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Authors
Deryugina, E I
Bourdon, M A
Luo, G X
et al.

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Matrix metalloproteinase-2 activation modulates glioma cell migration

Elena I. Deryugina[1], Mario A. Bourdon[1], Guang-Xiang Luo[2], Ralph A. Reisfeld[2] and Alex Strongin[1,*]

[1]La Jolla Institute for Experimental Medicine, La Jolla, CA, USA
[2]The Scripps Research Institute, La Jolla, CA, USA

*Author for correspondence (e-mail: strongin@ljiem.org)

SUMMARY

Stable transfection of U251.3 glioma cells with cDNA encoding MT-MMP-1 resulted in increased cell surface expression of MT-MMP-1 and TIMP-2, constitutive activation of MMP-2 proenzyme and increased collagen degradation. In tumor spheroid outgrowth assays, cell migration of MT-MMP-1 transfectants relative to control was enhanced on collagen and decreased on vitronectin and fibronectin. These effects were reversed by TIMP-2 and were not associated with any substantial changes in cell adhesion. Binding of U251.3 cells to the C-terminal domain of MMP-2 was specifically inhibited by anti-$\alpha_v\beta_3$ integrin blocking antibody indicating that MMP-2 interacts with $\alpha_v\beta_3$ through the enzyme's C-terminal portion at or near the integrin's matrix adhesion sites. We propose that these mechanisms could govern directed matrix degradation in the tumor cells' microenvironment by sequestration of active MMP-2 on the cell surface. Our data suggest that activation of MMP-2 and its proteolytic activity localized to the cell surface could differentially modulate tumor cell migration in response to particular matrix proteins by altering both composition of the extracellular matrix and expression of adhesion receptors on the cell surface.

Key words: Matrix metalloproteinase, MMP-2, MT-MMP-1, TIMP-2, Extracellular matrix, Integrin, Cell migration, Cell adhesion

INTRODUCTION

The integrity of the extracellular matrix (ECM) is of critical importance for the maintenance of normal tissues. Matrix metalloproteinases (MMPs) have been implicated in remodeling of the ECM and penetration of normal and tumor cells through tissue barriers (Birkedal-Hansen et al., 1993; Stetler-Stevenson et al., 1993; Behrendtsen and Werb, 1997). MMPs including MMP-2, the most abundant among all MMPs, are secreted in a latent zymogen form that requires activation (Woessner, 1994). This activation is a critical event in the regulation and the subsequent ECM degradation by MMPs (Aznavoorian et al., 1993; Yamamoto et al., 1996).

The activity of MMPs is primarily regulated by tissue metalloproteinase inhibitors-1,-2,-3,-4 (TIMPs) (Greene et al., 1996, and references therein). TIMP-2 exerts activity against several MMPs, although preferentially against MMP-2, by binding both its latent and activated forms (Goldberg et al., 1989; Howard et al., 1991). In contrast to all other secretory MMPs, MMP-2 proenzyme is resistant to soluble proteinases (Strongin et al., 1993) and is converted to the mature enzyme by mechanisms involving the recently identified membrane-type matrix metalloproteinases-1,-2,-3 (MT-MMPs) associated with the cell surface (Sato et al., 1994; Okada et al., 1995; Takino et al., 1995; Will and Hinzmann, 1995). The MT-MMP-1-driven activation of MMP-2 critically depends on a specific binding of TIMP-2 to cell surface MT-MMP-1 generating a heteromolecular receptor for MMP-2 proenzyme (Strongin et al., 1995).

MMP-2 activation has been directly correlated with the aggressiveness of tumor cells (Birkedal-Hansen et al., 1993; Coussens and Werb, 1996; Yamamoto et al., 1996). Inhibition of endogenous MMP-2 by an overexpression of TIMP-2 in human melanoma cells resulted in altered cell behavior in vitro (Ray and Stetler-Stevenson, 1994, 1995) and reduced tumor growth in mice (Montgomery et al., 1994). However, the effects of MMP-2 activation and its regulation at the level of MT-MMP-1 expression and complex formation with TIMP-2 have not been thoroughly addressed in cell functional assays.

Degradation of the ECM in the cells' microenvironment is thought to be critical for tumor migration and invasion. Thus, the role of integrins that mediate cell-ECM interactions by transducing multiple complex signals from the extracellular compartment into cell is particularly intriguing. These signals trigger changes in gene expression, including genes of the MMP family (Ashkenas et al., 1994; Tremble et al., 1995). In this regard, it was demonstrated that signaling by $\alpha_5\beta_1$ and $\alpha_4\beta_1$ integrins in response to fibronectin play a dominant role in regulating metalloproteinase genes in sinovial fibroblasts (Huhtala et al., 1995). The level of fibroblast collagenase in tumor cells is regulated by the collagen receptor $\alpha_2\beta_1$ integrin (Riikonen et al., 1995). Treatment of SNB19 and U251 glioblastoma cell lines with $\alpha_3\beta_1$ specific antibodies increased the MMP-2 activity and Matrigel invasion (Chintala et al., 1996). Recently, Brooks et al. (1996) demonstrated that proteolytically active MMP-2 directly binds integrin $\alpha_v\beta_3$, thereby inhibiting adhesion of $\alpha_v\beta_3$-transfected melanoma cells to vitronectin. Consequently, binding of activated MMP-2 to cell surface receptors may preferentially affect the recognition of specific ECM components by tumor cells and substantially modify cellular responses to the ECM.

Here, we study how the MT-MMP-1/TIMP-2 regulated MMP-2 activation affects the migration of U251.3 glioma cell line. Recently, this cell line has been characterized in adhesion and migration assays (Deryugina and Bourdon, 1996; Deryugina et al., 1996) and shown to produce MMP-2 proenzyme which is not activated under regular culture conditions. The constitutive activation of MMP-2 proenzyme was triggered by stable transfection of U251.3 cells with MT-MMP-1. The MT-MMP-1 cell transfectants and control cells were characterized for the expression of surface receptors and in cell function assays including matrix degradation, cell adhesion and cell migration. The results of our studies demonstrate that activation of MMP-2 proenzyme and accumulation of activated MMP-2 differentially modulate the tumor cell motility in response to major ECM components as collagen type I, vitronectin, and fibronectin. In addition, we show that U251.3 cells bind the C-terminal domain of MMP-2 through the functionally active site(s) of $\alpha_v\beta_3$ integrin. We conclude that the modulatory effects of MMP-2 activation on tumor cell migration could be attributed to both matrix degradation and alterations of adhesion receptors on the cell surface.

MATERIALS AND METHODS

Antibodies and reagents

Murine function-blocking monoclonal antibodies (mAbs) against integrins α_2 (P1E6), α_3 (P1B5), α_5 (P1D6), $\alpha_v\beta_3$ (LM609), and $\alpha_v\beta_5$ (P1F6), and non-blocking β_1 integrin specific mAb (JB1a) were from Chemicon International (Temecula, CA). Blocking anti-β_1 integrin mAb (P4C10) was from Gibco BRL (Gaithersburg, MD). Murine anti-α_v blocking mAb L230 (ATCC) was purified from serum-free supernatant. Control mAb produced by murine myeloma 45.6 (ATCC) was purified from ascitic fluid. FITC-conjugated F(ab′)$_2$-fragments of sheep anti-mouse and goat anti-rabbit IgG antibodies were from Sigma (St Louis, MO). Murine mAb specific for human TIMP-2 was from Calbiochem (San Diego, CA). Affinity purified rabbit anti-human MT-MMP-1 polyclonal antibodies generated against a peptide sequence of the hinge region of the enzyme were a kind gift of Triple Point Biologics (Forest Grove, OR). Human tenascin and fibronectin were purified from culture supernatant conditioned by U251.3 cells (Sriramarao et al., 1993) and human plasma (Ruoslahti et al., 1982), respectively. Collagen type I and vitronectin were from Sigma. ^{3}H-labeled collagen type I (0.2 mCi/mg) was from Du Pont NEN (Boston, MA) and human recombinant TIMP-2 was from Calbiochem (San Diego, CA).

Purification and N-terminal amino acid sequencing of MMP-2

MMP-2 proenzyme produced by U251.3 cells was purified by Red-agarose and gelatin-agarose affinity chromatography from conditioned medium (Strongin et al., 1993). Prior to chromatography, the medium was ultrafiltered through a 300 kDa cut-off membrane in a Pellicon tangential flow apparatus (Millipore, Bedford, MA). The isolated proenzyme was dialyzed against 5 mM Tris-HCl buffer, pH 7.5, 0.1 mM $CaCl_2$, 0.005% Brij-35 (Pierce, Rockford, IL) and stored at −70°C. For further analysis, the purified preparation was electrophoresed under non-reducing conditions on 14% acrylamide gel and transferred to a PVDF membrane (Millipore). After staining, the major bands were excised and sequenced using a 470A protein sequencer (Applied Biosystems).

Cell lines and cultures

The glioma U251.3 cell line was cloned from the parental U251MG line by limiting dilution. Cells were routinely maintained in DME medium (Gibco) supplemented with 10% FCS (Tissue Culture Biologicals, Tulare, CA) (DMEM/FCS). For experiments, 3×10^6 cells were seeded in a 100 mm Petri dish in DMEM/FCS. After overnight incubation, the medium was changed for 6 ml serum-free DMEM. Following an 18 hour incubation, conditioned medium was collected and analyzed immediately or stored at −20°C. Cultures were washed with PBS, rocked on a gyratory shaker in PBS at 37°C for 15-20 minutes. Thereafter, cells were detached by gentle pipetting. Where indicated, serum-free AIM-V medium (Gibco) was used in further cell assays as it supports cell growth, does not cause apoptosis or contain MMPs and TIMPs.

Expression of the MT-MMP-1 and the C-terminal domain of MMP-2

The MT-MMP-1 cDNA was synthesized by reverse-transcription using a total mRNA pool from HT-1080 human fibrosarcoma cells. Amplification of MT-MMP-1 coding sequences was performed by PCR with Pfu DNA polymerase (Stratagene, La Jolla, CA). The full length MT-MMP-1 cDNA was obtained by inserting the 3′-end cDNA *Eco*RI/*Xho*I fragment and 5′-end cDNA *Xho*I/*Xba*I fragment into the *Eco*RI/*Xba*I site of pBluescript SK plasmid (Stratagene). The sequence of the full length cDNA was verified and submitted to GenBank (accession number U41078). The cDNA fragment encoding the full-length MT-MMP-1 was inserted into the *Eco*RI/*Xba*I sites of the eukaryotic pcDNA3 plasmid (Invitrogen, La Jolla, CA) under the control of CMV promoter and the resulting pcDNA3-MT-MMP-1 plasmid was used for cell transfections.

The coding sequence of the C-terminal domain of MMP-2 was amplified by PCR from the MMP-2 full length cDNA (Goldberg et al., 1989) with a forward primer, starting from Leu-444, and a reverse primer starting from the stop codon. After DNA sequencing, the proper cDNA fragment encoding the C-terminal domain of MMP-2 was recloned into the *Hin*dIII/*Xho*I sites of the pFLAG-ATS expression vector (IBI, New Haven, CT). The resulting vector coding the fusion protein FLAG/C-terminal domain of MMP-2 was transfected into *Escherichia coli* DH5a cells (Gibco) and the fusion protein was purified from the periplasmic fraction by immunoaffinity chromatography (Strongin et al., 1995).

Cell transfection

Stable transfections with the pcDNA3-MT-MMP-1 plasmid (2 μg per 1.5×10^5 cells seeded in a well of 6-well plate) were done with the Lipofectamine reagent (20 μl per culture) as recommended (Gibco). Individual colonies of cells resistant to 0.5 mg/ml of G418 (Sigma) were picked up 10-20 days after transfection and transferred into wells of a 24-well plate. After reaching confluency, medium in the wells was changed for 0.3 ml serum-free DMEM supplemented with 0.25 mg/ml G418 (DMEM/G418). Following overnight incubation, the conditioned medium of each individual culture was analyzed by gelatin zymography. The MT-MMP-1 cell transfectants efficient in MMP-2 activation were selected and cloned by limiting dilution. In parallel, a control cell line was generated by stable transfection of parental U251.3 cells with the pcDNA3 vector. Gelatin zymography confirmed that control cells did not activate MMP-2. Transfectants were routinely grown in DMEM/FCS supplemented with 0.25 mg/ml G418.

Cell adhesion assay

Cell adhesion assays were done as described (Deryugina and Bourdon, 1996). Wells of high-binding 96-well plates (Costar) were coated with the individual ECM proteins at 4°C overnight at 1-10 μg/ml PBS and blocked with 1% BSA. A total of 4×10^4 cells were added per well in 100 μl of DMEM/1% BSA. Cells were allowed to attach for 45 minutes at 37°C in a CO_2 incubator. In the assays employing the recombinant C-terminal domain of MMP-2, the protein was coated onto the wells at 20 μg/ml. Subsequently, 7.5×10^4

cells/well in 100 µl serum-free AIM-V medium were plated with and without anti-integrin blocking mAbs and allowed to attach for 24 hours. After incubation, plates were gently washed with Dulbecco's PBS containing Ca^{2+}/Mg^{2+} (DPBS). Adherent cells were fixed and stained with 0.2% Crystal Violet in 10% ethanol. Following three washes with PBS, the dye was extracted with 50 mM sodium phosphate, pH 4.5, in 50% ethanol and the absorbance was measured at 540 nm.

Spheroid cell migration assay

Two-dimensional spheroid outgrowth assays were performed as described (Deryugina and Bourdon, 1996). Briefly, tumor spheroids were prepared from single cell suspensions. Spheroids with similar diameter were selected and placed in 100 µl serum-free AIM-V medium per well of 96-well Costar plates pre-coated overnight at 4°C with individual ECM proteins at 10 µg/ml. Where indicated, TIMP-2 was added to the medium at a final concentrations of 300 ng/ml. As spheroids with diameter of ~200 µm comprised of ~1×10^3 cells and 5 to 8 spheroids were plated per well, the amount of exogenous TIMP-2 in migration assays corresponded to ~4-6 pg/cell. Following overnight incubation, the radial distance of migration was determined after subtraction of the initial spheroid diameter at time zero from the diameter of the area covered with cells migrated from spheroids. There was no cell migration on BSA coated matrices.

Flow cytometry

Fluorescence-activated cell sorting (FACS) was performed as described (Deryugina et al., 1996). Where indicated, cultures were incubated in serum-free DMEM in the presence of 1 µg/ml (48 nM) TIMP-2 corresponding to ~1-2 pg TIMP-2 per cell. Staining procedures were done on ice in DPBS, 1% BSA, 0.02% sodium azide. After an overnight incubation in DMEM/G418, cells were detached with PBS and incubated with primary antibodies for 45-60 minutes. Following washing, cells were incubated for 30 minutes with the corresponding secondary FITC-conjugated $F(ab')_2$ fragment of sheep anti-mouse or goat anti-rabbit IgG antibodies. After washing, cells were resuspended in the above buffer supplemented with 3 µg/ml of propidium iodide (Sigma) and analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Population gates were set using cells incubated with control murine myeloma mAb 45.6.

Gelatin zymography

Gelatin zymography was done as described (Strongin et al., 1995). Cell conditioned medium was mixed 1:1 with 2× SDS sample buffer and 10 µl of the mixture was loaded into a well of the precast 10% zymogram gel copolymerized with 0.1% gelatin (Novex, San Diego, CA). After electrophoresis, gels were washed in 2% Triton X-100 for 30-60 minutes at room temperature, incubated overnight at 37°C in 50 mM Tris buffer, pH 8.0, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, and stained to visualize bands of gelatinolytic activity.

Collagen type I degradation assay

Wells of high binding 96-well plates (Costar) were coated with 0.25 µg/50 µl PBS of ^{3}H-labeled collagen type I (specific activity 0.2 mCi/mg) and dried overnight at room temperature. Plates were washed with PBS until free cpm reached basal levels. Cell cultures were seeded at 1×10^6 cells per 60 mm Petri dish in DMEM/FCS/G418. Following overnight incubation, the medium was changed for serum-free DMEM for 2 hours, then cultures were washed with PBS, and cells were detached after a short incubation in PBS at 37°C. A total of 7.5×10^4 cells in 150 µl DMEM/G418 per well of 96-well plate were incubated for 4 hours in a CO_2 incubator at 37°C. Where indicated, TIMP-2 was added at final concentrations of 0.004-1 µg/ml at the time of plating. Collagen degradation was assessed by measuring the cpm released in 50 µl of conditioned medium. Net collagen degradation was determined by subtracting mean cpm release into medium in the absence of cells.

Data analysis and presentation

For quantitative analysis the mean and s.e.m. were calculated. The significance of the data and the data interpolation were done with the Student's *t*-test using SigmaStat and Sigmaplot software programs (Jandel Corporation, Corte Madera, CA), respectively.

RESULTS

MMP-2 proenzyme produced by U251.3 glioma cells is non-covalently complexed with TIMP-2

U251.3 glioma cells secrete relatively high amounts of MMP-2 proenzyme which was revealed as a 68 kDa band by gelatin zymography (Fig. 1, lane A). As compared to a gelatinolytic activity of the purified MMP-2, 10^6 U251.3 cells produce about 10 ng MMP-2 proenzyme into serum-free DMEM per 1 hour. No other bands not related to the MMP-2 gelatinolytic activity were detected. Two protein bands with molecular masses of 68 and 21 kDa were revealed by SDS-PAGE of MMP-2 purified from the medium conditioned by U251.3 cells (Fig. 2). After transferring to a PVDF-membrane followed by N-terminal amino acid sequencing, the 68 kDa (X_1-X-A-P-S-P-I-I-K-F-P-G-D-V-A-P-K) and 21 kDa proteins (X_1-S-X-S-P-V-H-P-Q-Q-A-F) were identified as MMP-2 proenzyme and TIMP-2, respectively. Thus, MMP-2 proenzyme secreted by U251.3 cells formed a non-covalent complex with TIMP-2 and is not activated under regular culture conditions. Fall-through fractions from gelatin-agarose column contained minute amounts of free TIMP-2 while TIMP-2/MMP-2 proenzyme complex was retained by the column, suggesting that MMP-2 proenzyme in the medium conditioned by U251.3 cells was almost fully saturated with TIMP-2.

U251.3 cells transfected with MT-MMP-1 efficiently activate MMP-2 proenzyme

As MT-MMP-1 triggers cell-associated MMP-2 activation (Sato et al., 1994; Strongin et al., 1995), we decided to overexpress MT-MMP-1 to generate cell lines able to convert MMP-2 proenzyme into mature enzyme forms. Thus, we stably transfected U251.3 parental cells with cDNA encoding the full length human MT-MMP-1 and specifically selected two MT-MMP-1 cell clones differing in degree of MMP-2 activation. The most efficient was UMTG1 cell clone which activated up to 95% of MMP-2 proenzyme. Less efficient MMP-2 activation was char-

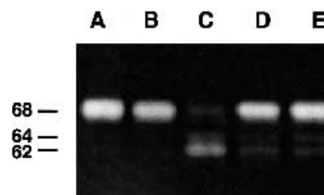

Fig. 1. U251.3 cells activate MMP-2 proenzyme upon transfection with the MT-MMP-1 cDNA. Samples of the medium conditioned by U251.3 cells were analyzed by gelatin zymography. Lane A, parental cell line. Lane B, control Uneo cells (U251.3 cell line transfected with a vector carrying the *neo* gene). Lanes C and D, the UMTG1 and UMT41 cell clones selected after transfection of U251.3 cells with MT-MMP-1 cDNA, respectively. Lane E, UMTG1 cells cultured overnight in the presence of 1 µg/ml TIMP-2. Size markers (kDa) are shown at the left.

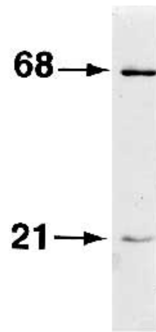

Fig. 2. U251.3 glioma cells produce MMP-2 as a non-covalent complex of the proenzyme and TIMP-2. MMP-2 was purified from the medium conditioned by U251.3 cells by Red- and gelatin-agarose chromatography. Purified material was electrophoresed under non-reducing conditions on 14% acrylamide gel and transferred to a PVDF membrane. Following sequencing, two protein bands with molecular masses of 68 and 21 kDa, were identified as MMP-2 proenzyme and TIMP-2, respectively.

acteristic of UMT41 cells which were able to convert about 15% of produced MMP-2 into active enzyme (Fig. 1, lanes C and D, respectively; Table 1). The Uneo control cell line obtained after transfection with the original vector lacking MT-MMP-1, did not activate MMP-2 (Fig. 1, lane B). All transfected cell lines produced MMP-2 in amounts similar to parental cells (Fig. 1; Table 1) and media conditioned by neo control and MT-MMP-1 transfected cells did not show any bands of gelatinolytic activity not related to MMP-2 including those which would correspond to MMP-9 or soluble MT-MMP-1.

MT-MMP-1 and TIMP-2 cell surface expression is increased in MT-MMP-1 cell transfectants

We further analyzed whether MMP-2 activation by U251.3 cell transfectants correlated with MT-MMP-1 expression. To this end, control Uneo and MT-MMP-1 transfected UMTG1 and UMT41 cells were each incubated with MT-MMP-1 specific antibodies followed by FACS analysis. The level of MT-MMP-1 cell surface expression was higher in the UMTG1 clone while Uneo cells expressed the lowest amounts of the enzyme and the UMT41 cell clone revealed intermediate expression of MT-MMP-1 (Table 1). Thus, the expression levels of MT-MMP-1 correlated well with the ability of each cell transfectant to activate MMP-2 proenzyme.

As TIMP-2 binds to cell surface MT-MMP-1 forming the MT-MMP-1/TIMP-2 heteromolecular complex essential for subsequent binding of MMP-2 proenzyme and MMP-2 activation (Strongin et al., 1993, 1995), we next assessed whether TIMP-2 levels on the cell surface of U251.3 transfectants correlated with MT-MMP-1 expression and MMP-2 activation. The levels of surface-bound TIMP-2 in UMTG1, UMT41, and Uneo cells were compared after staining with TIMP-2 specific mAb followed by FACS analysis. As shown in Table 1, the lowest level of TIMP-2 was observed on control Uneo cells characterized by lowest MT-MMP-1 expression and inability to activate MMP-2. In contrast, the UMTG1 cells distinguished by highest MT-MMP-1 expression and efficient MMP-2 activation, revealed about a seven-fold increase in cell bound TIMP-2. Accordingly, the UMT41 cells characterized by intermediate levels of MT-MMP-1 and partial activation of MMP-2 proenzyme, showed only a slight increase in bound TIMP-2 over control (Table 1). In addition, the preincubation of UMTG1 cells with an excess of exogenous TIMP-2 followed by staining with anti-TIMP-2 mAb, resulted in a further two-fold increase of cell bound TIMP-2, suggesting that TIMP-2-binding sites in the MT-MMP-1 overexpressing U251.3 cell transfectants were not fully saturated (Table 1).

MT-MMP-1 cell transfectants efficiently degrade collagen type I

We next examined whether MMP-2 activation by MT-MMP-1 transfected cells correlated with their efficiency of matrix degradation. The Uneo, UMTG1, and UMT41 cells were incubated for 4 hours in wells coated with ^{3}H-labeled collagen type I. Collagen cleavage was monitored by counting the radioactive soluble protein fragments released into the medium. The UMTG1 cells were 2.2-fold more efficient in collagen degradation while UMT41 cells showed only a 20%

Table 1. Detection of cell surface MT-MMP-1, TIMP-2, and $\alpha_v\beta_3$ integrin in U251.3 cell lines transfected with MT-MMP-1 or neo control cDNA

Cell transfectants*	MMP-2 activation†	Cell surface expression of		
		MT-MMP-1‡	TIMP-2‡	$\alpha_v\beta_3$‡
Uneo	0	54.7±8.1	25.5±13.1	195.2±11.0
UMT41	7.5	89.2±3.0 (§)	46.8±4.7	217.0±32.5
UMTG1	92.5	176.6±38.6 (§)	178.5±15.0 (§)	137.6±12.0 (§)
UMTG1+TIMP-2¶	15.0	nd‖	395.2±1.0 (§)	166.7±2.7

*U251.3 cells transfected with MT-MMP-1 cDNA (UMTG1 and UMT41) or control cDNA carrying the neo gene only (Uneo), were cultured overnight in serum-free DMEM and thereafter the cell surface MT-MMP-1, TIMP-2 and $\alpha_v\beta_3$ were detected by FACS, as described in Materials and Methods.

†Relative MMP-2 gelatinolytic activity in the medium conditioned by U251.3 cells was estimated by gelatin zymography. After densitometry of the gels, MMP-2 activation was expressed as percentage of the 68 kDa MMP-2 proenzyme converted to the 64-62 kDa enzyme forms.

‡Due to the differences in the intensity of staining, the data for anti-MT-MMP-1 antibodies are presented as mean ± s.e.m. of log fluorescence intensity determined in two independent experiments, and the data for TIMP-2 and $\alpha_v\beta_3$ specific mAbs are mean ± s.e.m. of linear fluorescence intensity determined in two to five independent experiments after subtracting the values obtained for staining with control mAb 45.6.

§Significance of difference ($P<0.05$) was estimated by comparing data for the MT-MMP-1 transfected cells with the Uneo control cells in the independent *t*-test.

¶UMTG1 cells were incubated in serum-free DMEM in the presence of 1 μg/ml TIMP-2.

‖Not determined.

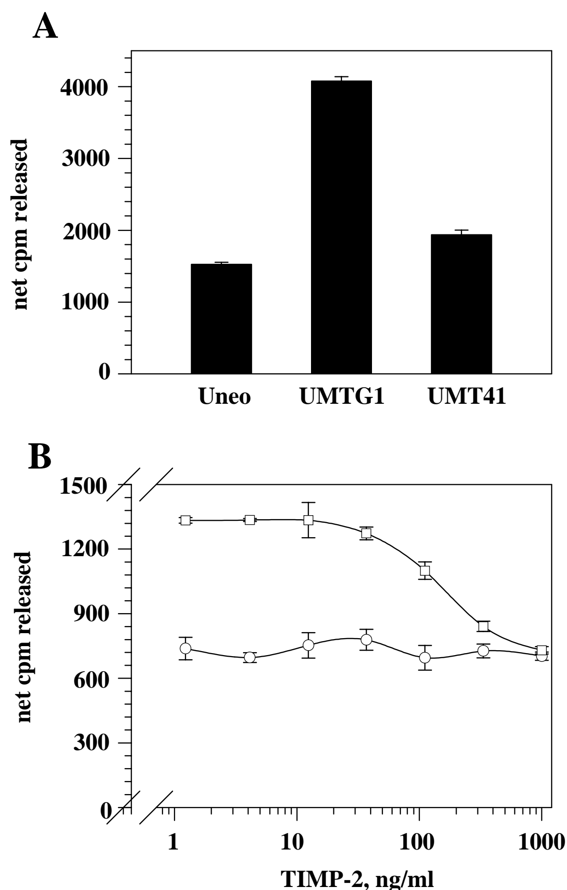

Fig. 3. U251.3 cell clones transfected with the MT-MMP-1 cDNA are efficient in collagen type I degradation. (A) Control Uneo cells and the MT-MMP-1 transfected cells, UMT41 and UMTG1, were incubated for 4 hours in wells coated with ^{3}H-labeled collagen type I. Collagen degradation was quantified by subtracting the cpm released into 50 µl of medium in control wells incubated without cells from data for each cell variant. Data represent mean ± s.e.m. from one of four independent experiments performed in quadruplicate.
(B) Increased degradation of collagen by UMTG1 cells is abolished by exogenous TIMP-2. Uneo control cells (open circles) and UMTG1 cells (open boxes) were incubated for 4 hours in wells coated with ^{3}H-labeled collagen type I with or without TIMP-2. Collagen degradation was quantified as described above.

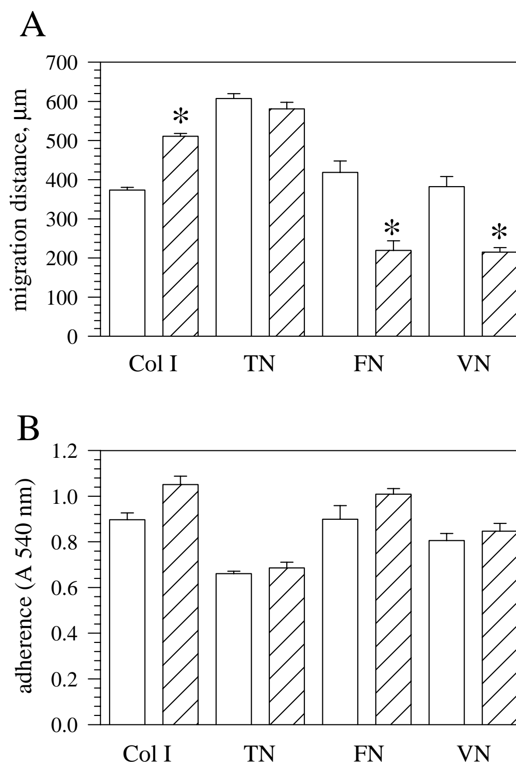

Fig. 4. MMP-2 activation by MT-MMP-1 transfectants differentially affects migration of U251.3 cells on individual ECM proteins. Tumor spheroids of Uneo (open bars) and UMTG1 (hatched bars) cell lines were plated into wells coated with 10 µg/ml of collagen type I (Col I), tenascin (TN), fibronectin (FN), and vitronectin (VN). Following overnight incubation, migration was measured as a distance traveled by cells from the edge of spheroid. Presented are data (mean ± s.e.m.) from one of four independent experiments. Significance of difference (*) was estimated by comparing data for the MT-MMP-1 transfected cells with the Uneo control cells in the independent *t*-test for $P<0.05$.

increase in collagen release compared to control Uneo cells (Fig. 3A). Thus, the efficiency of collagen degradation correlated well with levels of MT-MMP-1 expression, amounts of cell surface bound TIMP-2 and ability of U251.3 cell transfectants to activate MMP-2 proenzyme (Table 1).

We next assess whether an increased collagen degradation by MT-MMP-1 transfected cells could be attributed to activated MMP-2. As TIMP-2 is a potent inhibitor of MMP-2, we used it to monitor the effects of MMP-2 inhibition on collagen degradation. Exogenous TIMP-2 at concentrations from 1.4 to 1,000 ng/ml had no effect on Uneo cells plated on ^{3}H-labeled collagen but inhibited collagen degradation by UMTG1 cells in a dose-dependent manner to control levels (Fig. 3B). Even the highest concentration of TIMP-2 used in this study (corresponding to 2 pg/cell) did not affect collagen degradation by Uneo cells.

Migration of MT-MMP-1 transfected U251.3 cells on ECM proteins

Previously, we showed that U251.3 cells are highly motile and capable of migration on tenascin, fibronectin, and vitronectin (Deryugina and Bourdon, 1996; Deryugina et al., 1996). To evaluate the effects of MMP-2 activation on tumor cell migration, we compared UMTG1 and Uneo cell migration on several ECM proteins in a two-dimensional tumor spheroid outgrowth assay. Spheroids of UMTG1 and control Uneo cells were plated on matrices coated with collagen, tenascin, fibronectin, and vitronectin. The radial migration for the UMTG1 cells was over 120 µm higher on collagen compared to Uneo cells. In contrast, the migration of UMTG1 cells on fibronectin and vitronectin was significantly slower than Uneo cells, by 44% and 40%, respectively (Fig. 4). Notably, the migration mediated by tenascin which is relatively resistant to MMP-2 degrading activity (Siri et al., 1995) was similar for both Uneo and UMTG1 cell transfectants (Fig. 4). The pattern of migration observed for UMTG1 cells was reproduced in the preliminary assays with noncloned MT-MMP-1 transfectants confirming that the observed effects were not associated with

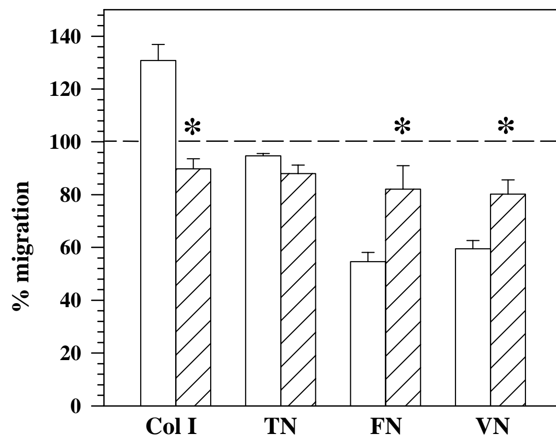

Fig. 5. TIMP-2 reverses differential effects of MMP-2 activation on UMTG1 cell migration. Tumor spheroids of Uneo and UMTG1 cells were plated into wells coated with 10 μg/ml of collagen type I (Col I), tenascin (TN), fibronectin (FN), and vitronectin (VN) either without (open bars) or with 300 ng/ml TIMP-2 (hatched bars). Following overnight incubation, migration distances were measured for each culture variant as a distance traveled by cells from the edge of spheroid. Data are presented as percent (mean ± s.e.m.) of UMTG1 cell migration compared to Uneo control cell migration (100%) determined in two to five independent experiments. Significance of difference (*) was estimated by comparing data for the MT-MMP-1 transfected cells cultured with and without TIMP-2 in the independent *t*-test for $P<0.05$.

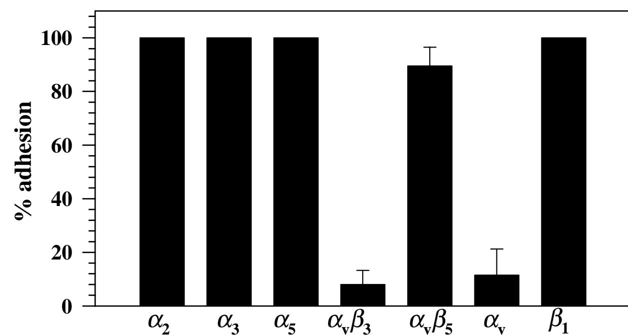

Fig. 6. Binding of U251.3 cells to the C-terminal domain of MMP-2 is inhibited by function blocking $\alpha_v\beta_3$ and α_v integrin specific mAbs. Uneo cells were incubated with and without specific integrin-blocking mAbs in wells coated with 20 μg/ml of the C-terminal domain of MMP-2. Following overnight incubation, adherent cells were stained with Crystal Violet, lysed and the absorbance measured at 540 nm. Maximal absorbance values ranged from 0.12 to 0.24 in independent experiments. The specificity of integrin specific mAbs is indicated at the bottom of the figure. Data are presented as percent of adherence determined for each integrin specific mAb in comparison with control mAb 45.6 (100%) and expressed as mean ± s.e.m. from one to three independent experiments.

unique features of the cell clone but rather with the acquired ability of cells to activate MMP-2 upon transfection.

To assess whether differences in migration of UMTG1 and Uneo cells were attributable to changes in adhesive properties of the transfectants, we assessed the adhesion of these cells to collagen, tenascin, vitronectin, and fibronectin in a standard cell adhesion assay. The results demonstrated a similar extent of adhesion by the UMTG1 and control Uneo cells on the substrata tested (data not shown). In addition, FACS analysis did not reveal any significant changes in the expression of integrins including α_2, α_3, α_5, α_v, $\alpha_v\beta_5$, and β_1 in both the UMTG1 and Uneo cells (data not shown). However, the $\alpha_v\beta_3$ integrin expression, as detected by the $\alpha_v\beta_3$ binding site specific mAb LM609, was reduced by 30-34% in the UMTG1 cells vs. Uneo control cells (Table 1).

Effects of TIMP-2 on migration of MT-MMP-1 transfected cells

We further determined whether inhibition of MMP-2 activation with TIMP-2 could reverse a rate of UMTG1 cell migration on ECM proteins. Thus, the Uneo and UMTG1 spheroids were plated on collagen, tenascin, fibronectin and vitronectin without and with TIMP-2 at 300 ng/ml. This concentration was considered to effectively block the activation of MMP-2 proenzyme produced by UMTG1 cells since it corresponded to ~4-6 pg/cell and thus, exceed 2-3 times the TIMP-2 concentration which completely blocked increased collagen degradation by UMTG1 cells over Uneo level (Fig. 3).

TIMP-2 did not alter migration of the Uneo control cells on any substrata tested, indicating that in our assays effects of TIMP-2 that were not associated with the inhibition of MMP-2 activation, were minor. However, when same amounts of TIMP-2 were added to UMTG1 cells, their migration on collagen decreased to a level characteristic for that of Uneo control cells (Fig. 4). In contrast, TIMP-2 partially increased migration of UMTG1 cells on vitronectin and fibronectin by 20 and 40%, respectively. Glioma migration on tenascin was unchanged in MT-MMP-1 cell transfectants with or without exogenous TIMP-2 (Fig. 5).

Activated MMP-2 competes for the adhesion site of $\alpha_v\beta_3$ integrin on UMTG1 cells

It has been shown recently that activated MMP-2 enzyme bound directly to $\alpha_v\beta_3$ integrin and that this interaction depended on the presence of the enzyme's C-terminal portion (Brooks et al., 1996). In our attempt to examine if MMP-2 binding inhibits cell adhesion sites of $\alpha_v\beta_3$ integrin, we first determined whether U251.3 cells could interact with the C-terminal domain of MMP-2 and, if so, whether $\alpha_v\beta_3$ integrin is essential for this interaction. To this end, we assessed the adhesion of Uneo control cells to the recombinant C-terminal domain of MMP-2 in the presence of integrin-specific blocking mAbs. As shown in Fig. 6, adhesion of the Uneo cells was almost completely blocked by anti-$\alpha_v\beta_3$ mAb LM609 and anti-α_v mAb L230 (92% and 90%, respectively). Other blocking mAbs directed against α_2, α_3, α_5, $\alpha_v\beta_5$, and β_1 integrin binding sites did not interfere with Uneo cell adhesion to the C-terminal domain of MMP-2. These results indicate that tumor cells could interact with the C-terminal domain of MMP-2 through $\alpha_v\beta_3$ and that the site on the integrin involved in this interaction is functionally important in mediating cell adhesion since it overlaps with the binding site for mAb LM609.

To show that the reduction in the number of $\alpha_v\beta_3$ sites available for binding anti-$\alpha_v\beta_3$ mAb LM609 on UMTG1 cells is associated with MMP-2 activation, we specifically compared $\alpha_v\beta_3$ expression on the surface of the UMTG1 cells incubated with and without exogenous TIMP-2 (Table 1).

TIMP-2 was used at 1 μg/ml (corresponding to ~2 pg/cell) which significantly decreased MMP-2 activation according to gelatin zymography (Fig. 1, lane E). FACS analysis showed that the addition of TIMP-2 increased the detected cell surface $\alpha_v\beta_3$ by 20% as compared to cells cultured without TIMP-2 (Table 1). Thus, activated MMP-2 appeared to bind $\alpha_v\beta_3$ on the cell surface and interfere with LM609 mAb binding to this integrin.

DISCUSSION

A coordination between proteolytic remodeling of the ECM and changes in cell adhesion receptors provide unique mechanisms for regulating directional cell motility. In this context, MMPs by potentiating ECM turnover and integrins by mediating cell adhesion to ECM proteins, should combine effectively to facilitate cell migration and invasion under normal and pathological conditions.

Cell surface-associated mechanisms involving MT-MMP-1 and TIMP-2 govern the activation of MMP-2 (Strongin et al., 1993, 1995; Sato et al., 1994). In the MMP-2 activation cascade, MT-MMP-1 was found to specifically bind TIMP-2 generating the MT-MMP-1/TIMP-2 complex which, in turn, functions as a receptor for MMP-2 proenzyme. The latter should be TIMP-2-free since it binds the heterodimer through the C-terminal domain (Murphy et al., 1992; Strongin et al., 1995; Ailenberg and Silverman, 1996). Upon activation, functionally active MMP-2 enzyme is released from the complex and could rebind cell surface by complexing $\alpha_v\beta_3$ integrin (Brooks et al., 1996). These unique mechanisms localize both MMP-2 activation and its activity to the vicinity of the cell surface making the overall amount of soluble MMP-2 physiologically irrelevant to the ability of cells to accomplish such functions as migration and invasion (Fig. 7).

Here, we determined the effects of constitutive activation of MMP-2 on tumor cell migration. These studies were done using the highly migratory human glioma cell line U251.3 (Deryugina and Bourdon, 1996). Similar to other gliomas (Nakano et al., 1995; Yamamoto et al., 1996), U251.3 cells express MT-MMP-1 and produce relatively high levels of MMP-2 and TIMP-2. All MMP-2 proenzyme secreted by U251.3 cells was found to be non-covalently complexed with TIMP-2. Since the TIMP-2/MMP-2 proenzyme complex resists the activation through cell surface MT-MMP-1 (Strongin et al., 1995), full saturation of MMP-2 proenzyme

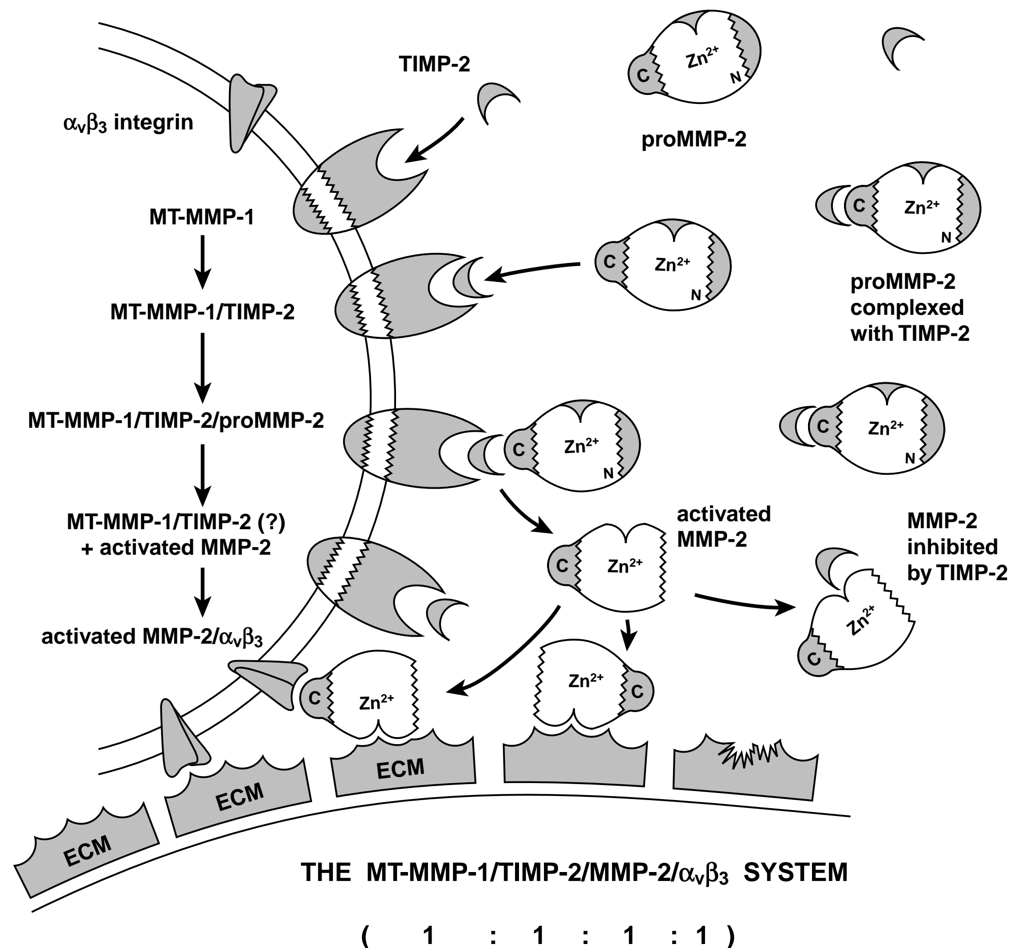

Fig. 7. Mechanisms of activation and regulation of MMP-2. The diagram schematically presents an interplay between molecules involved in the MMP-2 activation cascade and regulation of MMP-2 activity. MMP-2 proenzyme (proMMP-2) and TIMP-2 secreted by cells make the TIMP-2/proMMP-2 complex resistant to the activation through cell surface MT-MMP-1. TIMP-2 binds MT-MMP-1 generating the MT-MMP-1/TIMP-2 complex. This heteromolecular receptor binds the TIMP-2-free MMP-2 proenzyme via the proenzyme's C-terminal domain (C) forming the MT-MMP-1/TIMP-2/proMMP-2 complex. MT-MMP-1 initiates multistep cleavage which results in the deletion of N-terminal portion (N) of the proenzyme and activation of MMP-2. The fate of TIMP-2 in MT-MMP-1/TIMP-2 complex after release of activated MMP-2 is still unknown. Mature MMP-2 enzyme could directly degrade ECM potentiating cell migration and invasion. Alternatively, TIMP-2 binds catalytic domain of the activated enzyme (Zn^{2+}) thus inhibiting MMP-2 enzymatic activity. Through the enzyme's C-terminal domain, activated MMP-2 can also bind cell surface receptors, in particular $\alpha_v\beta_3$ (Brooks et al., 1996) competing adhesion sites of the integrin. This binding modulates integrin/ECM interactions and facilitate condensing of activated MMP-2 molecules on cell surfaces thus localizing its enzymatic activity to the tumor cell's microenvironment.

with TIMP-2 explains the inability of U251.3 cells to activate MMP-2.

Efficient activation of MMP-2 proenzyme by U251.3 cells was achieved by overexpression of MT-MMP-1 upon stable transfection of the parental cells with cDNA encoding human MT-MMP-1. In accordance with a concept of MMP-2 activation through the cell surface MT-MMP-1/TIMP-2 heteromolecular complex, the increased MT-MMP-1 expression in UMTG1 and UMT41 cells was associated with a proportional increase in cell surface bound TIMP-2. If total TIMP-2 production has not been changed in MT-MMP-1 cell transfectants, the sequestration of TIMP-2 on the cell surfaces accompanied by reduction in soluble TIMP-2, could lead to the appearance of TIMP-2-free MMP-2 proenzyme available for the activation through MT-MMP-1/TIMP-2. Western blotting confirms lower levels of TIMP-2 in media conditioned by MT-MMP-1 transfectants (data not shown).

In correlation with the level of activated MMP-2, MT-MMP-1 expression and cell surface bound TIMP-2, MT-MMP-1 transfected glioma cells were more efficient in degrading collagen than neo controls. MMP-2 has been shown to cleave native and denatured collagen type I (Aimes and Quigley, 1995). The basal levels of collagen degradation by Uneo cells were not affected by exogenous TIMP-2 even at the highest concentration used in this study apparently indicating the activity of TIMP-2-independent proteinases, for example of the uPA system found in U251 glioma (Rao et al., 1993; Salonen et al., 1996). This finding also demonstrates a lack of substantial contribution of other TIMP-2-sensitive MMPs which have been implicated in direct degradation of matrix proteins.

In contrast, the increased collagen degradation by MT-MMP-1 cell transfectants was TIMP-2 sensitive. Recently, transmembrane deletion mutants of MT-MMP-1 and soluble MT-MMP-1/TIMP-2 complex were shown to directly degrade different ECM molecules (Imai et al., 1996; Pei and Weiss, 1996). However, extensive gelatin zymography of medium conditioned by MT-MMP-1 transfected cell lines did not reveal any activity which would correspond to that of soluble MT-MMP-1. This clearly excludes putative soluble MT-MMP-1 from contributing to direct collagen degradation by MT-MMP-1 transfected cells. Moreover, the Uneo cells express MT-MMP-1 but were not affected even by the highest concentration of TIMP-2 which provided its tremendous excess on a molar level over cell surface MT-MMP-1. This finding apparently rules out also cell surface expressed MT-MMPs which would directly degrade collagen in our cell assay. We hope that a cell model in which the expression of all MMPs except MT-MMP-1 is shut down, would allow us to evaluate the ability of cell surface MT-MMP-1 to directly degrade ECM proteins. Thus, our data strongly suggest that activated MMP-2 is a major enzyme responsible for the increased collagen degradation as well as for TIMP-2 sensitive effects observed for MT-MMP-1 cell transfectants in other cell functional assays.

In tumor spheroid outgrowth assay, the migration of MT-MMP-1 cell transfectants was differentially altered on different ECM proteins as compared to neo control. When spheroids were plated on collagen, UMTG1 cells migrated more efficiently than Uneo cells. It has been proved that both too high and too low protein concentrations impede cell migration due to too tight and too weak cell adhesion, respectively (DiMilla et al., 1993). As collagen degradation by UMTG1 cells was more efficient, our results suggest that partial degradation of the collagen substratum could promote the rate of glioma cell migration. It can not be ruled out that the exposure of $\alpha_v\beta_3$ cryptic sites in degraded collagen (Montgomery et al., 1994) might also contribute to a higher migration rate of UMTG1 cells. In agreement with the above considerations, UMTG1 cell migration on native, full-length tenascin which is not extensively cleaved by MMP-2 enzyme (Siri et al., 1995), remained unchanged. While UMTG1 cell migration increased on collagen, the α_v integrin-mediated migration on vitronectin and fibronectin was substantially inhibited relative to Uneo cells.

The observed migration pattern of MT-MMP-1 transfected glioma cells was specifically reversed to that of neo control by exogenous TIMP-2 which abolished the increased migration of the UMTG1 cells on collagen and partially restored their migration on vitronectin and fibronectin. Since TIMP-2 did not affect migration of Uneo cells, our results strongly suggest that TIMP-2 modulated the migration of UMTG1 cells through the inhibiting activation of MMP-2 proenzyme and not through MMP-independent pathways (Murphy et al., 1993; Corcoran and Stetler-Stevenson, 1995).

Importantly, the observed effects in cell migration assays were not associated with changes in adhesion of the MT-MMP-1 transfectants to the ECM proteins tested. Accordingly, there were no significant differences in the integrin expression on Uneo and UMTG1 cells including α_2, α_3, α_5, $\alpha_v\beta_5$, total α_v and β_1 integrins. The only exception was $\alpha_v\beta_3$ integrin which expression was moderately but statistically significantly decreased not only in MT-MMP-1 glioma transfectants but also in other MT-MMP-1 transfected tumor cells, fibrosarcoma HT-1080 and breast carcinoma MCF-7, which achieved the ability to activate MMP-2 proenzyme (Deryugina et al., 1997). Since activated MMP-2 was shown to complex with $\alpha_v\beta_3$ (Brooks et al., 1996), both findings suggested rather a decreased detection of the integrin by LM609 mAb than significantly affected expression. Inhibition of MMP-2 activation by exogenous TIMP-2 partially restored the $\alpha_v\beta_3$ detection on UMTG1 cells. As $\alpha_v\beta_5$ but not $\alpha_v\beta_3$, is the major integrin mediating adhesion of glioma U251.3 cells to vitronectin and fibronectin in a standard short-term adhesion assay, this would explain the apparent contradiction between the unchanged adhesion of MT-MMP-1 cell transfectants and their reduced migration on these proteins since both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are important in mediating an overnight U251.3 migration on fibronectin and vitronectin (Deryugina et al., 1996). These findings correlate well with the results of other studies showing that different sets of integrins mediate the adhesion and migration of glioma cells, thus supporting the view that these processes may not be directly related (Friedlander et al., 1996, and references therein).

A direct interaction between purified $\alpha_v\beta_3$ and activated MMP-2 is dependent on the presence of the C-terminal domain of MMP-2 because such an interaction was not observed for the C-terminal domain truncated mutants of MMP-2 (Brooks et al., 1996). We extended these results by demonstrating that adherence of U251.3 glioma cells to the recombinant C-terminal domain of MMP-2 was $\alpha_v/\alpha_v\beta_3$ integrin-dependent. Moreover, since this binding was sensitive to inhibition with function blocking anti-$\alpha_v\beta_3$ integrin mAb LM609 and anti-α_v mAb L230 and was totally resistant to any of the other anti-integrin mAbs tested, we propose that functionally important

region(s) of $\alpha_v\beta_3$ integrin involved in cell adhesion are crucial for binding the C-terminal domain of MMP-2. Thus, the impaired α_v-dependent migration of the UMTG1 cells on fibronectin and vitronectin could be, at least in part, caused by the occupancy of $\alpha_v\beta_3$ integrin receptors with activated MMP-2 enzyme. In addition, our most recent results demonstrate that function-blocking α_v integrin mAbs decrease α_v-independent migration of UMTG1 cells on collagen whereas this effect was not observed for Uneo cells.

The results of our cell migration experiments suggest that the entire MMP-2 system which involves multiple and complex interactions between cell surface receptors, matrix proteins, MMPs and their inhibitors should be stoichiometrically balanced to achieve 1:1 molar ratios necessary for the stepwise complex interplay of at least 4 known components: MT-MMP-1, TIMP-2, MMP-2, and $\alpha_v\beta_3$ to maintain its optimal performance and facilitate efficient cell migration (Fig. 7). It appears that these mechanisms may be essential for a precise selection of activated MMP-2 from excess of MMP-2 proenzyme and necessary for the sequestration of functionally active enzyme forms on cell surfaces to control and maintain ECM proteolysis in the tumor cells' microenvironment. Thus, activation of MMP-2 and its proteolytic activity near the cell surface could differentially modulate the migratory and, consequently, invasive ability of tumor cells in response to particular ECM proteins both by altering the composition of the ECM surrounding the cells and the expression of cell surface adhesion receptors.

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