Differential Modulation of Human Melanoma Cell Metalloproteinase Expression by $\alpha_2\beta_1$ Integrin and CD44 Triple-helical Ligands Derived from Type IV Collagen*

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Tumor cell binding to components of the basement membrane is well known to trigger intracellular signaling pathways. Signaling ultimately results in the modulation of gene expression, facilitating metastasis. Type IV collagen is the major structural component of the basement membrane and is known to be a polyvalent ligand, possessing sequences bound by the $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ integrins, as well as cell surface proteoglycan receptors, such as CD44/chondroitin sulfate proteoglycan (CSPG). The role of $\alpha_2\beta_1$ integrin and CD44/CSPG receptor binding on human melanoma cell activation has been evaluated herein using triple-helical peptide ligands incorporating the a1(IV)382–393 and a1(IV)1263–1277 sequences, respectively. Gene expression and protein production of matrix metalloproteinases-1 (MMP-1), -2, -3, -13, and -14 were modulated with the $\alpha_2\beta_1$-specific sequence, whereas the CD44-specific sequence yielded significant stimulation of MMP-8 and lower levels of modulation of MMP-1, -2, -13, and -14. Analysis of enzyme activity confirmed different melanoma cell proteolytic potentials based on engagement of either the $\alpha_2\beta_1$ integrin or CD44/CSPG. These results are indicative of specific activation events that tumor cells undergo upon binding to select regions of basement membrane collagen. Based on the present study, triple-helical peptide ligands provide a general approach for monitoring the regulation of proteolysis in cellular systems.

Melanoma is one of the most rapidly increasing malignancies in the world in both young and old patients, with over 50,000 newly diagnosed patients each year (1).1 Mortality from cancer is frequently due to metastasis; given that surgical excision of the primary tumor considerably enhances the prognosis of a patient and prolongs survival (2). Metastasis is a complex series of finely coordinated events that results in cancer cells circulating in lymph and blood vascular systems to invade remote tissues and establish secondary sites of tumor growth (3).

Extravasation of the tumor cell into secondary tissues requires alterations in cellular behaviors, resulting from specific adhesion to components of the basement membrane. Tumor cells respond to each of the various components of the ECM,2 including the collagens; noncollagenous glycoproteins, such as fibronectin and laminin; and proteoglycans, such as decorin and syndecan (4). These interactions are known to occur through several families of cell surface receptors, including the integrins and cell surface proteoglycans.

Integrins are heterodimeric proteins composed of one $\alpha$ and one $\beta$ subunit and are the best described of the cell surface adhesion molecules. To date, there are 18 different $\alpha$ subunits and 8 distinct $\beta$ subunits identified that combine to form at least 24 heterodimers (5–7). Although the specific integrin expression profile can fluctuate with tumor type and stage of progression, highly metastatic melanoma cells are known to up-regulate expression of $\alpha_2\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_3\beta_1$, $\alpha_3\beta_1$, $\alpha_3\beta_1$, and $\alpha_3\beta_1$ integrins while down-regulating the expression of other integrins (2, 8). Integrins are frequently capable of recognizing more than one ligand from the ECM; in turn, ECM components such as collagen and fibronectin are known to bind several different integrins. Ligand binding by integrins triggers a series of intracellular signaling events that ultimately result in the release of cytokines and proteases. Increased proteolysis eventually results in basement membrane degradation, liberation of cell surface receptors, and cytokine activation, all of which are often beneficial for tumor cell progression (9).

The collagen-binding integrins include $\alpha_2\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, (10–15), and $\alpha_4\beta_1$, $\alpha_4\beta_1$, $\alpha_4\beta_1$ are overexpressed by melanoma cells (10, 16). More precisely, the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins are up-regulated in metastatic melanoma, whereas the $\alpha_2\beta_1$ integrin is up-regulated in both primary and metastatic melanoma (2, 17–19). The collagen-binding integrins have numerous roles in melanoma progression. For example, the $\alpha_2\beta_1$ integrin is the primary melanoma cell adhesion

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4 ‡ See also www.cancer.org/docroot/STT/stt_0.asp.

The abbreviations used are: ECM, extracellular matrix; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; $\alpha$1V436–447 fTHP, (Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Pro-Lys(Mca)-Gly-Pro-Pro-Lys(Mca)-Gly-Pro-Pro-Lys(Mca); ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; $\alpha$1V436–447 fTHP, (Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Pro-Gly–Val–Val–Val–Glu–Lys(Dnp)-Gly–Glu–Gln–(Gly–Pro–Hyp)–NH$_2$; CS, chondroitin sulfate; CSPG, chondroitin sulfate proteoglycan; Dnp, 2,4-dinitrophenyl; ELISA, enzyme-linked immunosorbent assay; FAK, focal adhesion kinase; fTHP-4, (Gly–Pro–Hyp)–Gly–Pro–Gly–Gln–Gly–Leu–Arg–Gly–Gln–Lys(Dnp)-Gly–Val–Arg–(Gly–Pro–Hyp)–NH$_2$; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hyaluronic acid; Mca, (7-methoxyxoumarin-4-yl)acetyl; MMP, matrix metalloproteinase(s); PBS, phosphate-buffered saline; pH 7.4; SSP, single-stranded peptide; THP, triple-helical peptide; TIMP, tissue inhibitor of metalloproteinase; MAPK, mitogen-activated protein kinase; mAb, monoclonal antibody; RT, reverse transcriptase; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
molecule for type IV collagen and is used to contract and remodel type I collagen (17, 20, 21). The α2β1 integrin also participates in migration of metastatic melanoma on type IV collagen (22). The α2β1 integrin up-regulates the α isoform of p38 MAPK in a three-dimensional collagen matrix, resulting in stimulation of MMP-1 and type I collagen synthesis (23). Engagement of the α2β1 integrin has also been linked to induction of MMP-1 and MMP-14 (24, 25) and implicated in MMP-9 regulation (26). Furthermore, MMP-1 binds to the α2β1 integrin via the I domain of the αv subunit (27).

The cell surface proteoglycans are a second class of receptors implicated in tumor metastasis. Melanoma cells have been shown to possess at least two distinct cell surface chondroitin sulfate proteoglycans, CD44 and the melanoma-associated proteoglycan/melanoma chondroitin sulfate proteoglycan (4, 28). CD44 binds directly to type IV collagen, and this binding is dependent upon chondroitin sulfate (CS) (21). In general, melanoma cell invasion of the basement membrane is inhibited by the selective removal of the CS (21). The nonspliced variant of CD44, CD44s (also known as CD44H), is the sole form of CD44 expressed by human melanoma cells (29).

Several lines of evidence indicate that CD44s plays an important role in melanoma progression. Up-regulation of CD44s mRNA transcription and cell surface expression has been found in highly metastatic melanoma as compared with lowly metastatic melanoma or nontransformed melanocytes (29–32). Enhanced expression of CD44s is also found in endothelial cells within the vasculature of tumors compared with endothelial cells from normal tissue (33). Transfection of CD44 isolated from metastatic cells into nonmetastatic tumor cells results in conferment of metastatic behavior (34). In addition, tumor cells expressing CD44s display accelerated tumor growth and metastatic spread in immunodeficient mice compared with parental cells (34), whereas a knockout of the CD44 gene virtually eliminates metastasis in mice (35). Approaches that interfere with CD44 binding to its ligand, such as administration of high molecular weight hyaluronic acid (HA), anti-CD44 mAb, or a CD44 receptor globulin, reduce tumor formation in the lung for animal models established from CD44-expressing tumor cell lines (36). Finally, proteolytic removal of CD44 inhibits the growth of primary tumors and curtails metastasis in a mouse B16 melanoma model (37).

In an attempt to dissect the roles of the α2β1 integrin and CD44 cell surface receptors in melanoma progression, we constructed type IV collagen-derived triple-helical peptide (THP) ligands for these receptors. The Gly–Phe–Hyp–Gly–Glu–Arg motif, in triple-helical conformation, has been shown to bind to the α2β1 integrin (38–40). This motif is found within type IV collagen at α1(IV)382–393 (39, 41).3 The type IV collagen α1(IV)1263–1277 sequence Gly–Val–Lys–Gly–Asp–Lys–Gly–Asn–Pro–Gly–Trp–Pro–Gly–Ala–Pro is bound by melanoma cell CD44 receptors in the chondroitin sulfate proteoglycan (CSPG) form (42–44). Loss of triple-helical structure dramatically reduces melanoma cell adhesion, spreading, and signaling mediated by this ligand (45–47). These THP ligands were employed individually, along with their linear counterparts (see Fig. 1), to study temporal changes in gene expression, at the levels of both mRNA and protein. RT-PCR was used to measure the levels of MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13, heparanase A, ADAMTS-1, ADAMTS-2, ADAMTS-3, and ADAMTS-4 gene expression over 24 h. Protein expression of targets modulated by either ligand was examined by ELISA analysis. Finally, fluorescent THP substrates were used to quantify active protease production. The molecular consequences of metalloprotease regulation by these two receptors were then considered in light of metastatic progression.

**EXPERIMENTAL PROCEDURES**

**Materials**—The parental human melanoma cell line M14 was obtained as a generous gift from Dr. Barbara M. Mueller (La Jolla Institute for Molecular Medicine, La Jolla, CA). Tissue culture reagents were obtained from Fisher unless otherwise stated. The reagents for RT-PCR were all purchased through Invitrogen, and all of the immunological supplies were purchased from Chemicon International (Temecula, CA). MMPs used as standards were expressed, purified, or purchased as recombinant proteins (34, 41). 2-β-Hexosaminidase was a generous gift of Dr. Michael Jones (Tufts), 1,4-butanediol di-(2-ethylhexyl)-hexafluorophosphate, 1-hydroxybenzotriazole, and 9-fluorenylmethoxycarbonyl-amino acid derivatives were obtained from Novabiochem (San Diego, CA). Amino acids are of the L-configuration (except for Gly). Hexanoic acid (CH3(CH2)5-CO2H, designated CH3 and decanoic acid (CH3(CH2)7-CO2H, designated C10) were purchased from Sigma. Isopropyl alcohol, N,N-dimethylformamide, and 380 Da molecular weight hyaluronic acid (HA), anti-CD44 mAb, and a CD44 receptor globulin, reduce tumor formation in the lung for animal models established from CD44-expressing tumor cell lines (36).

Preparation of Peptide and Peptide Amphiphile Ligands—Peptide resin assembly was performed on an Applied Biosystems 433A peptide synthesizer using 9-fluorenylmethoxycarbonyl solid phase methodology, as previously described (50, 51). All of the standard peptide synthesis chemicals and solvents were analytical grade or better. Edman degradation sequence analysis was performed on each peptide resin with an Applied Biosystems 477A protein and peptide sequencer, as previously described for “embedded” (noncovalent) sequencing (52). Lipidation of the peptide resin involved condensing a 4-fold molar excess of hexanoic or decanoic acid to the N′-deprotected resin, with a 3.8-fold molar excess each of 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and 1-hydroxybenzotriazole in N,N-dimethylformamide for 2 h. Cleavage and side chain deprotection of the lipidated peptide resins was accomplished by treating the resin for 1–2 h with either H2O–TFA (1:190) or trifluoroacetic acid (TFA) (2.5:5:5:8:2.5) (for SSP-C, SSP-D, and C10-(Gly–Pro–Hyp), Gly–Pro–Lys(Mca)–Gly–Pro–Gln–Gly–Cys(4-methoxybenzyl)-Arg–Gly–Lys–Gly–Asp-(Gly–Val–Arg–Gly–Pro–Hyp)–NH2). The resulting cleavage products were purified by preparative reverse phase HPLC and analyzed by analytical reverse phase HPLC and matrix-assisted laser desorption ionization time-of-flight mass spectrometry as previously described (53, 54). [M+H]+ values were: SSP-A, 1469.9 Da (theoretical 1470.7 Da); SSP-B, 3610.7 Da (theoretical 3609.3 Da); SSP-C, 1658.3 Da (theoretical 1671.0 Da); SSP-D, 3811.5 Da (theoretical 3813.3 Da); and C10-(Gly–Pro–Hyp), Gly–Pro–Lys(Mca)–Gly–Pro–Gln–Gly–Cys(4-methoxybenzyl)-Arg–Gly–Lys–Gly–Asp-(Gly–Val–Arg–Gly–Pro–Hyp)–NH2, 4846.2 Da (theoretical 4853.2 Da). Individual peptide sequences were assigned by mass spectrometry with a JASCO 6000 spectrometer, with readings collected in the range of λ = 190–250 nm. The individual thermal transitions were determined in a thermostatted 1-cm quartz cell over the range of 5–80 °C at λ = 225 nm, with lipidated peptide concentrations of 10–20 μM.

**Cell Culture**—Parental M14 cells were routinely cultured in 75-cm2 flasks or 100-mm plates with RPMI 1640, supplemented with 10% fetal bovine serum (BioWhittaker, Inc., Walkersville, MD) and the following antibiotics: 0.1 mg/ml gentamicin sulfate, 50 units/ml penicillin, and 0.05 mg/ml streptomycin sulfate. The cells were cultured for up to eight passages and then replaced by frozen stocks to minimize phenotypic drift. All of the M14 cultures were maintained at 37 °C in a humidified incubator containing 5% CO2.

**Cell Adhesion**—Cell adhesion assays were performed to quantify cell binding to ligands. Melanoma cell adhesion to substrate-coated ProBind™ 96-well plates (B & D Biosciences) was performed as described previously (44, 55). Lipidated peptides dissolved in PBS were diluted in 7% FCS, added to the 96-well plate, and allowed to adsorb overnight at room temperature with mixing. Non-specific binding sites were blocked with 2 mg/ml bovine serum albumin in PBS for 1 h at 37 °C. The cells were released with 50 mM EDTA in PBS, washed twice with adhesion medium (20 mM HEPES, 2 mg/ml bovine serum albumin in RPMI 1640) and labeled with 5- or 6-carboxyfluorescein diacetate. Unincorporated fluorophore was removed by repeated washings with adhesion medium. The cells were then resuspended in adhesion medium and added to the plate. The plate was incubated 80 min at 37 °C. Nonadherent cells were removed by washing three times with adhesion medium. Adherent cells were lysed with 0.2% SDS and quantified using λexcitation = 485 nm and λemission = 538 nm with a SpectraMAX Gemi-
niEM 96-well plate spectrophuorometer and SoftMax Pro 4.3LS software (both from Molecular Devices).

**Induction of Melanoma Cells**—Pro-Bind\textsuperscript{TM} 96-well assay plates were conditioned at room temperature overnight prior to initiation of the induction experiment with the appropriate concentration of ligand (see “Results”). The plates were then blocked by adding 2 mg/ml bovine serum albumin in PBS and incubated overnight at room temperature. M14 cultures used for induction experiments were typically 60–80% confluent before release from growth flasks with PBS containing 5 mM EDTA, pH 7.3. Subsequent to release, the cells were conditioned at room temperature overnight prior to initiation of all of the reagents were from Invitrogen\textsuperscript{TM}.

**Primer-dropping** PCR was performed on 10% of the resulting cDNA in Pro-Bind\textsuperscript{TM} 96-well Pro-Bind\textsuperscript{TM} assay plates following the manufacturer’s recommendations. The cycling program was UDG inactivation at 50 °C for 2 min, 95 °C for 2 min, followed by 45 cycles of (a) 5 s denaturation at 94 °C, (b) annealing at 60 °C for 10 s, and (c) extension at 72 °C for 15 s. Melting curve analysis was performed using continuous acquisition and a slope of 0.1 °C/s. The human GAPDH-certified LUX\textsuperscript{TM} primer set was used as a housekeeping control for all amplifications.

**Melanoma Cell-specific Protein Expression**—Conditioned medium was isolated by withdrawal of the media from growing cells, centrifuging at 1000 × g (to remove any floating cells). For MMP-1 and MMP-3, ELISA was performed as described (62) using appropriate antibodies purchased from Chemicon. MMP-8 and MMP-13 ELISA kits were obtained from R & D Systems (Minneapolis, MN). ELISA was used to measure protein expression in the conditioned media from cells grown on surfaces coated with lipitated peptides.

**Cellular MMP Assays**—Two assays were utilized to evaluate active MMP production by melanoma cells. The first assay involved MMP immobilization (106). A 96-well plate was incubated with the appropriate MMP mAb for at least 18 h at 4 °C. Nonspecific binding sites were blocked by incubating with phosphate-buffered saline containing 0.05% Tween\textsuperscript{TM} 20 and 2 mg/ml bovine serum albumin for at least 4 h at 4 °C and washed three times with enzyme assay buffer (50 mM Tricine, 50 mM NaCl, 10 mM CaCl\textsubscript{2}, 0.05% brij-35). Either MMP standards or
FIG. 1. Sequences of linear and triple-helical ligands. The four peptide amphiphiles used in this study are C_{16}(α1(IV)382–393)-NH₂ SSP ligand for the α₁β₁ integrin (SSP-A), C₁₆(α₁(IV)382–393)-NH₂ THP ligand for the α₁β₁ integrin (THP-B), C₁₆(α₁(IV)1263–1277)-NH₂ SSP ligand for CD44/CSPG (SSP-C), and C₁₆(α₁(IV)1263–1277)-NH₂ THP ligand for CD44/CSPG (THP-D).

FIG. 2. Relative mRNA expression levels for MMP-1 (open diamonds), MMP-8 (open triangles), MMP-13 (open circles), and MMP-14 (open squares) following melanoma cell adhesion to the α₁β₁ integrin-specific THP-B (A) or CD44/CSPG specific THP-D (B). M14 melanoma cells were allowed to adhere to 10 μM THP-B or THP-D for 60 min at 37 °C. Three washes of adhesion medium were used to remove nonadherent cells, and the cells were grown for a total of 24 h. DNase I-treated total RNA was isolated from 90,000 cells/time point and used in RT-PCR with gene-specific primers. All of the values are expressed as a ratio of target gene to GAPDH and normalized to the initial time point. The results are the mean of duplicate experiments and gels, with standard deviations typically on the order of 10% or less.
up-regulation followed by down-regulation; 

Table II

| Target | Receptor engaged | mRNA | Protein | Active enzyme |
|--------|------------------|------|---------|---------------|
| MMP-1  | α_{2β1} integrin  | +    | +       | +             |
| MMP-2  | α_{2β1} integrin  | – → + | ND      | +             |
| MMP-3  | α_{2β1} integrin  | +    | + → –   | ND            |
| MMP-8  | α_{2β1} integrin  | NM   | +       | ND            |
| MMP-13 | α_{2β1} integrin  | +    | +       | ND            |
| MMP-14 | α_{2β1} integrin  | + → – | ND      | + → –         |

Fig. 3. Relative mRNA expression levels for MMP-8 following melanoma cell adhesion to the CD44/CSPG specific THP-D (open triangles) and SSP-C (open squares). M14 melanoma cells were allowed to adhere to 10 μM THP-D or 50 μM SSP-C for 60 min at 37 °C. Three washes of adhesion media were used to remove nonadherent cells, and the cells were grown for a total of 24 h. DNase I-treated total RNA was isolated from 90,000 cells/time point and used in RT-PCR with gene-specific primers. All of the values are expressed as a ratio of target gene to GAPDH and normalized to the initial time point. The results are the means of duplicate experiments and gels, with standard deviations typically on the order of 10% or less.

unknown samples were added to each well, and the plate was mixed for at least 18 h at 4 °C. All of the liquid was removed, and 2 μl 4-aminophenylmercuric acetate was added to each well (where applicable). This was followed by a 2-h incubation at 37 °C. The wells were washed three times with enzyme assay buffer, and the appropriate fluorogenic substrate was added to each well. The plate was incubated at 37 °C in a humidified atmosphere for at least 18 h. Fluorescence readings (λ_{excitation} = 325 nm and λ_{emission} = 393 nm) were taken at appropriate intervals, and a standard curve was created by plotting the percentage of increase in fluorescence versus concentration of MMP standard. This standard curve can be used to calculate the active enzyme concentration in the conditioned media.

Protein Electrophoresis/Gelatin Zymography—Proteins in the cell-conditioned medium were resolved by SDS-PAGE with 4–15% pre-cast Tris-HCl gels (Bio-Rad) and silver stained (BioRad). Images of the gels were captured and analyzed on a BioRad Fluor-S TM MultiImager using Quantity One®, version 4.3.0 software package. To prepare the conditioned medium samples, the samples were first concentrated 15 times using spin concentrators (10,000 molecular weight cut-off) at 3000 rpm for 90 min. Fifteen μl of sample was mixed with 5 μl of 2× sample buffer (0.125 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol). Fifteen μl was loaded and then electrophoresed for 1 h at 180 V on SDS-polyacrylamide gels containing 1 mg/ml gelatin (type A, porcine skin). After electrophoresis, the gels were washed once with deionized water, three times with 2.5% (w/v) Triton X-100 to remove SDS, and three times with the collagensen buffer (50 mM Tris-HCl, 200 mM NaCl, 5 mM CaCl2, and 0.1% Brij-35, pH 7.6). The gels were then incubated in collagenase buffer at 37 °C for 16 h, stained with Coomassie brilliant blue, and destained in 20% methanol and 10% acetic acid until the lysis zones were clear against a blue background. Inhibition studies to determine protease class were performed by incubating gels in collagenase buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 mM N-ethylmaleimide, or 0.1 mM 1,10-phenanthroline.
RESULTS

The present study has examined the changes induced by melanoma αβ1 integrin and CD44/CSPG binding using well defined “peptide amphiphile” substrates. Peptide amphiphiles consist of peptide sequences covalently linked to hydrocarbon chains and are designed to house discrete binding sites (such as those found in various ECM proteins) (41, 44, 47, 56 – 59, 63, 64). These small biomolecules assume stable “mini-protein” structures (α-helical or triple-helical) at physiological temperatures, allowing for the study of cellular behaviors in response to discreet binding sites that maintain the three-dimensional structure of the parent protein. CD spectroscopic analysis of two of the peptide amphiphiles used in the present study, THP-B and THP-D (Fig. 1), indicated Tm values of 55.0 and 45.0 °C, respectively (data not shown). Thus, both peptide amphiphiles were triple-helical under assay conditions. SSP-A and SSP-C (Fig. 1) did not form triple-helical structures (data not shown). Assays were then performed to determine the ligand concentrations at which cell adhesion was optimal and equivalent. The ligand concentrations that provided high levels (>80%) of comparable adhesion were 100 μM for SSP-A, 10 μM for THP-B, 50 μM for SSP-C, and 10 μM for THP-D (data not shown). These ligand concentrations were used for all subsequent induction experiments.

Expression profiles were initially examined for the M14 melanoma cell line induced by the αβ1 integrin specific THP-B and the linear peptide model SSP-A. RT-PCR analysis was performed for several members of the MMP family (MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13, and MMP-14), heparanase A, and several members of the ADAMTS family (ADAMTS-1, ADAMTS-2, ADAMTS-3, and ADAMTS-4). Semi-quantitative PCR amplicons were gel-purified and sequenced, whereas real time PCR amplicon sequences were verified by melting curve analysis. DNA sequences matched the respective gene sequences in GenBank™ (data not shown). The expression levels of heparanase A and ADAMTS-1, -2, -3, and -4 gene products remained unchanged relative to the internal GAPDH standard (data not shown). However, melanoma cells displayed differential expression of several MMPs relative to an internal GAPDH control (Fig. 2A and Table II) and as compared with a linear version of the ligand (data not shown). The expression of MMP-8 (Fig. 2A) and MMP-9 (data not shown) was unaffected by exposure to the triple-helical ligand, whereas the expression of MMP-3 increased slightly over the initial expression levels (1.8-fold after 6 h; data not shown). In contrast, the relative expression of MMP-1 increased 2-fold by 12 h, whereas MMP-14 expression increased 2-fold after 2 h, decreased by 6 h, and then increased back to initial levels after 8 h (data not shown). The relative expression of MMP-13 increased nearly 2.5-fold by 12 h and continued over the induction period until a final value of over 4-fold maximum expression by the 24-h time point was achieved (Fig. 2A). The mounting expression of the MMP-13 gene may be due to either prolonged gene activation or by additional stimulation by type IV collagen normally pro-

![Fig. 4.](image-url)
duced by the melanoma cell. Comparison of induction profiles showed only slight variations between M14 and SK-Mel2, another highly metastatic human melanoma cell line. For example, MMP-1 and MMP-14 expression was stimulated by THP-B at similar times and levels by M14 and SK-Mel2 cell lines (65).

Expression profiles were subsequently examined for the M14 melanoma cell line induced by the CD44-specific THP-D and the linear peptide model SSP-C. RT-PCR analysis was performed for several members of the MMP family (MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13, and MMP-14). Melanoma cells displayed differential expression of several MMPs relative to an internal GAPDH control (Table II) and as compared with a linear version of the ligand. MMP-8 expression, for example, was stimulated 3.5-fold by THP-D after 6 h (Figs. 2B and 3) and was only slightly stimulated by the linear SSP-C (Fig. 3). MMP-1 showed a slight sustained increase in relative expression, whereas MMP-14 was up-regulated at 2 h and then sharply down-regulated at 6 and 8 h (Fig. 2B). MMP-13 expression showed a slight increase at 6 h (Fig. 2B). MMP-2 modulation followed a similar trend as for the αβ1 integrin ligand, in that initial expression levels decreased over time and then increased at 8 h (data not shown).

Protein expression was next examined for MMPs whose mRNA levels were modulated by either the αβ1 integrin or CD44 ligand. Conditioned media from M14 melanoma cells grown on the αβ1 integrin-specific THP-B or CD44/CSPG-specific THP-D were subjected to ELISA analyses (Fig. 4 and Table II). For MMP-2 and MMP-14, ELISA was found to be unreliable, so fluorogenic substrates selective for MMP-2/MMP-9 (41) and MMP-14 (66) were used for evaluation of active enzyme (see below). MMP-1 was slightly induced by both ligands, with increases in the range of −1 to 2 ng/ml over 24 h (Fig. 4, A and B). ELISA for MMP-8 induction showed a slight decrease in protein because of αβ1 integrin binding (−1 ng/ml) and an increase in protein from CD44 engagement (−6 ng/ml) (Fig. 4, A and B), consistent with relative mRNA expression (Fig. 2). ELISA for MMP-13 showed a substantial increase in protein in response to the αβ1 integrin ligand and a lesser increase in response to the CD44 ligand (Fig. 4C). The relative MMP-13 protein levels induced by the αβ1 integrin (−60 pg/ml) and CD44 (−25 pg/ml) ligands were consistent with mRNA expression (Fig. 2).

The prior analyses have revealed trends in MMP mRNA and protein expression but have not addressed the production of active enzyme. MMPs initially exist as zymogens and may also be rendered inactive by binding to TIMPs or other, more general protease inhibitors. For MMP-1, active enzyme was quantified by solid phase mAb immobilization of the enzyme followed by reaction with the fluorogenic substrate fTHP-4 (49). The solid phase assay showed more activity induced by the αβ1 integrin than by CD44 (Fig. 4D). Treatment of samples with an activator of proMMPs (4-aminophenylmercuric acetate) resulted in a further increase in MMP-1 activity (Fig. 5), indicative of the production of both MMP-1 and proMMP-1 by engagement of either the αβ1 integrin or CD44. For other MMPs, calibration of the solid phase assay was problematic and not further pursued.

An additional approach to monitor active enzyme is the use of discriminatory substrates. Unfortunately, such substrates do not yet exist for MMP-1, MMP-8, and MMP-13. General triple-helical peptidase activity can be evaluated using fTHP-4. Engagement of either the αβ1 integrin or CD44 resulted in significant triple-helical peptidase activity detected in melanoma cell conditioned media (Fig. 6A), with greater activity found in response to CD44. This activity was completely inhibited by EDTA (data not shown), suggesting metalloproteinase activity (41). The MMP-14 selective substrate C17-D-(Gly-Pro-Hyp)2-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly-Pro-Hyp(4-methoxybenzyl)-Arg-Gly-Gln-Lys(Dnp)-(Gly-Pro-Hyp)_2-NH2 was used for comparison with general triple-helical peptidase activity. Soluble MMP-14 activity, which can be generated by nonautocatalytic shedding of MMP-14 (67, 68), was significant at early time points and then decreased over 8 h in response to the αβ1 integrin and CD44 ligands (Fig. 6B). The MMP-14 activity profiles correlate well with the mRNA expression profiles in that MMP-14 is induced at early time points. The subsequent decrease in MMP-14 activity may be due to degradation of MMP-14. Other MMP activity profiles do not decrease at later time points (Fig. 6, A and C), and exogenous MMP activity is not significantly effected over a 24-h period by melanoma conditioned media (106). These results suggest that degradation of MMP-14 is specific, which is not surprising considering the multitude of MMP-14 shedding processes (68).

Gelatinase activity was initially evaluated using the MMP-2/MMP-9 selective substrate αV(436–447) fTHP (41). Gelatinase activity showed virtually identical increases in response to the αβ1 integrin and CD44 ligands (Fig. 6C). Because the MMP-2/MMP-9 selective substrate does not differentiate between the two gelatinases, additional activity assays were performed using gelatin zymography. Zymography indicated that the αβ1 integrin and CD44 ligands produced predominantly MMP-2 (Fig. 7). Treatment with 1,10-phenanthroline resulted in complete loss of gelatinolysis (data not shown), indicative of metalloproteinase activity.

**DISCUSSION**

Engagement of the αβ1 integrin and CD44 by ECM ligands results in intracellular signaling (25, 69, 70). However, the precise nature of this signaling within melanoma cells has not been documented. MMP-1, -2, -3, -8, and -13 are expressed in numerous human melanoma cell lines but not melanocytes.
with MMP-2 and MMP-8 expression at higher constitutive levels than other MMPs (71, 72). Conversely, MMP-14 is expressed in both melanocytes and melanoma (71). The present study has used \( \alpha_2\beta_1 \) integrin- and CD44-specific ligands to examine the regulation of several members of the MMP family associated with melanoma cell invasion.

Triple-helical ligands for the \( \alpha_2\beta_1 \) integrin and CD44/CSPG promote expression of relatively low levels MMP-1 mRNA and protein (Table II and Figs. 2 and 4, A and B). However, more active MMP-1 was found in response to \( \alpha_2\beta_1 \) integrin engagement (Fig. 5). This result may be related to increased MMP-3, which is an activator of proMMP-1 (73), seen in response to the \( \alpha_2\beta_1 \) integrin ligand. The triple-helical ligand for the \( \alpha_2\beta_1 \) integrin also promotes expression of MMP-13 and MMP-14 (Table II and Figs. 2A, 4C, and 6B). The profile seen here in response to \( \alpha_2\beta_1 \) integrin engagement is consistent with high invasion potential, in that the MMPs up-regulated can participate in the dissolution of basement membrane (type IV) and type I collagen.

In addition to MMP-1, the triple-helical ligand for CD44/CSPG promotes expression of MMP-8, MMP-13, and MMP-14 (Table II and Figs. 2B, 3, 4B, and 6B). The overall greater triple-helical peptidase activity seen in response to CD44 (Fig. 6A) is probably due to MMP-8, because the other MMPs that could contribute to general triple-helical peptidase activity are equivalently expressed in response to the \( \alpha_2\beta_1 \) integrin and CD44 (MMP-2 and MMP-14) (Fig. 6, B and C) or more highly expressed in response to the \( \alpha_2\beta_1 \) integrin (MMP-1 and MMP-13) (Figs. 2 and 4). MMP-8 is only slightly modulated if the ligand is non-triple-helical (Fig. 3).

Examination of gene expression by RT-PCR analysis indicated initial down-regulation, followed by up-regulation, of MMP-2 by either the \( \alpha_2\beta_1 \) integrin or CD44 (Table II), whereas enzyme activity is clearly increased (Figs. 6C and 7). Prior studies had shown that neither type I collagen nor HA induce MMP-2 expression in melanoma cells (74). The results seen here appear to represent high constitutive levels of MMP-2, as previously reported for human metastatic melanoma (74). Enhanced MMP-2 activity could also be achieved via MMP-14 up-regulation as well as TIMP modulation, because MMP-14 and TIMP-2 participate in proMMP-2 activation (73). MMP-14 mRNA was initially up-regulated by both the \( \alpha_2\beta_1 \) integrin and CD44 ligands (Fig. 2), whereas soluble active enzyme was significantly present up to 4 h (Fig. 6B).

CD44 binding to two different ligands, \( \alpha_1(IV)1263–1277 \) and HA, has been well characterized (34, 44, 75). HA binds to the amino-terminal globular domain of CD44, using motifs of two basic amino acids separated by seven non-acidic amino acids (B[X7]B) (34). These motifs are found within CD44 residues 21–45 (34). Several distant residues also contribute to HA binding, such as Lys\(^{158} \) and Arg\(^{162} \) (34). CD44 binding to type IV collagen and \( \alpha_1(IV)1263–1277 \) is dependent upon CS (21, 44).
CS interaction with collagen requires a minimum oligosaccharide chain length and distinct sulfation pattern (76). Conversely, CS interferes with CD44 binding to HA (34). It appears that α1/IV1263–1277 and HA bind to different regions of CD44, and thus these ligands may induce different CD44 signaling pathways. Consistent with this proposal, HA signaling through FAK and extracellular signal-regulated kinase 1/2 results in elevated expression of MMP-3, MMP-9, MMP-14, and CD44 (78). These HA-induced signaling outcomes are clearly different from the results in the present study. However, it should be noted that the identity of the receptor in the prior HA binding study (77), either CD44 or receptor for hyaluronan-mediated motility, was not determined. Regardless, it will be of great interest to determine whether different CD44 extracellular environments result in different cellular responses.

The in vitro activation of melanoma cells with selective ligands serves as a means to dissect specific pathways resulting from engagement of a particular receptor. Triple-helical ligands for CD44 and the αβ1 integrin promote expression of MMPs with differing downstream events. The intracellular signaling pathways engaged by these individual receptors have been somewhat defined but do not explain the differing downstream events. For example, FAK induction has long been somewhat defined but do not explain the differing downstream events. The intracellular CD44 fragment subsequently generated signaling by liberated CD44 domains.

It is possible that CD44 works in concert with the αβ1 integrin to efficiently bind to type IV collagen and subsequently up-regulate cell signaling pathways. Melanoma-associated proteoglycan/melanoma chondroitin sulfate proteoglycan has been shown to mediate αβ1 integrin binding to fibronectin (80). Prior studies showed that removal of cell surface CS by chondroitinase ABC digestion inhibits β1-integrin-dependent adhesion to type IV collagen (81). The CD44 α1/IV1263–1277 ligand had the same effect as chondroitinase ABC, implicating CD44/CSPG as the receptor working in concert with the integrin. Adhesion was reestablished by a β1-integrin subunit stimulating mAb. Other studies have also shown interactions between membrane-bound carbohydrates and integrins. Tumor gangliosides enhance αβ1 integrin-dependent platelet activation upon binding to collagen (82). Several transmembrane proteins are known to associate with the αβ1 integrin (5, 83). A CD44/αβ1 integrin interaction may be indirect, because the CD44 cytoplasmic domain binds to ankyrin, which in turn is linked to actin by fodrin/spectrin (84, 85). Another possible interaction between the αβ1 integrin and CD44 relates to prior studies showing that CD44 can associate with active MMP-14 at the tumor cell migration front, potentially enhancing invasion of the basement membrane (86). MMP-14 can shed CD44, and the intracellular CD44 fragment subsequently generated by presenilin/γ-secretase action may function as a transcription factor (70, 86). In turn, the soluble CD44/CSPG may modulate tumor growth and spreading (87, 88) in an analogous fashion to other liberated tumor cell surface proteoglycans (89, 90). Because MMP-14 is initially up-regulated by the αβ1 integrin, this integrin may modulate CD44 shedding and subsequent signaling by liberated CD44 domains.

The MMP proteolytic profiles identified here can in turn lead to differential processing of type IV collagen. For example, the sites of hydrolysis within type IV collagen are different for

![Gelatin zymographic analysis of MMP-2 and MMP-9 in conditioned medium following melanoma cell adhesion to the αβ1 integrin-specific THP-B (lanes 2 and 5) and the CD44/CSPG-specific THP-D (lanes 1 and 4). M14 melanoma cells were allowed to adhere to 10 μM THP-B or THP-D for 60 min at 37 °C. Three washes of adhesion medium were used to remove nonadherent cells and cells were grown for 12 (lanes 1 and 2) or 24 (lanes 4 and 5) h. Cell supernatants were added either without treatment or following 100 μM 1,10-phenanthroline treatment (data not shown). Lane 3 is an activated MMP-2 standard, which has been processed to the 50-kDa form. The majority of gelatinolytic activity corresponded to MMP-2 in the 52- and 50-kDa active forms. A small amount of gelatinolytic activity was seen at 81 kDa, which corresponded to active MMP-9 (standard not shown).](https://example.com/gelatin-zymography.png)
MMP-2/MMP-9 compared with MMP-3 (91, 92). Generation of different cleavage products may then influence tumor progression in several ways. First, liberated type IV collagen fragments can modulate tumor angiogenesis (93). Second, proteolysis may reveal formerly “cryptic” binding sites that promote migration (94). Third, decreased triple-helical structure negatively influences binding and signaling through collagen-specific receptors, altering cellular phenotypes (47, 57). Fourth, the loss of basement membrane integrity results in a barrier less resistant to invasion (95). Thus, the MMP proteolytic profiles induced by engagement of different cellular receptors (i.e., the αβ integrin and CD44/CSPG) may have profound consequences on metastatic potential.

It is noteworthy to consider how the activities induced by the αβ integrin and CD44 ligands compare with either constitutive or type IV collagen-induced melanoma behaviors. We have recently examined the constitutive production of active MMPs by M14 melanoma cells and found that levels of active MMP-1, MMP-3, MMP-13, and soluble MMP-14 are low (106). As documented here, the engagement of either the αβ integrin or CD44 specifically increases active protease production. Prior studies had shown that binding of the αβ integrin to collagen results in increased production of MMP-1, MMP-13, and MMP-14 (23, 25, 96), analogous to the MMP profile induced by the αβ integrin triple-helical ligand. The present study provides the first correlation between CD44 binding and MMP production (see prior discussion). Human melanoma cell binding to type IV collagen induces an inositol 1,4,5-trisphosphate-independent release of intracellular Ca²⁺ stores (97); inhibition of this release decreases expression of MMP-2 (98). In related studies with human fibrosarcoma cells, calcium ionophores inhibit the release of pro-MMP-2 and pro-MMP-9 but no modulation of MMP-14. This clearly shows that binding of the /H9251/H9252 integrin triple-helical ligand. The present study provides the first correlation between CD44 binding and MMP production (see prior discussion). Human melanoma cell binding to type IV collagen induces an inositol 1,4,5-trisphosphate-independent release of intracellular Ca²⁺ stores (97); inhibition of this release decreases expression of MMP-2 (98).

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