Activation of Matrix Metalloproteinase-9 (MMP-9) via a Converging Plasmin/Stromelysin-1 Cascade Enhances Tumor Cell Invasion*

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Matrix metalloproteinase-9 (MMP-9) may play a critical catalytic role in tissue remodeling in vivo, but it is secreted by cells as a stable, inactive zymogen, pro-MMP-9, and requires activation for catalytic function. A number of proteolytic enzymes activate pro-MMP-9 in vitro, but the natural activator(s) of MMP-9 is unknown. To examine MMP-9 activation in a cellular setting we employed cultures of human tumor cells (MDA-MB-231 breast carcinoma cells) that were induced to produce MMP-9 over a 200-fold concentration range (0.03–8.1 nM). The levels of tissue inhibitors of metalloproteinase (TIMPs) in the induced cultures remain relatively constant at 1–4 nM. Quantitation of the zymogen/active enzyme status of MMP-9 in the MDA-MB-231 cultures indicates that even in the presence of potential activators, the molar ratio of endogenous MMP-9 to TIMP dictates whether pro-MMP-9 activation can progress. When the MMP-9/TIMP ratio exceeds 1.0, MMP-9 activation progresses, but through an interacting protease cascade involving plasmin and stromelysin 1 (MMP-3). Plasmin, generated by the endogenous urokinase-type plasminogen activator, is not an efficient activator of pro-MMP-9, neither the secreted pro-MMP-9 nor the very low levels of pro-MMP-9 associated with intact cells. Although plasmin can proteolytically process pro-MMP-9, this limited action does not yield an enzymatically active MMP-9, nor does it cause the MMP-9 to be more susceptible to activation. Plasmin, however, is very efficient at generating active MMP-3 (stromelysin-1) from exogenously added pro-MMP-3. The activated MMP-3 becomes a potent activator of the 92-kDa pro-MMP-9, yielding an 82-kDa species that is enzymatically active in solution and represents up to 50–75% conversion of the zymogen. The activated MMP-9 enhances the invasive phenotype of the cultured cells as their ability to both degrade extracellular matrix and transverse basement membrane is significantly increased following zymogen activation. That this enhanced tissue remodelling capability is due to the activation of MMP-9 is demonstrated through the use of a specific anti-MMP-9 blocking monoclonal antibody.

Degradation of connective tissue extracellular matrix

1 The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinase; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; uPA, urokinase-type plasminogen activator; PMA, phorbol 12-myristate 13-acetate.
tease furin (10). Activation of interstitial collagenase (MMP-1) and stromelysin (MMP-3) occurs extracellularly and can be mediated by the serine protease, plasmin (11, 12). Cell surface activation of an MMP can occur when gelatinase A (MMP-2) is brought into contact with a membrane-associated MMP, MT-1 MMP (13, 14). Interestingly, gelatinase B (MMP-9), a close structural homologue of MMP-2 does not appear to be activated by the same mechanism, as it remains a zymogen under identical cellular conditions where a majority of co-expressed pro-MMP-9 is activated via the cell surface mechanism (15, 16).

MMP-9 is produced by mesenchymal, epithelial, and hematopoietic cells and also by distinct tumor cell types (17–20). While MMP-2 appears to be constitutively expressed by many cell types in culture, MMP-9 expression is induced by cytokines (21, 22), growth factors (23), and cell/stroma interactions (19, 20, 24). MMP-9 expression has been correlated with a number of physiological and pathological processes, including trophoblast implantation (25), bone resorption (26), inflammation (18, 19), and arthritis (27). Tumor cell invasion in a number of instances also has been linked with MMP-9 activity (5–7, 20). Furthermore, Muschel and colleagues (28, 29) have shown by both transfection and ribozyme-based approaches that MMP-9 is directly involved in tumor metastasis and more recently MMP-9 activity has been linked with the process of tumor cell invasation (30). Thus in these malignant cell systems, the regulatory controls that maintain MMPs as inactive zymogens were circumvented, since conversion of pro-MMP-9 to active enzyme had clearly occurred. The exact mechanism of MMP-9 activation in malignant tissue, however, has not been defined. A number of purified proteases, including trypsin (31, 32), chymase (33), MMP-2 (34), tissue kallikrein (35), trypsin-2 (36), plasmin (37, 38), MMP-7 (39), MMP-13 (40), and MMP-3 (31–33, 37, 38, 41, 42) have been reported to activate pro-MMP-9 in vitro. MMP-3, based on in vitro kinetic and catalytic parameters, appears to be the most efficient activator of pro-MMP-9 (32) and may be a natural activator in vivo. It is not clear, however, if MMP-3 will always be present in the tumor tissues where pro-MMP-9 is expressed. Furthermore, even if MMP-3 is available in the tissues, it is produced as a zymogen and also requires activation. In addition, the inhibitory potential of the TIMPs must be circumvented in the tumor tissue to allow for catalytic manifestations of both the activating MMP-3 and the activated MMP-9.

To demonstrate a possible tumor-associated mechanism of MMP-9 activation and the circumvention of TIMP-mediated control, we have examined cultures of a breast carcinoma cell line, MDA-MB 231, which expresses both MMP-9 and TIMP-1 and TIMP-2. MDA-MB-231 cultures do not appear to express MMP-2, also a potent gelatinase. Since MMP-9 activation is monitored by the generation of gelatinase activity, the absence of any interfering MMP-2 gelatinase activity in the cultures was critical for quantitating zymogen activation. Our results indicate that MMP-9 produced by the breast tumor cells is a stable zymogen even when exceptionally high levels of MMP-9 are induced and when active plasmin is generated in the cultures. However, when pro-MMP-3 is introduced into the system at concentrations that stoichiometrically exceed the endogenous TIMP levels, plasmin activates the MMP-3 which in turn efficiently processes and activates the pro-MMP-9. The activated MMP-9 contributes significantly to tumor cell-mediated ECM degradation and basement membrane invasion, and the specificity of these mechanisms is demonstrated through the use of neutralizing anti-MMP-9 monoclonal antibodies.

MATERIALS AND METHODS

Cell Culture—MDA-MB-231 cells, HT1080 cells, and MDA-MMP-9 cells were cultured at 37°C in 8% CO2 in DMEM containing 10% heat inactivated fetal bovine serum (FBS) supplemented with 0.1 mM non-essential amino acids, 2 mM glutamine, 1 mM sodium pyruvate, and 100 units/ml penicillin with 100 μg/ml streptomycin. For harvesting supernatant conditioned medium, the cell cultures were washed with DMEM and incubated for 24 h with serum-free DMEM that included the above mentioned supplements.

**Activation of MMP-9 by a Plasmin/Stromelysin-1 Cascade**

**MMP-9-expressing Cell Line—** Cultures of MDA-MB-231 (5 × 10⁶ cells/cm² culture dish) were transfected with a pRC/RSV/MMP-9 cDNA construct (20 μg) in calcium phosphate buffer (125 mM CaCl₂, 140 mM NaCl, 25 mM HEPES, 0.75 mM Na₂HPO₄, pH 7.1). The MMP-9 cDNA construct was provided by Dr. Ruth Muschel, University of Pennsylvania, Philadelphia, PA (28). After 48 h, the transfected cells were replated (5 × 10⁶ cells/10-cm dish) in supplemented DMEM, 10% FBS containing 600 μg/ml Geneticin (Life Technologies, Inc.). After 2 weeks in culture, the supernatants from the cloned cultures were analyzed by enzyme-linked immunosorbent assay for secreted pro-MMP-9. A number of clones expressed increased levels of pro-MMP-9 over that of the nontransfected, parental cultures. One stable cell line, MDA-MMP-9, was propagated under standard culture conditions and analyzed in detail.

**MMP-9 Enzyme-linked Immunosorbant Assay—** Microtiter 96-well plates were precoated with an anti-MMP-9 monoclonal antibody (6-6B IgG₂a, 1 μg/ml, 50 μl/well) in PBS for 1 h at room temperature and blocked with 1% bovine serum albumin/PBS (1 h at room temperature). Conditioned medium (50 μl/well) was added for 1 h at 37°C followed by washing and the addition of a secondary anti-MMP-9 monoclonal antibody, 7-11C IgG₁, (1 μg/ml, 50 μl/well) in 1% bovine serum albumin/PBS for 1 h at 37°C. The cells were washed with PBS, 0.5% Tween, incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (1:1000 in 1% bovine serum albumin/PBS), followed by washing and the addition of p-nitrophenyl phosphate substrate and the development of color according to manufacturer (Kirkgaard and Perry, Gaithersburg, MD) instructions.

**Western Blot Analysis—** Samples of conditioned medium were first separated by SDS-polyacrylamide gel electrophoresis and then electrophoresed onto nitrocellulose membranes (Millipore, Burlington, MA). The membranes were blocked in 5% non-fat milk in Tris-buffered saline, 0.5% Tween, washed, and incubated overnight with purified monoclonal antibodies (1–5 μg IgG/ml). The blots were then washed and incubated for 2–4 h with secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (Kirkgaard and Perry) at 1:1000 dilution. The blots were developed using the ECL chemiluminescence detection system (Amersham Pharmacia Biotech).

**Gelatinolytic and Reverse Zymography—** Gelatin substrate zymography was performed in SDS-polyacrylamide (10%) gels copolymerized with gelatin, as described previously (21). Reverse zymography, used to detect and quantitate TIMP levels, was performed in SDS-polyacrylamide (15%) gels copolymerized with gelatin (60 μg/ml) and conditioned medium (1.0 ml) from a cell line expressing MMP-2 (43). After electrophoresis, the gels were washed for 2 h in Trition X-100 (2.5%) and incubated overnight in buffer (50 mM Tris, pH 7.25, 200 mM NaCl, 0.5% Brij-35, 0.02% NaN₃). The gels were stained with Coomassie Brilliant Blue, and dark zones marked the TIMP-mediated inhibition of gelatin degradation.

**Activation of Pro-MMP-9 in Culture—** The MDA-MB-231 cells and MDA-MMP-9 cells were plated at 1 × 10⁵ cells/cm² in DMEM, 10% FBS. After 24 h, the cells were rinsed once with 2 mM 6-amino-n-capric acid in serum-free DMEM to remove any cell surface plasminogen. The cells were rinsed twice with serum-free DMEM and incubated in the same medium in the presence or absence of the following components: plasminogen (25 nM), pro-MMP-3 (2–16 nM), active MMP-3 (5 nM), apronin (40 μg/ml), TIMP-1 (20 nM), anti-MMP-9 IgG (200 nM), and normal mouse IgG (200 nM) added singularly or in the indicated combinations. The cell cultures were incubated for 24–48 h and the conditioned medium collected and stored at −70°C before analysis. Pro-MMP-3 was expressed as a recombinant protein in Chinese hamster ovary K-1 cells and purified from conditioned medium as described previously (44). Active MMP-3 (MMP-3C) was a C-terminal truncated derivative of MMP-3 that spontaneously activated and yielded a specific activity equivalent to native MMP-3. It was expressed in Escherichia coli and purified as described previously (45). Anti-MMP-9 monoclonal antibody 7-11C blocks activation of pro-MMP-9 and was purified from hybridoma conditioned medium as described previously (46).

**Preparation of Cell Extracts—** MDA-MMP-9 cells were grown and confluent to 100-mm dishes (1.5–2 × 10⁵ cells/plate in DMEM, 10% FBS). Each plate was washed twice with serum-free DMEM with apronin 40 μg/ml and once with serum-free DMEM.
Enhanced Expression of MMP-9 from Human Tumor Cells That Do Not Express MMP-2—To examine the activation of pro-MMP-9 in a cellular setting that is devoid of MMP-2, cultures of the human breast carcinoma cell, MDA-MB-231, were treated with cytokines known to stimulate the production of pro-MMP-9. Gelatin substrate zymography, a method that can detect subnanogram amounts of gelatinase, was used to monitor levels of MMP-9 in the conditioned medium harvested from the cultures. A parallel culture of HT1080, a human fibrosarcoma cell line that produces both MMP-2 and MMP-9 was used as control. Treatment of MDA-MB-231 cells with PMA and interleukin-1 resulted in increased levels of MMP-9 expression while platelet-derived growth factor treatment yielded no change in MMP-9 levels (Fig. 1). There was no evidence of MMP-2 expression in the treated cultures. Although PMA stimulation resulted in a substantial increase in MMP-9 expression, an 82-kDa form was not observed, indicating that the induced enzyme was not activated but remained in the 92-kDa zymogen form.

Generation of Active Stromelysin (MMP-3) in MDA-MB-231 Cultures Fails to Activate Pro-MMP-9—To determine if a previously proposed MMP-3-dependent mechanism of pro-MMP-9 activation (41) could occur in a complex cell culture system, a proteolytic cascade that would yield active MMP-3 was initiated in the MDA-MB-231 cultures. These cultures produce uPA at levels sufficient to catalyze the conversion of plasminogen to plasmin. Plasmin is a known activator of pro-MMP-3 (12), and active MMP-3 had been shown to activate pro-MMP-9 under in vitro conditions using purified preparations of MMP-3 and pro-MMP-9 (32, 38). The proteolytic cascade was initiated by supplementation of the PMA-treated MDA-MB-231 cultures with pro-MMP-3 (16 nM) and plasminogen (25 nM). The activation of pro-MMP-3 was monitored by immunoblot analysis and activation of pro-MMP-9 was measured by zymography (Fig. 2). In the absence of plasminogen, the addition of pro-MMP-3 had no effect on the status of the pro-MMP-9 as it remained in the 92-kDa zymogen form (Fig. 2, zymograph, lane 2). The pro-MMP-3 also was maintained as the 55-kDa zymograph (Fig. 2, immunoblot, lane 2). Addition of plasminogen plus pro-MMP-3 caused a conversion of the 55-kDa pro-MMP-3 to the 45-kDa active MMP-3 (immunoblot, lane 3) but, unexpectedly, little or no activation of pro-MMP-9 occurred; only trace levels of an 82-kDa form of MMP-9 appeared in the zymograph (lane 3), and no gelatinase activity was detectable. Plasmin was generated in the culture system since plasmin activity as measured by a specific peptide hydrolysis assay was detected only in the plasminogen-containing cultures (data not shown). The plasmin that was generated in this system was responsible for the conversion of pro-MMP-3 because the addition of aprotinin, a
specific inhibitor of plasmin, blocked the conversion of pro-MMP-3 from the 55-kDa form to the 45-kDa form (Fig. 2, immunoblot, lane 4).

The absence of conversion of pro-MMP-9 to active MMP-9 in the presence of a functioning proteolytic cascade, which clearly had generated active MMP-3, was further investigated. The relative concentrations of pro-MMP-9, TIMP-1, and TIMP-2 in the cultures were determined (Table I). Interestingly, the levels of pro-MMP-9 in the PMA-stimulated cultures were increased 8-fold over the levels in the unstimulated culture (0.25 nM versus 0.03 nM), but the endogenous TIMP concentrations still remained 10–20-fold higher than the MMP-9 concentration in these cultures (3–4 nM versus 0.25 nM). These data suggested that TIMP levels were controlling the progression to active MMP-9 and that activation might only occur when the total MMP concentration exceeded the TIMP concentration in the immediate environment. Within the MDA-MB-231 cell culture system, the molar concentration of pro-MMP-9 would have to be increased at least 20-fold to exceed the endogenous TIMP levels.

An MDA-MB-231-transfected Cell Line Expressing Increased Levels of MMP-9—To achieve increased levels of MMP-9 expression, MDA-MB-231 cells were transfected with an MMP-9 cDNA construct (28), selected, cloned, and analyzed for MMP-9 expression by zymography and immunoblot analysis. A stable cell line that secreted substantially increased levels of MMP-9 was isolated and designated MDA-MMP-9 (Fig. 3, A and C). Like the parent MDA-MB-231 cells, this cell line did not express MMP-2 (Fig. 3, B and C), MMP-3 (Fig. 3D), or TIMP-1 (Fig. 3E) and did express significant levels of uPA (Fig. 3F). A direct enzyme assay indicated that the uPA concentration in the cell culture supernatant was 0.1–0.2 ng/ml, which could very effectively activate microgram quantities of plasminogen. Although this cell line expressed increased levels of MMP-9, the TIMP levels were the same as the parent cell line and slightly less than the TIMP levels expressed by the PMA-treated MDA-MB-231 cells (Fig. 3, F and G). Quantitation of MMP-9 levels expressed by the MDA-MMP-9 cell line showed that the MMP-9 concentration now exceeded the concentration of both TIMP-1 and TIMP-2 (8 nM versus ~3 nM) (Table I, third column). However, the MMP-9 in the transfected cultures remained in the 92-kDa zymogen form despite the significant increase in the MMP-9:TIMP ratio (Fig. 3, A and C), and no gelatinase activity was manifested by the transfected cultures (data not shown). Furthermore, when the transfected cultures were incubated in the presence of plasminogen, which was rapidly converted to plasmin, the 92-kDa zymogen form was maintained (see Fig. 4, inset, lane I).

MMP-3-dependent Activation of the Pro-MMP-9 Secreted by the MDA-MMP-9 Cells—The activation of pro-MMP-9 in the tumor cell culture system was re-analyzed using the trans-
fected MDA-MMP-9 cells. By adding plasminogen (2.5 μg/ml) and pro-MMP-3 at increasing concentrations to the cell culture system, the activation of MMP-9 occurred in a dose-dependent manner (Fig. 4). The appearance of a gelatinolytic zone with an apparent molecular mass of 82 kDa was observed upon the addition of 8–16 nM pro-MMP-3 (Fig. 4, zymograph inset), and this change in molecular mass was accompanied by a corresponding increase in solution phase gelatinolytic activity (Fig. 4, line graph). In the presence of 16 nM pro-MMP-3, more than half of the 92-kDa form of the enzyme had been processed, and significant gelatinolytic activity was observed.

The presence in the cell culture system of active MMP-3 generated from the added pro-MMP-3 was measured using a fluorogenic peptide assay that is specific for MMP-3 (49). Enzyme activity above background was observed only in cultures containing pro-MMP-3 at concentrations ≥8 nM (Fig. 4, bar graph), which corresponded with the appearance of both the 82-kDa form of MMP-9 and gelatinase activity. At concentrations less than 8 nM pro-MMP-3, MMP-3 activity as well as conversion of MMP-9 and gelatinase activity were at background levels. These data suggested that the effective generation of gelatinolytic activity was dependent on concentrations of MMP-3 and MMP-9 that exceeded the endogenous TIMP concentrations.

To determine the pro-MMP-3 concentration necessary to activate MMP-9 in the absence and presence of stoichiometric levels of TIMP-1, the plasmin-induced proteolytic cascade was reconstituted in vitro using purified components (Fig. 5). Purified pro-MMP-3 was added at 8 nM, the concentration present in the MDA-MMP-9 cell culture system (Table 1). Plasmin was added at 10 nM, the concentration determined to be generated from 25 nM plasminogen in a uPA-containing culture, and pro-MMP-3 was added at increasing concentrations (0–16 nM). Plasmin alone failed to convert pro-MMP-9 (Fig. 5A, lane 1). The conversion of pro-MMP-9 to the 82-kDa form of the enzyme was observed with as little as 2 nM of pro-MMP-3 in the absence of TIMP (Fig. 5A, lanes 2–5). However, the addition of purified TIMP-1 at a concentration of 4 nM prevented the conversion of pro-MMP-9 until 8 nM pro-MMP-3 was added and then the 82-kDa form is observed (Fig. 5B). Almost complete conversion of the enzyme occurred at a pro-MMP-3 concentration of 16 nM, which yields an effective concentration of 6–8 nM active MMP-3 (40–50% conversion). This activation pattern of MMP-9 closely resembled the pattern observed in the crude cell culture system (Fig. 4) and provided additional evidence that the activation of MMP-9 was regulated by the levels of TIMP in the system.

The requirements of the proteolytic cascade for MMP-9 activation and the contribution of the individual components were evaluated by the addition of specific inhibitors to the supplemented MDA-MMP-9 cell cultures. Pro-MMP-3 and plasminogen were employed at concentrations that would yield 50–75% conversion of pro-MMP-9, and samples of culture supernatants were analyzed by zymography for conversion of pro-MMP-9 and solution phase gelatinase activity for appearance of active enzyme (Fig. 6). Only background levels of gelatinase activity were generated when either plasminogen or pro-MMP-3 was added. Interestingly, some processing of pro-MMP-9 to an intermediate 84–86-kDa form was present (zymograph, lanes 2 and 3), but this form was not enzymatically active in solution (bar graph, lanes 2 and 3). Substantial gelatinase activity was
generated only in plasminogen-containing cultures with added pro-MMP-3 (bar graph, lane 4) and was accompanied by near full conversion of pro-MMP-9 to the 82-kDa form of the enzyme (zymograph, lane 4). The generation of plasmin from plasminogen was essential for MMP-9 activation, because the addition of aprotinin resulted in background levels of gelatinase activity and only partial conversion of pro-MMP-9 to the 86-kDa intermediate form (bar graph and zymograph, lane 5). The activation of pro-MMP-9 was also blocked by the addition of TIMP-1 at concentrations (20 nM) in excess of MMP-3 and MMP-9 (lane 6). The most effective inhibitor of pro-MMP-9 processing and activation was an anti-MMP-9 monoclonal antibody 7-11C. The purified 7-11C IgG completely blocked the plasmin-plus MMP-3-dependent activation of MMP-9 (lane 7), while control IgG allowed for full processing and activation (lane 8). A partner monoclonal antibody, 6-6B, isolated from the same fusion as 7-11C, had been shown previously to block organomercurial mediated activation of pro-MMP-9 (46).

The presence in the cultures of both plasmin and pro-MMP-3 are required for active MMP-9 to be generated, and neither one alone is sufficient (Fig. 6). Plasmin efficiently activates pro-MMP-3 (Fig. 2), and presumably the active MMP-3 directly converts 92-kDa pro-MMP-9 to 82-kDa active MMP-9. However, it was possible that plasmin, in addition to activating pro-MMP-3, could proteolytically modify the pro-MMP-9, thereby allowing it to be efficiently processed and activated by MMP-3. Plasmin thus could have a dual requirement in the system. To address this possibility, serum-free conditioned medium from MDA-MMP-9 cells containing endogenous pro-MMP-9 (8 nM) was preincubated in the absence or presence of plasminogen (2 μg/ml). After 16 h of incubation, the plasminogen-containing sample had a measured concentration of active plasmin of 0.9 μg/ml (10 nM), a significant level of active enzyme. Aprotinin was added to the samples, completely inhibiting any further action of plasmin, and then 5 nM active MMP-3 was added to both samples and incubation at 37°C was continued. At various times, aliquots were withdrawn from both samples and analyzed by gelatin zymography to monitor the pro-MMP-9 conversion process and by radiolabeled gelatin degradation to monitor the appearance of active MMP-9. Fig. 7 demonstrates that the processing of pro-MMP-9 by MMP-3 is nearly identical for plasmin-pretreated or -untreated samples (Fig. 7, A and B). Plasmin pretreatment also did not enhance the rate of appearance of active MMP-9 and in fact slightly retarded the appearance of gelatinase activity (Fig. 7C). These results also illustrate that prior to MMP-3 addition, plasmin alone at physiological concentrations does not generate any detectable gelatinase activity or conversion to 82-kDa forms (Fig. 7B, lane 1). Furthermore, the requirement for plasmin in the activation process appears to be solely to activate pro-MMP-3, since 5 nM active MMP-3 alone can replace both plasminogen and the 16 nM pro-MMP-3 in generating full conversion to the 82-kDa gelatinase (Fig. 7A, lane 6). Interestingly, 2.5 nM MMP-3, in contrast to 5 nM MMP-3, was unable to generate any active gelatinase in the treated or untreated conditioned media (data not shown), consistent with the need to overcome the 3–4 nM TIMP present in the conditioned medium.

Activation of the Cell-associated MMP-9 Appears to Have Similar Plasmin/MMP-3 Requirements—It has been reported that MMP-2 and MMP-9 are associated with the cell surface and can be activated independently of other MMPs directly via a cell surface uPA/plasmin cascade (52). The association of pro-MMP-9 with cells, and its activation requirements were examined in the overexpressing MDA-MMP-9 cultures. The cells were incubated for 24 h in serum-free medium in the absence or presence of either plasminogen, pro-MMP-3, or both together. The conditioned medium (5 ml) was harvested from the four cultures, and a total cell and membrane extract was prepared from the same harvested cultures. The extract was passed over and eluted from gelatin-Sepharose in order to concentrate all the cell-associated MMP-9 free of any extraneous cellular proteins and inhibitors (0.15 ml). Aliquots (1 μl) from each conditioned medium and extract were examined by zymography, and aliquots (75 μl) from each were tested for active gelatinase (Fig. 8). The total gelatinase activity in each sample was calculated to provide a measure of how much active MMP-9 was cell-associated compared with the amount of secreted, soluble MMP-9. The zymographic analysis indicates that a lower molecular mass form of MMP-9 (~80–85 kDa) exists in the cells from the untreated cultures (lane 5), but it would not appear to be the 82-kDa active gelatinase, since no enzymatic activity can be detected assaying as much as 50% (75 μl) of the total cell extract. The 80–85 kDa band may be a variant glycosylated form of pro-MMP-9 found associated only with cells and not secreted (53). Such a cell-associated MMP-9
MMP-3 following pretreatment with plasmin. Serum-free conditioned medium was collected after 24 h from 1.5 l labeled gelatin (20 of plasmin, and then 5 nM active MMP-3 was added along with radioactivity, active plasmin was present at 0.9 conversion to plasmin was monitored, and at the end of the preincubation absence (conditioned medium (3 ml) was incubated at 37 °C for 16 h in the degradation (zymography and 100-mg/ml) was added to the conditioned media to block any further activity of plasmin, and then 5 nM active MMP-3 was added along with radiolabeled gelatin (20 μg/ml, 2000 cpm/μg), and incubation was continued at 37 °C. At the indicated times, 1-μl aliquots were removed for gelatin zymography and 100-μl aliquots removed for measuring [3H]gelatin degradation (C).

variant would exist as a zymogen and thus would manifest little or no soluble enzymatic activity but yield a zone of lysis in the zymograph. Alternatively, the 80–85-kDa species could be an active form of MMP-9 that is tightly complexed with a cell-associated inhibitor and thus in solution unable to express gelatinase activity, but following dissociation in SDS-polyacrylamide gel electrophoresis would yield a zone of lysis in the zymograph. This band is present in all of the cell extracts; however, an enhanced zymographic signal at 80–85 kDa appears in the extracts prepared from cells treated with pro-MMP-3, and plasminogen (Fig. 8, lane 6). This enhanced transmigration was accompanied by a small but significant increase in the soluble gelatinase activity of the sample. The cell-associated gelatinase activity (659 cpm) is much lower than the activity (14,056 cpm) manifested by a similar 82-kDa band generated in the corresponding conditioned medium (lane 4), possibly indicating the presence of an inhibitor in the cell extract sample. When the gelatinase activity present in the total cell extract is calculated, the amount of active enzyme present (0.30 unit) represents only 0.15% of the total activity of the culture; 99.8% of the activity (197.6 units) is present in the conditioned medium. Thus only a very small level of active MMP-9 is associated with the tumor cells, possibly on the cell surface. However, even that small level of active enzyme appears to require the combined action of plasmin and pro-MMP-3 for its generation. With no pro-MMP-3 present, the presence of plasmin alone causes little or no increase in cell-associated active MMP-9 (Fig. 8, lane 7).

MMP-9 Activation via the Plasmin/MMP-3 Cascade Enhances ECM Degradation—The unique reactivity of the monoclonal antibody, 7-11C, to human MMP-9 (46) and that it could prevent the generation of active MMP-9 (Fig. 6) indicates that the antibody could be used to assess the specific involvement of MMP-9 activation in human tumor cell invasive behavior. To assess the functional activity of the MMP-9 generated in the MDA-MMP-9 tumor cell culture system, progressive matrix degradation by these cells was analyzed in the absence and presence of the monoclonal antibody. The ECM is coated with glycoproteins that protect the fibrillar collagens from the catalytic activity of MMPs; thus examination of the role of specific MMPs in ECM degradation requires removal of this glycoprotein layer (54). To evaluate the role of MMP-9 activity in ECM degradation, MDA-MMP-9 cells were cultured on a smooth muscle cell matrix that was radiolabeled and partially depleted of glycoproteins by trypsin treatment (Fig. 9). Incubation of the cells alone for 3 days resulted in a low level of ECM degradation (Fig. 9, condition A), and addition of plasminogen to this system resulted in a small but significant increase of released radiolabeled ECM fragments over the three days (condition B). The addition of plasminogen and pro-MMP-3, however, enhanced the ECM degradation 3-fold over the culture of cells alone (C). This enhanced ECM degradation was due to MMP-9 activation as shown by the resulting inhibition using the specific anti-MMP-9 monoclonal antibody (condition D) and TIMP-1 (condition E). The enhanced solubilization of radiolabeled ECM that is sensitive to the anti-MMP-9 antibody is apparently the result of active MMP-9 degrading the collagens components of the ECM. Since the smooth muscle ECM does not contain type IV or type V collagen, the known native substrates of MMP-9, the observed degradation is likely the result of MMP-9 acting catalytically on unfolded or partially denatured collagens I and III, the major collagens found in this ECM.

MMP-9 Activation Results in Enhanced Tumor Cell Invasion of Basement Membrane—Transmigration of cells across a complex basement membrane is often used as an indicator of the invasive behavior of cells. The migratory and invasive ability of MDA-MMP-9 cells were examined in a series of Matrigel invasion assays to determine the relative contribution of MMP-9 activation to this translocation process (Fig. 10). MMP-9 conversion and the generation of MMP-9 gelatinase activity were monitored using samples of culture medium that were removed directly from the upper compartment of the invasion chamber during the assay (Fig. 10, top). In the absence of plasminogen and pro-MMP-3, 2600 cells, approximately 5% of the inoculated MDA-MMP-9 cells, transmigrated across the filter in 48 h (Fig. 10, column 1). In the presence of either plasminogen or pro-MMP-3, 7–8% of the inoculated cells transmigrated across the filter (columns 2 and 3). These levels of cellular invasion were accompanied by a small generation of gelatinase activity (418 and 595 cpm, respectively) above background levels (267 cpm). When plasminogen and pro-MMP-3 were both added to the culture chamber, 6000 cells or ~12% of the inoculated cells transmigrated across the filter in 48 h (column 4). This enhanced transmigration was accompanied by conversion of pro-
MMP-9 to the 82-kDa form of the enzyme (Fig. 10, zymograph) and a substantial increase in gelatinase activity (3056 cpm). The cellular transmigration and gelatinolytic activity were inhibited by the anti-MMP-9 monoclonal antibody (column 5) and TIMP-1 (column 6), indicating a specific role for MMP-9 and its activation in the invasion process.

**DISCUSSION**

In the present study, we have utilized a cell culture model in an attempt to recapitulate some of the biochemical events that might take place in an invasive tumor when active MMP-9 is generated. A critical feature of the model is that the natural regulators of MMP function, the TIMPs, are present endogenously in nanomolar amounts. A second feature of the model is that it has the capacity to generate an additional protease system, namely the uPA/plasmin cascade, that has been closely linked to the migratory, invasive phenotype (7) and also linked functionally to the activation of a number of MMPs including MMP-9 (55). Our initial analysis of this culture system (Figs. 1 and 2) illustrates the inherent stability of the pro-MMP-9zymogen and its apparent resistance to activation even when the levels of MMP-9 are increased by cytokine treatment, when plasminogen is added to the cultures and when active MMP-3 is generated. Since active plasmin was demonstrated to be present in the cultures and at sufficient catalytic levels to activate pro-MMP-3, plasmin did not appear to be an effective activator of pro-MMP-9. However, MMP-3 had been shown in vitro to be an efficient pro-MMP-9 activator (32), and the question arose as to why the newly generated MMP-3 did not activate the available pro-MMP-9. Determining the total TIMP (4 nM) present in the cultures and comparing it with the amount of available pro-MMP-9 (0.25 nM) indicated that sufficient TIMP-1 was present to complex all of the pro-MMP-9 and MMP-9 to the 82-kDa form of the enzyme (Fig. 10, zymograph) and a substantial increase in gelatinase activity (3056 cpm). The cellular transmigration and gelatinolytic activity were inhibited by the anti-MMP-9 monoclonal antibody (column 5) and TIMP-1 (column 6), indicating a specific role for MMP-9 and its activation in the invasion process.

**FIG. 8.** The cell-associated MMP-9 appears to require the same proteolytic cascade for generating a low level of activated gelatinase. Cultures of MDA-MMP-9 (2 x 10⁷ cells/plate) were incubated in serum-free DMEM in the absence or presence of the indicated components. After 24 h, the conditioned media (5 ml) were harvested, and cell extracts were prepared and concentrated by gelatin-Sepharose chromatography (to 0.15 ml) as described under “Materials and Methods.” All samples were analyzed for pro-MMP-9 processing by subjecting 1 ml to gelatin zymography. Samples were analyzed for pro-MMP-9 activation by subjecting 75 ml to a 44-h [³H]gelatin degradation assay. Total gelatinase activity in enzyme units was calculated for each conditioned medium and cell extract. Gelatinase units are defined as 100 cpm released per hour.

**FIG. 9.** Degradation of ECM by MDA-MMP-9 cells is enhanced upon induction of the protease cascade that activates endogenous pro-MMP-9. MDA-MMP-9 cells were plated onto radiolabeled, modified ECM (see “Materials and Methods”) and incubated in the absence or presence of plasminogen (plg., 2.5 µg/ml), pro-MMP-3 (16 nM), anti-MMP-9 IgG (30 µg/ml), and TIMP-1 (40 nM) singly or in the indicated combinations. Samples of conditioned medium (50 µl) were collected on days 1, 2, and 3, and the soluble [³H]-labeled peptides released from the ECM were measured in a β scintillation counter. The bar graphs represent the mean counts/min ± S.D. released from triplicate samples in a single experiment.

MMP-9 to the 82-kDa form of the enzyme (Fig. 10, zymograph) and a substantial increase in gelatinase activity (3056 cpm). The cellular transmigration and gelatinolytic activity were inhibited by the anti-MMP-9 monoclonal antibody (column 5) and TIMP-1 (column 6), indicating a specific role for MMP-9 and its activation in the invasion process.
for the conversion of 55-kDa pro-MMP-3 to the 45-kDa active MMP-3. When both plasminogen and pro-MMP-3 were added to the cultures, the tumor cell uPA activated the plasminogen, the generated plasmin activated the pro-MMP-3, and then active MMP-3, when it exceeded the concentration of TIMP, converted 92-kDa pro-MMP-9 to 82-kDa active MMP-9.

In studies of zymogen activation by proteases, it would seem important to distinguish true enzyme activation from proteolytic conversion of the proenzyme to smaller but catalytically inactive forms. Enzyme activity measurements in solution were critical in the present study, since distinct lower molecular mass forms of MMP-9 were generated upon addition of plasminogen alone or pro-MMP-3 alone (Fig. 6). These processed forms were active in gelatin substrate gels (zymographs) but were not active in solution, and thus true activation of MMP-9 did not occur under these conditions. In addition, a lower molecular mass form of MMP-9 was observed in tumor cell extracts (Fig. 8), and its similar zymographic position to the 82-kDa active MMP-9 suggested that it represented a cell-associated form that had been activated. However, enzyme activity measurements on the isolated cellular MMP-9 indicated that this lower molecular mass form was not an active enzyme but behaved like a zymogen. Other laboratories also have shown that certain lower molecular weight forms of MMP-9 are not enzymatically active (41, 42, 53). Toth et al. (53) demonstrated that a cell-associated 85-kDa species of MMP-9 was active in zymographs but enzymatically inactive in solution. The 85-kDa form represented a different glycosylated variant of pro-MMP-9. Ogata et al. (41) showed that an 86-kDa processed form of MMP-9 was zymographically active but not enzymatically active in solution. This 86-kDa form represented a species that had been proteolytically processed at the Glu41-Met42 site in pro-MMP-9, 47 residues upstream of the actual activation site. A number of studies (38, 56–58), however, have relied mainly on zymographic or immunoblot detection methods and implied that the appearance of distinct lower molecular mass forms of MMP-9 represented activation of the zymogen. In some of these studies plasmin was implicated as a direct activator of pro-MMP-9 (57, 58). In the culture system described herein, plasmin indeed can generate lower molecular mass forms of MMP-9 but does not yield activated enzyme. The plasmin involvement in MMP-9 activation in this system is only indirect; it functions within an interacting cascade to generate active MMP-3. This indirect role of plasmin and the apparent requirement for plasminogen in the cascade is more clearly illustrated when already activated MMP-3 is added directly to the tumor cell culture medium (Fig. 7). The requirement for plasminogen and pro-MMP-3 in the cascade is circumvented as active MMP-3 directly generates the 82-kDa MMP-9 and in turn generates significant gelatinase activity.

In contrast to the present studies, Mazzieri et al. (52), using HT1080 cultures, concluded that plasmin could process and activate pro-MMP-9 (and also pro-MMP-2) in the absence of any detectable MMP-3. The plasmin that appeared to be responsible for this MMP activation was cell surface-activated plasmin, and the gelatinases that were activated were apparently associated with the cell surface. Since most of our studies involve the secreted, soluble pro-MMP-9, which could be readily activated by MMP-3 and could not be activated at all by plasmin, the apparent contrasting results may reflect cell-associated activation versus solution phase activation. However, when the tumor cell-associated MMP-9 was examined (Fig. 8), it was found that little if any active MMP-9 was associated with the cells, that plasmin did not enhance the level of active MMP-9 that became cell-associated, and that when a detectable level of active MMP-9 was found associated with the cells...
very favorable catalytic parameters describe MMP-3's efficient activation in the tissues where MMP-9 activation occurs. However, since we noted, however, that MMP-9 activation in (62) was monitored to be equal to the level observed in the injured rather than normal stromal tissue, Heppner et al. (1996) employed levels of MMP-3 often found with inflammation, wound repair, and tumor infiltration (1–3) might represent a stromal source of this zymogen. Indeed the concentration of MMP-3 was higher than that which might be found in normal stromal cells do not produce MMP-3 (Fig. 3), and indeed a majority of cells do not produce MMP-3 (Fig. 3), and indeed a majority of cells do not produce MMP-3 (Fig. 3), and indeed a majority of cells do not produce MMP-3 (Fig. 3), and indeed a majority of

It should be emphasized that MMP-3 may not be the sole natural activator of pro-MMP-9. In the injured arteries of homozgyous MMP-3-deficient mice, some MMP-9 activation occurs approximately equal to the level observed in the injured arteries of wild-type mice, and it was concluded that MMP-9 activation does not depend solely on MMP-3 (62). It should be noted, however, that MMP-9 activation in (62) was monitored by the appearance of lower molecular mass forms of MMP-9. Nevertheless, MMP-3 probably will not be found in all the tissues where MMP-9 activation occurs. However, since very favorable catalytic parameters describe MMP-3's efficient activating ability in vitro (32), and since active MMP-3 can be generated by a widely distributed serine protease cas-cade (Fig. 6), and since MMP-3 can effectively generate a pro-

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