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FAST TRACK

PRINOMASTAT, A HYDROXAMATE INHIBITOR OF MATRIX METALLOPROTEINASES, HAS A COMPLEX EFFECT ON MIGRATION OF BREAST CARCINOMA CELLS

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Membrane type-1 matrix metalloproteinase (MT1-MMP) and αvβ3 integrin have been directly implicated in tumor cell dissemination and metastasis. We have demonstrated that in the case of breast carcinoma MCF7 cells co-expressing MT1-MMP and αvβ3 integrin, the proteinase processes the pro-αv integrin subunit, thus facilitating αvβ3 integrin maturation and cell migration on vitronectin. Our findings show that cell surface MT1-MMP is a short-lived protein with a life span in the range of several hours. In contrast, turnover of αvβ3 integrin is much slower. The half-life of αvβ3 heterodimer is about 24 hr. This large difference in life span allowed us to distinguish between the effects of MT1-MMP on cell migration brought by matrix proteolysis from those imposed through αvβ3 integrin maturation. We then modulated the enzyme's activity by a potent hydroxamate MMP inhibitor, Prinomastat (AG3340), to analyze the divergent effects of MT1-MMP on cell migration. Although Prinomastat immediately blocked MT1-MMP-mediated matrix degradation, the pool of MT1-MMP-modified αvβ3 integrin molecules was still capable of mediating cell-matrix interactions. To our considerable surprise, inhibition of MT1-MMP-dependent vitronectin proteolysis by Prinomastat allowed a several-fold increase in migration of MCF7 cells co-expressing MT1-MMP and αvβ3 integrin. In contrast, long-term Prinomastat inhibition of MT1-MMP-dependent pro-αv cleavage and thus αvβ3 integrin maturation strongly inhibited cell motility. Our studies suggest that MT1-MMP could actually promote cell migration via modification of the cell surface receptors, including αvβ3 integrin, rather than facilitate cell migration through direct cleavage of the matrix proteins.

Key words: *matrix metalloproteinases; membrane type-1 matrix metalloproteinase; hydroxamates; breast carcinoma; cell migration*

Matrixins or matrix metalloproteinases (MMPs) are multidomain zinc-dependent endopeptidases that share a common domain structure.[1] It is well documented that MMPs take an integral part in tumor dissemination and the metastatic cascade.[2] To exert enzymatic activity *in vivo*, individual MMPs require activation by Golgi network-associated proprotein convertases (PCs), such as furin, or by other members of the matrixin family.[3,4] About 20 MMPs are secreted as soluble enzymes into the extracellular milieu. However, recent evidence suggests that membrane-tethered MMPs, named the membrane-type MMPs (MT-MMPs), rather than soluble proteases are critical to directional cell migration and invasion.[5–7]

A transmembrane domain, anchoring the protease to the cell surface, and a carboxyl-terminal cytoplasmic tail, connecting the molecule with the intracellular compartment, characterize MT1-MMP, the most common enzyme of the MT-MMP subfamily.[8–10] The amino-terminal furin recognition motifs (RXK/RR) are believed to be important, but apparently are not absolutely essential, to the activation mechanisms of the latent zymogen of MT1-MMP.[3,11–13] The physiologic significance of MT1-MMP may involve its discrete surface localization in migrating cells that facilitates focalized pericellular proteolysis of a broad spectrum of extracellular matrix (ECM) components, including type I and II collagens, vitronectin, fibronectin, laminin, fibrin and proteoglycans.[14] MT1-MMP has also been also shown to induce activation of soluble proMMP-2 and proMMP-13[9,15] and through these MMPs to indirectly affect matrix degradation and cell locomotion.[16,17]

αvβ3 integrin has a unique role in normal and pathologic conditions, especially in cancer.[18–20] Similarly to many α integrin subunits, maturation of pro-αv chain (pro-αv) involves its cleavage by furin and other functionally related PCs to form the disulfide-bonded heavy and light α chains.[21] Recently, we have identified a PC-independent, MT1-MMP-mediated pathway of pro-αv maturation. Thus, MT1-MMP co-expressed with αvβ3 in MCF7 breast carcinoma cells directly processes pro-αv, thereby contributing to accumulation of the mature integrin.[22] Although MT1-MMP cleavage of pro-αv does not affect the RGD-ligand binding efficiency of αvβ3, the MT1-MMP-processed integrin is characterized by the enhanced outside-in signaling through a focal adhesion kinase (FAK) pathway.[23] Increased phosphorylation of FAK has been associated with high levels of migration on vitronectin of MT1-MMP/αvβ3-positive MCF7 cells.

To analyze the divergent effects of MT1-MMP on vitronectin-mediated migration of breast carcinoma MCF7 cells co-expressing MT1-MMP with αvβ3 integrin, we modulated activity of the enzyme by a potent hydroxamate MMP inhibitor Prinomastat (AG3340).[24,25] This MMP inhibitor is characterized by one of the lowest K_is for MMP-2 and MMP-9 (50–150 pM)[26] and MT1-MMP (300 pM).[25] Surprisingly, there was a bimodal relationship between the MT1-MMP activity, inhibited by AG3340, and the levels of MCF7 cell migration. Our results suggest the existence of 2 distinct biochemical mechanisms, *i.e.,* matrix degradation and integrin processing, by which the inhibition of membrane-associated MT1-MMP regulates cell motility. Thus, immediate inhibition by AG3340 of MT1-MMP-mediated vitronectin proteolysis was associated with an increase in cell motility, which continued until the MT1-MMP-processed αvβ3 integrin was internalized and replaced with conventional αvβ3 integrin, at which time cell migration significantly decreased.

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MATERIAL AND METHODS

Inhibitors, proteins and antibodies

ProMMP-2 was purified from medium conditioned by p2AHT2A72 cells (a derivative of HT-1080 fibrosarcoma cell line doubly transfected with E1A and proMMP-2).[9] Vitronectin purified from human plasma was a kind gift of Richard DiScipio (La Jolla Institute for Molecular Medicine, La Jolla, CA). Control murine and rabbit IgGs and rabbit anti-MT1-MMP antibodies (M3927) against the hinge region of human MT1-MMP were obtained from Sigma (St. Louis, MO), as were protease inhibitors (PMSF, leupeptin, pepstatin and aprotinin). Murine MAb LM609 specific to integrin αvβ3 was acquired from Chemicon (Temecula, CA). A recombinant catalytic domain of MT1-MMP (rMTcat) was purified and refolded as reported previously.[27,28] A broad-range hydroxamate inhibitor of MMP activity, Prinomastat (AG3340) was a helpful gift of Peter Baciu (Allergan Pharmaceuticals, Irvine, CA).

Cell culture

Breast carcinoma MCF7 cells were obtained from ATCC (Rockville, MD) and cultured in DMEM supplemented with 10% FCS and 10 μg/ml gentamicin (DMEM/FCS). The full-length human β3 integrin subunit (GenBank™ accession no. P05106) and the wild-type MT1-MMP (MT-WT; GenBank™ accession no. U41078) cDNAs were cloned into the pcDNA.3-neo (neo) and pcDNA.3-zeo (zeo) mammalian expression vectors (Invitrogen, San Diego, CA), respectively.[29] The catalytically inactive MT1-MMP mutant (MT-E240A) was generated by replacing the essential Glu-240 of the enzyme's active site with the Ala residue.[13] Transfection of cells was performed using Lipofectamine (Life Technologies, Rockville, MD) according to the manufacturer's instructions. To generate double transfectants, the parental MCF7 cells were first transfected with the full-length β3 integrin chain cDNA in the pcDNA.3-neo plasmid or with the original neo plasmid. Stably transfected cells were selected in DMEM/FCS supplemented with 0.2–0.8 mg/ml G418 (Life Technologies). Following staining with MAb LM609, cells expressing αvβ3 integrin (β3 cells) were sorted on a FACStar cell sorter (Becton Dickinson, Mountain View, CA). The β3 cells were next transfected with the original pcDNA.3-zeo plasmid or with the zeo plasmid carrying MT-WT or mutant MT-E240A to generate β3/zeo and β3/MT-WT or β3/E240A doubly transfected cells, respectively. Stable double transfectants were selected in DMEM/FCS supplemented with 0.6–0.8 mg/ml zeocin and 0.2 mg/ml G418, followed by sorting on a flow cytometer after staining with anti-MT1-MMP antibodies. Efficient expression of MT1-MMP and αvβ3 integrin in selected cells was additionally confirmed by immunoprecipitation and Western blotting. Transfection of neo cells with the original zeo vector was used to generate control neo/zeo cells. Routinely, doubly transfected cell lines were maintained in DMEM/FCS supplemented with 0.2 mg/ml each of G418 and zeocin.

Immunoprecipitation and western blotting

Cells were plated in DMEM/FCS and, where indicated, incubated for 24–72 hr with 10 μM AG3340 or 0.1% DMSO (solvent control). After incubation, cells were washed with Dulbecco's PBS (DPBS) supplemented with 1 nM $CaCl_2$ and 1 nM $MgCl_2$ and surface biotinylated with 0.2 mg/ml sulfo-NHS-LC-biotin in DPBS (Pierce, Rockford, IL). After incubation for 60 min, cells were washed with ice-cold DPBS and lysed with 50 mM *N*-octyl-β-D-glucopyranoside (Amresco, Solon, OH) in TBS supplemented with 1 mM $CaCl_2$, 1 mM MgCl2, and protease inhibitor cocktail containing 1 mM PMSF and 2 μg/ml each of aprotinin, pepstatin and leupeptin (OG buffer). The lysates were precleared with Pansorbin (Calbiochem, San Diego, CA). The precleared lysates (each containing 1.0 mg of total protein) were mixed with 3 μg of antiαvβ3 or anti-MT1-MMP antibodies and 15 μl Protein G Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ). After incubation for 14 hr at 4°C, Sepharose beads were washed with the OG buffer, then with PBS supplemented with 0.05% Tween 20 (PBS/Tw) and 0.5 M NaCl, and with PBS/Tw. Immune complexes were released by boiling the beads for 5 min in 2× SDS sample buffer (20 μl) supplemented with 50 mM DTT. After centrifugation, solubilized proteins were subjected to SDS-PAGE. Separated proteins were transferred to an Immobilon P membrane (Millipore, Bedford, MA). After blocking with 1% casein in DPBS/Tw, the membrane was probed with Extravidin-HRP (Sigma) and the TMB/M substrate (Chemicon).

Gelatin zymography

To evaluate the efficiency of MCF7 cells in MMP-2 activation, cells were plated in DMEM/FCS at 2×10^5 per well of a 24-well cluster. After incubation for 16 hr, the medium was exchanged for 0.3 ml/well serum-free DMEM supplemented with purified proMMP-2 (100 ng/ml). Where indicated, β3/MT-WT cells were incubated for 30 min in 0.15 ml serum-free DMEM supplemented with increasing concentrations of AG3340 (0.2–600 μM). Then, 0.15 ml of proMMP-2 (200 ng/ml) was added into each well, yielding the final concentrations of proMMP-2 100 ng/ml and AG3340 0.1–300 μM. After an 18–24 hr incubation, the aliquots of conditioned medium were mixed with an equal volume of 2× SDS sample buffer and 12 μl of the mixture were loaded per lane of a precast zymography gel (Novex, San Diego, CA). After electrophoresis, the gels were incubated in 2.5% Triton X-100 and then kept overnight at 37°C in the developing buffer (Novex). The bands of gelatinolytic activity were revealed after staining the gels with Coomassie blue.

Cell migration in transwells

The haptotactic migration of cells in Transwells (Costar, Cambridge, MA) was analyzed in serum-free AIM-V medium (Gibco, Gaithersburg, MD) essentially as described.[29,30] Briefly, the undersurface of a 6.5 mm insert membrane with an 8 μm pore size was coated overnight at 4°C with vitronectin at the concentrations indicated in the text. Cells detached with an enzyme-free buffer (Specialty Media, Lavalette, NJ) were washed twice in AIM-V medium and then plated into the insert at $5–50 \times 10^4$ cells in 0.15 ml AIM-V medium with 10 μM AG3340 or 0.1% DMSO. The outer chamber was filled with 0.6 ml of AIM-V medium supplemented with the same ingredient as used for the inner chamber. Where indicated, the cells were preincubated for 24–72 hr in DMEM/FCS with 10 μM AG3340 or 0.1% DMSO and then plated into Transwells in AIM-V medium. The same concentrations of inhibitor or DMSO were added both into the inner and outer chambers of the Transwells. After incubation for 48 hr, cells that migrated to the membrane's undersurface were detached with trypsin/EDTA and counted.

Where indicated, after coating with vitronectin (2 μg/ml) or BSA, β3/zeo or β3/MT-WT cells (5×10^4) were plated onto the coated surface of the insert in the presence of 10 μM AG3340 or 0.1% DMSO. Control inserts were incubated in cell-free AIM-V medium with or without rMTcat (10 μg/ml). After an overnight incubation, cells were detached with 0.5 mM EDTA (control inserts were also treated with EDTA). After extensive washing with PBS and AIM-V medium, β3/MT-WT cells (5×10^4) were plated into each insert in AIM-V medium without any additives. After incubation for 48 hr, the transmigrated cells were detached and counted.

Adhesion assay

Cell adhesion was performed in high-binding 96-well plates (Corning, Corning, NY) precoated overnight at 4°C with 2 μg/ml vitronectin. Cells were plated at 5×10^4 per well in 0.1 ml of DMEM/1% BSA/20 mM HEPES buffer, pH 7.2 (DMEM/BSA) and allowed to bind vitronectin-coated plastic for 1 hr at 37°C in a CO_2 incubator. Attached cells were stained with Crystal Violet. The incorporated dye was extracted with 100 mM sodium phosphate/50% ethanol, pH 4.5, and the absorbance of the samples was measured at 540 nm.

Immunofluorescence confocal microscopy

Cells were plated in 8-well LabTek II glass chambers (Nalge Nunc International, Naperville, IL) at 1.0–1.5 × 10^4 cells/well. After incubation for 48 hr, cells were washed with DPBS and fixed for 20 min in 4% paraformaldehyde in DPBS (pH 7.4). The slides were incubated for 30 min at room temperature in DPBS containing 10% goat serum and 5% BSA and then stained for 1 hr with 10 μg/ml MT1-MMP-specific antibodies or control rabbit antibodies. After washing in DPBS, the slides were incubated with 20 μg/ml goat anti-rabbit IgG antibodies conjugated with Alexa 568 (Molecular Probes, Eugene, OR). After washing, the cells were embedded into VectaShield (Vector Laboratories, Burlingame, CA) and examined on an MRC 1024 scanning confocal microscope (Bio-Rad, Hercules, CA). Acquisition and processing of images were performed with the Lasersharp (Bio-Rad) and Adobe Photoshop software (San Jose, CA).

Hydrolysis of vitronectin by rMTcat

Native vitronectin (5 μg) was cleaved by rMTcat (20 ng) in 50 mM HEPES, pH 7.0, 5 mM $CaCl_2$, 50 μM $ZnCl_2$ for 16 hr at 37°C. The reactions were stopped with 20 mM EDTA. The samples were mixed with an equal volume of 2× SDS-PAGE sample buffer containing 20 mM DTT, boiled for 5 min at 100°C and resolved on 4–20% SDS PAGE (Novex).

AG3340 inhibition of MT1-MMP activity in vitro

Titration of the rMTcat activity with AG3340 was performed as described previously.[28] After pre-incubation, the steady-state rate of hydrolysis of the fluorogenic substrate Mca-PLGL-Dnp-AR-NH_2 (Bachem, King of Prussia, PA) was determined at ambient temperature using an fMax fluorescence microplate reader (Molecular Devices, Sunnyvale, CA). The steady-state rate was plotted as a function of inhibitor concentration and fitted with the equation $V = SA(E_0 - 0.5\{(E_0 + I + K_i) - [(E_0 + I + K_i)^2 - 4\,E_0\,I]^{0.5}\})$, where V is the steady-state rate of substrate hydrolysis, SA is specific activity (rate per unit enzyme concentration), E_0 is enzyme concentration, I is inhibitor concentration and K_i is the dissociation constant of the enzyme-inhibitor complex.

RESULTS

Expression of αvβ3 integrin and MT1-MMP in MCF7 cells

To analyze the effects of individual and joint expression of MT1-MMP and αvβ3 integrin on migration of breast carcinoma MCF7 cells, the parental cells deficient in MMP-2, MT1-MMP and β3 integrins, were transfected with the MT1-MMP and/or αvβ3 integrin cDNAs in the plasmids conferring geneticin (neo) and zeocin (zeo) resistance, respectively.[29] The resulting doubly transfected cell lines stably expressed the wild-type MT1-MMP (neo/MT-WT cells), αvβ3 integrin (β3/zeo cells) or both molecules (β3/MT-WT cells) on cell surfaces. Furthermore, we employed MCF7 cells co-expressing αvβ3 with the catalytically inert mutant MT1-MMP bearing the E240A mutation in the enzyme's active site (β3/MT-E240A cells).[13] Transfection of parental MCF7 cells with both original plasmids was used to generate the control neo/zeo cells. High levels of surface expression of αvβ3 integrin and MT1-MMP in the corresponding cell lines were confirmed by flow cytometry (data not shown) and immunoprecipitation followed by Western blotting (Fig. 1*a*).

No MT1-MMP was detected in the lysates of surface biotinylated control cells. However, MT1-MMP was readily identified in the cells transfected with MT-WT and mutant MT-E240A constructs. In agreement with our earlier observations,[13,22,23] MT-WT was predominantly represented by the 39 kDa and 42 kDa stable, catalytically inactive ectodomain forms. The cells expressing MT-E240A exhibited the 60 kDa species since self-proteolysis of the enzyme was significantly suppressed (Fig. 1*a*, upper panel).

Consistent with the presence of functionally active MT1-MMP, neo/MT-WT and β3/MT-WT cells were potent in the activating exogenous proMMP-2. Thus, incubation of these cells with the 68 kDa MMP-2 proenzyme generated the 64 kDa activation intermediate and the mature 62 kDa enzyme of MMP-2. In contrast, β3/zeo and β3/MT-E240 cells, both deficient in functionally active MT1-MMP, failed to process proMMP-2 (Fig. 1*b*).

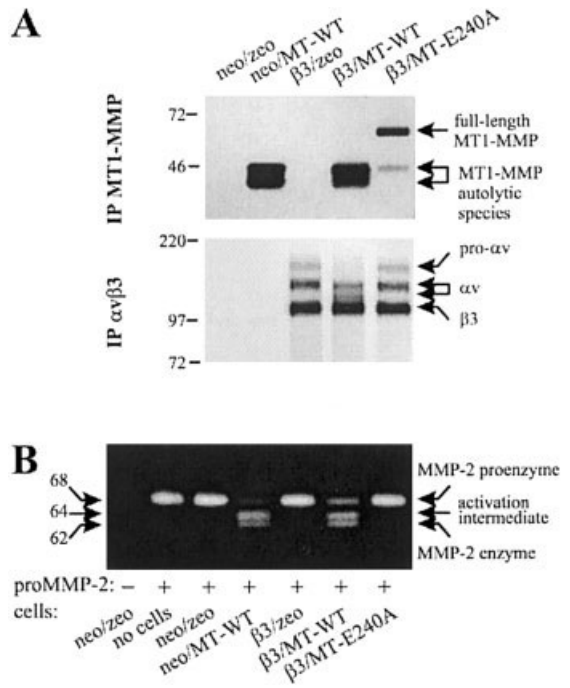

FIGURE 1 – Expression of MT1-MMP and αvβ3 integrin in MCF7 cells. *(a)* Immunoprecipitation and Western blot analysis of MT1-MMP (upper panel) and αvβ3 integrin (lower panel) in doubly transfected MCF7 cells. neo/zeo, control cells transfected with the original pcDNA.3-neo and -zeo plasmids; neo/MT-WT, cells transfected with the original neo plasmid and the wild-type MT1-MMP in the zeo plasmid; β3/zeo, cells transfected with the β3 integrin subunit in the neo plasmid and the original zeo plasmid; β3/MT-WT, cells transfected with β3 and the wild-type MT1-MMP; β3/MT-E240A, cells transfected with β3 and the catalytically inert MT1-MMP mutant. Cells were surface biotinylated and lysed with OG buffer. MT1-MMP and αvβ3 were immunoprecipitated (IP) from the cell lysates with the specific Ab M3927 and MAb LM609, respectively. Precipitates were analyzed by reducing SDS-PAGE on 10% (MT1-MMP) or 8% (αvβ3) gels, followed by Western blotting with Extravidin-HRP. Positions of molecular weight markers (kDa) are indicated on the left. *(b)* Zymographic analysis of proMMP-2 activation. ProMMP-2 (30 ng per 2 × 10^5 cells) was added (+) to MCF7 cell transfectants deficient in MMP-2 production (the lack of proMMP-2 is depicted for control neo/zeo cells, first lane on the left). After incubation for 16 hr, the aliquots of conditioned media were analyzed by SDS-PAGE on 10% gelatin gels. Conversion of proMMP-2 (68 kDa) into the activation intermediate (64 kDa) and the active enzyme (62 kDa) was detected in neo/MT-WT and β3/MT-WT cells expressing the wild-type MT1-MMP.

Immunoprecipitation of αvβ3 integrin identified no specific protein bands in control and neo/MT-WT cells (Fig. 1*a*, lower panel). In contrast, the β3/zeo, β3/MT-WT and β3/MT-E240A cells demonstrated high levels of αvβ3 integrin on the cell surface. The 105 kDa β3 integrin chain was readily detected in the lysates from all β3-transfected cell lines. Notably, in the β3-positive cells expressing no MT1-MMP (β3/zeo cells) or expressing functionally inert protease (β3/MT-E240A cells), α chain of αvβ3 was represented by the 150 kDa pro-αv and the 125 kDa αv heavy chain. In contrast, almost entire pro-αv pool was processed in β3/MT-WT cells, making the 125 kDa αv heavy chain a major αv species in these cells. In addition to the 125 kDa αv heavy chain, an unusual αv heavy chain with the lower molecular weight (115 kDa) was also present in β3/MT-WT cells. These observations

support our earlier results and suggest a direct cleavage of pro-αv by MT1-MMP in the cells co-expressing αvβ3 integrin and the wild-type MT1-MMP enzyme.[22,23]

Migration of MCF7 cells co-expressing MT1-MMP and αvβ3 integrin

We next studied the influence of the catalytic activity of MT1-MMP on vitronectin-mediated cell migration. Since the parental MCF7 cells did not exhibit high levels of migration on vitronectin,[29] we first identified the optimal conditions for Transwell migration using β3/MT-WT cells, including the number of cells plated per insert and the concentration of vitronectin for precoating of the membrane undersurface.

To identify the optimal number of cells for migration experiments, increasing amounts of β3/MT-WT cells were plated into Transwells precoated with saturating amounts of vitronectin (20 μg/ml). The percentage of cells capable of transmigrating to the membrane's undersurface declined sharply with the increasing numbers of cells plated per insert (Fig. 2*a*). Since 5.0–7.5 × 10^4 cells plated per insert were sufficient to provide reliable counting of transmigrated cells, these cell numbers were used in our further migration experiments.

Concentrations of vitronectin optimal for cell migration were determined by plating 5 × 10^4 cells into each Transwell with the membrane undersurface precoated with increasing amounts of the protein (from 0.08–20 μg/ml). Figure 2*b* shows that migration of β3/MT-WT cells was most efficiently promoted by 1–3 μg/ml vitronectin, thereby supporting previously published findings that both insufficient and excessive densities of matrix proteins could impair cell migration.[31] Accordingly, 2 μg/ml vitronectin was used for coating the undersurface of Transwell membranes in our subsequent migration experiments.

Next, we examined whether expression of MT1-MMP alone or jointly with αvβ3 integrin affects migration of MCF7 cells on vitronectin. As shown in Figure 3*a*, β3/MT-WT cells were about 6-fold more migratory relative to MT1-MMP-deficient β3/zeo cells. Inactivation of the proteolytic activity of MT1-MMP by an E240A mutation reduced the migration efficiency of β3/MT-E240A cells to the levels observed in β3/zeo cells. Migration of either β3/zeo or neo/MT-WT cells was relatively low and comparable with that of control neo/zeo cells lacking both αvβ3 and MT1-MMP. These findings suggest that the presence of αvβ3 integrin and the proteolytic activity of MT1-MMP are both essential for the enhanced migration of β3/MT-WT cells.

The observed differences in migration of MCF7 cell transfectants were not attributable to any substantial differences in their

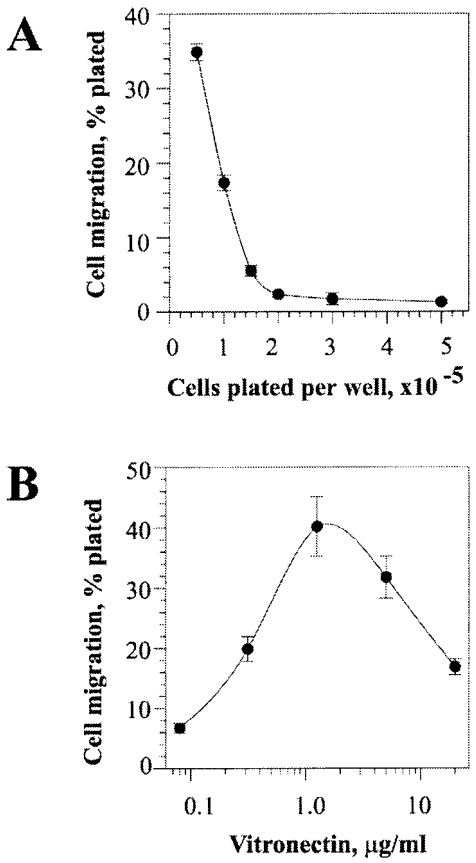

FIGURE 2 – Migration of β3/MT-WT cells in Transwells. Cells were plated in serum-free AIM-V medium into the Transwell inserts with the membrane undersurface precoated with vitronectin. After incubation for 48 hr, cells transmigrated onto the membrane undersurface were detached and counted. Cell migration (the percentage of plated cells) is depicted as a function of the number of cells plated per insert precoated with saturating amount of vitronectin (20 μg/ml) *(a)* or the coating concentrations of vitronectin with 5 × 10^4 cells per insert *(b)*. Data are means ± SE from a representative experiment performed in triplicate.

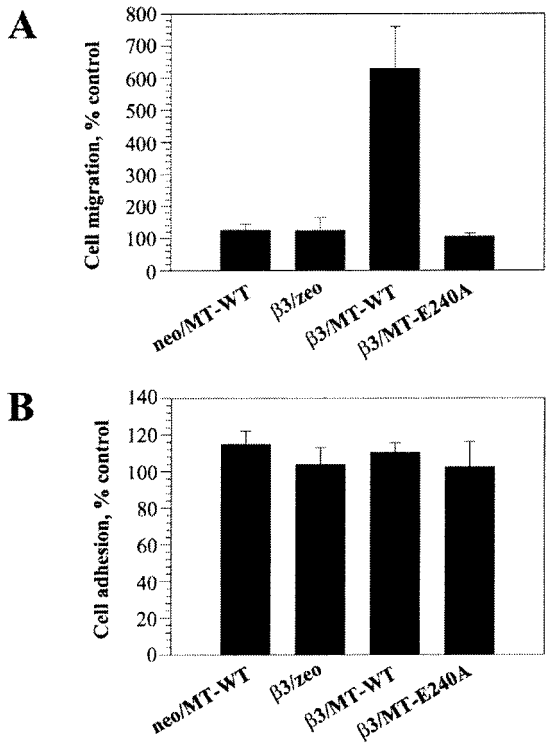

FIGURE 3 – Co-expression of αvβ3 integrin and MT1-MMP induces cell migration. *(a)* Migration of MCF7 cell transfectants on vitronectin. Migration efficiencies of neo/MT-WT, β3/zeo, β3/MT-WT and β3/MT-E240A cells were assayed in Transwells with the membrane undersurface precoated with 2 μg/ml vitronectin. Cells were plated at 5 × 10^4 per insert and allowed to migrate for 48 hr. Cells transmigrated to the membrane undersurface were detached and counted. Migration is depicted as the percentage of migration of control neo/zeo cells (100%). Data are means ± SE from 4–17 independent experiments performed in triplicate. *(b)* Adhesion of MCF7 cell transfectants to vitronectin. Cells (5 × 10^4) were plated per well of a 96-well cluster and allowed to attach to plastic precoated with 2 μg/ml vitronectin. Following incubation for 1 hr, adherent cells were fixed and stained with Crystal Violet. The incorporated dye was extracted and the absorbance was measured at 540 nm. Adhesion is presented as the percentage of adhesion of control neo/zeo cells (100%). Data are means ± SE from 3–11 individual experiments performed in triplicate.

adhesion to vitronectin coated at 2 μg/ml. As shown in Figure 3*b*, all tested MCF7 cell lines, including neo/MT-WT cells lacking αvβ3 but expressing high levels of αvβ5 integrin, demonstrated highly similar adhesion efficiency.

Inhibition of MT1-MMP catalytic activity by AG3340 promotes cell migration

AG3340 is a highly potent MMP inhibitor with K_i values against gelatinases in the low picomolar range.[26] Using the fluorogenic Mca-PLGL-Dnp-AR-NH_2 substrate, we have shown that AG3340 inhibits proteolytic activity of rMTcat with the K_i value of about 0.3 nm (Fig. 4*a*). Thus, AG3340 also is a potent inhibitor of MT1-MMP activity, which is in agreement with the previous published data.[25]

To determine the range of AG3340 concentrations inhibitory to the functional activity of cell surface MT1-MMP, β3/MT-WT cells were incubated with proMMP-2 (100 ng/ml, 30 ng per 4×10^5 cells) in the presence of increasing concentrations of the inhibitor. Following incubation for 16 hr, aliquots of conditioned medium were analyzed by gelatin zymography (Fig. 4*b*). At 0.1 μM, AG3340 was capable of inhibiting the autolytic step of MMP-2 maturation, *i.e.,* conversion of the 64 kDa activation intermediate of MMP-2 into the 62 mature enzyme of MMP-2. In contrast, at the 0.1–1 μM range of inhibitor concentrations, the 68 kDa proenzyme was still converted into the 64 kDa intermediate. However, this MT1-MMP-dependent step of proMMP-2 activation was fully blocked by 10 μM AG3340, thereby indicating that the functional activity of cell surface MT1-MMP was completely abolished.

To determine whether inhibition of the MT1-MMP catalytic activity by AG3340 affects cell migration, β3/MT-WT, neo/MT-WT and β3/zeo cells were allowed to migrate in the presence of increasing concentrations of the inhibitor added both to the upper and lower chambers of Transwells. At 1 μM and lower concentrations, AG3340 did not influence migration of any tested cells (Fig. 4*c*). Surprisingly, at a 3–10 μM range, AG3340 increased migration of cells expressing MT-WT. Specifically, 10 μM AG3340 caused a 4-fold and a 2.5-fold increase in migration

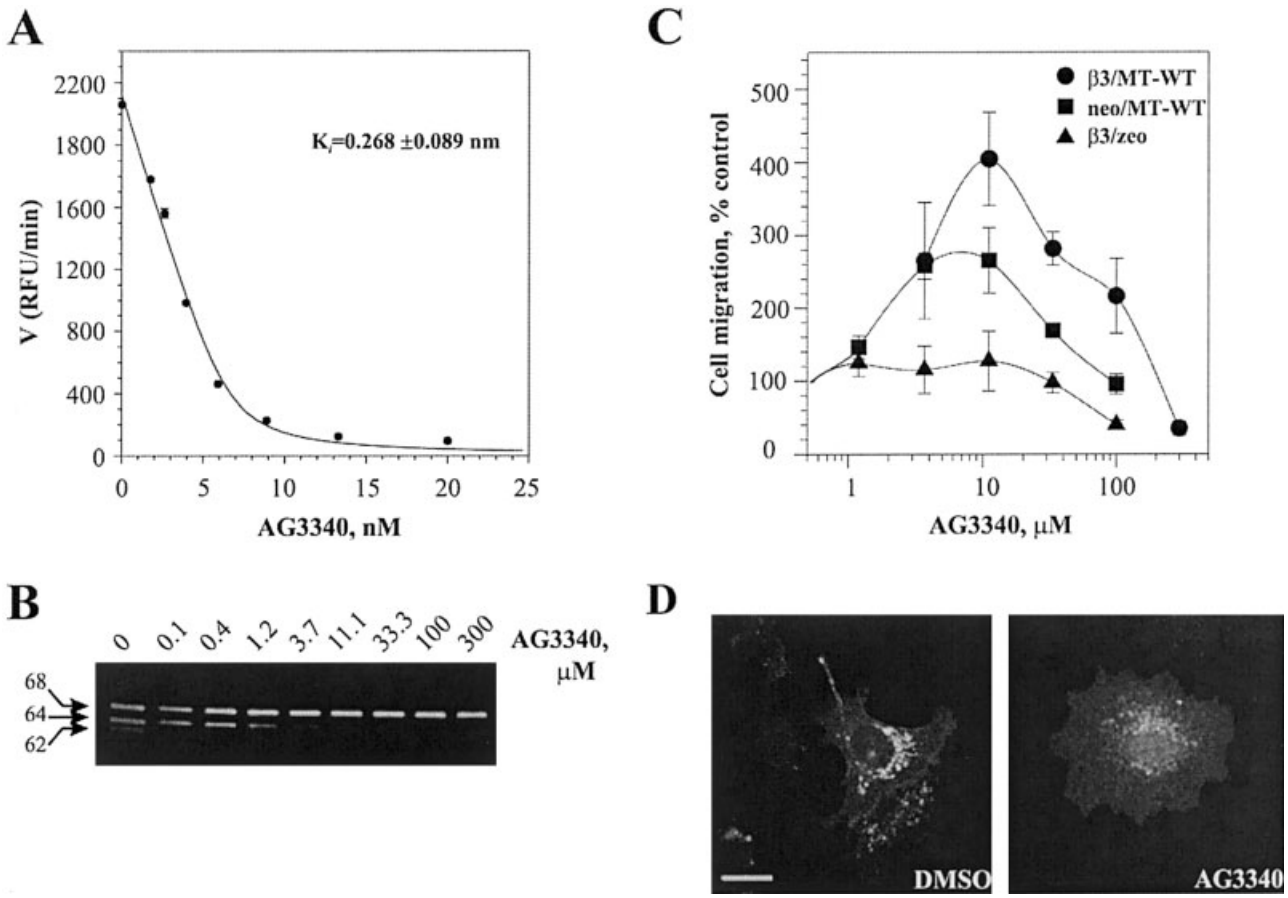

FIGURE 4 – *(a)* AG3340 is a potent inhibitor of MT1-MMP. Hydrolysis of the fluorogenic substrate Mca-PLGL-Dnp-AR-NH_2 by rMTcat was performed in the presence of increasing concentrations of AG3340. The steady-state rate of hydrolysis (V) is plotted as a function of inhibitor concentration. Data are presented as relative fluorescence units (RFU) per min and are means ± SE from a representative experiment performed in duplicate. K_i, the dissociation constant of the enzyme-inhibitor complex. *(b)* AG3340 inhibits activation of proMMP-2 by β3/MT-WT cells. Cells (2×10^5 per well) were incubated in serum-free DMEM in the presence of 30 ng exogenous proMMP-2 and increasing concentrations of AG3340. After incubation for 16 hr, the aliquots of conditioned medium were analyzed by gelatin zymography. The positions of proMMP-2 (68 kDa), the activation intermediate (64 kDa) and the mature MMP-2 enzyme (62 kDa) are indicated on the left. *(c)* AG3340 modulates migration of MCF7 cells expressing MT1-MMP. The undersurface of the Transwell membrane was coated with 2 μg/ml vitronectin. Cells were plated at 5×10^4 per Transwell in serum-free AIM-V medium supplemented with the increasing concentrations of AG3340. After incubation for 48 hr, migrated cells were detached and counted. Migration is presented as the percentage of migration of control neo/zeo cells (100%). Data are means ± SE from the representative experiments performed in triplicate. *(d)* AG3340 modifies morphology of β3/MT-WT cells. Cells were plated in DMEM/FCS with or without 10 μM AG3340 (right panel) or 0.1% DMSO (solvent control) (left panel). After incubation for 48 hr, cells were fixed and processed for confocal microscopy as described in Material and Methods. MT1-MMP was revealed with specific rabbit antibodies followed by staining with goat anti-rabbit IgG conjugated with Alexa 568. Scale bar = 20 μm.

efficiency of β3/MT-WT and neo/MT-WT cells, respectively. In contrast, migration of β3/zeo cells was not affected at the same range of AG3340 concentrations. A significant decrease in migration of all tested cells was observed at 30–300 μM AG3340. Thus, 100 μM AG3340 caused about a 2-fold decrease in migration efficiency of all cell types. This inhibition was likely nonspecific and apparently could be attributed to cytotoxic effects of these extremely high concentrations of the inhibitor.

In agreement, confocal microscopy confirmed that AG3340 induced changes in cell morphology. Figure 4*b* shows that β3/MT-WT cells appeared more flattened and exhibited fewer plasma membrane protrusions after treatment for 48 hr with 10 μM AG3340. These findings support our suggestion that at higher concentrations AG3340 may cause toxic effects, thereby nonspecifically decreasing cell migration.

Matrix degradation by MT1-MMP impairs cell migration

Our observations indicated that while inhibiting the activity of cell surface MT1-MMP (Fig. 4*b*), 10 μM AG3340 caused a substantial increase in vitronectin-mediated migration of neo/MT-WT and β3/MT-WT cells (Fig. 4*c*). Since MT1-MMP inactivated by AG3340 would be incapable of cleaving matrix proteins, we speculated that stimulation of cell motility in the presence of AG3340 could be attributed to the inhibition of vitronectin degradation by cell surface MT1-MMP.

To specifically evaluate the role of MT1-MMP-mediated degradation of vitronectin in cell migration, we examined the motility of β3/MT-WT cells on vitronectin pretreated with rMTcat. For these purposes, the undersurface of Transwell membranes were precoated with 2 μg/ml vitronectin. Then, the inserts were incubated overnight with rMTcat (10 μg/ml). After washings to remove rMTcat and hydrolysis products, the β3/MT-WT cells were allowed to migrate on hydrolyzed vitronectin. Figure 5*a* demonstrates that the rMTcat treatment decreased migration of β3/MT-WT cells to the levels observed with BSA-coated membranes. Thus, excessive proteolysis of vitronectin by MT1-MMP appears to be capable of completely inhibiting cell migration.

To confirm the ability of MT1-MMP to degrade vitronectin, we incubated native vitronectin with rMTcat for 16 hr at 37°C at the enzyme to protein molar ratio of about 1:60. As shown in Figure 5*b*, the soluble rMTcat efficiently hydrolyzed vitronectin. These findings agree well with the similar observations by other authors[32] and confirm susceptibility of vitronectin to MT1-MMP proteolysis.

To further corroborate these findings, we next examined whether cell membrane-associated MT1-MMP is able to degrade vitronectin, thus inhibiting cell migration. In these experiments, the β3/MT-WT cells (5×10^4) were plated with or without AG3340 (10 μM) onto the Transwell membranes precoated with vitronectin (2 μg/ml). To assess the levels of MT1-MMP-independent matrix proteolysis, the same numbers of β3/zeo cells were plated into a separate set of Transwells. Control inserts were incubated in cell-free medium. After incubation for 16 hr, the cells were detached with 0.5 mM EDTA. The control inserts were also treated with EDTA. After washing with PBS and then with AIM-V medium, β3/MT cells (5×10^4) were plated per each insert and allowed to migrate for 48 hr.

As shown in Figure 5*a*, incubation of vitronectin-coated membranes with the β3/zeo cells did not significantly affect the levels of subsequent migration of β3/MT-WT cells. In contrast, preincubation of the inserts with the β3/MT-WT cells caused a sharp decrease in migration of the newly plated cells. Consistent with inactivation of MT1-MMP, the subsequent migration of β3/MT-WT cells was partially restored if AG3340 was included during incubation of β3/MT-WT cells on the vitronectin-coated membranes.

Taken together, these findings support our opinion that excessive degradation of vitronectin by the cell membrane-associated MT1-MMP negatively affects cell locomotion because of insufficient density of the matrix (Fig. 5*a*). Arguably, deposition of matrix components by β3/MT-WT cells during their incubation on vitronectin-coated inserts might have affected the subsequent cell migration. However, pre-incubation of BSA-coated membranes with β3/MT-WT cells failed to induce migration of the newly plated cells, making deposition of any significant quantities of *de novo* synthesized matrix components unlikely (Fig. 5*a*).

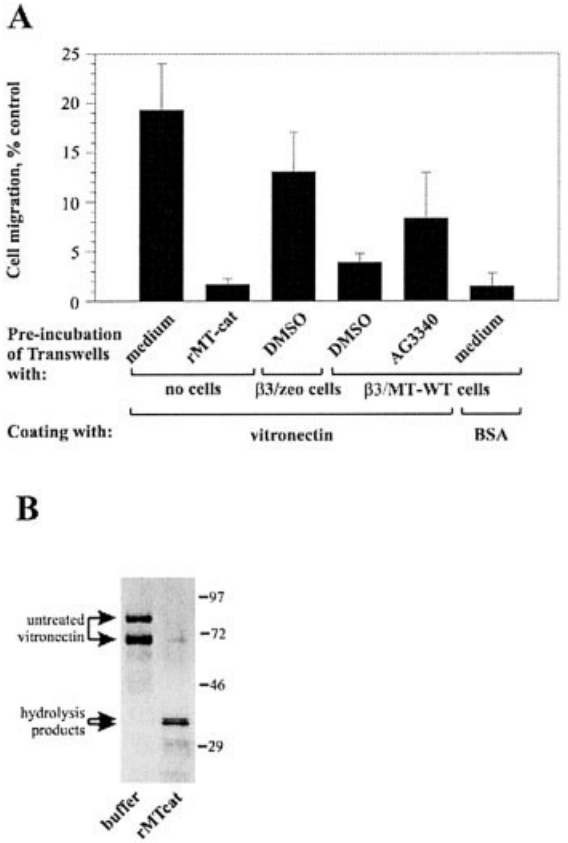

FIGURE 5 – Hydrolysis of vitronectin by MT1-MMP inhibits migration of MCF7 cells co-expressing αvβ3 integrin and MT1-MMP. *(a)* Pretreatment of vitronectin-coated Transwell membranes with cells expressing MT1-MMP decreases subsequent migration of the newly seeded μ3/MT-WT cells. The membrane undersurface was coated with vitronectin or BSA (both at 2 μg/ml). Thereafter, 5×10^4 β3/zeo or β3/MT-WT cells were plated onto the coated surface of the inserts in 0.1 ml AIM-V medium supplemented with 10 μM AG3340 or 0.1% DMSO (solvent control). Control inserts were incubated with cell-free AIM-V medium with or without rMTcat (10 μg/ml). After incubation for 16 hr, the inserts were treated with EDTA and then β3/MT-WT cells (5×10^4) were plated into the inner chamber in AIM-V medium and allowed to migrate to the membrane undersurface. After incubation for 48 hr, the transmigrated cells were detached and counted. Migration is presented as the percentage of plated cells. Data are means ± SE from the representative experiments performed in triplicate. *(b)* MT1-MMP efficiently hydrolyses vitronectin. Native vitronectin was incubated with rMTcat or buffer, followed by SDS-PAGE on a 4–20% gel under reducing conditions. The positions of molecular weight markers are indicated on the right.

MT1-MMP-dependent maturation of αvβ3 integrin facilitates cell migration on vitronectin

The mature αv integrin subunit is generated by the PC endoproteolytic cleavage of the pro-αv precursor.[21] Recently, we have demonstrated that MT1-MMP is also involved in the maturation of pro-αv in β3/MT-WT cells.[22,23] Our earlier findings and the results of other groups suggested a relatively long life span (approximately 24 hr) of αvβ3 integrin on cell surfaces. In contrast, MT1-MMP

in β3/MT-WT cells is characterized by a relatively short half-life (of perhaps several hours).[22,23] Whereas the MT1-MMP catalytic activity would be instantly inhibited by potent hydroxamate inhibitors, a prolonged, 48–72 hr incubation of β3/MT-WT cells with AG3340 (10 μM) was required to substitute the MT1-MMP-processed αvβ3 integrin on the cell surface with the *de novo* synthesized integrin species conventionally processed by furin. This significant difference in the life span of MT1-MMP and of αvβ3 integrin in the β3/MT-WT cells allowed us to specifically distinguish the role of MT1-MMP-dependent processing of αvβ3 integrin in cell migration.

For these purposes, β3/MT-WT cells were incubated for 24–72 hr with or without AG3340 (10 μM) followed by cell surface biotinylation and immunoprecipitation of αvβ3 integrin and MT1-MMP. As shown in Figure 6*a*, AG3340 at 10 μM efficiently blocked MT1-MMP activity and self-proteolysis of MT1-MMP. In the cells pre-incubated with the inhibitor for 24 hr, the full-length 60 kDa MT1-MMP form completely replaced the autolytic forms of the protease, confirming a short life span of MT1-MMP on the cell surface. Blocking the autocatalytic conversion of MT1-MMP by AG3340 correlated with the inhibition of the MT1-MMP-dependent pathway of pro-αv maturation in β3/MT-WT cells. However, incubation with AG3340 for 48–72 hr was needed to substitute the cell surface αvβ3 containing the 115 kDa and 125 αv heavy chains for the integrin represented by the 150 kDa pro-αv and the 125 kDa mature αv species (Fig. 6*a*). In agreement, this treatment caused an increase in the levels of pro-αv and a reciprocal decrease in the amounts of the 115 kDa heavy αv-chain. After incubation with AG3340 for 72 hr, αvβ3 in β3/MT-WT cells was highly similar to that observed in MT1-MMP-negative β3/zeo cells or β3/MT-E240A cells expressing functionally inactive MT1-MMP (Fig. 6*a*). These results suggest a relatively long residence of αvβ3 integrin on the cell surface and agree with the earlier estimates for other integrins.[33]

To elucidate whether inhibition of the MT1-MMP-dependent pathway of pro-αv maturation affects cell motility, we evaluated migration of the cells pretreated with AG3340 (10 μM) for 24–72 hr. AG3340 had no effect either on the pattern of αvβ3 integrin or migration of β3/zeo cells (data not shown). In contrast, pre-incubation with the inhibitor decreased in a time-dependent manner the migration efficiency of β3/MT-WT cells to the levels observed for β3/zeo and β3/MT-E240A cells (Fig. 6*b*). Since MCF7 cells exhibit a cohort-like migration 20–30 hr after plating into Transwells (data not shown), while a complete turnover of αvβ3 on the cell surface requires about 72 hr (Fig. 6*a*), the increase in migration of β3/MT-WT cells plated in the presence of AG3340 (Fig. 4*c*) does not contradict the inhibited migration of β3/MT-WT cell pretreated with the inhibitor (Fig. 6*b*).

Altogether, the results of our study allowed us to conclude that the processing of αvβ3 integrin rather than direct cleavage of the vitronectin matrix by MT1-MMP facilitates tumor cell locomotion.

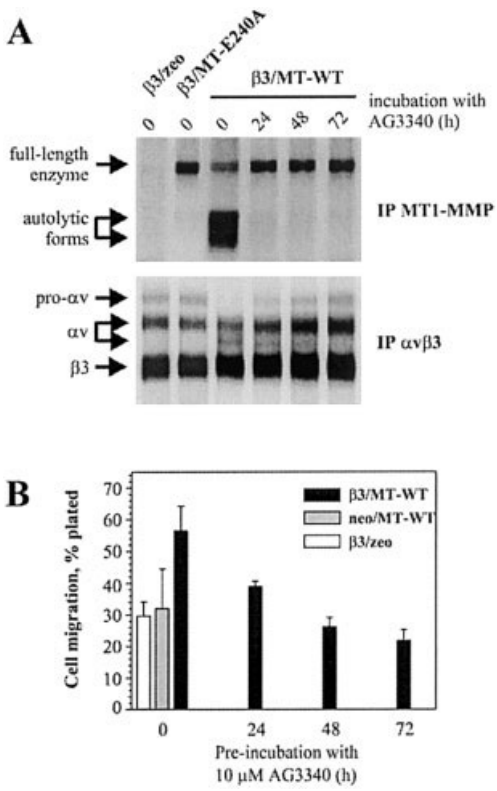

FIGURE 6 – AG3340 inhibits the MT1-MMP-dependent processing of αvβ3 integrin and decreases migration of β3/MT-WT cells on vitronectin. Cells were first incubated with or without AG3340 (10 μM) for 24–72 hr and then analyzed by immunoprecipitation *(a)* for MT1-MMP (upper panel) and αvβ3 (lower panel) or assessed for migration efficiency in Transwells. *(b)* Cells were pre-incubated for the indicated time with AG3340 and then plated into Transwells with the inhibitor added to both the inner and outer chambers. Migration is expressed as the percentage of plated cells. Data are means ± SE from 4 independent experiments performed in triplicate.

DISCUSSION

Since there is a general agreement that MMPs play a key role in matrix degradation associated with cancer cell invasion, metastasis and angiogenesis, these proteases are potential targets for anticancer pharmaceuticals.[2,16,17,34–36] By blocking MMP activity, broad-range hydroxamate inhibitors were supposed to suppress tumor progression and metastasis. However, the desired effects of MMP inhibitors have not been achieved *in vivo*.[16,17] Thus, Batimastat promoted liver metastasis of T-cell lymphoma in mice.[37] In clinical trials with nonsmall-cell lung and prostate cancers, Prinomastat provided no survival benefit for patients.[38] The unfavorable outcome of clinical trials allowed speculations that some critical functions of MMPs and especially MT1-MMP, an enzyme specifically associated with tumor cell motility, may not be directly associated with ECM degradation.[6,7,39–41]

Emerging evidence indicates that MT1-MMP is involved in the endoproteolytic cleavage of cell surface receptors, thereby enabling cells to adjust their receptor profile in the continually changing ECM environment. The proteolytic modification of cell receptors by MT1-MMP was demonstrated to change significantly the efficiency of tumor cell migration and invasion. Here, we present evidence that in breast carcinoma cells inhibition of cellular MT1-MMP affects both matrix degradation and maturation of αvβ3 integrin.

Our data demonstrate that αvβ3 integrin has a relatively slow turnover rate and consequently a long life span (approximately 24–48 hr) on the surface of MCF7 cells.[22,33] Therefore, although the activity of cell surface MT1-MMP and MT1-MMP-mediated matrix cleavage were efficiently inhibited by AG3340, a prolonged incubation of cells co-expressing MT1-MMP and αvβ3 with AG3340 for 48–72 hr was needed to fully substitute MT1-MMP-modified αvβ3 for the integrin molecules processed by conventional PCs. This treatment with the inhibitor caused a significant decrease in cell locomotion on vitronectin, an extracellular matrix ligand of αvβ3 integrin. In contrast to αvβ3, the life span of MT1-MMP in MCF7 cells is a few hours. This difference allowed us to distinguish the roles of MT1-MMP in matrix degradation and in αvβ3 integrin maturation.

The results of our study indicate that vitronectin is highly sensitive to MT1-MMP proteolysis. Thus, excessive degradation of vitronectin by the recombinant catalytic domain of the protease or by cell-associated MT1-MMP diminished the subsequent migration of newly seeded cells. Shortly after addition of AG3340 to the cells co-expressing MT1-MMP and αvβ3, the matrix-degrad-

ing activity of the protease is likely to be the initial target of hydroxamate inhibition. Accordingly, blocking MT1-MMP-dependent degradation of the vitronectin matrix by Prinomastat could significantly facilitate cell migration.

These findings corroborate the hypothesis that modification of cell receptors could be the primary role of MT1-MMP in tumor cells. A few of these receptors have been identified and include precursors of αv, α3 and α5 integrin subunits, tissue transglutaminase and CD44.[22,23,42,43] Proteolysis of these and other surface molecules by MT1-MMP may strongly affect signal transduction pathways such as the FAK pathway in cells overexpressing the protease. In turn, this may lead to changes in the adhesive, migratory, proliferative and other important physiologic characteristics of cells. Our results agree with and extend the earlier observations of other authors,[6,7] supporting the primary role of membrane-tethered proteinases of the MT-MMP subfamily in promoting cell migration and invasion.

In conclusion, our study clearly demonstrates that an excessive or uncontrolled cleavage of the ECM by MT1-MMP may negatively affect cell locomotion. Conversely, inhibition of MT1-MMP activity might promote tumor cell migration and invasion. This is consistent with clinical observations and may partially explain the relatively low efficiency of the hydroxamate inhibitors *in vivo*.

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