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Direct evidence linking expression of matrix metalloproteinase 9 (92-kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells

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ABSTRACT **Members of the matrix metalloproteinase (MMP) family have been implicated in the metastasis of tumor cells, but no direct evidence linking any given member of the MMP family to metastatic behavior has been presented. Rat embryo cells transformed by the Ha-*ras* and v-*myc* oncogenes or by Ha-*ras* alone are metastatic in nude mice and release the 92-kDa gelatinase/collagenase (MMP-9), whereas those transformed by Ha-*ras* plus the adenovirus *E1A* gene are not metastatic and do not release MMP-9. Here we demonstrate that MMP-9 expression can be induced in these tumorigenic but nonmetastatic rat cells by transfection with an MMP-9 expression vector. Transfection of a MMP-9 expression vector, but not control DNAs, conferred metastatic capacity on the nonmetastatic cells. The majority of colonies isolated after continued passage either *in vivo* or *in vitro* had lost the MMP-9 expression vector. However, occasional cells were isolated from metastases which retained MMP-9 expression after passage. These cells retained metastatic capacity. In contrast, cells isolated after losing MMP-9 expression also lost the ability to metastasize. These results provide direct evidence that MMP-9 has a role in tumor metastasis.**

To investigate the role of matrix metalloproteinase 9 (MMP-9) in metastasis, we have used rat embryo cells transformed by either Ha-*ras* plus v-*myc* or Ha-*ras* plus *E1A*. Each of these types of transformants was equally and highly tumorigenic in nude mice and expressed similar levels of Ha-*ras*. However, cells transformed by Ha-*ras* plus v-*myc* were highly metastatic in nude mice, whereas those transformed by Ha-*ras* plus *E1A* were not (1, 2). Furthermore, introduction of *E1A* into the metastatic cells transformed by Ha-*ras* plus v-*myc* resulted in nonmetastatic cells (3). The metastatic cells transformed by Ha-*ras* plus v-*myc* produced conditioned medium which contained readily detectable type IV collagenolytic activity after activation, whereas the rat embryo cells transformed by Ha-*ras* plus *E1A* did not (2). Substrate gel electrophoresis using gelatin revealed that the metastatic cells secreted gelatinolytic activity at 92 kDa (MMP-9), but the nonmetastatic cells did not. Gelatinase activity present at other molecular masses was similar between the metastatic and nonmetastatic cells (4). These experiments established a correlation between metastasis and release of MMP-9, suggesting that the importance of MMP-9 for metastasis by transformed rat embryo cells should be explored.

The human 92-kDa gelatinase/collagenase (MMP-9) was cloned by Wilhelm *et al.* (5). Comparison of the predicted amino acid sequence of the cDNA of the human MMP-9 with other members of the metalloproteinase family showed the closest homology to the 72-kDa gelatinase/collagenase (MMP-2). Huhtala *et al.* (6, 7) have determined the genomic structure of both MMP-2 and MMP-9 and found both to contain 13 exons. The divergent region of MMP-9 is encoded within exon 9. The promoters of these two genes differ markedly, with the MMP-9 promoter having several putative AP-1 binding sites not found in the MMP-2 promoter (6–8).

The MMPs are attractive candidates for enzymes required for tumor metastasis (9–11). The MMP family includes interstitial collagenase (MMP-1), three types of stromelysin (MMP-3, -10, and -11), neutrophil collagenase (MMP-8), Pump-1 (MMP-7), and the 72- and 92-kDa type IV gelatinases/collagenases (MMP-2 and MMP-9). All of these enzymes are secreted in a latent form and are activated by cleavage of an amino-terminal propeptide at a conserved sequence. Activation can be mediated by proteases, including plasmin, trypsin, kallikreins, neutrophil elastase, cathepsins, and cellular activities or by organomercurials (12, 13). The substrate specificities of the various MMPs overlap. Stromelysin can degrade fibronectin, casein, proteoglycans, and laminin, as well as collagens of types III and V and the nonhelical domains of collagen types IV and IX. This enzyme also has weak activity on elastin and gelatin but does not cleave intact type I collagen (14). Both the 72-kDa and the 92-kDa gelatinase have similar substrate specificities, and both can degrade elastin, casein, and types IV, VII, and X collagens in the helical domains and gelatin, but not intact type I collagen (15–17).

The most convincing evidence for the involvement of the MMP family in metastasis comes from studies using inhibitors. Both of the two naturally occurring inhibitors of this family, TIMP-1 and TIMP-2 (tissue inhibitors of metalloproteinases), inhibit enzymatic activity of all members of the MMP family which have been tested. Both TIMPs also inhibit metastasis and invasion. When added during *in vitro* invasion assays, TIMP-1 results in inhibition of invasion by B16F10 melanoma cells, and TIMP-1 infused into mice immediately after injection of these cells or transformed rat embryo cells diminished experimental metastasis (18, 19). Introduction of an expression vector for TIMP-1 inhibited metastasis by a human gastric cell line (20), and introduction of expression vectors for TIMP-2 inhibited metastasis by transformed rat embryo cells (21). Other inhibitors of MMPs, including estramustine and SC 44463, which inhibit activity of most members of the gelatinase family, also inhibit experimental metastasis (22, 23). Expression of antisense RNA to TIMP-1 has been found to augment metastasis by BALB 3T3 cells (24). Taken together, these experiments indicate the involvement of the MMP family in metastasis but do not define which particular member(s) of the MMP family plays a critical role, since all are inhibited with these methods.

MATERIALS AND METHODS

Cells and Cell Culture. Cells were maintained at 37°C at 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with

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Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases.

10% fetal bovine serum (Flow Laboratories) and penicillin/streptomycin (GIBCO). The RA3 cell line was derived from primary rat embryo fibroblasts by cotransfection with the Ha-*ras* and adenovirus *E1A* oncogenes, as described (25). Subclone RA3.1 was derived by limiting-dilution cloning of RA3. Cells from metastases were placed into culture by excising individual metastases from sacrificed animals, mincing the metastases, and then plating them in medium. Such cultures used in the experiments described were called RA3D11 met2, RA3D11 met5, RA3.1S7 met1, RA3.1S7 met5, and RA3.1 D11 met1.

The MMP-9 gene was excised from the pBluescript (Stratagene) vector with either *Spe* I and *Not* I, for construction of sense-orientation vectors, or *Not* I and *Xba* I, for antisense constructs. The resulting fragments were ligated into pRc/RSV (Invitrogen) cut with the same combination of enzymes. The orientation of the MMP-9 gene inserts was confirmed by restriction mapping.

Transfection of expression vectors or control DNA into RA3 was accomplished by calcium phosphate-mediated DNA transfer of 30 μg of expression vector together with 3 μg of pSV2neo as a selectable marker. Selection for DNA integration was begun 48 hr after transfection by including Geneticin (G418 sulfate; GIBCO) at 300 μg/ml in the medium. Transfectants were maintained under selection with Geneticin at 200 μg/ml throughout these experiments. Integration of MMP-9 expression vector was verified and the clonal nature of isolated transfectants was established by Southern blot analysis of *Eco*RI-digested genomic DNA isolated with a model 340A nucleic acid extractor (Applied Biosystems). *Eco*RI cleaves the pRc/RSV sense construct twice at sites flanking the MMP-9 gene. Southern blots were hybridized to probes generated by random-primer [α-^{32}P]dCTP labeling of the human MMP-9 cDNA clone.

Substrate Gels. Substrate PAGE was accomplished by a modification (4) of the protocol of Heussen and Dowdle (26). Serum-free conditioned medium samples were obtained by 24-hr culture of cells at confluence in serum-free DMEM (0.1 ml/cm^2 of culture area). Samples were stored at −80°C and clarified by centrifugation at 12,000 × *g* immediately before electrophoresis. Prestained molecular weight standards (BRL) were used to monitor protein migration and were removed prior to development of substrate gels.

Mice. Four-week-old female NCR-*nu/nu* mice were obtained from the National Cancer Institute and housed aseptically in the laboratory animal facilities of the Wistar Institute. Cells were grown to subconfluence, subjected to brief trypsin treatment, washed twice, and suspended in serum-free DMEM. Mice were given injections in the tail vein with 5 × 10^4 cells suspended in 0.05–0.1 ml. Cells used for injection were negative for mycoplasma contamination. Animals were sacrificed when they exhibited labored breathing or after 8 weeks and were examined for metastases. Lungs were inflated and fixed in buffered formalin prior to counting of lung and mediastinal metastases, which was accomplished with the aid of a dissecting microscope.

RNA Blot Analysis. Samples (20 μg) of total cellular RNA (27) were electrophoresed in a formaldehyde/agarose gel and transferred to a Duralon-UV membrane (Stratagene). Northern blot hybridization was performed with a probe derived from the 1.3-kb *Eco*RI insert fragment released from cDNA clone p8P2a (unpublished work) and encoding a fragment of the rat homologue of MMP-9 and spanning nucleotides 125–1408 of the published sequence of the homologous human gene. This sequence is 84% identical to the human MMP-9 DNA sequence with 94% homology at the protein level, including conservative substitutions. The blot was subsequently hybridized to a 2.5-kb rat MMP-2 probe derived from pCol IV 3.12 (unpublished data). The sequence of this cDNA is 86% identical at the nucleotide level, with 96% amino acid homology to the human 72-kDa gelatinase/collagenase (MMP-2) deduced at the protein level.

RESULTS

Differential Expression of MMP-9. Substrate SDS/PAGE with gelatin incorporated into the gels was used to monitor the presence of gelatinase in the conditioned medium from the cells. After electrophoresis, gels were processed to allow the gelatinases to renature. The gels were then stained with Coomassie blue and gelatinase activity was revealed by zones of clearing. The conditions under which the gels were run resulted in dissociation of the gelatinases from TIMP and also in autoactivation of latent forms. The metastatic cell lines 2.10.10 and 4.1, derived from rat embryo fibroblasts transformed by Ha-*ras* plus v-*myc*, released the 92-kDa MMP-9 into the medium, whereas the nonmetastatic but tumorigenic cell lines RA3 and RA1, rat embryo fibroblast lines transformed by Ha-*ras* plus *E1A*, did not release detectable 92-kDa gelatinase under the same conditions (Fig. 1*A*). The amounts of lower molecular weight gelatinases in conditioned medium were equivalent from both types of transformants. RNA blotting indicated that 2.10.10 has markedly higher levels of MMP-9 mRNA than RA3, which reflects the difference between amounts of gelatinase released by the two cell lines (Fig. 1*B*, MMP-9). The two lines contained equivalent levels of MMP-2 mRNA (Fig. 1*B*, MMP-2). Thus RA3, a tumorigenic but nonmetastatic cell line which has no detectable MMP-9 activity, was selected as a recipient for transfection experiments.

Transfection of an Expression Vector for MMP-9. To test the hypothesis that increased expression of MMP-9 was important for the metastatic potential of transformed rat embryo cells, MMP-9 levels were augmented in RA3 cells

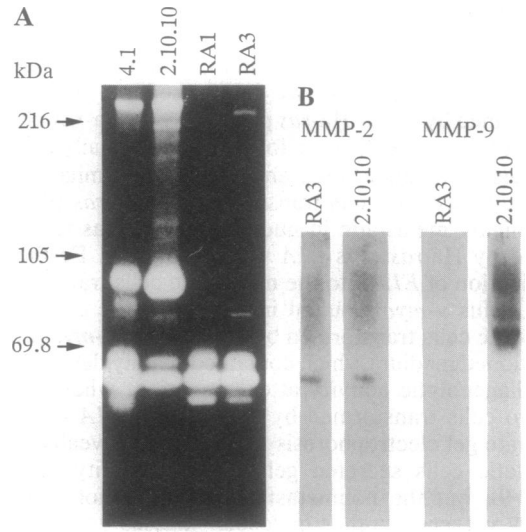

FIG. 1. Expression of gelatinase activity by cell lines transformed by Ha-*ras* plus v-*myc* or Ha-*ras* plus *E1A*. The RA3 and RA1 cell lines were derived from primary rat embryo fibroblasts by cotransfection with the Ha-*ras* and adenovirus *E1A* genes, and the 2.10.10 and the 4.1 cell lines were derived by cotransfection with the Ha-*ras* and v-*myc* genes (25). (*A*) Gelatinase activity released into conditioned medium. Zones of clearing after electrophoresis in gels containing substrate (gelatin) indicate gelatinase activity (4). Prestained molecular weight standards (BRL, MD) were used to monitor protein migration. (*B*) RNA blot analysis of mRNA for MMP-2 and MMP-9. Membranes were probed first with a rat MMP-9 probe and subsequently with a probe for the rat MMP-2 gene (see *Materials and Methods*). The MMP-9 blot was overexposed to indicate that even after long exposures only trace amounts of MMP-9 mRNA could be detected in RA3 cells but that under similar conditions MMP-9 mRNA was readily detected in 2.10.10 cells.

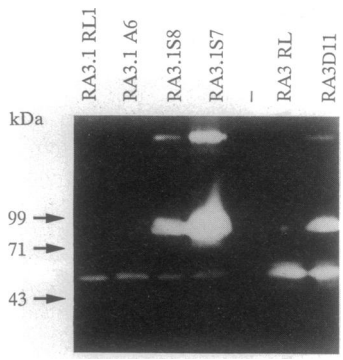

FIG. 2. Induction of MMP-9 release in RA3 cells by transfection with an MMP-9 expression vector. Conditioned media from confluent cultures of RA3.1 transfected with either rat liver genomic DNA (RL1), an MMP-9 antisense expression vector (A6), or an MMP-9 sense expression vector (S8 and S7) were tested for gelatinase activity as in Fig. 1*A*. RA3.1 is a subclone of RA3. Also shown is RA3 gelatinase release 2 weeks after transfection with rat liver DNA (RA3 RL) or the MMP-9 sense expression vector for MMP-9 (RA3 D11). Zones of clearing indicate gelatinase activity. Migration of molecular size markers is indicated.

using a eukaryotic expression vector for MMP-9 that is not influenced by *E1A*. The pRc/RSV vector uses a Rous sarcoma virus promoter to drive expression and contains a growth hormone polyadenylylation site and the selectable marker for neomycin resistance. The cDNA for human MMP-9, as cloned by Wilhelm *et al.* (5) and generously provided by them, was inserted into the pRc/RSV vector in both the sense and the antisense direction. The expression vector for MMP-9 in the sense direction (D11) was introduced into RA3 by calcium phosphate-mediated transfer. Selection with Geneticin was applied for 2 weeks to reduce the number of nontransfected cells. The expression vector resulted in the secretion of MMP-9 by the cells into which it was introduced, whereas RA3 cells transfected with either rat liver DNA admixed with pSV2neo or the vector containing MMP-9 in the antisense direction did not release detectable MMP-9 under the same conditions (Fig. 2).

Duplicate populations of the transfected cells were tested for gelatinase release and then were injected into nude mice in an experimental metastasis assay to determine whether the augmentation of MMP-9 expression would alter their metastatic potential. The populations of RA3.1 cells into which the MMP-9 expression vector had been introduced and which released MMP-9 were found to generate significantly more lung colonies than the cells which had undergone control transfections (Table 1).

Table 1. Metastatic potential of RA3 after transfection with an MMP-9 expression vector

Exp.	Transfected DNA	No. of metastases
1	D11	18 (9, 4, 3, 2)
	RL	0 (0, 0, 0, 0)
2	D11	31 (16, 6, 5, 4)
	RL	5* (2, 2, 1, 0)

Cells surviving 2 weeks after transfection and selection were harvested and 5×10^4 cells in single-cell suspension were injected into the tail vein of 5- to 7-week-old nude mice. Metastases were scored at necropsy 8 weeks after injection. For each group $n = 4$. A χ^2 test for positive versus negative mice reached statistical significance ($P = 0.008$), as did Fisher's exact test ($P = 0.0256$). The distribution of metastases in the mice was significantly different by a Mann–Whitney test ($P = 0.0015$).

*In Exp. 2, all of the metastases from the D11 transfectants had diameters of >5 mm, whereas the metastases from the control transfectants were all <2 mm in diameter.

To attempt to generate colonies of RA3 cells which had stably incorporated the expression vector for MMP-9 and continued to release MMP-9, selection with Geneticin was maintained after the transfection of D11 until individual colonies could be isolated and expanded. Ten of these colonies were tested for MMP-9 release. Conditioned media from only 2 of the 10 showed any detectable MMP-9, and in both of those the level of gelatinase activity was low (Fig. 3*A*). These same 2 colonies were the only 2 which retained the introduced MMP-9 cDNA as shown by Southern blot analysis (Fig. 3*B*). Thus, permanent expression of MMP-9 and retention of the expression vector appear to exert an unfavorable effect on growth even in the presence of selection for Geneticin resistance, and this suggested that growth of the cells *in vivo*, where selection could not be applied, would be even less conducive to continued expression of MMP-9.

Retention of MMP-9 Expression Predicts Metastatic Potential. The difficulty of maintaining the expression vector for MMP-9 in permanent lines raised the question of whether the

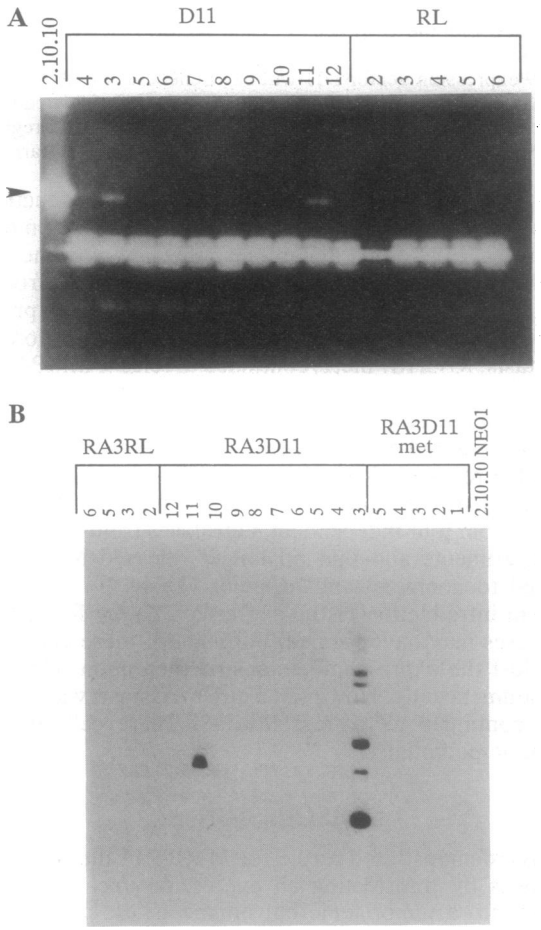

FIG. 3. Gelatinases expressed by MMP-9 transfected clones after selection in tissue culture. (*A*) After transfection with either the expression vector (D11) or control rat liver DNA (RL) and Geneticin selection, individual clones were isolated and expanded. Cultures of these clones were tested for release of gelatinase activity. MMP-9 activity seen as clearing at 92 kDa is indicated (arrowhead). (*B*) The clones were also analyzed for MMP-9 expression vector integration by Southern blotting. DNA was isolated from control clones (RA3 RL clones 2, 3, 5, and 6), or from MMP-9 transfectant clones derived in tissue culture (RA3 D11 clones 3–12), or from cultures of individual metastases which had arisen after intravenous injection of the RA3 D11 bulk transfectant culture (RA3 D11 met clones 1–5). DNA was digested with *Eco*RI and the fragments were separated in a 0.7% agarose gel. After transfer, the membrane was hybridized with a probe from the human MMP-9 cDNA (5).

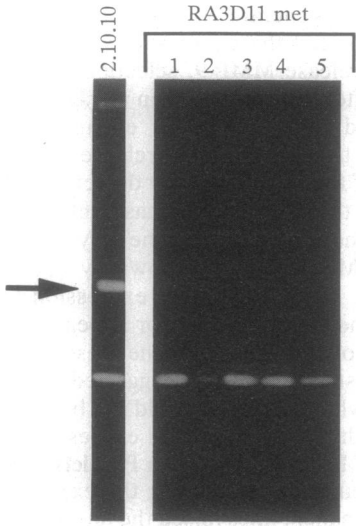

FIG. 4. Gelatinases released by metastases derived from RA3 transfected with the MMP-9 expression vector. Five individual lung colonies were isolated after injection of RA3 transfected with the MMP-9 expression vector D11 without Geneticin. Conditioned medium samples from confluent cultures of each clone isolated from a metastasis were analyzed by substrate SDS/PAGE. The region of MMP-9 activity (seen in the 2.10.10 control) is indicated (arrow).

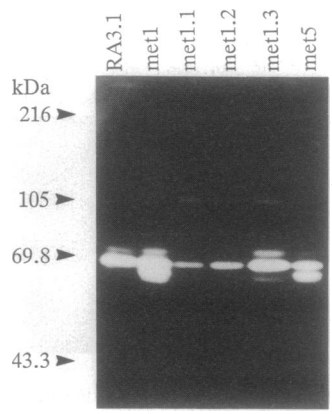

FIG. 5. Clones of RA3 transfected with the MMP-9 expression vector and screened for continued MMP-9 expression after metastasis. RA3.1S7 met1 was derived from a metastasis from RA3.1 transfected with D11. These cells were reinjected and three of the resultant metastases were isolated: met1.1, met1.2, and met1.3. RA3.1S7 met5 was also isolated from a metastasis from RA3.1 transfected with D11. The metastases were isolated in medium without Geneticin. Serum-free conditioned media from these cultures were analyzed by substrate SDS/PAGE. Molecular size markers are indicated.

metastases which were isolated after transient introduction of the vector still retained the vector after the formation of the metastases. Five of the metastases produced after the injection of RA3 cells transfected with D11 were isolated from the lungs and reestablished in culture. None of these expressed MMP-9 (Fig. 4). However, from a second transfection, one metastasis, RA3.1S7 met1, continued to release MMP-9 (Fig. 5). The cells cultured from these metastases were retested in the experimental metastasis assay. Several metastases which were isolated and did not release the MMP-9 after isolation were also retested. RA3.1S7 met1 cells were highly metastatic upon reinjection in nude mice, but cells that were isolated from four independent metastases from three different experiments and that no longer released MMP-9 had reverted to nonmetastatic behavior (Table 2). Thus, after transient introduction of the expression vector for MMP-9, metastases may have formed, but most of these cells eventually lost the expression vector and then upon reinjection were nonmetastatic. However, a rare metastasis was isolated which continued to express MMP-9. These cells retained metastatic potential.

DISCUSSION

We have demonstrated a role for MMP-9 in the process of metastasis by transfecting an expression vector encoding MMP-9 into a tumorigenic but nonmetastatic cell line and demonstrating a resultant increase in metastatic capacity. The results presented here directly demonstrate the contribution of a specific member of the MMP family to metastasis.

Expression of MMP-9 has been repeatedly shown in malignant cells. The 92-kDa gelatinase has been detected in metastatic transformed NIH 3T3 cells, murine colonic carcinoma cells, and transformed rat embryo cells, but not in their nonmetastatic counterparts, in sarcoma cell lines, or in human brain tumors (4, 28–31). It has also been found in invasive variants of HT1080 and C3H/10T½ cells (32). MMP-9 activity has been implicated in other physiological processes as well. MMP-9 activity was identified in the basal layer of the regenerating epithelium after wounding in a corneal repair model (33). MMP-9 activity also increases during trophoblast invasion. Librach *et al.* (34) showed that an antibody to the 92-kDa gelatinase blocked matrix degradation and invasion by early-trimester trophoblasts in tissue culture, suggesting its importance for invasion in the placenta (34, 35). Monocytes in circulation also express MMP-9 (36). In these instances, increased MMP-9 expression is a transient phenomenon correlating with either remodeling of tissue or invasion.

The expression of MMP-9 in RA3 transfectants was rapidly lost in most cases, either after continuous culture or during metastasis in nude mice. This did not appear to result from down-regulation of the transfected MMP-9 gene but was associated with loss of the expression vector. We have also noted a loss of MMP-9 expression after long-term culture of transformed cells that constitutively express this enzyme, presumably as a result of down-regulation of expression. Thus it appears that continuous expression of MMP-9, either as a result of endogenous gene expression or as a result of expression from a transfected gene, may be selected against. This may account for the lack of constitutive expression of MMP-9 in certain clones of metastatic cells seen by ourselves and other investigators (4, 37).

Stromelysin, or transin (MMP-3), is also down-regulated by *E1A* and its expression is also greatly decreased in the rat embryo cell transformants which contain *E1A* (37). It remains to be determined what the role of transin is in these cells, but

Table 2. Cells from metastases that retain MMP-9 expression retain metastatic potential

Cell line	MMP-9 expression	No. of metastases per mouse	(mean ± SD)
RA3.1S7 met1	+	69, 48, 45, 43, 33, 26, 24, 12	(38 ± 16)
RA3.1S7 met5	−	2, 1, 0, 0, 0, 0, 0, 0	(0.4 ± 0.8)
RA3D11 met2	−	1, 0, 0, 0	(0.25 ± 0.5)
RA3D11 met5	−	1, 0, 0, 0	(0.25 ± 0.5)
RA3.1D11 met1	−	2, 1, 0, 0, 0	(0.25 ± 0.9)

Cells were isolated from metastases of the RA3.1S7 transfectant. They were called RA3.1S7 met1 and met5. Additional cells isolated from metastases from two other experiments, which did not release detectable MMP-9 activity, were also tested. They were RA3D11 met2 and met5 and RAD3.1D11 met1. Cells (5×10^4) were injected intravenously to test for metastatic potential as in Table 1. Results of two experiments are pooled in this table.

in pilot experiments, introduction of a transin expression vector did not alter metastatic potential (E.J.B. and R.J.M., unpublished data).

The findings presented here demonstrate that transient expression of the MMP-9 enzyme at the time of injection in an experimental metastasis assay promotes metastases. This suggests that MMP-9 is a critical contributor to the ability of these cells to undergo metastatic spread and that transient up-regulation of endogenous MMP-9 might act *in vivo* to influence invasion. Such up-regulation could occur either in tumor cells or in normal tissue via signaling through cytokines or lymphokines or as a result of tissue injury. The finding that increased MMP-9 is frequently lost in metastatic colonies implies that the contribution of this enzyme to metastatic spread is essential during early phases of this process. This is consistent with a role for this enzyme in extravasation and penetration into tissues at the site of metastasis, but not in tumor growth. Previous work showed that MMP-9 expression was not only diminished after long-term culture but could also be regained in certain metastatic cells when subjected to stimuli resulting from *in vivo* growth (4, 38). We have shown that induction of expression of MMP-9 in rat embryo cells transformed by Ha-*ras* plus *E1A* results in acquisition of metastatic potential. It remains to be determined whether MMP-9 is a critical determinant of metastasis in other tumor cells.

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