

Intact Vitronectin Induces Matrix Metalloproteinase-2 and Tissue Inhibitor of Metalloproteinases-2 Expression and Enhanced Cellular Invasion by Melanoma Cells*

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The initial site of melanoma cell metastasis is frequently the regional lymph nodes, and the appearance of lymph node metastasis correlates with poor prognosis. Lymph node adhesion is mediated by an interaction between the tumor cell integrin $\alpha\beta3$ and lymph node vitronectin. In this study, we explored the relationship between adhesion and proteolysis by examining the direct effect of vitronectin receptor ligation on matrix metalloproteinase-2 (MMP-2) production by B16F1 and B16F10 melanoma cells. We report a dose-dependent increase in secretion of both MMP-2 and tissue inhibitor of metalloproteinases-2 (TIMP-2) in response to vitronectin. Cellular invasiveness was also enhanced by vitronectin, as shown by the increased ability of vitronectin-treated cells to invade a synthetic basement membrane (Matrigel). Both the vitronectin-induced MMP-2 production and vitronectin-enhanced invasion were blocked by the peptide ligand Arg-Gly-Asp-Ser (RGDS). Furthermore, neither plasmin-degraded vitronectin nor the peptide ligand RGDS stimulated MMP-2 secretion or invasiveness, indicating that a multivalent ligand-receptor interaction rather than simple receptor occupancy was required for MMP-2 induction. MMP-2 and MMP-2/TIMP-2 interaction with the plasma membrane of melanoma cells resulted in enhanced catalytic activity against ^{14}C -labeled gelatin, suggesting that membrane association may function in posttranslational regulation of MMP-2 activity. This is supported by data showing increased cellular invasion by cells containing membrane-bound MMP-2. Binding of proMMP-2 and proMMP-2/TIMP-2 to melanoma cells was not inhibited by RGDS, and melanoma cell adhesion to vitronectin was unaffected by pro- or active MMP-2, indicating that MMP-2 did not interact with the murine vitronectin receptor. Together, these data provide evidence for a functional link between adhesion and proteolysis and suggest a potential mechanism whereby adhesion of an invasive cell to the extracellular matrix regulates subsequent invasive behavior.

adhesion to the regional lymph nodes, which correlates with poor prognosis, is mediated via interaction of specific integrins on the melanoma cell surface with lymph node vitronectin (3). Previous data have demonstrated a relationship between elevated levels of vitronectin-binding integrins and increased melanoma cell invasiveness (4–6). Furthermore, ligation of the $\alpha\beta3$ integrin on melanoma cells by anti- $\alpha\beta3$ antibodies enhances secretion of matrix metalloproteinase-2 (MMP-2, gelatinase A, 72-kDa type IV collagenase), resulting in increased cellular invasiveness (7). Together, these data suggest that integrin-mediated binding of tumor cells to a specific matrix-associated protein, such as vitronectin, can promote tumor cell invasion by increasing the levels of a matrix-degrading proteinase.

The majority of integrins recognize multiple extracellular matrix ligands (1, 2), and precise biologic responses may be regulated by differential integrin ligation with distinct extracellular matrix proteins. In this study, we have explored the direct effect of the matrix-associated ligand vitronectin on production of MMP-2 by melanoma cells. We report a dose-dependent increase in secretion of both MMP-2¹ and tissue inhibitor of metalloproteinases-2 (TIMP-2), as well as enhanced cellular invasiveness, in response to vitronectin. Intact vitronectin is required for MMP-2 induction because neither plasmin-treated vitronectin nor a peptide ligand (Arg-Gly-Asp-Ser (RGDS)) alters MMP-2 secretion, indicating the requirement for a multivalent ligand-receptor interaction. Furthermore, MMP-2 interacts with the plasma membranes of melanoma cells, exhibits enhanced catalytic activity relative to the solution phase enzyme, and increases cellular invasive activity. Membrane binding of MMP-2 is unaffected by RGDS, and melanoma cell adhesion to vitronectin is not inhibited by MMP-2, indicating that MMP-2 does not bind the vitronectin receptor on murine melanoma cells. These data suggest a potential physiologic mechanism whereby the relative integrity of the adhesive substratum may differentially regulate secretion and activity of a matrix-degrading proteinase and subsequent cellular invasive behavior.

EXPERIMENTAL PROCEDURES

Cell Culture—The murine B16F10 and B16F1 melanoma cell lines were obtained from Dr. I. J. Fidler (The University of Texas M. D. Anderson Hospital) and were cultured in Eagle's minimal essential medium supplemented with 5% fetal calf serum, nonessential amino acids, L-glutamine, and vitamins. Prior to each experiment, cells were washed with calcium and magnesium-free Dulbecco's phosphate-buffered saline (PBS) and incubated for 2 min at 25 °C with 1 mM EDTA in serum-free Eagle's minimal essential medium to release cells from the culture flask. Cells were seeded in 1 ml of serum-free Eagle's minimal essential medium at a density of 1×10^6 cells/ml and incubated 18 h at

Adhesion of tumor cells to specific extracellular matrix macromolecules is an initial component of the metastatic process (reviewed in Refs. 1 and 2). In metastatic melanoma, tumor cell

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¹ The abbreviations used are: MMP-2, matrix metalloproteinases-2; TIMP, tissue inhibitor of metalloproteinases; PBS, phosphate-buffered saline; APMA, amino-phenylmercuric acetate.

37 °C in the presence of vitronectin, peptide, or both as described below. In some experiments, colchicine (0.2 μ M) was included for inhibition of secretion. Conditioned media were removed and concentrated 4-fold at 4 °C using a Centricon 10 (Amicon) concentrator and analyzed immediately as described below.

Proteins and Antibodies—Native vitronectin was purified from pooled human plasma according to the procedure of Dahlback and Podack (8). Briefly, pooled plasma was subjected to salt fractionation followed by ion exchange, dye affinity, and gel filtration chromatography. Presence and purity of vitronectin in each fraction was assessed by both dot blot and electrophoretic analysis on 5–15% gradient SDS-polyacrylamide gels. ProMMP-2 and proMMP-2/TIMP-2 complex were purified as described previously (9, 10). Plasminogen was purified from human plasma by affinity chromatography on L-lysine-Sepharose and separated into isoforms 1 and 2 as described previously (11). Plasmin was generated by incubating 100 μ g of plasminogen with 100 μ l of urinary-plasminogen activator-Sepharose (Calbiochem) in 10 mM Hepes, pH 7.4, at 25 °C for 12 h followed by centrifugation to remove the resin. Protein concentrations were determined spectrophotometrically at 280 nm using an $A_{1\text{cm}}^{1\%}$ value of 16.8 and molecular masses of 92 and 81 kDa for plasminogen and plasmin, respectively. Endoproteinase Glu-C from *Staphylococcus aureus* strain V8 (endoproteinase V8) and soybean trypsin inhibitor were purchased from Sigma and were coupled to cyanogen bromide-activated Sepharose 4B (Sigma) according to the manufacturer's specifications. The synthetic peptide Arg-Gly-Asp-Ser (RGDS), which blocks α v β 3 and α v β 5-mediated adhesion to vitronectin (12), and the noninhibitory control peptide Gly-Asp-Glu-Ser (RGES) were purchased from Sigma.

Analysis of MMP-2 and TIMP-2 Secretion—Latent MMPs in concentrated conditioned medium were activated by incubation at 37 °C for 60 min in the presence of 1.0 mM amino-phenylmercuric acetate (APMA). Zymographic analysis of secreted MMPs was performed using 9% SDS-polyacrylamide gels containing co-polymerized gelatin as described previously (13). Reverse zymography for detection of TIMPs was performed using 15% SDS-polyacrylamide gels containing co-polymerized gelatin and reagents purchased from University Technologies International (Calgary, Alberta, Canada) according to the manufacturer's specifications. Inactivation of the TIMP(s) in conditioned medium by reductive carboxymethylation was carried out as described by Salvesen and Nagase (14). Briefly, conditioned medium was treated with 2 mM dithiothreitol for 1 h at 37 °C followed by incubation with iodoacetamide (5 mM) for 15 min at 37 °C.

In Vitro Invasion Assays—*In vitro* invasive activity was assessed by determining the ability of cells to invade a synthetic basement membrane (Matrigel, Becton Dickinson, Bedford, MA). Polycarbonate filters (8- μ m pore size, Becton Dickinson) were coated with Matrigel (11 μ g/filter) and placed in modified Boyden chambers. Cells (1×10^5) were added to the top of the chamber in serum-free medium in the presence or absence of vitronectin, plasmin-treated vitronectin, RGDS, or RGES. Following incubation for 8–24 h, noninvading cells were removed from the top surface of the membrane filter with a cotton swab, and filters were removed and stained with Diff-Quik (Fisher). Cells on the lower surface of the filter were enumerated using an ocular micrometer and counting a minimum of six high-powered fields. In some experiments, untreated cells (1×10^5) were preincubated for 90 min with MMP-2 (50 nM) in serum-free culture medium containing 3% bovine serum albumin. Cells were pelleted, washed twice with PBS to remove unbound MMP-2, and enumerated, and invasiveness was assayed as described above.

Limited Proteolysis of Vitronectin—Limited plasmin proteolysis of vitronectin was carried out by incubating vitronectin and plasmin in a 20:1 (w/w) vitronectin:plasmin ratio for various time periods from 10 min to 3 h at 37 °C. After incubation, plasmin was removed from the reaction by the addition of soybean trypsin inhibitor-Sepharose, followed by centrifugation to remove the plasmin-soybean trypsin inhibitor-Sepharose complex. Complete removal of plasmin was confirmed by addition of the synthetic plasmin substrate D-Val-Leu-Lys-p-nitroanilide (Sigma) and monitoring absorbance at 405 nm. Limited proteolysis of vitronectin by endoproteinase V8 was performed by incubating vitronectin (200 μ g) with 200 μ l of endoproteinase V8-Sepharose for various time periods from 30 min to 18 h at 25 °C, followed by centrifugation to remove the proteinase resin. Cleavage of vitronectin by MMP-2 was assessed by incubating vitronectin with activated MMP-2 in a 20:1 vitronectin:MMP-2 (w/w) ratio in 50 mM Tris-HCl, 0.2 M NaCl, 10 mM CaCl₂, pH 7.6 at 37 °C for 18 h. Intact vitronectin and vitronectin treated with plasmin, endoproteinase V8, or MMP-2 were analyzed by electrophoresis on 5–15% SDS-polyacrylamide gradient gels followed by staining with Coomassie Blue.

Adhesion Assay—Vitronectin was passively adsorbed to 24-well culture plates as described previously (15), blocked by incubating with PBS containing 2% bovine serum albumin for 2 h at 25 °C, and utilized for cell attachment assays as described by Pierschbacher and Ruoslahti (16). Briefly, cells were plated at a constant of 1.5×10^5 cells/ml in a total volume of 0.5 ml and incubated for 30–60 min on vitronectin-coated plates. After incubation, plates were washed twice with PBS, and bound cells were enumerated using an ocular micrometer and counting six high-powered fields. The effect of RGDS, RGES, proMMP-2, and active MMP-2 on adhesion to vitronectin was determined by addition of cells in the presence of various concentrations of peptide or proteinase to vitronectin-coated wells.

Analysis of MMP-2 Membrane Association—To analyze binding of proMMP-2 or proMMP-2/TIMP-2 to intact melanoma cells, proteins were labeled with ¹²⁵I using Iodogen (Pierce) according to the manufacturer's specifications. Labeled protein (100 nM in minimal essential medium containing 0.1% bovine serum albumin) was added to B16F10 cells (5×10^4) for 1 h at 4 °C in the presence of increasing concentrations of RGDS or RGES peptide (0–1000 μ g/ml). Cells were washed three times with Hanks' balanced salt solution, and cell associated radioactivity was measured in a γ -counter. To assess the interaction of proMMP-2 or proMMP-2/TIMP-2 with isolated melanoma cell membranes, melanoma cells were cultured overnight in serum-free medium, released from the flask with EDTA, and washed three times with PBS. The plasma membrane fractions of B16F1 (2×10^8) and B16F10 (4×10^8) cells were isolated using nitrogen cavitation (350 p.s.i., 20 min) and differential centrifugation as described previously (17). Cell lysates were treated with soybean trypsin inhibitor (20 μ g/ml), leupeptin (25 μ g/ml), elastatinal (25 μ g/ml), 3,4 dichloroisocoumarin (0.05 mM), and *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64, 0.01 mM) to prevent proteolysis of membrane proteins. B16F1 and B16F10 plasma membranes were incubated (2 h, 37 °C) with purified proMMP-2/TIMP-2 or proMMP-2 (100 nM) in 20 mM Tris, 5 mM CaCl₂, 25 mM sucrose, pH 7.4 (Tris-Ca-sucrose). Control samples contained buffer alone. The membranes were recovered by centrifugation (50,000 \times g for 30 min), supernatants were discarded, and pellets were washed twice in Tris-Ca-sucrose. Membrane extracts were prepared by incubating aliquots of membrane fractions with 1% Triton X-100 at 4 °C for 16 h followed by clarification by centrifugation (50,000 \times g for 30 min) as described previously (17). After resuspension in Tris-Ca-sucrose, aliquots of membranes were analyzed for bound activity by gelatin zymography and by ¹⁴C-gelatin degradation (17, 18). ¹⁴C-Gelatin was incubated for 22 h at 37 °C with treated membrane aliquots in Tris-Ca-sucrose containing 50 mM NaCl and 0.5 mM APMA. Parallel experiments contained 1 mM *o*-phenanthroline. Reactions were stopped by trichloroacetic acid precipitation, and soluble radioactivity was determined by scintillation counting. Gelatin degradation by membrane-associated proteinase was determined relative to membrane-free controls. The effect of membrane association on the gelatinolytic activity of pre-activated MMP-2 was also determined as described previously (17).

RESULTS

It has been previously reported that treatment of A375M melanoma cells with antibodies directed against the vitronectin-binding integrin α v β 3 enhanced MMP-2 secretion and stimulated cellular invasion through Matrigel (7). To analyze the direct effect of vitronectin on melanoma MMP-2 secretion, B16F1 and B16F10 cells were incubated with increasing concentrations of vitronectin, and secretion of gelatin-degrading MMPs was analyzed by zymography. A dose-dependent increase in secretion of a gelatinolytic metalloproteinase was observed in the conditioned medium of vitronectin-treated B16F1 cells (Fig. 1) and B16F10 cells (Fig. 2A). The gelatinolytic enzyme was activated by treatment with APMA, inhibited by the zinc chelator *o*-phenanthroline (data not shown), and co-migrated with authentic MMP-2, suggesting its identity as MMP-2. MMP-2 secretion was not detectable by zymography in untreated B16F1 or B16F10 cells (Fig. 1, lane 1, and Fig. 2, lane 1), but it was observed in cells treated with as little as 10 μ g/ml vitronectin. Addition of vitronectin in the presence of RGDS peptide inhibited the vitronectin-induced MMP-2 secretion, demonstrating that direct interaction of vitronectin with its cellular receptor is required for MMP-2 induction (Fig. 1). Furthermore, vitronectin-induced MMP-2 secretion was inhibited

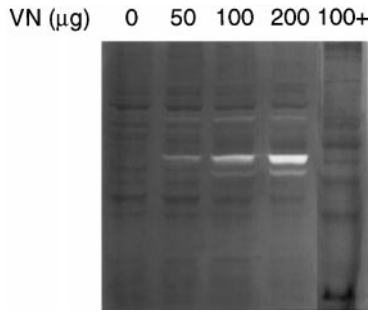


FIG. 1. Zymogram depicting MMP-2 activity in B16F1-conditioned medium. B16F1 cells (1×10^6) were cultured for 18 h in serum-free medium containing increasing amounts of vitronectin (0–200 $\mu\text{g/ml}$), and conditioned media were analyzed for MMP activity by gelatin zymography on 9% SDS-polyacrylamide gels. The lane designated 100+ contained conditioned medium from cells cultured in the presence of 100 $\mu\text{g/ml}$ of vitronectin and 500 $\mu\text{g/ml}$ of RGDS.

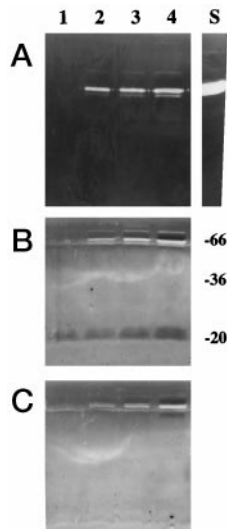


FIG. 2. MMP and TIMP activity in B16F10-conditioned medium. B16F10 cells (1×10^6) were cultured for 18 h in serum-free medium containing 0 (lane 1), 100 (lane 2), 200 (lane 3), or 300 (lane 4) $\mu\text{g/ml}$ vitronectin, and conditioned media were analyzed for MMP activity by gelatin zymography on 9% SDS-polyacrylamide gels (A) and for TIMP activity by reverse zymography on 15% SDS-polyacrylamide gels (B and C). Samples in C were subjected to reductive carboxymethylation to inactivate TIMPs as described under "Experimental Procedures" prior to reverse zymography. Lane S, MMP-2 standard.

ited by colchicine, demonstrating that vitronectin does not displace MMP-2 from a common cell surface receptor such as $\alpha v \beta 3$ (data not shown). Analysis of TIMP-2 activity by reverse zymography revealed a metalloproteinase inhibitor that co-migrated with TIMP-2 (M_r 21,000) in the conditioned medium of untreated cells (Fig. 2B, lane 1), which was increased in vitronectin-treated cells (Fig. 2B, lanes 2–4). Incubation of the conditioned medium samples with dithiothreitol and iodoacetamide, a procedure shown to denature TIMP to a noninhibitory form (14), removed the TIMP-2 activity from the conditioned medium (Fig. 2C). Vitronectin treatment also enhanced cell surface-associated MMP-2. B16F1 and B16F10 cells cultured with vitronectin (50 μg) displayed a 17 and 20% increase in cell surface MMP-2, respectively, as determined by cell surface enzyme-linked immunosorbent assay using an anti-MMP-2 antibody and an alkaline-phosphatase-conjugated secondary antibody (15). To assess the functional effect of MMP-2 induction, the ability of melanoma cells to penetrate a synthetic basement membrane (Matrigel) was analyzed. Both B16F1 and B16F10 cells displayed enhanced *in vitro* invasive behavior when cultured in the presence of vitronectin (Fig. 3).

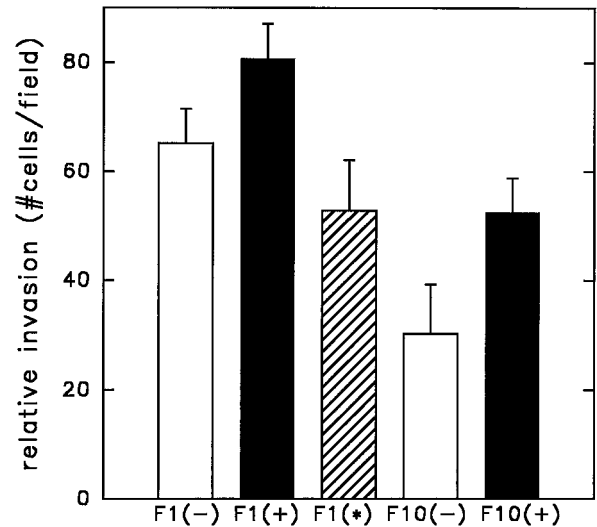


FIG. 3. Effect of vitronectin on Matrigel invasion. B16F1 or B16F10 cells (1×10^5) were added to 8- μm pore size polycarbonate filters coated with Matrigel basement membrane extract as described under "Experimental Procedures." Following incubation for either 8 (B16F10) or 17 (B16F1) h, membranes were removed and stained, and the invading cells were enumerated. Open bars, control B16F1 (designated F1(-)) or B16F10 (designated F10(-)); solid bars, B16F1 (designated F1(+)) or B16F10 (designated F10(+)) in the presence of 100 $\mu\text{g/ml}$ vitronectin. Hatched bar (designated F1(*)), B16F1 cells in the presence of 100 $\mu\text{g/ml}$ V8-degraded vitronectin. Experiments were performed in triplicate, and error bars represent S.D.

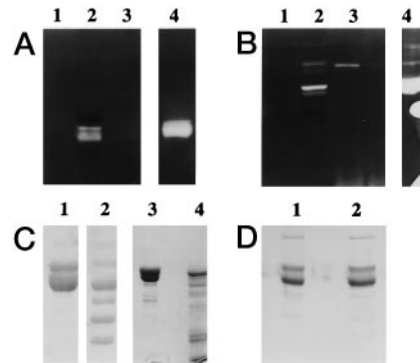


FIG. 4. Limited proteolysis of vitronectin. Vitronectin (200 μg) was subjected to limited proteolysis with plasmin (A and C) or endoproteinase V8 (B and C) and incubated with B16F10 cells (1×10^6) in serum-free medium (1 ml) for 18 h. Conditioned media were analyzed for MMP activity by gelatin zymography on 9% SDS-polyacrylamide gels. A, lane 1, cells only; lane 2, cells + intact vitronectin; lane 3, cells + plasmin-degraded vitronectin; lane 4, MMP-2 standard. B, lane 1, cells only; lane 2, cells + intact vitronectin; lane 3, cells + endoproteinase V8-degraded vitronectin; lane 4, MMP-2 standard. Panel C, control showing limited proteolysis of vitronectin. Vitronectin was incubated with plasmin (37 $^{\circ}\text{C}$ for 30 min) (lane 2) or endoproteinase V8 (37 $^{\circ}\text{C}$ for 2 h) (lane 4) as described under "Experimental Procedures," and reaction products were analyzed by electrophoresis on 5–15% gradient SDS-polyacrylamide gels and stained with Coomassie Blue. Lanes 1 and 3, intact vitronectin (20 μg); lane 2, plasmin-degraded vitronectin (20 μg); lane 4, endoproteinase V8-degraded vitronectin (20 μg). D, effect of MMP-2 on vitronectin. Vitronectin (20 μg) was incubated with purified APMA-activated MMP-2 (1 μg) for 18 h at 37 $^{\circ}\text{C}$. Reaction products were analyzed by electrophoresis on 5–15% gradient gels and stained with Coomassie Blue. Lane 1, vitronectin; lane 2, vitronectin + MMP-2.

In addition to metalloproteinases, B16 melanoma cells also secrete the serine proteinase tissue-type plasminogen activator, which converts the plasma zymogen plasminogen to the active proteinase plasmin (13). Plasmin is a broad spectrum serine proteinase that degrades numerous extracellular matrix proteins, including vitronectin (19). To determine the effect of

TABLE I

Effect of RGDS on adhesion of B16F1 and B16F10 cells to vitronectin

Increasing concentrations of RGDS were added to melanoma cells, and adhesion to vitronectin-coated wells was quantitated as described under "Experimental Procedures." Results are expressed relative to wells containing no RGDS.

RGDS	Inhibition	
	B16F1	B16F10
$\mu\text{g/ml}$		
0	0	0
50	21 \pm 3.5	16 \pm 9.0
100	44 \pm 1.7	40 \pm 11.9
200	89 \pm 0.6	72 \pm 16.0

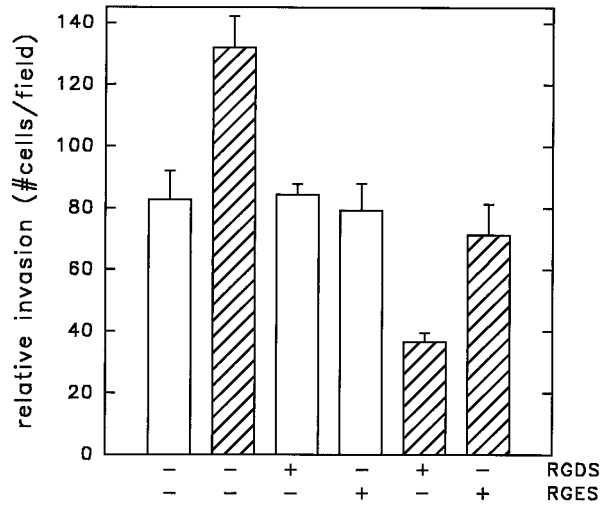


FIG. 5. Effect of RGD peptides on vitronectin-enhanced Matrigel invasion. B16F1 cells (1×10^5) were added to 8- μm pore size polycarbonate filters coated with Matrigel in the presence (hatched bars) or absence (open bars) of 100 $\mu\text{g/ml}$ vitronectin. RGDS or RGES (100 $\mu\text{g/ml}$) was added to control or vitronectin-containing samples as indicated. Following incubation for 23 h, membranes were removed and stained, and invading cells were enumerated. Experiments were performed in triplicate, and error bars reflect S.D.

plasmin-degraded vitronectin on melanoma cell MMP-2 secretion, vitronectin was subjected to limited proteolysis with plasmin (Fig. 4C, lane 2) prior to incubation with melanoma cells. In contrast to results observed with intact vitronectin (Fig. 4A, lane 2), plasmin-degraded vitronectin did not induce MMP-2 secretion (Fig. 4A, lane 3). Decreasing the extent of proteolysis did not reinstate the stimulatory effect (data not shown). Because plasmin has trypsin-like specificity and therefore may cleave within the RGD site utilized by melanoma cells for integrin-mediated vitronectin binding, limited proteolysis of vitronectin with endoproteinase V8 (which has Glu specificity (20)) was also performed (Fig. 4C, lane 4). Similar to results obtained with plasmin, endoproteinase V8-cleaved vitronectin also failed to induce melanoma cell MMP-2 production (Fig. 4B, lane 3). Furthermore, Matrigel invasion was not stimulated by endoproteinase V8-cleaved vitronectin (Fig. 3, hatched bar). To determine whether MMP-2 itself may initiate vitronectin degradation, vitronectin was incubated with APMA-activated MMP-2 for 18 h at 37 $^{\circ}\text{C}$ at a 1:20 MMP-2: vitronectin ratio and analyzed by electrophoresis on a 5–15% SDS-polyacrylamide gel. No change in the electrophoretic migration of MMP-2-treated vitronectin was observed (Fig. 4D, lane 2), demonstrating that vitronectin is not susceptible to proteolysis by MMP-2.

To analyze further the effect of ligating the melanoma cell vitronectin receptor on MMP-2 secretion, the peptide RGDS, which interacts with both the $\alpha\text{v}\beta 3$ and $\alpha\text{v}\beta 5$ vitronectin receptors (12), was utilized. Control experiments demonstrated

TABLE II

Effect of RGDS on binding of proMMP-2 and proMMP-2/TIMP-2 to B16F10 cells

^{125}I -labeled proMMP-2 or proMMP-2/TIMP-2 (100 nM) was added to wells containing 5×10^4 B16F10 cells in the presence of increasing concentrations of RGDS or RGES peptide. Bound ligand was determined as described under "Experimental Procedures." Results are expressed as % bound relative to control wells containing no added peptide (designated 100%). ProMMP-2 binding was analyzed in triplicate, whereas proMMP-2/TIMP-2 data are the average of duplicate experiments.

[Peptide]	ProMMP-2 bound		ProMMP-2/TIMP-2 bound	
	RGDS	RGES	RGDS	RGES
$\mu\text{g/ml}$				
0	100.0 \pm 2.9	100.0 \pm 2.9	100.0	100.0
1	92.8 \pm 0.8	97.4 \pm 2.1	105.0	94.0
10	91.5 \pm 9.4	95.1 \pm 3.3	89.6	98.0
100	91.8 \pm 11.1	94.5 \pm 4.9	85.0	96.0
1000	94.5 \pm 6.2	91.5 \pm 0.5	90.6	96.0

TABLE III

Effect of MMP-2 on melanoma cell adhesion to vitronectin

B16F10 cells (1.5×10^5) were added to 24-well culture plates coated with vitronectin in the presence or absence of pro- or active MMP-2, as indicated. After incubation for 40 min at 37 $^{\circ}\text{C}$, plates were washed with PBS and fixed, and bound cells were enumerated using an ocular micrometer. Experiments were performed in triplicate, and S.D. is indicated.

Treatment	Concentration	Adhesion
	ng/ml	$\text{No. of cells/field}$
Control		70.6 \pm 17.5
ProMMP-2	350	84.2 \pm 8.2
	700	76.0 \pm 9.4
Active MMP-2	350	72.4 \pm 25.0
	700	65.8 \pm 8.3

binding of RGDS to vitronectin receptors on B16F1 and B16F10 cells, as evidenced by the ability of the peptide to inhibit melanoma cell adhesion to vitronectin in a concentration-dependent manner (Table I). Incubation of either B16F1 or B16F10 cells with up to 1 mg/ml of RGDS or RGES did not induce MMP-2 secretion (data not shown). However, simultaneous addition of vitronectin and RGDS inhibited vitronectin-induced MMP-2 secretion as shown in Fig. 1 (lane designated 100+). Furthermore, inhibition of vitronectin-enhanced Matrigel invasion was 50% greater using RGDS peptide as compared with RGES (Fig. 5), further demonstrating that interaction of the RGD sequence of vitronectin with a cellular integrin(s) is necessary for MMP-2 induction.

Because recent data indicate that MMP activity may be regulated posttranslationally by interaction with the cell surface (17, 22–27), the ability of MMP-2 to associate with the plasma membrane of B16F1 or B16F10 cells was determined. Analysis of MMP-2 binding to the membranes of intact melanoma cells was assessed using ^{125}I -labeled proMMP-2 or proMMP-2/TIMP-2. Attempts to determine an equilibrium dissociation constant for binding of either proMMP-2 or proMMP-2/TIMP-2 to intact cells were unsuccessful because saturation of binding sites was not achieved in the presence of ^{125}I -labeled ligand concentrations up to 500 nM (data not shown). These data suggest that proMMP-2 and/or proMMP-2/TIMP-2 interacts with a prevalent cell-associated protein such as collagen or fibronectin (25–26) or that binding is mediated by a receptor present in low abundance such that specific binding is obscured by nonspecific interactions. To determine whether proMMP-2 or proMMP-2/TIMP-2 may associate with vitronectin binding integrins, binding experiments were performed in the presence of RGDS. No significant change in the amount of bound proMMP-2 or proMMP-2/TIMP-2 was observed in the presence

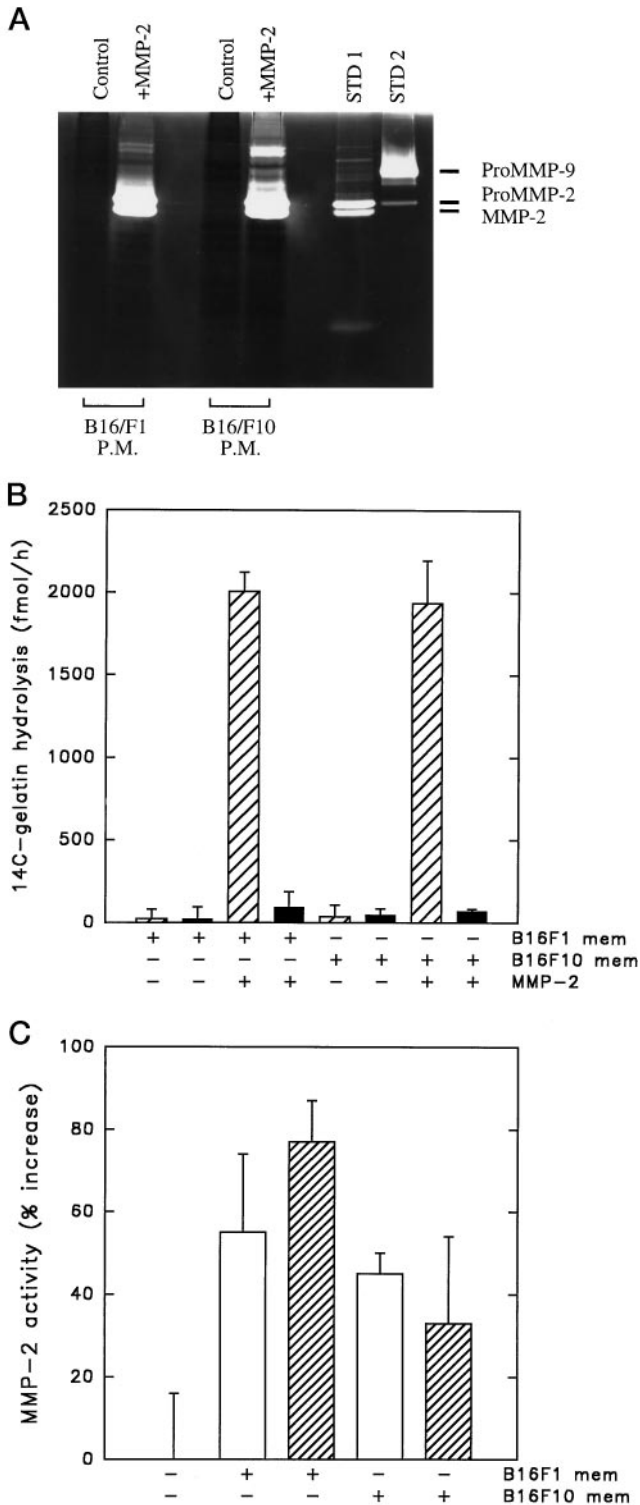


FIG. 6. MMP-2 association with melanoma cell plasma membranes. A, B16F1 (2×10^8) and B16F10 (4×10^8) cells were fractionated as described under "Experimental Procedures," and plasma membranes (P.M.) were incubated for 2 h at 37 °C in the absence (control) or presence (+MMP-2) of purified proMMP-2/TIMP-2 (100 nM). Membranes were washed to remove unbound complex, treated with APMA, and analyzed by gelatin zymography to detect bound enzyme. B, plasma membranes (mem) from B16F1 or B16F10 cells were incubated in the presence (+) or absence (-) of proMMP-2/TIMP-2 as indicated, and bound enzyme was analyzed by incubating membrane aliquots with ^{14}C -gelatin in Tris-Ca-sucrose containing 50 mM NaCl and 0.5 mM APMA in the presence (solid bars) or absence (hatched bars) of 1 mM *o*-phenanthroline. Reactions were terminated by trichloroacetic acid precipitation, and soluble radioactivity was determined by scintillation counting. Experiments were performed in quadruplicate, and error bars

TABLE IV
Effect of cell-associated MMP-2 on Matrigel invasion

B16F1 cells were incubated for 90 min in serum-free medium with 3% BSA containing 50 nM proMMP-2 and washed twice with PBS to remove unbound proMMP-2, and cells (1×10^5) were added to 8 μm pore size polycarbonate filters coated with Matrigel. Following incubation for 17 h, membranes were removed and stained, and invading cells were enumerated.

Sample	Relative invasion
	No. of cells/field
Control	22.0 ± 5.9
+50 nM proMMP-2	68.4 ± 11.6

of up to 1 mg/ml (2.3 mM) RGDS or control peptide RGEs (Table II), suggesting that interaction of proMMP-2 and proMMP-2/TIMP-2 with intact murine melanoma cells is not mediated by vitronectin-binding integrins. This was confirmed by adhesion experiments that demonstrated that MMP-2, whether in proenzyme or active form, was unable to inhibit melanoma cell adhesion to vitronectin (Table III).

In addition to binding to intact cells, exogenous proMMP-2 (data not shown) and proMMP-2/TIMP-2 bound to isolated plasma membranes from both B16F1 and B16F10 cells and retained hydrolytic activity against a macromolecular gelatin substrate (Fig. 6, A and B). Gelatinolytic activity was not detected in either clone of B16 plasma membranes prior to the addition of exogenous proMMP-2 or proMMP-2/TIMP-2 (Fig. 6), which is consistent with the lack of MMP-2 secretion observed in unstimulated cells (Figs. 1 and 2). The membrane-associated gelatinolytic activity was fully inhibited by addition of *o*-phenanthroline (Fig. 6B). Furthermore, membrane association enhanced the catalytic activity of activated MMP-2/TIMP-2. Incubation of MMP-2/TIMP-2 with either plasma membranes or detergent soluble extracts from B16F1 and B16F10 cells resulted in a 33–76% increase in gelatinolytic activity (Fig. 6C), suggesting that membrane association may function as a mechanism for posttranslational regulation of MMP-2/TIMP-2 activity in melanoma cells. To assess the potential functional consequence of MMP-2 cellular association on invasive behavior, cells were incubated with 50 nM MMP-2 in serum-free culture medium containing 3% bovine serum albumin, washed to remove unbound MMP-2, and analyzed in a Matrigel invasion assay. A 3-fold increase in invasiveness was observed in cells containing bound MMP-2 relative to controls (Table IV), demonstrating that cellular association of MMP-2 contributes functionally to enhanced invasiveness.

DISCUSSION

The initial site of melanoma cell metastasis *in vivo* is frequently the regional lymph nodes, and the appearance of lymph node metastases is correlated with poor prognosis (3). Studies of experimental metastasis using B16F1 and B16F10 cells demonstrated similar incidence of lymph node metastases, although animals injected with B16F10 cells were more likely to develop pulmonary metastases (28). Expression of the vitronectin-binding integrin $\alpha v \beta 3$ is enhanced in metastatic melanoma cells relative to parental nonmetastatic variants and recent

represent S.D. C, effect of membrane association on MMP-2 catalytic activity. ^{14}C -gelatin cleavage by APMA-activated MMP-2/TIMP-2 (10 nM) was determined in the absence of membranes or in the presence of B16 plasma membranes (1 μg) (open bars) or Triton X-100 detergent extracts (100 ng) (hatched bars). Data are presented as the percent increase in gelatin hydrolysis relative to the activity of control MMP-2/TIMP-2 determined in the absence of membranes. Experiments were performed in triplicate, and error bars represent S.D. In additional controls, the membrane preparations were incubated with the gelatin substrate in the absence of enzyme (data not shown).

experiments have demonstrated that adhesion of metastatic melanoma cells to lymph node sections is blocked by either anti- $\alpha\beta 3$ or RGD-containing peptides (3). In related experiments, ligation of melanoma cell $\alpha\beta 3$ using an anti-integrin antibody was shown to enhance cellular invasiveness *in vitro*, and conditioned medium from cells treated with anti- $\alpha\beta 3$ displayed increased MMP-2 activity (7). This is particularly interesting in light of previous data that indicate that MMP-2 expression by melanoma cells correlates with increased invasiveness, and it provides a biochemical mechanism whereby invasion may be enhanced (29–31).

These observations are supported by data from the present study that demonstrate a direct dose-dependent increase in MMP-2 secretion in vitronectin-treated B16F1 and B16F10 melanoma cells. As a functional consequence of increased MMP-2 levels, cellular invasiveness is also enhanced. The vitronectin-induced increase in MMP-2 secretion and invasive activity was abolished by RGDS peptide, providing evidence that vitronectin interaction with cellular integrins regulates invasive behavior. Concomitant with MMP-2 secretion, levels of TIMP-2 were also increased by vitronectin treatment. Previous studies have shown that MMP-2 is secreted as a proenzyme in complex with TIMP-2 by melanoma cells and other cell types, and additional reports suggest that the presence of TIMP-2 in this proenzyme-inhibitor complex is required for cellular activation of the MMP-2 zymogen by membrane-type MMPs (24, 32–36).

Recent evidence indicates that cell surface association may function as a mechanism for posttranslational regulation of MMP activity (17, 21–26). The present data demonstrate that MMP-2/TIMP-2 associates with the plasma membrane fraction of murine melanoma cells and exhibits enhanced catalytic activity against macromolecular substrates relative to the solution phase enzyme supporting the role of membrane association in MMP regulation. The mechanism of interaction of proMMP-2 and proMMP-2/TIMP-2 with B16F1 and B16F10 cells is currently unknown. However, it has recently been reported that recombinant chick MMP-2 binds to hamster melanoma cells transfected with the $\beta 3$ integrin subunit via a direct interaction with $\alpha\beta 3$, which is inhibited by RGD peptides, as well as to purified $\alpha\beta 3$ immobilized on microtiter wells (37). This is in contrast to the results of the present study, which show that binding of human proMMP-2 and proMMP-2/TIMP-2 to intact murine melanoma cells is not RGD-mediated. This is supported by the observation that MMP-2 secretion by vitronectin-treated cells was inhibited by colchicine, demonstrating that the enhanced proteinase levels observed were not due simply to displacement of MMP-2 by vitronectin from a common cell surface receptor. Furthermore, neither the peptide RGDS nor endoproteinase V8-treated vitronectin, both of which bind the cellular vitronectin receptor, increased the level of MMP-2 in conditioned medium. In addition, neither pro- nor active MMP-2 inhibited cell adhesion to vitronectin. The discrepancy between results reported in the current study and those of Brooks *et al.* (37) may reflect differences in the distinct model systems employed. Alternatively, differences in the activation state of the enzymes may also influence cellular association because proteolytic activity may alter (or confer) cell binding ability.

The complex array of functions attributed to integrins, including cell-matrix adhesion, cytoskeletal organization, and signal transduction, results from a cellular ability to discriminate functionally between transmembrane signals induced by simple ligand occupancy, receptor aggregation, or simultaneous occupancy and aggregation (38). In the present study MMP-2 secretion was induced by intact vitronectin in both

B16F1 and B16F10 cells; however, limited proteolysis of vitronectin removed the stimulatory effect, regardless of whether the RGD site was disrupted (plasmin) or remained intact (endoproteinase V8). Furthermore, simple ligation of the vitronectin receptor with the peptide RGDS also failed to induce MMP-2 activity at concentrations well in excess of that required for inhibition of cell adhesion. However, antibody ligation of melanoma cell $\alpha\beta 3$, which can induce receptor aggregation, stimulated MMP-2 production (7). Together, these data indicate that a multivalent ligand-receptor interaction, rather than simple ligand occupancy, is required for induction of MMP-2. In light of these results, it is interesting to consider recent biophysical data that demonstrate that under physiologic conditions, vitronectin can exist in both monomeric and multimeric forms (39). Vitronectin in extravascular sites (*i.e.* matrix and tissue-associated) is predominantly in the multimeric form, suggesting that multivalent ligand-receptor interactions may prevail *in vivo* (39, 40). Furthermore, the current data suggest a biologic control mechanism whereby the stimulus for MMP-2 induction, *i.e.* intact vitronectin, may be removed. It is interesting to speculate that vitronectin-adherent melanoma cells, which also catalyze tissue-type plasminogen activator-mediated plasmin generation (13), may initiate plasmin-dependent proteolysis of vitronectin, thereby disrupting the multivalent signal necessary for MMP-2 induction.

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REFERENCES

- Ruoslahti, E., and Giancotti, F. G. (1989) *Cancer Cells* **1**, 119–126
- Hynes, R. O. (1992) *Cell* **69**, 11–25
- Nip, J., Shibata, H. R., Loskutoff, D. J., Cheresch, D. A., and Brodt, P. (1992) *J. Clin. Invest.* **90**, 1406–1413
- Albelda, S. M., Mette, S. A., Elder, D. E., Stewart, R., Damjanovich, L., Herlyn, M., and Buck, C. A. (1990) *Cancer Res.* **50**, 6757–6764
- Sanders, L. C., Felding-Habermann, B., Mueller, B. M., and Cheresch, D. A. (1992) *Cold Spring Harbor Symp. Quant. Biol.* **57**, 233–240
- Nip, J., and Brodt, P. (1995) *Cancer Metastasis Rev.* **14**, 241–252
- Seftor, R. E. B., Seftor, E. A., Gehlsen, K. R., Stetler-Stevenson, W. G., Brown, P. D., Ruoslahti, E., and Hendrix, M. J. C. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 1557–1561
- Dahlback, B., and Podack, E. R. (1985) *Biochemistry* **24**, 2368–2374
- Young, T. N., Rodriguez, G. C., Moser, T. L., Bast, R. C., Jr., Pizzo, S. V., and Stack, M. S. (1994) *Am. J. Obstet. Gynecol.* **170**, 1285–1296
- Itoh, Y., Binner, S., and Nagase, H. (1995) *Biochem. J.* **308**, 645–651
- Gonzalez-Gronow, M., and Robbins, K. C. (1984) *Biochemistry* **23**, 190–197
- Felding-Habermann, B., and Cheresch, D. A. (1993) *Curr. Opin. Cell Biol.* **5**, 864–868
- Stack, M. S., Gray, R. D., and Pizzo, S. V. (1993) *Cancer Res.* **53**, 1998–2004
- Salvesen, G. S., and Nagase, H. (1989) in *Proteolytic Enzymes: A Practical Approach* (Beynon, R. J., and Bond, J. S., eds), pp. 83–104, IRL Press, Oxford
- Moser, T. L., Pizzo, S. V., Bafetti, L. M., Fishman, D. A., and Stack, M. S. (1996) *Int. J. Cancer* **67**, 695–701
- Pierschbacher, M. D., and Ruoslahti, E. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 5985–5988
- Young, T. N., Pizzo, S. V., and Stack, M. S. (1995) *J. Biol. Chem.* **270**, 999–1002
- Harris, E. D., and Krane, S. M. (1972) *Biochim. Biophys. Acta* **258**, 566–576
- Preissner, K. T. (1991) *Annu. Rev. Cell Biol.* **7**, 275–310
- Houmard, J., and Drapeau, G. R. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69**, 306–309
- Ward, R. V., Atkinson, R. J., Reynolds, J. J., and Murphy, G. (1994) *Biochem. J.* **304**, 263–269
- Emonard, H. P., Remacle, A. G., Noel, A. C., Grimaud, J. A., Stetler-Stevenson, W. G., and Foidart, J. M. (1992) *Cancer Res.* **52**, 5845–5848
- Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. (1994) *Nature* **370**, 61–65
- Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A., and Goldberg, G. I. (1995) *J. Biol. Chem.* **270**, 5331–5338
- Toth, M., Gervasi, D. C., and Fridman, R. (1997) *Cancer Res.* **57**, 3159–3167
- Wallon, U. M., and Overall, C. M. (1997) *J. Biol. Chem.* **272**, 7473–7481
- Allan, J. A., Docherty, A. J. P., Barker, P. J., Huskisson, N. S., Reynolds, J. J., and Murphy, G. (1995) *Biochem. J.* **309**, 299–306
- Nathanson, S. D., Haas, G. P., Mead, M. J., and Lee, M. (1986) *J. Surg. Oncol.* **33**, 41–45
- Turpeenniemi-Hujanen, T., Thorgeirsson, U. P., Hart, I. R., Grant, S. S., and Liotta, L. A. (1985) *J. Natl. Cancer Inst.* **75**, 99–103
- Liotta, L., Rao, C. N., and Wewer, U. M. (1986) *Annu. Rev. Biochem.* **55**,

1037-1057

31. Stetler-Stevenson, W. G. (1990) *Cancer Metastasis Rev.* **9**, 289-303
32. Stetler-Stevenson, W. G., Krutzsch, H. C., Wachter, M. P., Margulies, I. M. K., and Liotta, L. A. (1989) *J. Biol. Chem.* **264**, 1353-1356
33. Goldberg, G. I., Marmer, B. L., Grant, G. A., Eisen, A. Z., Wilhelm, S., and He, C. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 8207-8211
34. Cockett, M. I., Birch, M. L., Murphy, G., Hart, I. R., and Docherty, A. J. P. (1994) *Biochem. Soc. Trans.* **22**, 55-57
35. Emmert-Buck, M. R., Emonard, H. P., Corcoran, M. L., Krutzsch, H. C., Foidart, J. M., and Stetler-Stevenson, W. G. (1995) *FEBS Lett.* **364**, 28-32
36. Nagase, H., Suzuki, K., Itoh, Y., Kan, C.-C., Gehring, M. R., Huang, W., and Brew, K. (1996) in *Intracellular Protein Catabolism* (Suzuki, K., and Bond, J., eds), pp.23-31, Plenum Press, New York
37. Brooks, P. C., Stromblad, S., Sanders, L. C., von Schalscha, T. L., Aimes, R. T., Stetler-Stevenson, W. G., Quigley, J. P., and Cheresch, D. A. (1996) *Cell* **85**, 683-693
38. Miyamoto, S., Akiyama, S. K., and Yamada, K. M. (1995) *Science* **267**, 883-885
39. Zhuang, P., Blackburn, M. N., and Peterson, C. B. (1996) *J. Biol. Chem.* **271**, 14323-14332
40. Preissner, K. T., Grulich-Henn, J., Ehrlich, H. J., Declerck, P., Justus, C., Collen, D., Pannekoek, H., and Muller-Berghaus, G. (1990) *J. Biol. Chem.* **265**, 18490-18498