Melanoma Chondroitin Sulfate Proteoglycan Regulates Matrix Metalloproteinase-dependent Human Melanoma Invasion into Type I Collagen*

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Tumor cell adhesion and proteolysis of the extracellular matrix proteins surrounding the cells are tightly linked processes in tumor invasion. In this study, we sought to identify components of the cell surface of a vertical growth phase melanoma cell line, WM1341D, that mediate invasive cellular behavior. We determined by antisense inhibition that melanoma chondroitin sulfate proteoglycan (MCSP) and membrane-type 3 matrix metalloproteinase (MT3-MMP) expressed on WM1341D are required for invasion of type I collagen and degradation of type I gelatin. MT3-MMP co-immunoprecipitated with MCSP in WM1341D melanoma cells cultured on type I collagen or laminin. The association between MT3-MMP and MCSP was largely disrupted by removing chondroitin sulfate glycosaminoglycan (CS) from the cell surface, suggesting CS could mediate the association between the two cell surface core proteins. Recombinant MT3-MMP and MT3-MMP from whole cell lysates of WM1341D cells were specifically eluted from CS-conjugated affinity columns. The results indicate that MT3-MMP possesses the potential to promote melanoma invasion and proteolysis and that the formation of a complex between MT3-MMP and MCSP may be a crucial step in activating these processes.

Cell adhesion and degradation of ECM proteins are tightly linked processes in tumor invasion and metastasis (1). Melanoma progression from radial growth phase to vertical growth phase (VGP) is characterized in part by enhanced invasion into the surrounding dermis (2). Invasion requires that tumor cells bind to and degrade ECM components within the dermal/epidermal junction (i.e. basal lamina containing type IV collagen) and the dermis (rich in types I and III collagen). Many studies have documented the importance of adhesion receptors such as integrins and cell surface proteoglycans in melanoma cell invasion through basement membrane or into type I collagen matrices in vitro (3–7). Furthermore, it has also been demonstrated that certain cell adhesion receptors (e.g. αvβ3 integrin and CD44) interact with proteolytic enzymes on cell surfaces, and these interactions facilitate proteolysis of ECM proteins and cell migration and/or invasion (8–11). It has been proposed that at least one consequence of integrin/ protease interaction on the surface of tumors may be to focus the delivery of extracellular matrix-degrading proteases, allowing for the fine control of ECM degradation and tumor invasion.

Melanoma chondroitin sulfate proteoglycan (MCSP) is a cell surface proteoglycan expressed relatively early in melanoma progression (12), and MCSP has been implicated in the malignant properties of advanced melanoma. Melanoma adhesion to endothelial cells and chemotactic responses to fibronectin are inhibited by monoclonal antibodies directed against the MCSP core protein (13), and MCSP has been associated with melanoma invasion in vitro (14). Furthermore, recent studies demonstrate that mouse melanoma cells transfected with NG2 (the rat homologue of human MCSP) are more tumorigenic and metastatic than control counterparts in vivo (15). Although the exact mechanisms by which MCSP may facilitate metastasis are unknown, we have previously demonstrated that MCSP can enhance ligand binding by αvβ3 integrin, and MCSP activation can enhance integrin-mediated cell spreading by cdc42 and p130cas-dependent mechanisms, indicating that MCSP can directly transduce signals important for tumor adhesion and extravasation (16–18). Thus, MCSP may be involved at multiple discrete but overlapping steps in tumor progression, acting through several distinct mechanisms to enhance melanoma invasion and metastasis.

Membrane-type matrix metalloproteinases (MT-MMPs) comprise a family of transmembrane metalloproteinases that regulate proteolysis of ECM proteins at the plasma membrane (19). MT-MMPs degrade various ECM proteins (i.e. collagens, fibronectin, and laminin) and proteolytically activate gelatinase A (MMP-2) on the cell surface. The localization of MT-MMPs within the plasma membrane uniquely situates them in position to promote tumor invasion (19). MT1-MMP is highly expressed in cancerous tissues compared with tissues that are either normal or contain benign tumors (20–24). Furthermore, overexpression of MT1-MMP stimulates tumor cell invasion through basement membranes, and it has been immunolocalized within cell-to-ECM adhesion sites (25, 26). MT1-MMP has...
been shown to directly degrade type I collagen and to activate MMP-2 and MMP-13 (collagenase-3) (27), indicating that it may facilitate tumor invasion by acting on multiple substrates. As a result, MT1-MMP could facilitate the degradation of denatured and native collagen fibers in the vicinity of invading tumors. Recent studies show that MT1-MMP and MMP-2 are expressed at the tumor-stroma interface at the invasive front of primary melanomas (28), suggesting these enzymes may be linked in promoting melanoma progression and invasion into surrounding tissues. MT3-MMP, a related transmembrane type MMP, has been identified in malignant oral melanoma cells (29) but not in breast or thyroid cancer cells (22, 24). Although purified MT3-MMP degrades various ECM proteins, including denatured collagen, it is relatively ineffective at degrading native type I collagen (30). The potential role of MT3-MMP in tumor invasion and proteolysis has not been studied.

In this study, we have used antisense mRNA inhibition to demonstrate that both MCSP and MT3-MMP are critical factors for promoting the invasion of WM1341D VGP melanoma cells into type I collagen gels in vitro. Co-immunoprecipitation was employed to identify an association between MCSP and MT3-MMP through chondroitin sulfate (CS). Recombinant MT3-MMP was able to bind directly to CS affinity columns, suggesting that MCSP may specifically localize MT3-MMP to ECM adhesion sites on the surface of melanoma cells. Finally, removal of cell surface CS resulted in impaired type I collagen degradation by adherent tumor cells. These results suggest that MCSP may facilitate the invasion of aggressive primary tumors within the dermis by enhancing the local concentration and/or activation of specific MMPs at sites of contact between melanoma cells and the underlying ECM.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The vertical growth phase (VGP) human melanoma cell line WM1341D was maintained in RPMI 1640 supplemented with 10% FCS (31–33). MDCK cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS.

**Reagents**—Rat type I collagen was purchased from Collaborative Biomedical Products (Bedford, MA). A synthetic MMP inhibitor, BB94, was obtained from British Biotech (Oxford, UK). NHS-biotin was purchased from Pierce (Rockford, IL). Plasmid pBKRSV was purchased from Stratagene (La Jolla, CA). Lipofectin and G418 were purchased from Life Technologies (Gaithersburg, MD). Human TIMP-1 and TIMP-2 were purchased from Chemicon (Temecula, CA). α-Type I collagen (specific activity, 0.28 mCi/mg of protein) was purchased from PerkinElmer Life Sciences (Boston, MA). Anti-FLAG M2 affinity gel and a synthetic FLAG peptide were purchased from Sigma Chemical Co. (St. Louis, MO).

**Antibodies**—Anti-MCSP monoclonal antibody (mAb) (9.2.27) was generously provided by Dr. Ralph Reifeld (The Scripps Research Institute, La Jolla, CA) (34). Anti-integrin mAbs: αβ1 (clone P1B5), αβ3 (clone P1B5) and αβ4 (clone LM609) were purchased from Chemicon (Temecula, CA). Anti-β1, mAb (clone P5D2) was given by Dr. Elizabeth Wayner (University of Washington, Seattle, WA) (35). Anti-human CD44 (clone Hermes 3) was purchased from ATCC. Anti-MT1-MMP mAb (IM57L clone 113–57B), anti-MT2-MMP polyclonal antibody (catalogue number 475930), and anti-MT4-MMP polyclonal antibody (catalogue number 475934) were purchased from Calbiochem (San Diego, CA). Anti-MT3-MMP polyclonal antibody was generated in rabbit as described previously (36). The specificities of anti-MT1-MMP and anti-MT3-MMP antibodies were determined by immunoprecipitation and immunodepletion assays using MDCK cells transfected with MT1-MMP or MT3-MMP. Biotinylated anti-FLAG mAb was purchased from Sigma.

**Antisense Oligonucleotides (Oligos)**—Full-length human brain MT3-MMP was ligated into pCR3.1-uni (Invitrogen) (36). The MT3-MMP-encoding fragment (1300 bp) was liberated by EcoRI/HindIII digestion and subcloned in antisense orientation via HindIII/EcoRI into pBKRSV. Melanoma cells were transfected with this plasmid, termed pBKRSV(MT3AS), using Lipofectin according to manufacturer’s protocol. Transfectants were selected by culturing in the presence of 1 μg/ml G418, and characterized for MT3-MMP expression by using specific anti-MT3-MMP antibodies in immunoprecipitation (see text). Antisense oligos for human MCSP were synthesized based on previously reported sequence information (37). Antisense MCSP (5′-AAGTGGGGGGCCGCGGGGACTGCACT-3′) and sense MCSP (5′-ATGGGCGCGCGCGCGGACTGCACT-3′) oligos were synthesized by the Microchemical Facility (University of Minnesota, Minneapolis, MN). These oligos were incubated with melanoma cells as described below without any chemical modification. To inhibit MCSP expression on melanoma cells, melanoma cells were cultured with 30 μM sense or antisense MCSP oligo for 48 h, with fresh doses given every 12 h, prior to assays and oligos were included during assays. The effectiveness of specific MCSP oligo on expression of the antagonist was measured by flow cytometry using 9.2.27 (see “Results” section).

**Flow Cytometry**—Expression of MCSP, CD44, and integrins was measured by FACS analysis as described previously (5). Aliquots of 1 ml of cell suspension were incubated with 1 μg/ml mAbs for 30 min at 4 °C with tapping of samples every 5 min. Cells were washed three times with the FACS buffer and resuspended in 200 μl of PBS containing 2% formaldehyde. Cells were analyzed on a FACS Star (Becton-Dickinson).

**Migration Assays**—Migration assays were performed in modified Boyden chambers (Neuroprobe, Bethesda, MD) with 8-μm pore size polystyrolidone-free polycarbonate filters (Nucleopore, Pleasanton, CA) as described previously (5). Melanoma cells were harvested and resuspended in RPMI-1640 at a final concentration of 5 × 10⁶ cells/ml. Lower wells of the chambers were filled with RPMI-1640 containing 20 μg/ml of rat type I collagen. Cells (50 μl of suspension) were added to the upper wells with or without appropriate mAbs or inhibitors in corresponding assays. Migration assays were performed for 4 h at 37 °C. Data are shown as mean number of cells migrated ± S.D. per mm² from one representative experiment.

** Invasion Assays**—Human melanoma cell invasion was evaluated using type I collagen gels cast in 24-well Transwell culture inserts with 8-μm pore size (catalogue number 3422, Costar). Rat tail type I collagen was diluted in RPMI 1640-serum free (RPMI-SF) to a final concentration of 100 μg/ml. 100 μl of the solution was gelled in the upper chamber of 24-well transwells by incubating at 37 °C for 4–5 h. Gels were washed twice with prewarmed RPMI-1640-SF. WM1341D cells were harvested by brief treatment with trypsin/EDTA, washed three times with RPMI 1640–10% FCS, and resuspended in RPMI-1640–1% FCS at a concentration of 1 × 10⁴ cells/ml to ensure the viability of melanoma cells during assay periods. The cell suspension (100 μl) was placed in the upper chamber of each transwell. The lower chambers were filled with 600 μl of RPMI-1640-SF containing 10 μg/ml human plasma fibronectin as an adhesive matrix. The chambers were incubated for 20 h at 37 °C. For inhibiting melanoma cell invasion, MMP inhibitors (BB94, TIMP-2, TIMP-1) or control PBS were dispensed into both the cell suspension and the collagen gel. Polycarbonate membranes were stained with Diff-Quick (Baxter, Miami, FL), and non-invaded cells were scraped from the upper side of the membranes. Invaded cells were visualized microscopically (50×) and counted by randomly selecting three areas from one membrane. Each assay condition was performed in triplicate, and results are expressed as mean ± standard deviation (S.D.) per mm² from one representative experiment.

**Cell Adhesion Assays**—Cell adhesion assays were performed as described with minor modifications (16). Briefly, type I collagen was diluted in PBS, and 50-μl aliquots were dispensed in triplicate into Immulon-1 polystyrene microtiter wells. The wells were incubated at 37 °C overnight, and blocked with 200 μl of PBS containing 2% BSA. Subconfluent VGP human melanoma cells radiolabeled overnight with [3H]thymidine (specific activity 6.7 Ci/mmol, PerkinElmer Life Sciences, Boston, MA) were harvested with 1 mM EDTA. The released cells were washed twice with RPMI 1640 containing 2 mg/ml BSA and 15 mM Hepes, pH 7.2, and adjusted to a concentration of 1 × 10⁶ cells/ml in the same medium. Aliquots of 100 μl of the cell suspension were dispensed into the wells, and the cells were incubated at 37 °C for 30 min. These wells were terminated by adding 0.5 ml of 50 mM Tris-HCl, pH 8.0, and unbound cells from the wells. Bound radioactivity, determined in a Beckman Model 3801 liquid scintillation counter, was used to calculate the percentage of cells that remained adherent. Experiments were repeated a minimum of three times.

**Type I Collagen Degradation Assays**—96-well plates were coated with 100 μg/ml collagen (2 × 10⁵ dpm/50 μl well) overnight and then blocked

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with PBS-BSA. Melanoma cells were harvested by trypsin-EDTA, washed twice with RPMI 1640, and resuspended in RPMI-SF at a density of 2 × 10^5 cells/ml. Aliquots of 100-μl cell suspension were seeded in each well and incubated for 8 h at 37 °C. Cell culture supernatants were then harvested and centrifuged to remove cell debris. Released extravasation activity, corresponding to degraded type I collagen, was measured in 50 μl of each supernatant. Under these experimental conditions, over 90% of radioactivity was released into medium by trypsin, suggesting that a large part of type I collagen is denatured as gelatin. We therefore considered these experiments to be type I gelatin degradation assays. For inhibiting proteolysis, BB94 (5 μM), rTIMP-1 (0.5 μM), rTIMP-2 (50 μM) was included in the cell suspension. We performed these assays with six replicates/point.

**Immunoprecipitation**—Melanoma cells (5 × 10⁵) were cultured in RPMI 1640–1% FBS on Petri dishes coated with 100 μg/ml type I collagen. Cells were harvested with 1 ml EDTA, washed, and labeled with 100 μg/ml of NHS-biotin in PBS for 20 min at 4 °C. Cells were extensively washed and solubilized in 50 mM Tris-HCl (pH 7.5) containing 50 mM β-mercaptoethanol, 150 mM NaCl, 1 mM CaCl₂, 1 mM MnCl₂, 1 mM MgCl₂, 1 mM NEM, and 1 mM PMSF. Cell lysates were precleared by nonimmune rabbit IgG, isotype-matched IgG-conjugated protein A-Sepharose, and then immunoprecipitated with specific primary antibodies (17). Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to an Immobilon-P membrane (Millipore, Bedford, MA), and detected with horseradish peroxidase (HRP)-conjugated streptavidin followed by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Piscataway, NJ). Alternatively, unlaeled cells were briefly washed with PBS and then lysed in 50 mM Tris-HCl (pH 7.5) containing 1% Brij58, 150 mM NaCl, 1 mM CaCl₂, 1 mM MnCl₂, 1 mM MgCl₂, 1 mM NEM, 1 mM PMSF. Cell lysates were precleared and then immunoprecipitated with primary monoclonal antibodies against MCSP (9.2.27), α integrin (P2δ5), or αβ integrin (LM609). The immunocomplexes were blotted with anti-MT3-MMP antibody followed by HRP-conjugated goat anti-rabbit IgG and detected by ECL.

**Co-immunoprecipitation**—Melanoma cells (5 × 10⁵) were cultured overnight in RPMI 1640 containing 1% FBS on Petri dishes coated with 100 μg/ml type I collagen. Cell lysates prepared as described above were immunoprecipitated with anti-MT3-MMP antibody. Beads were extensively washed with lysis buffer, and bound proteins were released in 50 μl of PBS containing 1% SDS for 10 min at room temperature and diluted in 1 ml of PBS containing 1% Triton-X-100, 100 mM NaCl, 1 mM CaCl₂, 1 mM MnCl₂, 1 mM MgCl₂, 1 mM NEM, 1 mM PMSF. MT3-MMP-associated proteins were re-immunoprecipitated with control isotype-matched IgG, anti-β, mAb (P5D2), anti-αβ, mAb (LM609), or anti-MCSP mAb (9.2.27). The mixture was then extensively washed with 50 mM Tris-HCl (pH 7.5) containing 1% Triton-X-100, 1 mM CaCl₂, 1 mM MnCl₂, 1 mM MgCl₂, 1 mM NEM, 1 mM PMSF and washed three times with PBS. Bound proteins on the beads were labeled with 1 μM NHS-biotin for 15 min at 4 °C, because neither anti-β, nor anti-αβ, mAb is compatible with Western analysis. The beads were washed with PBS five times to remove NHS-biotin, and incubated in SDS-sample buffer under reducing conditions to release the proteins from the beads. Blotted proteins were detected with HRP-streptavidin and ECL. Chondroitin Sulfate Glycosaminoglycan (CS) Affinity Chromatography—CS affinity beads were prepared as described previously (17). Biotinylated melanoma cell lysates were prepared as for immunoprecipitation, precleared on a mock column, and applied to a CS affinity column. The mixture was incubated overnight at 4 °C and then extensively washed with the lysis buffer. Bound proteins were eluted with Tris-HCl (pH 7.5) containing 50 mM β-mercaptoethanol, 0.4 mM NaCl, 1 mM CaCl₂, 1 mM MnCl₂, 1 mM MgCl₂, 1 mM NEM, 1 mM PMSF. Aliquots of eluted protein were separated and detected as described above. Eluted proteins were further immunoprecipitated with anti-MT3-MMP antibody and MT3-MMP was detected as described.

**Purification of MT3-MMP**—MDCK cells were transfected with an expression construct encoding FLAG-tagged MT3-MMP as described previously (36). Transfectants were lysed in 100 mM Tris-HCl (pH7.5) containing 1% Triton-X-100, 0.14 mM NaCl, 10 mM EDTA, 1 mM NEM, and 1 mM PMSF and then incubated with anti-FLAG M2-agarose at a final concentration of 1 mg/ml overnight at 4 °C. Beads were washed four times with the lysis buffer, and bound proteins were eluted with lysis buffer containing 100 μg/ml FLAG peptide. The eluted MT3-MMP was incubated with 50 μl (bed volume) of CS beads or mock-activated beads prepared as described above for 4 h at 4 °C. The beads were washed four times in lysis buffer, bound MT3-MMP was released in SDS sample buffer, and blotted with anti-FLAG and HRP-goat anti-mouse IgG.

### Table I

| Inhibition by monoclonal antibodies* | αβ | αβ1 | αβ2 |
|-------------------------------------|-----|-----|-----|
| Adhesion                            | 80  | 12  | 5   |
| Migration                           | 75  | 6   | 3   |
| Invasion                            | 80  | 10  | 6   |

* Melanoma cell adhesion, migration, and invasion of type I collagen was assayed in the presence of function-blocking anti-integrin monoclonal antibodies P1E6 (anti-αβ), P1B5 (anti-αβ), and LM609 (anti-αβ) as described under "Experimental Procedures."

**RESULTS**

MCSP Mediates Melanoma Invasion into Type I Collagen—VGP melanoma cells invasively invade the collagen-rich dermis in vivo as a presage to metastasis. To model this process in vitro, we examined the adhesion, migration, and invasion of WM1341D melanoma cells on type I collagen-coated surfaces. Based on the use of inhibitory anti-integrin antibodies in short-term adhesion or Boyden chamber migration assays, adhesion and migration to type I collagen was determined to depend primarily on αβ integrin (and to a lesser extent on αβ integrin) (Table I). By contrast, anti-αβ integrin antibodies had no inhibitory effect on melanoma cell adhesion or migration to type I collagen (Table I). The invasion of VGP cells through type I collagen gels was similarly dependent on αβ integrin, with no detectable involvement of αβ integrin. We previously reported that cell surface chondroitin sulfate proteoglycans facilitate melanoma invasion into type I collagen gels and/or basement membrane matrices in vitro (4, 5). WM1341D melanoma cells express two cell surface chondroitin sulfate proteoglycan core proteins (MCSP and CD44) as evaluated by flow cytometry (Fig. 1, A and D). Both MCSP and CD44 have been implicated in melanoma metastasis, because antibodies generated against either of these cell surface proteoglycans inhibit invasion (5, 14). To further assess the importance of MCSP in the invasion of WM1341D cells, we utilized antisense oligonucleotides (oligos) to specifically inhibit expression of the MCSP core protein. Melanoma cells cultured in the presence of sense or antisense MCSP oligos correspond to the first 27 nucleotides of coding sequence were evaluated by FACS analysis for cell surface expression of MCSP (Fig. 1, A–C), CD44 (Fig. 1, D–F), and the β integrin subunit (Fig. 1, G–I). Expression of MCSP was essentially completely inhibited by the antisense but not by the sense MCSP oligos (Fig. 1, B and C). Neither sense or antisense MCSP oligos had a significant effect on CD44 expression (Fig. 1, E and F), nor did they affect the morphology of WM1341D melanoma cells in tissue culture and on type I collagen coated surfaces (not shown). Oligos were not cytotoxic as evaluated by trypan blue exclusion. Although antisense MCSP oligos inhibit expression of β integrin (and to a lesser extent on β integrin), the oligos do not inhibit WM1341D melanoma cell adhesion (not shown) or migration to type I collagen (Fig. 2A), indicating that functional β integrin is unaltered.

WM1341D melanoma cells pretreated with antisense MCSP oligos were assayed for their invasive abilities. Antisense MCSP oligos significantly suppress invasion of type I collagen compared with control or sense MCSP oligo-treated melanoma cells (Fig. 2B), suggesting that MCSP core expression is important for mediating melanoma invasion into type I collagen. Furthermore, MCSP appeared to be involved specifically in mediating invasive potential of the cells, because oligo treatment had no effect on adhesion (not shown) or migration (Fig. 2A) to this substrate.
WM1341D Melanoma Invasion into Type I Collagen Requires Chondroitin Sulfate—To further explore the requirement for chondroitin sulfate glycosaminoglycan (CS) in human WM1341D melanoma invasion into type I collagen, melanoma cells were treated with chondroitinase ABC, beta-D-xylopyranoside (bDX) or its inactive analogue (aDX) to remove CS from the cell surface. Under these conditions, the relative amount or type of integrins on the cell surface was not affected (not shown). Removal of CS either by enzymatic digestion with cABC (Fig. 3A) or by preventing its attachment to cell surface core proteins with bDX (Fig. 3B) effectively inhibited melanoma invasion into type I collagen relative to control untreated cells. As was observed using the antisense MCSP oligos, melanoma CS was not required for adhesion or migration on type I collagen (not shown). Other MMPs either were not detected (MT1-MMP and MT2-MMP) or were detected at extremely low levels (MT4-MMP) by this method.

MMPs Are Required for WM1341D Melanoma Cell Invasion of Type I Collagen—Because metalloproteinases (MMPs) have been shown to mediate melanoma invasion (1), we evaluated the potential involvement of MMPs in WM1341D collagen gel invasion by using a specific synthetic MMP inhibitor, BB94 (38). BB94 significantly inhibited invasion of type I collagen by VGP melanoma cells (Fig. 4). To identify potential MMPs involved in this process, we also tested tissue inhibitor of metalloproteinases-1 (TIMP-1) and TIMP-2 for their ability to inhibit invasion (39). Melanoma invasion into type I collagen was significantly inhibited by TIMP-2 but not by TIMP-1 (Fig. 4). The reported specificity of these inhibitors suggested that the invasion of WM1341D cells into collagen gels may be mediated by a specific membrane type MMP (MT-MMP) expressed on WM1341D melanoma cell surfaces.

WM1341D Melanoma Cells Express MT3-MMP, which Mediates Invasion of Type I Collagen—We next evaluated melanoma cells for surface expression of MT-MMPs. Melanoma cells were surface-biotinylated, and cell lysates were immunoprecipitated with antibodies against MT1-, MT2-, MT3-, or MT4-MMPs. The immunoreactivity of the antibodies for their specific MT-MMPs was verified by immunoprecipitating each MT-MMP from MDCK cells transfected with the respective MT-MMP cDNA (not shown). As shown in Fig. 5A, WM1341D cells predominantly expressed MT3-MMP on the cell surface. Other MT-MMPs either were not detected (MT1-MMP and MT2-MMP) or were detected at extremely low levels (MT4-MMP) by this method.
To determine if MT3-MMP is required for VGP melanoma invasion of type I collagen, we stably transfected WM1341D cells with a plasmid encoding full-length antisense mRNA for MT3-MMP. Untransfected cells, vector-transfected (mock) or antisense MT3-MMP transfectants were cultured on type I collagen-coated transwells for invasion assays as described. Data are presented as mean ± S.D., *p < 0.001.

Degradation of Type I Gelatin Requires MT3-MMP and MCSP—MT3-MMP has been shown to degrade various ECM proteins, including type I gelatin (30). We reasoned, therefore, that MT3-MMP expressed on melanoma cells could be promoting invasion of the cells by directly or indirectly proteolyzing type I gelatin (the partially denatured form of collagen). We assayed gelatinolytic activity of untransfected WM1341D cells and transfectants expressing vector (mock) or antisense MT3-MMP constructs. Antisense MT3-MMP-inhibited transfectants released significantly reduced quantities of 3H-labeled proteolytic fragments than control cells, indicating that MT3-MMP expression is important for the innate gelatinolytic activity of these melanoma cells (Fig. 6A). To further support the notion that melanoma-mediated proteolysis of type I collagen is mediated by MT3-MMP, BB94, rTIMP-1, and rTIMP-2 were added exogenously as inhibitors for MMP-catalyzed degradation of type I gelatin. As was observed for tumor invasion, BB94 and rTIMP-2 (but not rTIMP-1) significantly inhibited melanoma cell-mediated degradation of type I gelatin. These results are consistent with an active role for MT3-MMP in proteolysis of type I collagen required for invasion of a three-dimensional gel (Fig. 6C).

Because MCSP was also required for invasion of type I collagen, we next assayed gelatinolytic activity of wild type WM1341D cells in the absence or presence of sense and antisense MCSP oligos. Antisense (but not sense) MCSP oligo treatment significantly inhibited degradation of type I gelatin in this assay (Fig. 6B), leading to the speculation that MCSP expression may be required to mediate MT3-MMP proteolysis of type I gelatin, possibly via its covalent CS moieties. To address this question, we assayed for gelatinolytic activity of wild type WM1341D cells in the absence or presence of βDX and αDX to inhibit CS modification. βDX, but not αDX, significantly inhibited release of 3H-type I gelatin fragments (Fig. 6C). Neither βDX nor αDX treatment inhibited MT3-MMP expression on the melanoma cell surface (not shown). MCSP-mediated collagen invasion and proteolysis may therefore occur...
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FIG. 6. WM1341D melanoma cell proteolysis of type I collagen requires MT3-MMP and chondroitin sulfated MCSP. Melanoma cells were analyzed for gelatinase activity in 96-well plates coated with 3H-type I gelatin. Following an 8-h incubation, media were removed and radioactivity released as a result of proteolysis was quantified by liquid scintillation counter. A, gelatinase activity was determined for wild type, mock-transfected, and pBKRSV(MT3AS)-transfected cells. B, assays were performed in the absence (WT) or presence of 48-h pretreatment with 30 μM MCSP antisense or sense oligos. C, assays were conducted in the absence (control) or presence of 1 mM DX and αDX, or MMP inhibitors BB94 (5 μM), rTIMP-1 (50 nM), or rTIMP-2 (50 nM). *, p < 0.001.

by association with MT3-MMP through CS at the melanoma cell surface.

MT3-MMP Associates with MCSP and the Interaction Is CS-dependent—To identify potential direct interactions between MT3-MMP and other cell surface proteins, lysates from WM1341D melanoma cells cultured on type I collagen were immunoprecipitated with mAbs against adhesion receptors: MCSP (9.2.27), β1 integrins (P5D2), and αvβ3 integrin (LM609). The immunoprecipitated proteins were analyzed by Western blot probed with anti-MT3-MMP antibody. MT3-MMP was found to co-immunoprecipitate most efficiently with MCSP, whereas anti-β1 integrin co-precipitated very low amounts (Fig. 7A). No detectable MT3-MMP was co-immunoprecipitated with αvβ3 integrin (Fig. 7A). As a control, cell lysates prepared from melanoma cells cultured on laminin-coated plates were also immunoprecipitated with anti-MCSP, anti-β1, and anti-αvβ3 integrin mAbs. Under these conditions, MT3-MMP is again co-precipitated with MCSP, and in lesser amounts with β1 integrin, but not with αvβ3 integrin (Fig. 7B).

To further demonstrate the specificity of the interaction between MT3-MMP and MCSP, we performed reciprocal immunoprecipitations. Cell lysates prepared from WM1341D cells cultured on type I collagen were first immunoprecipitated with anti-MT3-MMP, and then immune complexes were released and re-immunoprecipitated with anti-MCS (anti-β1 integrin, or anti-αvβ3 integrin mAbs. Proteins were then biontylated and blotted with HRP-streptavidin. Using this approach, only MCSP was found to co-precipitate with MT3-MMP (Fig. 8A), indicating a specific interaction exists between MT3-MMP and MCSP. We then investigated the importance of CS modification for this interaction. The inhibition of CS expression by βDX (but not αDX) significantly diminished the association of MCSP with MT3-MMP (Fig. 8B) in co-immunoprecipitations, indicating that CS on the MCSP core protein is a critical component of MCSP-MT3-MMP interactions.

To determine if MT3-MMP can interact directly with CS, we performed affinity chromatography with CS immobilized on Sepharose beads (17). Biotinylated melanoma cell surface proteins were applied to a CS-affinity column as described under “Experimental Procedures.” Precleared cell lysates were immunoprecipitated with antibodies against MCSP (9.2.27), β1 integrin (P5D2), or αvβ3 integrin (LM609). Immunocomplexes were separated by SDS-polyacrylamide gel electrophoresis, transferred to Immobilon-P membrane, and blotted with anti-MT3-MMP. Molecular masses are shown on the left (kDa).

FIG. 7. MT3-MMP is co-immunoprecipitated with MCSP. Melanoma cells were cultured on type I collagen (A) or laminin (B) overnight and then lysed as described under “Experimental Procedures.” Precleared cell lysates were immunoprecipitated with antibodies against MCSP (9.2.27), β1 integrin (P5D2), or αvβ3 integrin (LM609). Immunocomplexes were separated by SDS-polyacrylamide gel electrophoresis, transferred to Immobilon-P membrane, and blotted with anti-MT3-MMP. Molecular masses are shown on the left (kDa).
FIG. 8. CS mediates the interaction between MT3-MMP and MCSP. A, WM1341D melanoma cells cultured on type I collagen were lysed and lysates immunoprecipitated with anti-MT3-MMP antibody. Associated proteins were released and then re-immunoprecipitated with control IgG, PS2D (β1 integrin), LM609 (αvβ3), or 9.2:27 (MCSP). Final immunoprecipitated proteins were biotinylated and detected by Western blot with HRP-conjugated streptavidin. Molecular masses (kDa) are shown on the left. B, melanoma cells were cultured on type I collagen in the absence (control) or presence of αDX or δDX and then lysed and immunoprecipitated by anti-MT3-MMP antibody as described above. Immunoprecipitated proteins were released and re-immunoprecipitated with 9.2:27. Immuno complexes were biotinylated and analyzed by Western blot with HRP-conjugated streptavidin.

![Diagram](image)

FIG. 9. MT3-MMP binds chondroitin sulfate glycosaminoglycan (CS)-conjugated Sepharose. A, WM1341D melanoma cells cultured on type I collagen were cell surface-labeled with biotin and lysed as described under “Experimental Procedures.” Pre cleared cell lysates were applied to a CS-affinity column. Molecular masses (kDa) are indicated. B, melanoma cells were cultured on type I collagen and then lysed and immunoprecipitated with biotinylated anti-FLAG mAb and horseradish peroxidase-conjugated beads were washed, and associated proteins were detected by Western blot using biotinylated anti-FLAG mAb and horseradish peroxidase-conjugated streptavidin. The arrowhead designates MT3-MMP, demonstrating the specificity of anti-MT3-MMP antibody. Purified MT3-MMP-FLAG was incubated with CS-conjugated beads or mock (no ligand was immobilized) and washed extensively. MT3-MMP-FLAG bound to CS-conjugated beads but not to mock beads (Fig. 9C). Together, the studies demonstrate that MT3-MMP directly interacts with CS and this interaction may be required for WM1341D melanoma invasion and proteolysis of type I collagen.

**DISCUSSION**

Cell adhesion and proteolysis are tightly linked processes in the migration and invasion of normal and transformed cells. Cell surface adhesion receptors bind directly to specific proteolytic enzymes involved in degradation of the extracellular matrix. The interaction of MMP-2 with αvβ3 integrin is among the first such protease/adhesion receptor combinations identified (9, 10). Binding of MMP-2 by αvβ3 integrin is thought to provide a mechanism by which invading tumor cells can focus and activate MMPs within adhesion sites in the vicinity of the cell surface. More recently, it has been reported that MMP-9 interacts with CD44, leading to enhanced tumor invasion and survival (11). Urokinase plasminogen activator binds to a cell surface receptor that has also been localized with αvβ3 integrin to caveolin-rich domains on cells (8). Finally, other transmembrane proteases, termed ADAMs, interact directly with specific integrins and modify the activation of those integrins on adherent or migrating cells (40–42). Therefore, proteases and adhesion receptors may enhance invasion both by promoting ECM degradation and/or modifying the activation state of adhesion receptors.

MMPs degrade native and denatured collagen by their collagenolytic and gelatinolytic activities (43). In the current report, we have used antisense RNA inhibition to demonstrate that MT3-MMP expression is required for invasion of type I collagen by WM1341D VGP melanoma cells. Other reports have documented the importance of MT3-MMP expression in the growth, tubulogenesis, and the formation of cyst-like structures in MDCK cells cultured in type I collagen gels (36, 44). MT3-MMP does not act as a typical collagenase (i.e. it does not cleave native collagen into the typical 3 quarter/1 quarter fragments). Rather, MT3-MMP has been shown to cleave the telopeptides from type I collagen at specific sites, leading to the partial denaturation of non cross-linked collagen (30). Our results may, therefore, suggest that MT3-MMP expression in vertical growth phase melanomas in vivo could promote the degradation of newly formed collagen, partially denatured collagen, or non-collagenous ECM proteins in the vicinity of the growing tumor, providing space in which the tumor can expand (45).

Whether MT3-MMP acts directly on the surrounding matrix is not clear from our current studies. TIMP-2, which inhibits MT3-MMP catalytic activity (30), significantly inhibits invasion. However, MT-MMPs can act to directly promote degradation of the extracellular matrix or they can facilitate the activation of other MMPs. For example, MT1, MT2, MT3, and MT5 MMP have all been documented to activate pro-MMP-2 (25, 29, 46, 47). Active MMP-2 has been associated with the invasion of multiple tumor types, including melanoma (48). MMP-2 in solution is inhibited by both TIMP1 and TIMP2 (39). In our system, only TIMP-2 is effective at inhibiting WM1341 melanoma invasion. This observation might argue for a minimal involvement of MMP-2 in the invasion of these cells. Alternatively, the failure of TIMP-1 to inhibit invasion might be explained by the inability of TIMP-1 to access MMP-2 in the vicinity of the cell surface, as has been suggested by Deryugina, et al. (49). We do have preliminary data to indicate that this cell line expresses MMP-2, and we are cur-
rently addressing the importance of MMP-2 expression and activation in the VGP cell line.

In addition to MMP-2, it is also quite possible that MT3-MMP could activate other proteases (e.g. MMP-13) that are more effective at degrading fibrillar collagens (50). By analogy, MT1-MMP can activate MMP-13 alone or in concert with MMP-2 (27). In contrast to either MT3-MMP or MMP-2, MMP-13 is a potent collagenolytic and gelatinolytic enzyme that cleave types I, II, and III collagens (50). MMP-13 may be an important protease for stimulating connective tissue remodeling associated with tumor invasion and recently increased amounts of MMP-13 have been associated with melanoma progression (28). Given the similar structural homologies between MT1-MMP and MT3-MMP (25, 29), MT3-MMP may activate MMP-13 directly or indirectly via an MMP-2 mediated mechanism.

MMP activity is not required for the migration of WM1341D cells on type I collagen. This is in contrast to other studies documenting the importance of MMP activation in cell migration. For example, Pilcher et al. (51) demonstrated that the catalytic activity of collagenase-1 (MMP-1) is important for the migration of primary keratinocytes, because TIMP-1 inhibits the migration of these cells. In our system, neither TIMP-1 nor TIMP-2 inhibited melanoma migration, nor did BB94 (not shown). Other studies indicate that MT1-MMP and several other MMPs do not enhance migration of cells on type I collagen (44). The relevance of MMPs to cell migration may be cell-type-specific, although additional studies are required to understand the basis for such specificity.

MCSP interaction with MT3-MMP in WM1341D cells requires the expression of an intact chondroitin sulfate proteoglycan. Agents that interfere with the expression of the MCSP core protein or with the addition of CS onto the core protein can effectively inhibit invasion and proteolysis of surface-bound type I collagen in our degradation assay. Removal of either the core protein or the CS has no effect on the adhesion of these cells to surface-bound type I collagen nor is the expression of MT3-MMP inhibited by these treatments. Furthermore, recombinant epitope-tagged MT3-MMP interacts directly with CS-affinity columns. Collectively, these results suggest that the CS on the proteoglycan may alter the localization and/or activity of MT3-MMP. Other members of the MMP protease family have been documented to bind the glycosaminoglycan heparin, and this interaction has been shown to activate certain MMPs (52). Our results are consistent with an analogous role for melanoma cell surface CS with respect to MMP function.

The co-precipitation of the protease and MCSP is not dependent on the substrate on which the cells are cultured. This suggests that the interaction of these two plasma membrane molecules is not driven by the extracellular matrix, but rather may be dependent on intrinsic features of the two molecules. Understanding the molecular structures within MT3-MMP that interact with CS will lead to further defining the molecular basis for the interaction of these two molecules and for understanding the importance of this interaction for MT3-MMP activation.

In contrast to other melanoma cells (53), the adhesion, migration, and invasion of these cells on or within type I collagen is independent of $\alpha_\beta_3$ integrin. MT3-MMP and MCSP interactions may enhance VGP melanoma cell invasion by an alternative mechanism to that previously described for $\alpha_\beta_3$ integrin and MMP-2. Our results argue that MT3-MMP interaction with MCSP is largely dependent on the presence of CS, but we cannot definitely rule out a contribution from the MCSP core protein. Small amounts of MT3-MMP are also observed in immunoprecipitates of $\beta_1$ integrin, but there is no detectable interaction between $\alpha_\beta_3$ and MT3-MMP. It is not yet clear if the small amounts of MT3-MMP detected in the $\beta_1$ integrin immunoprecipitates reflect a biologically meaningful interaction between integrin and this MT-MMP. We are addressing this issue with other approaches (i.e. different solubilization conditions, different anti-integrin antibodies, etc.). It is possible that the small amount of MT-MMP in the $\beta_1$ integrin immunoprecipitates may result from MCSP-$\alpha_\beta_3$ integrin complexes on the cell surface, by a mechanism analogous to what we have previously shown for $\alpha_\beta_1$-MCSP interactions (17).

Our results clearly demonstrate that the invasion of this VGP melanoma cell line requires the action of specific MMPs. However, our results do not preclude the involvement of MMP-independent mechanisms in melanoma invasion. There is some residual invasion of these cells even in the presence of specific MMP inhibitors (e.g. BB94 and TIMP-2), as has previously been demonstrated for the invasion of certain mammary carcinomas (54). The removal of cell surface CS is almost completely effective at inhibiting invasion in our system, and it is possible that such MMP-independent mechanisms might be CS-dependent.

Early melanoma progression involves distinct changes in the relationship(s) between keratinocytes and transformed melanocytes (2). Epidermal melanocytes are normally in contact with keratinocytes through an E-cadherin-dependent mechanism. This cell-cell adhesion results in the regulation of melanocyte growth and invasive phenotype. In contrast, progression to melanoma is accompanied by a loss in the keratinocyte/melanocyte intercellular adhesions, with a corresponding loss in the ability of the keratinocyte to down-regulate melanoma growth or expression of invasion-related adhesion receptors. The up-regulation of specific MMPs such as MT3-MMP may enhance the dissolution of keratinocyte/melanocyte adhesion structures and contribute to early stages of tumor progression. By using melanoma cells isolated from early primary melanomas, this and other questions related to the importance of protease activation in early tumor progression can be addressed directly. Such information may improve diagnostics or the development of more tailored therapies for melanoma patients.

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