasion.

Effect of Local Administration of Recombinant MI/TIMP-2 on Tumor Invasion. To further demonstrate that the formation of a fibrotic capsule around tumors expressing MI/TIMP-2 was due to high MI/TIMP-2 production and not to other genetic alterations that may have been associated with DNA transfection, recombinant MI/TIMP-2 was administered locally by daily s.c. injection in tumors formed by parental 4R cells. The recombinant inhibitor was initially administered at the site of injection of the tumor cells and later as a tumor developed (days 8 to 9), between the posterior aspect of the tumor mass and the underlying adjacent tissues. Control tumors on the other flank of the animal were given daily injections of 0.1 ml of sterile phosphate buffered saline (Fig. 7). Whereas these injections had no effect on the overall tumor growth (data not shown), histological analysis of the tumors after 14 days showed the formation of a fibrotic capsule between the tumor cells and the adjacent muscle layer in tumors treated with recombinant MI/TIMP-2. This fibrotic capsule was particularly prominent in the posterior part of the tumor near the site of inhibitor administration but was much less obvious in the anterior part of the tumor where invasion of the muscle layer was detected (Fig. 7, A and C). In control tumors the muscle layer was extensively invaded (Fig. 7B). These data therefore are consistent with our hypothesis that local production of MI/TIMP-2 in tumor can alter the protease/protease inhibitor balance in favor of the inhibitors. This change in balance results in the increased deposition of matrix proteins and the formation of a fibrotic capsule that limits tumor cell invasion.

DISCUSSION

We have demonstrated that it is possible to restore the metalloproteinase-metalloproteinase inhibitor balance in tumor cells and to markedly decrease the activity of their secreted metalloproteinases by transfection with the cDNA of an inhibitor of these enzymes. In addition, restoration of this balance has a significant impact on the behavior of these cells in vivo. In particular, we have observed that the local production of MI/TIMP-2 in 4R cells markedly limited their growth in vivo.
and completely suppressed their ability to invade surrounding tissues.

Our data show that despite induction of a significant change in the metalloproteinase-metalloproteinase inhibitor balance in 4R cells, the metastatic behavior of these cells examined with an experimental model was not completely suppressed. Considering the highly complex and multistep nature of metastasis (1), a complete suppression of this process in 4R cells overexpressing MI/TIMP-2 would be unexpected. Previous observations using similar experimental models have also demonstrated that administration of recombinant TIMP was unable to completely suppress metastasis in mice (26, 38). Since the inhibition of metalloproteinases by MI/TIMP-2 is of a stoichiometric nature (17, 28), it is anticipated that the degree of inhibition will depend on the amount of inhibitor produced. This is consistent with our observation showing that significant inhibition of metastasis could be achieved only in the presence of very high levels of secreted inhibitor. However, despite high levels of MI/TIMP-2 secretion, a significant number of tumor cells are able to successfully metastasize. This finding suggests the presence of an escape mechanism, perhaps involving compartmentalization of metalloproteinases and/or MI/TIMP-2 (39, 40). For example, preferential localization of collagenase at the cytoplasmic face of the plasma membrane of human pancreatic carcinoma cells has been reported (41) and a similar localization in 4R cells may have prevented the enzyme from interacting with the inhibitor. Alternatively, a proteolytic cascade other than metalloproteinases, such as the plasminogen activator-plasmin system, may have been responsible for this escape (36, 42). Our data suggest that in order to completely suppress metastasis, other steps and/or proteolytic pathways may have to be inhibited.

Khokha et al. (37) have previously shown that down-regulation of TIMP in nontumorigenic and nonmetastatic Swiss 3T3 cells makes these cells invasive, tumorigenic, and metastatic. Although their data initially suggested that the acquisition of one step (i.e., increased metalloproteinase activity) can allow cells to further advance through the last stage of tumor progression, their more recent clonal analysis of these cells indicates that down-regulation of TIMP is associated with further changes in gene expression that may have accounted for their tumorigenic potential (43). These data suggest that reduction in TIMP secretion enhances the oncogenic capacity of the cell not only by changing the metalloproteinase activity but also by altering the extracellular environment in multiple ways. It is therefore not surprising that in our studies inhibition of only one proteolytic pathway involved in local invasion and penetration through the basement membrane was insufficient to suppress distant metastasis in an experimental model.

The effect of MI/TIMP-2 expression on local tumor growth and invasiveness in vivo is a remarkable observation implying that this inhibitor may play an important role in an earlier stage of metastasis.
of tumor progression toward the metastatic phenotype. The data indicate that local production of this inhibitor causes a dense fibrotic reaction that prevents tumor cells from invading adjacent tissues and limits tumor growth in vivo. It is noteworthy that the accumulation of connective tissue proteins was specifically localized to the interface between the tumor cells and the normal tissue. This suggests that the predominant effect of MI/TIMP-2 was on increasing the deposition of ECM proteins by mesenchymal cells, most likely by impairing the proteolytic degradation of these proteins by metalloproteinases secreted by tumor cells and normal inflammatory cells. Since local invasion is the first requirement for distant metastasis, inhibition of this step may have a significant impact on the prevention of distant metastasis.

Fig. 6. Microscopic analysis of tumors derived from 4R cells and transfected clones injected s.c. into nude mice. Representative photomicrographs of thin sections of tumors stained with hematoxylin-eosin (original, × 100). A, 4R parental cells. Note the invasion of the muscle. B, clone 4.6. Note invasion of the kidney on the right side of the photomicrograph. C, clone 8.27. Note absence of invasion of the muscle and presence of a fibrotic capsule that separates the tumor (left) from the underlying muscle (right). D, clone 8.39. Note absence of invasion of s.c. tissue. E, clone 8.60. Note the presence of a similar fibrotic capsule between tumor cells (left) and muscle (right). F, clone 8.71. Note extensive invasion of the muscle as seen with parental 4R cells. Tumors from clone 4.17 (not shown) had invaded the muscle as seen with 4R parental cells, clone 4.6, and clone 8.71. Tumors derived from clone 8.68 (not shown) were similar to tumors from clone 8.60.
In this study, we have used cDNA transfection to express a metalloproteinase inhibitor in tumor cells and modulate the protease-protease inhibitor balance. Our data are consistent with a negative effect of MI/TIMP-2 expression on the invasive behavior of tumor cells. However, final proof of the direct involvement of MI/TIMP-2 in this process awaits the demonstration that the effect can be reversed by either antibody or antisense to MI/TIMP-2. Nonetheless, the presence of low levels of endogenous transcript for MI/TIMP-2 in these tumor cells suggests that our ability to modulate and enhance endogenous expression will result in a similar effect on proteolytic activity and may allow us to significantly alter tumor progression in vivo.

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