A Novel Host/Tumor Cell Interaction Activates Matrix Metalloproteinase 1 and Mediates Invasion through Type I Collagen*

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Ulrike Benbow‡, Matthias P. Schoenermark§§, Teresa I. Mitchell‡, Joni L. Rutter‡, Ken-ichi Shimokawa‡, Hideaki Nagase‡, and Constance E. Brinckerhoff***‡‡‡

From the Departments of ‡Medicine, §Pharmacology/Toxicology, and **Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755 and the Department of Biochemistry§, University of Kansas Medical Center, Kansas City, Kansas 66160

Along with degradation of type IV collagen in basement membrane, destruction of the stromal collagens, types I and III, is an essential step in the invasive/metastatic behavior of tumor cells, and it is mediated, at least in part, by interstitial collagenase 1 (matrix metalloproteinase 1 (MMP-1)). Because A2058 melanoma cells produce substantial quantities of MMP-1, we used these cells as models for studying invasion of type I collagen. With a sensitive and quantitative in vitro invasion assay, we monitored the ability of these cells to invade a matrix of type I collagen and the ability of a serine proteinase inhibitor and all-trans-retinoic acid to block invasion. Although these cells produce copious amounts of MMP-1, they do not invade collagen unless they are cocultured with fibroblasts or with conditioned medium derived from fibroblasts. Our studies indicate that a proteolytic cascade that depends on stromal/tumor cell interactions facilitates the ability of A2058 melanoma cells to invade a matrix of type I collagen. This cascade activates latent MMP-1 and involves both serine proteinases and MMPs, particularly stromelysin 1 (MMP-3). All-trans-retinoic acid (10⁻⁶ M) suppresses the invasion of tumor cells by several mechanisms that include suppression of MMP synthesis and an increase in levels of tissue inhibitor of metalloproteinases 1 and 2. We conclude that invasion of stromal collagen by A2058 melanoma cells is mediated by a novel host/tumor cell interaction in which a proteolytic cascade culminates in the activation of pro-MMP-1 and tumor cell invasion.

The ability of many tumor cells to invade their local environment and to metastasize from their primary site to vital organs such as liver, lung, and brain, is potentially life-threatening. Therefore, the critical event in tumor cell invasion is degradation of the extracellular matrix, because this process allows dissemination from the localized site (1–8). This matrix is composed of numerous structural macromolecules, including collagen types I, III, and IV (1–8). Most degradation is mediated by the matrix metalloproteinases (MMPs), a multigene family containing at least 16 members in humans (1–9). With the exception of membrane-type MMPs and stromelysin 3 (MMP-11), these enzymes are synthesized and secreted in a latent form, and activation is usually accomplished by proteolytic cleavage of a propeptide domain at the N terminus of the molecule. Both serine proteinases and MMP family members have been implicated in this activation (2, 3, 8, 9).

Experimental and clinical studies suggest that elevated expression of MMPs correlates with tumor invasiveness and with an unfavorable prognosis (1–3, 11–14). Considerable attention has focused on the role of the 72-kDa gelatinase (MMP-2) and the 92-kDa gelatinase (MMP-9), because of their ability to degrade type IV collagen in basement membrane (1–8). Production of these enzymes by numerous tumor cells has been documented (1–3, 8, 13, 14) and correlated with invasiveness (13–15). However, they may not be sufficient by themselves. In addition to basement membranes, tumor cells must traverse the interstitial stroma, which is made up of collagens I and III (1–3, 6, 7, 16, 17). Thus, degradation of interstitial collagen is an essential component of the three-step process of invasion/metastasis: adhesion, degradation, and migration (6, 7, 16, 17).

Of significance is the fact that this degradation is accomplished most effectively by the interstitial collagenases, MMP-1, MMP-8, and MMP-13, and to some extent by MMP-2 (20) and the membrane-type MMP, MT1-MMP (MMP-14), (8, 20, 21), but relatively little attention has been given to the role of collagenase in tumor invasion. MMP-1 (collagenase 1) is ubiquitously expressed and is often a major gene product of stimulated fibroblasts and of some tumor cells (5, 16 22–25), including A2058 melanoma cells (22).

Previous studies have described several types of host/tumor cell interactions that either mediate or augment tumor invasion by MMPs. These include secretion of MMPs by stromal cells in response to stimulation by tumor cells or, conversely, induction of MMP production by the tumor cells in response to host stimuli. Some of these mechanisms require direct contact between the stromal and tumor cells (27, 28), whereas others do not (29–31). The increase can occur at a pretranslational level (27), and in most instances, conditioned medium from macrophages or fibroblasts is an effective inducer (32). In this paper, we begin to investigate the mechanism(s) by which MMP-1 mediates the invasive behavior of tumor cells through a matrix of type I collagen. As models we used A2058 melanoma cells, which produce substantial amounts of MMP-1.
stutively and which are an aggressive and invasive line established from a brain metastasis in a 43-year-old man (22). Our studies indicate that a cascade that depends on proteinases produced by both stromal cells and by tumor cells facilitates tumor invasion through the collagen. Furthermore, invasion was inhibited by all-trans-retinoic acid by mechanisms that include down-regulation of MMP-1 synthesis and up-regulation of TIMP-1 and TIMP-2 gene expression.

MATERIALS AND METHODS

Cell Culture—Stock cultures of A2058 melanoma cells were grown in 150-mm-diameter culture dishes in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Inc.) containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (37 °C in 5% CO2). After 3–4 days, when the cells were confluent, they were passaged 1:7 with 0.25% trypsin. For most experiments, cells were grown to the desired level of confluence, washed with Hanks’ balanced salt solution (Life Technologies, Inc.) to remove traces of serum and placed in serum-free DMEM supplemented with 0.2% lactalbumin hydrolysate (DMEM/LH) (33). Human foreskin fibroblasts were obtained from the Birthing Pavilion at Mary Hitchcock Memorial Hospital (Lebanon, NH), and fibroblasts were isolated as described previously (33). They were grown in 150-mm-diameter culture dishes in DMEM, 10% fetal bovine serum, penicillin/streptomycin, and used between passages 4 and 12 (33).

Western Analysis—At confluence, cells were washed with Hanks’ balanced salt solution and placed in DMEM/LH. Culture medium (1 ml) was precipitated with 0.5 ml of 10% trichloroacetic acid for 30 min on ice. Proteins were pelleted and resuspended in SDS-polyacrylamide gel electrophoresis sample buffer, electrophoresed on 7.5% SDS-polyacrylamide gel electrophoresis minigels, transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a Trans-Blot Cell (BioRad). MMP-1 protein was detected as described previously (33). Monoclonal antibodies for MMP-3, TIMP-1, and TIMP-2 were obtained from Calbiochem (San Diego, CA), and Western analysis was performed using chemiluminescence reagents from Amersham Pharmacia Biotech.

Northern Blot Analysis—A2058 melanoma cells at confluence were placed in serum-free medium for 24 h, and total RNA was harvested with a Trizol kit. RNA (5–10 μg/lane) was subjected to electrophoresis on 1% formaldehyde agarose gels and transferred to a GeneScreen Plus membrane. Membranes were hybridized with denatured [α-32P]dCTP-cDNA labeled by random oligo priming to probes for MMP-1, MT1-MMP, MMP-3, MMP-2, MMP-9, and MMP-13. Hybridization with glyceraldehyde-phosphate dehydrogenase was used as a loading control.

Collagen Type I Zymography—Collagen zymography was performed as described previously (34). Briefly, culture medium (15 μl) was loaded on an 8% gel (7.5% containing 1 mg/ml type I collagen) that lacks the 100 μl of 10% SDS. Following electrophoresis, gels were washed twice with 5% Triton X-100 (30 min each). After washing, the gels were incubated for 24 h at 37 °C in the presence of 50 mM Tris-HCl, 5 mM CaCl2, 5 mM ZnCl2, pH 7.5, stained with Coomassie Brilliant Blue R-250 for 30 min and then destained.

Evaluation of Tumor Cell Invasiveness by Invasion Chamber Assay and Scanning Electron Microscopy—The assay utilizes a modified Boyden chamber (35, 36) with the two compartments separated by a nitrocellulose filter (Schleicher & Schuell AE100, 25-mm diameter, 12-μm pore size). Briefly, filters were autoclaved and coated with collagen type I (1 mg/ml; Sigma) diluted in sterile DMEM in the presence of 1% antibiotics, and successively applied to the membrane (3 × 150 μl, 1 × 550 μl, allowed to gel at 37 °C for 30 min, and air dried for 1 h at room temperature). The efficiency of coating was monitored by scanning electron microscopy at the electron microscopy faculty at Dartmouth Medical School. Single cell suspensions, consisting of 106 tumor cells alone, 106 fibroblasts alone, or the two cell types co-cultured in varying amounts up to equal numbers of 5 × 106, were washed three times in Hanks’ balanced salt solution, resuspended in serum-free medium, and counted. Cell viability was monitored by trypan blue exclusion. The lower chamber was filled with serum-free medium, and 106 cells in 1 ml were added to the upper chamber. In some experiments, two-compartment culture model was used, and fibroblasts were cultured on plastic in the lower compartment, while the tumor cells were cultured on type I collagen in the upper compartment. The chamber was cultured at 37 °C and 5% CO2 for 24 h, and the degree of cellular invasion was assessed by confocal laser scanning microscopy.

For scanning electron microscopy images, filters were washed with phosphate-buffered saline, fixed in 2.5% glutaraldehyde (30 min), washed with 0.1 M sodium cacodylate (2 × 5 min), and postfixed in 1% osmiumtetroxide (90 min). Then filters were dehydrated in 2-propanol and subjected to critical point drying using hexamethyldisilazane: HCl (9:1) (Sigma; 20 min) followed by hexamethyldisilazane alone. Filters were mounted and sputter coated (approximately 30 nm; Polaron E5400, 0.6kV, 20 mA). Scanning electron microscopy was carried out using a Zeiss SEM 962.

Quantitation of Invasion with Confocal Laser Scanning Microscopy—Confocal laser scanning microscopy (CLSM) was performed on invasion chambers (35, 36) at the Dartmouth Hitchcock Medical Center Imaging Unit (Lebanon, NH). Briefly, invasion assays were terminated after 48 h. The membranes were washed in phosphate-buffered saline and dehydrated with 2-propanol (10 min) followed by a 30-min RNase-treatment (1 mg/ml) at room temperature. After washing with phosphate-buffered saline, the filters were stained for 30 min with propidium iodide (0.01 μg/ml) at room temperature. Filters were washed with water and dehydrated with 2-propanol. The specimens were then treated with 100% xylene (four times for 4 min each), which leads to complete translucence of the filters. Samples were mounted on slides and sealed with Canada balsam (Sigma). A Bio-Rad MRC-1024 CLSM with a Zeiss Axioskop microscope and a Zeiss Plan Neofluor 40 × 1.3NA objective was used to assay propidium iodide fluorescence in the membranes (Carl Zeiss, Inc., Thornwood, NY). Excitation light was 488 + 568 nm with fluorescence measured through a 60×, 1.3NA objective using a 0.7 confocal iris. Images were 240 × 240 μm and were captured at 2-μm steps starting at the top of the collagen matrix. For analyses, the stack of images was processed with Molecular Dynamics Image Space software (Molecular Dynamics, Sunnyvale, CA) in a modification of the method described by Schoenermark et al. (35). A threshold was set to eliminate background fluorescence, and the total propidium iodide fluorescence (related to the total double stranded nucleic acid content) of each section was determined as the sum of the intensity of cell fluorescent pixels in that section. Results were plotted as percentages of total fluorescence in each section, which are equivalent to the numbers of cells in each section. Invasion assays were repeated at least four times. For each assay, a minimum of four fields/filter was taken. Minimal variability values were observed between the treatments in each set of experiments. Representatives of individual assays are shown in Figs. 4–6.

Preparation and Fractionation of Conditioned Medium from Human Dermal Fibroblasts—Fibroblast conditioned medium was fractionated using a range of Ultra-free-4 protein concentrators (Fisher). After concentration the individual fractions were adjusted to the same protein concentration and added to the upper invasion chamber.

Purification of Human Recombinant MMP-3 and Immunodepletion of HFF Conditioned Medium—Recombinant pro-MMP-3 was purified from the culture medium of Chinese hamster ovary K1 cells stably transfected with the expression vector pEE14, harboring the full-length human MMP-3 cDNA by immunoaffinity chromatography as described previously (37). Recombinant pro-MMP-3(ΔΔC) that lacks the C-terminal hemopexin-like domain (residue Met1 to Thr258) was expressed in Escherichia coli transformed with the pET3α expression vector, harboring the pro-MMP-3(ΔΔC) cDNA, and the recombinant protein in inclusion bodies was refolded and purified as described by Suzuki et al. (38). Both recombinant pro-MMP-3 and pro-MMP-3(ΔΔC) were homogeneous on SDS-polyacrylamide gel electrophoresis. The full-length pro-MMP-3 was activated to the 45-kDa MMP-3 species by incubation with 10 mg/ml chymotrypsin for 2 h at 37 °C followed by inactivation of chymotrypsin with 2 mM phenylmethylsulfonyl fluoride. Pro-MMP-3(ΔΔC) was activated to the 23.5-kDa MMP-3 by incubating with 1 mM 4-aminophenylmercuric acetate at 37 °C for 16 h. HFF conditioned medium was applied to the anti-MMP-3 affinity column as described above. Depletion of MMP-3 from the medium was monitored with a monoclonal antibody obtained from Amersham Pharmacia Biotech.

RESULTS

Profile of Matrix Metalloproteinase Expression in A2058 Melanoma Cells and Effect of All-trans-retinoic Acid—We used Northern and Western blot analyses to confirm and extend earlier reports of MMPs that are constitutively expressed by A2058 melanoma cells (22). Fig. 1A shows that A2058 cells constitutively produce MMP-1, MMP-2 (gelatinase A), MT1-MMP, and lesser amounts of MMP-9 (gelatinase B). In con-
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Invasion of Type I Collagen by A2058 Melanoma Cells—Because tumor cell invasion requires degradation of stromal collagen, we investigated the ability of the A2058 melanoma cells to invade a matrix of type I collagen. Fig. 2A shows A2058 melanoma cells cultured on a membrane coated with type I collagen for 24 h and illustrates the finely structured array of collagen fibrils. Surprisingly, despite the production of substantial amounts of MMP-1, A2058 cells are not invasive. We also cultured normal dermal fibroblasts on type I collagen (Fig. 2B). The cells are stretched out on the matrix and are not invasive. Because host/tumor cell interactions can mediate tumor cell invasion (28, 29, 32), we co-cultured the two cell types (Fig. 2C) and found that under these conditions, the A2058 invade the matrix, as seen by the “halo” of degraded collagen that surrounds the tumor cells. Thus, invasion is dependent on host/tumor cell interactions and on a factor(s) produced by the stromal cells. Furthermore, treating the co-cultures with all-trans-retinoic acid (10⁻⁶ M) for 24 h prevented invasion (Fig. 2D). We also investigated the possibility that retinoic acid was inducing apoptosis in these cells, which might have been responsible for decreased invasive behavior. However, we found that retinoic acid has no apoptotic effects on these tumor cells, nor does it decrease cell proliferation (data not shown).

To study the nature of this host/tumor cell interaction, we determined if cell-cell contact was necessary (28, 29, 32). Stromal fibroblasts were cultured on the bottom of the culture dish, whereas the tumor cells were placed on the collagen-coated filter (Fig. 2E). In this two-compartment system, where there is free diffusion of culture medium but no direct cell-cell contact, invasion still occurs. Quantitation of this invasion by CLSM revealed that 35% of the tumor cells had invaded the collagen to a depth of at least 10 μm as shown in Table I. This table summarizes the results of several experiments, some of which are also presented as figures. We then investigated whether the fibroblasts make this factor constitutively or whether it is synthesized in response to a signal from the tumor cells. The tumor cells were placed on collagen in the presence of serum-free conditioned medium derived from the stromal cells that had been cultured in the absence of the tumor cells. Fig. 2F

![Image](https://via.placeholder.com/150)

**Fig. 1. Effect of all-trans-retinoic acid on expression of matrix metalloproteinases by A2058 cells.** A, Northern analyses. Confluent cells were incubated in serum-free medium in the absence (un) or presence of RA (10⁻⁶ M). For Northern analysis, 5 μg of RNA was loaded, and the blots were probed for MMP expression as indicated. The 28 S RNA is shown as loading control. B, MMP-1 protein expression in the absence or presence of RA. Culture medium (200 μl) was trichloroacetic acid precipitated, and protein expression was visualized by Western analysis.

![Image](https://via.placeholder.com/150)

**Fig. 2. Invasion of type I collagen by A2058 melanoma cells.** Cells were seeded on a collagen type I matrix and scanning electron microscopy images were taken after 48 h. A, A2058 melanoma cells. B, dermal fibroblasts (HFFs). C, co-culture of A2058 melanoma cells and HFFs. D, co-cultures in the presence of RA (10⁻⁶ M). E, HFFs were seeded on the bottom of the tissue culture plate, while A2058 cells were placed on the collagen-coated filter, allowing free diffusion of serum-free culture medium. F, A2058 cells were placed on the collagen coated filter in the presence of serum-free fibroblast conditioned medium added to a final concentration of 10%.

| Type I collagen +/- treatment | Cells invadinga | Invasion depth |
|-----------------------------|-----------------|----------------|
| A2058 cells + LHb           | 5–10            | 4              |
| Co-culture of HFFs and A2058 cells | 35             | 10             |
| A2058 cells + HFF medium    | 33              | 10             |
| A2058 cells + Fraction 3c   | 20              | 10             |
| A2058 cells + recombinant   | 22              | 10             |
| MMP-3                       |                 | 4              |
| A2058 cells + immunodepleted HFF medium | 5–10 | 4 |
| A2058 cells + RAa           | 20              | 4              |
| A2058 cells + aprotininb    | 20              | 4              |

a % of cells invading to a depth of 4 or 10 μm, which defines the least and greatest levels of invasion.
b A2058 cells cultured in LH, serum-free DMEM.
c Fraction 3 corresponds to the molecular mass fraction of 30–50 kDa.
d RA, all-trans-retinoic acid (10⁻⁶ M).
e Aprotinin (100 μg/ml).
shows that under these conditions, the tumor cells also invade the collagen, with about 33% of the cells invading to a depth of 10 μm (Table 1). These data indicate that tumor cell invasion depends on a soluble factor(s) secreted constitutively by the stromal cells and are supported by the reproducibility of the number of cells invading to the 10-μm layer using either coculture or HFF conditioned medium.

Identification of a Soluble Factor in Fibroblast Conditioned Medium That Facilitates Invasion of Type I Collagen by A2058 Melanoma Cells—As a first step in characterizing this soluble factor(s), we fractionated fibroblast conditioned medium to separate proteins according to size. Each fraction was dialyzed, adjusted to approximately the same protein concentration, filtered sterilized, and then placed in the invasion chamber with the tumor cells, which were cultured on type I collagen. Fractions 1–5, corresponding to molecular masses of <20, 20–30, 30–50, 50–70, and >70 kDa, respectively, were tested. Fractions 1, 2, 4, and 5 did not facilitate invasion of the melanoma cells (Fig. 3A and data not shown). However, in the presence of fraction 3, which contains proteins ranging from 30 to 50 kDa, about 20% of the tumor cells invaded the collagen matrix (Fig. 3B and Table 1).

Although the tumor cells are producing collagenase 1 constitutively, it is secreted as a latent proenzyme (Fig. 1B) (22, 24, 33). One hypothesis is, therefore, that the fibroblasts secrete a proteinase that activates the latent collagenase, permitting collagen degradation and facilitating invasion. One candidate is stromelysin 1 (MMP-3), which is an activator of latent MMP-1 (9, 39). A2058 melanoma cells do not make detectable levels of this enzyme (Fig. 1A), whereas normal skin fibroblasts do (33, 41, 42). Consistent with this hypothesis are the facts that only the fraction containing material with molecular masses of 30–50 kDa facilitated invasion (Fig. 3B), and the size of proteins in this fraction corresponds to the molecular mass of an active form of MMP-3 (~45 kDa) (39).

To test the hypothesis we used purified recombinant human protein. Fig. 3C shows that an activated form of purified recombinant MMP-3 (10 μg) facilitates invasion of the tumor cells through the collagen. Quantitation revealed that approximately 22% of the tumors cells invaded to a depth of 10 μm (Table 1). In contrast, latent recombinant stromelysin failed to mediate invasion (data not shown). Thus, the interaction of these two MMPs provides one mechanism for facilitating the invasive behavior of A2058 melanoma cells. However, these results do not imply that MMP-3-mediated activation of latent MMP-1 is the only mechanism by which this enzyme is activated in these cells (see “Discussion”).

Immunodepletion of MMP-3 from Fibroblast Conditioned Medium—As a final test of this hypothesis, we immunodepleted MMP-3 from fibroblast conditioned medium and then measured the ability of the immunodepleted medium to facilitate invasion of the A2058 cells through a collagen matrix. The inset in Fig. 4A shows the presence of MMP-3 in the medium derived from cells transfected with the human stromelysin gene as a positive control (31) (lane 1) and also from fibroblasts (lane 2). Note the absence of this protein after the medium has been immunodepleted (lane 3). In the presence of the native conditioned medium more than 80% of the tumor cells invaded into the 10-μm layer and beyond (Fig. 4A). In contrast, immunodepleted conditioned medium, concentrated 5-fold, decreased the number of invading cells to the level seen when the melanoma cells are cultured on the collagen matrix in serum-free medium that has not been conditioned by fibroblasts (Table I and Fig. 4A, LH control, and Fig. 2A). A similar result was obtained in the presence of 1× concentrated immunodepleted medium (data not shown).

We used collagen type I zymography (34) to assess the collagenolytic activity of culture medium taken from the lower invasion chamber (Fig. 4B, Lane 1) represents a positive control of recombinant pro-MMP-1. Although the enzyme is predominantly in latent form, lower molecular mass, fully active, species are present (asterisk in Fig. 4B), probably because of freezing and thawing of the medium. Lanes 2 and 3 demonstrate pro-MMP-1 in medium from A2058 cells and HFFs, respectively, each in latent form (pro-MMP-1). Note that the A2058 cells also express pro-MMP-2 (Fig. 4B, lane 2, and Fig. 1A), which has some collagenolytic activity under these experimental conditions (20). Fig. 4B, lane 4, corresponds to the 5× concentrated, MMP-3-immunodepleted HFF medium; no active enzyme is seen. Interestingly, zymography indicates that both the native conditioned medium (Fig. 4B, lane 5) and the MMP-3 depleted medium that was used in the invasion assay (Fig. 4B, lane 6) showed processing of pro-MMP-1 to an intermediate species of MMP-1. However, this lower molecular mass form may not represent fully active collagenase, because activation occurs by sequential processing of the propeptide (39). This processing, even in the presence of stromelysin-depleted medium, suggests that fibroblast conditioned medium mediates partial activation of pro-MMP-1 produced by the A2058 cells. Full activation may be observed only in the presence of MMP-3 (31, 39) (see “Discussion”).

Role of All-trans-retinoic Acid in Suppressing Invasion of A2058 Melanoma Cells—Fig. 2B illustrates that treatment of cocultures of melanoma and stromal cells with retinoic acid blocked the invasion of the tumor cells through collagen. Because retinoic acid decreases MMP-1 synthesis in the tumor cells (Fig. 1) and MMP-3 synthesis in fibroblasts (41–44), it is not clear whether inhibition of invasion is affected at the level of MMP-1 synthesis, MMP-3 synthesis, or both. Therefore, we treated fibroblasts with retinoic acid to suppress MMP-3 and...
collected the conditioned medium, dialyzed it to remove the retinoic acid, and then tested its ability to facilitate tumor invasion (Fig. 5). First, as a positive control, we demonstrated that conditioned medium from untreated stromal cells allows about 75% of the total tumor cells to invade collagen to a depth of 8 μm or greater. Next the dialyzed conditioned medium from retinoic acid-treated stromal cells was tested for its ability to facilitate invasion. We found that only ~5% of the tumor cells invaded to a depth of 4 μm, similar to the inhibition seen when the co-cultures were treated with retinoic acid. The converse experiment, in which conditioned medium from untreated stromal cells was placed in the lower invasion chamber and retinoic acid is added at the beginning of the invasion assay, also prevented invasion.

**Effect of Aprotinin on the Invasive Potential of A2058 Melanoma Cells**—The processing of pro-MMP-1 produced by the A2058 cells to a partially active species, even in the presence of stromelysin-depleted fibroblast conditioned medium (Fig. 4B) is intriguing. Possibly a serine proteinase may also contribute to the activation of pro-MMP-1, and therefore, we addressed the question of whether aprotinin, a serine proteinase inhibitor, could impede invasion through type I collagen in the presence of fibroblast conditioned medium. In the absence of the inhibitor, about 60% of the total cells invaded the collagen to a depth of 10 μm or more, with 90% of the cells invading beyond the 4-μm layer. However, in the presence of aprotinin, only about 20% of the cells invaded into the 4-μm layer (Table I and Fig. 6A), suggesting that a serine proteinase, such as plasmin, might be involved in the activation of collagenase 1 and/or stromelysin 1, thereby facilitating the invasive behavior of the A2058 cells. It has been reported that urokinase-type plasminogen activator (uPA) is expressed in melanoma cells and that uPA-catalyzed plasminogen activation enhances tumor cell invasion (18, 45).

A role for a serine proteinase(s) is further supported by collagen type I zymography data (Fig. 6B). Lanes 1–3 represent controls, as described above (Fig. 4B). Culture medium taken from the invasion chamber indicates processing of the precursor pro-MMP-1 to several lower molecular mass species (lane 4), and this processing was greatly reduced in the presence of Aprotinin (lane 5). Furthermore, treatment of the cells with both aprotinin and retinoic acid resulted in greater accumulation of the precursor in the culture medium (lane 6), which was also associated with decreased invasiveness (Fig. 6A). We noted that the processing pattern of pro-MMP-1 differed between lane 4 and lanes 5 and 6. In lane 4 processing occurred from pro-MMP-1 (52 kDa) to an intermediate of 43 kDa and to the fully active form of 41 kDa, with further removal of the C-terminal domain (MMP-1, 22 kDa), as described for the activation of pro-MMP-1 by proteinases and MMP-3 (39). In the presence of aprotinin, two new bands of collagenolysis were observed. The higher molecular mass species (asterisk in Fig. 6B) of approximately 48–50 kDa may be the result of incomplete processing of the propeptide domain because of inhibition of the putative serine proteinase. Similarly, the lower molecular mass species (two asterisks in Fig. 6B) observed in lanes 5 and 6 may be the result of MMP-1 autolysis, which may occur in the presence of the serine proteinase inhibitor. Nonetheless, it is important to note again the similarity and reproducibility of the number of cell present in the 4-μm layer in the presence of MMP-3 depleted medium and RA or aprotinin treatment (Table I). Thus, the decrease in invasiveness may be due to the reduced processing of pro-MMP-1 and pro-MMP-3 and to a reduction in MMP synthesis by retinoic acid.

**Regulation of TIMP Expression by All-trans-retinoic Acid**—Along with suppression of MMP synthesis, retinoic acid increases the expression of the TIMPs in fibroblasts (19). The observation that conditioned medium from retinoic acid-treated HFFs did not prevent processing of procollagenase to an active form but still impeded tumor cell invasion led us to explore the possibility that retinoid treatments affected TIMP expression. We used Western blot analysis to determine the levels of TIMP-1 and TIMP-2 in both untreated and retinoic acid-treated HFFs in A2058 melanoma cells. We found that both cell types expressed TIMP-1 and TIMP-2 constitutively (Fig. 7). Retinoic acid treatment resulted in a modest increase in TIMP-1 levels in HFFs, whereas TIMP-2 expression was increased 3–4-fold in these cells. In contrast, a 3-fold increase in the level of TIMP-1 expression was observed in A2058 cells, whereas retinoic acid treatment had no effect on TIMP-2 expression. Therefore, the decreased invasive behavior seen in Fig. 6 might be due, at least in part, to an increase in the binding of TIMP-1 to the activated form of MMP-1.

**DISCUSSION**

In this paper, we describe a mechanism of tumor invasion through a matrix of type I collagen in which a serine/MMP proteolytic cascade culminates in the activation of pro-MMP-1, with subsequent invasion of type I collagen by tumor cells. We used A2058 melanoma cells because they produce MMP-1 and are an aggressive/invasive cell line (22). It is interesting, then, that these cells do not invade the collagen unless they are...
either co-cultured with normal fibroblasts or with conditioned medium derived from the fibroblasts. We have identified one member of this cascade as stromelysin 1/MMP-3, and our data suggest that the activation pathway may proceed as shown in Fig. 8. The latent MMP-1 is produced by the tumor cells, and it may be partially activated by a serine proteinase, also produced by these cells. However, this activation is incomplete, and collagen degradation/tumor invasion cannot occur. Nonetheless, our data suggest that the serine proteinase(s) can fully activate latent stromelysin 1, which is produced constitutively by the stromal cells. Active stromelysin 1 then completes the activation of collagenase 1, allowing invasion to proceed. Because the A2058 cells do not produce stromelysin 1, the interaction with fibroblasts/fibroblast conditioned medium is essential. The stromelysin 1 immunodepletion studies and experiments with recombinant active stromelysin 1 clearly demonstrate the critical role of this enzyme in the invasive process. However, this role is indirect in that it facilitates invasion by activating latent MMP-1, which then degrades collagen. The importance of serine proteinase(s) is evident from the fact that MMPs in the conditioned medium from fibroblasts remain in latent form unless this medium is exposed to the tumor cells. The source of the serine proteinase(s) is presently unknown. The A2058 melanoma cells may secrete this enzyme. Alternatively, it is possible that even though the cells were washed extensively to remove traces of serum which may contain serine proteinases, a minute amount may remain and be sufficient to initiate activation of latent MMPs.

The proteolytic activation cascade was blocked at a number of steps, thereby preventing invasion. All-trans-retinoic acid decreased production of MMP-1 (Fig. 1) and MMP-3 (41–43) and also increased TIMP levels. These changes altered the ratio of TIMP:MMP in favor of TIMP, increasing the amount of TIMP available to complex with active MMPs and interrupting invasion. Furthermore, depleting MMP-3 from the conditioned
medium may have provided an additional mechanism for increasing the amount of TIMP-1 that can complex with active MMP-1. We also found that aprotinin, a serine protease inhibitor, blocked both invasion and the enzymatic conversion of pro-MMP-1 to an active species, thus documenting a role for serine proteinases in this activation cascade. Finally, our data demonstrate that interstitial collagenase 1 is the ultimate target of this cascade, which culminates in the degradation of type I collagen and invasion by the tumor cells.

This proteolytic cascade and the invasion of collagen by the tumor cells is the product of a host/tumor cell interaction that has not been described previously. However, several other cellular mechanisms by which MMPs can mediate the invasive behavior of tumor cells have been noted. First, the tumor cells, themselves, may produce MMPs, degrade the matrix, and facilitate their own invasion (24, 36). Second, the neighboring stromal cells may produce matrix-degrading enzymes that allow the tumor cells to invade (28). Third, there may be interactions between the tumor cells and the surrounding stromal fibroblasts that increase MMP levels and enhance invasion. In many of these stromal/tumor cell interactions, MMPs are produced by the neighboring stromal cells in response to factors secreted by the tumor cells (30, 32). Examples of these factors are EMMPRIN (extracellular matrix metalloproteinase inducer), a 58-kDa protein that belongs to the immunoglobulin superfamily (29), and a less well characterized protein of 19 kDa that is produced by basal cell carcinomas (26). Alternatively, stromal cells can stimulate MMP production in several different types of tumor cells (27–28). In contrast, our data indicate that MMP-1 and MMP-3 are each produced constitutively by the tumor cells and stromal cells, respectively. The critical factor in invasion is the interaction of these constitutively expressed enzymes to fully activate MMP-1 rather than the stimulation of MMP synthesis by one or both cell types.

Only recently have we begun to understand the molecular basis of host/tumor cell interactions and of the proteolytic cascades they can create. One serine proteinase/MMP cascade seems to require only the tumor cells (45). An uPA/uPA receptor/MMP-9 (gelatinase-B) was essential for the intravasation (invasion of the blood vessel wall) of several types of tumor cells. Invasion was inhibited in the presence of marimastat, an inhibitor of MMP-3. The activated MMP-3 is a potent activator of pro-MMP-9 (40). Active MMP-9 then mediates the invasive ability of the tumor cells. These findings are similar to ours, with the exception that the final target in the activation cascade is pro-MMP-1 instead of pro-MMP-3. Possibly, then, with their ability to activate both pro-MMP-9 and pro-MMP-1, these activation cascades facilitate the invasion of tumor cells through both basement membrane and the interstitial collagens.

In keeping with other reports (35), we found that the A2058 cells readily invade Matrigel®, without the need for stromal factors and that this invasion was blocked by retinoic acid (data not shown). Matrigel® is a model for basement membrane and type IV collagen, and the gelatinases (MMP-2 and MMP-9) are dominant in basement membrane destruction (1–8). Because pro-MMP-2 can be activated by MT1-MMP, the A2058 cells possess a mechanism that allows them to degrade type IV collagen and invade through the Matrigel®. However, a recent report suggests that the degradation of type IV collagen may be accomplished by an interstitial collagenase (24). Human MIM melanoma cells, established from an inguinal metastasis, constitutively produce MMP-1 and MMP-2 (24). When MMP-1, but not MMP-2, production is specifically blocked by 90–96% through antisense mRNA, invasion through Matrigel® is significantly reduced. This finding has led the authors to the interesting speculation that degradation of type IV collagen, as well as type I, depends on MMP-1 gene expression by these cells.

In summary, tumor invasiveness, which requires the breakdown of the extracellular matrix by MMPs, is a critical factor in the morbidity and mortality of cancer. Degradation of collagens I and III is one step in this process, and it depends primarily on the interstitial collagens, such as MMP-1. MMP-1 is the dominant MMP expressed by several aggressive and invasive tumor cell lines, and expression of this enzyme is correlated

**Fig. 8. Model for proteolytic cascade of tumor cell invasion.** Pro-MMP-1 secreted by tumor cells is partially activated by a serine proteinase(s), and pro-MMP-3 secreted by the stromal cells is fully activated by the serine proteinase(s). Full activation of MMP-1 is achieved by MMP-3. Active MMP-1 degrades collagen types I and III, leading to the degradation of interstitial collagens and tumor cell invasion. Collagen degradation can be inhibited by the levels of TIMPs secreted by both tumor cells and fibroblasts.

cooperation between serine proteinases and MMPs is essential for invasion, and two models to explain the roles of these two proteinases have been postulated (46). In the first, different components of basement membrane may be degraded by different enzymes, and in the second, the two enzymes may be components of one proteolytic cascade.

This latter model is supported by recent studies describing the activation of pro-MMP-9 via a converging cascade that involves plasmin and stromelysin 1 (MMP-3) in tumor cells (40). In this case, plasmin, which is not derived from the tumor cells, activates pro-MMP-9 incompletely. It is, however, very efficient in activating/converting pro-MMP-3 to fully active MMP-3. The activated MMP-3 is a potent activator of pro-MMP-9 (40). Active MMP-9 then mediates the invasive ability of the tumor cells. These findings are similar to ours, with the exception that the final target in the activation cascade is pro-MMP-1 instead of pro-MMP-3. Possibly, then, with their ability to activate both pro-MMP-9 and pro-MMP-1, these activation cascades facilitate the invasion of tumor cells through both basement membrane and the interstitial collagens.
with a poor prognosis in several cancers (13, 14). Ultimately, the ability to stop tumor invasion mediated by MMP-1 may depend on the ability to block the action of this enzyme, either by interrupting the proteinase activation cascade or by specifically blocking the activity of the synthesis of this interstitial collagenase.

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