De-*N*-acetyl GM3 Promotes Melanoma Cell Migration and Invasion through Urokinase Plasminogen Activator Receptor Signaling–Dependent MMP-2 Activation

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Abstract

We have recently discovered that de-*N*-acetyl GM3 [NeuNH_2-LacCer, d-GM3], a derivative of ganglioside GM3, is specifically expressed in metastatic tumor cells and that its expression correlates with an enhanced metastatic phenotype. Although the classic *N*-acetylated form of GM3 (Neu*Ac*LacCer, c-GM3) is found in both normal and tumor cells, metastatic tumor cells (but not other cells) predominantly express d-GM3 (82–95% of total GM3). d-GM3 expression is mainly found in metastatic melanomas, but not in benign nevi or the majority of primary melanomas. Using metastatic (d-GM3–positive) and poorly invasive (d-GM3–negative) human melanoma cell lines, we found that d-GM3 stimulates cell migration and invasion by increasing the expression and activation of urokinase-like plasminogen activator (uPA). Further studies showed that d-GM3 activates matrix metalloproteinase-2 (MMP-2), but not MMP-9, when uPA receptor signaling is activated. These results implicate d-GM3 as a specific marker for metastatic melanoma and a novel therapeutic target for neoplastic diseases. [Cancer Res 2009;69(22):8662–9]

Introduction

The increasing prevalence of melanoma and the lack of effective treatments for metastatic disease position melanoma as one of the most devastating of malignances (1). Identifying molecular targets that distinguish metastatic from more indolent melanomas could improve our ability to prognosticate and potentially lead to new therapies. Gangliosides, sialylated glycosphingolipids located at the outer leaflet of the plasma membrane, are known to regulate cell proliferation, migration, invasion, and survival (2–14). Malignant tumors often show alterations in ganglioside expression and distribution that correlate with abnormal cell growth and motility, as well as oncogenic transformation (12–14). The accessibility of gangliosides on the cell surface makes them ideal targets for manipulating cell behavior through their binding to antibodies or other small molecules. The principal gangliosides of melanomas are GM3 (including *N*-acetylated classic GM3), GM2, GD3, and GD2 (15). GM3, a precursor of other gangliosides, is the simplest ganglioside (6). Increases in GM3 have been found in highly metastatic variants of colon adenocarcinomas and neuroectodermal tumors, including melanomas, suggesting that it may be a target for suppressing tumor metastasis (16–18). GM3-based vaccines and immunotherapy have decreased tumor load but not completely cleared or prevented the metastasis of neuroectodermal tumors (19–23). The widespread expression of *N*-acetylated GM3 (c-GM3) on normal cells, including immune cells, may contribute to the inefficiency in targeting GM3 for treatment. Although variant forms of GM3 are frequently found in neoplastic mammalian cells and tissues, no particular variant of GM3 has been specifically related to metastasis.

The urokinase plasminogen activator (uPA) system, consisting of the serine protease uPA, its two endogenous inhibitors (PAI-1 and PAI-2), and the uPA receptor (uPAR), has been implicated in cancer invasion and metastasis (for reviews, see refs. 24, 25). uPAR expression strongly correlates with a metastatic phenotype in human cancers, including melanoma and neoplasia of the breast, lung, and colon (24–27). Independent of its role in proteolysis, uPAR is also capable of initiating signaling (25). uPAR signaling promotes cell growth and migration during development, wound healing, vascular remodeling, and cancer cell spreading (28). In addition, targeting uPAR has practical implications in treating neoplastic diseases. For example, (*a*) downregulating uPAR expression leads to dormancy of cancer cells (29); (*b*) administrating uPAR antagonists markedly inhibits metastatic ability, including that of melanomas (30); (*c*) deficiency of plasminogen/uPA in mice slows tumor growth and progression (30); and (*d*) inhibiting uPA and/or its binding with uPAR prevents/reduces metastasis in animal models (31). uPAR and uPA are considered important in malignant human tumors but not essential for fertility or cell survival. The additional role of uPAR signaling in mediating ganglioside function (4, 10) makes uPA/uPAR, in addition to ganglioside, an attractive target for cancer therapy (for review, see ref. 25).

Matrix metalloproteinases (MMP), in particular, gelatinases MMP-2 and MMP-9, have been associated with tumor invasiveness and metastasis due to their ability to degrade type IV collagen in extracellular matrices surrounding tumor cells (32). The degradation of basal membrane by tumor cells involves secretion and activation of proteinases, such as MMPs, and plasminogen activation system components. Reduced activity of MMP inhibitors or enhanced activity of MMP activators, regulated by molecules such as gangliosides, further enhances this degradation, promoting cancer progression (12, 25).

We have discovered a novel variant of GM3, de-*N*-acetyl GM3 (d-GM3), which is highly expressed in metastatic melanomas, but not

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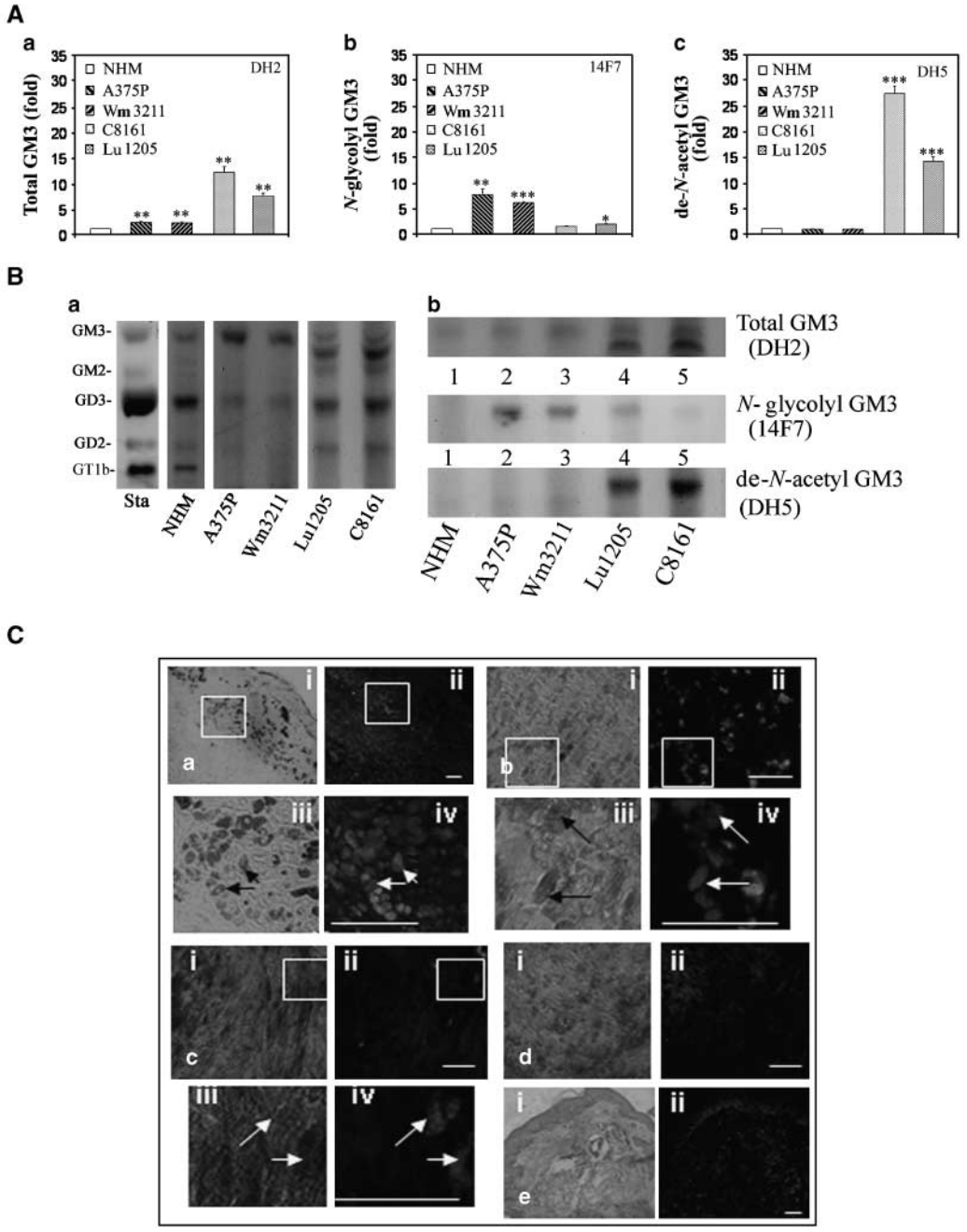

Figure 1. GM3 is deacetylated to d-GM3 in metastatic melanomas. The expression of GM3 in NHMs, poorly invasive primary melanoma cells (A375P and Wm3211), and metastatic melanoma cells (C8161 and Lu1205) was detected by both ganglioside ELISA (*A*) and TLC (*B*). *A,* antibodies directed against total GM3 (*DH2*; *a*), *N*-glycolyl GM3 (*14F7*; *b*), and de-*N*-acetyl GM3 (*DH5*; *c*), or the control anti-IgG antibody, were used as described in Materials and Methods. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; *B,* ganglioside profile of cultured cells was examined by routine TLC with resorcinol staining (*a*), and the differences in the expression level of total GM3 and two GM3 variants that are not found in normal human tissues were further detected by TLC immunostaining with antibodies used for ELISA assay (*b*). *C,* the expression of d-GM3 in human tissues of metastatic melanoma (*a, b*), primary melanoma (*c, d*), and benign nevi (*e*) was examined by routine immunofluorescence staining (*ii*). The images of nonstained same section or H&E-stained serial section (*i*) were presented to show tissue structure of *ii. iii* and *iv* are higher-magnification images of *i* and *ii*, respectively. *Arrows,* d-GM3–positive cells; *bar,* 120 μm. All experiments were performed three times.

in normal melanocytes, nevi, or poorly invasive melanomas. d-GM3 augments uPA and stimulates MMP-2 expression and activation, enhancing the melanoma metastatic phenotype. Given that (*a*) d-GM3 enhances melanoma metastatic potential mainly through uPAR activation and (*b*) the stimulation of cell migration and invasion by uPAR depends primarily on d-GM3, d-GM3 could be a novel tumor-related marker in metastatic neuroectodermal tumors (including melanomas) and an ideal target for tumor treatment.

Materials and Methods

Cells. Human cutaneous melanoma A375P and C8161 cells (courtesy of Drs. A. Hess and M. Hendrix, Chicago, IL) were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen). Human metastatic melanoma Lu1205 cells and poorly invasive Wm3211 cells (courtesy of Dr. M. Herlyn, Philadelphia, PA) were cultured in L15/MCDB medium with 5% FBS, 5% newborn calf serum, and 10 μg/mL insulin. C8161 and Lu1205 cells were used because they express high levels of d-GM3 (82–95% of total GM3), proliferate rapidly, and are highly invasive, whereas A375P and Wm3211 cells do not express d-GM3, proliferate slowly, and are poorly invasive. Normal human melanocytes were purchased from ScienCell Research Laboratories and maintained in melanocyte medium (ScienCell Research Laboratories).

Modulating ganglioside expression. The content of gangliosides in melanoma cell lines was modulated as previously described (4, 6, 11). Different variants of GM3 on the cell membrane were either augmented by pharmacologic addition of purified GM3 or depleted by treatment of cells with PPPP (P4, racemic *threo*-1-phenyl-2-hexadecanoyl-amino-3-pyrrolidinopropan-1-ol, HCl, Calbiochem), a chemical reagent that blocks the synthesis of GM3 without accumulating ceramide (6, 11). The content of ganglioside was detected by ganglioside ELISA (6, 7, 9, 11), routine TLC, and TLC immunostaining (3, 6, 33). Antibodies directed against total GM3 (DH2, Glycotech), *N*-glycolyl-GM3 (14F7, a generous gift from Dr. Krengel, Oslo, Norway; ref. 34), or d-GM3 (DH5, a generous gift from Dr. Hakomori, Seattle, WA; ref. 35) were used. For ganglioside ELISA, BM blue POD substrate (Roche Applied Science) was used, and the absorbance was read at 450 nm in a UVmax kinetic microplate reader (Molecular Devices). For routine TLC and TLC immunostaining, total lipids were extracted from cells (5×10^7) using 10 volumes of chloroform/methanol (2:1; ref. 3). The aqueous phase was separated and desalted, and the bands were separated by TLC in chloroform/methanol/water in 0.02% $CaCl_2$, 55:45:10 (v/v/v). Ganglioside was identified by resorcinol staining on silica gel plates for routine TLC or by immunostaining on aluminum-backed TLC plates, and bands were visualized by an ECL kit on X-Omat film. Band density was quantified using the NIH Image J program (3).

Immunofluorescence staining. Sections (5 μm) obtained from paraffin- or OCT-embedded patient tissues were either stained with H&E or immunostained using Texas red–labeled secondary antibody. DH5 (mouse monoclonal antibody, 1:100) was used as primary antibody. Patient tissues were obtained according to approval institutional review board protocols at Northwestern University, Dalian Medical University, and Loyola University. Staining was assessed in triplicate by three blinded observers. Images were captured under Zeiss Axioscope 2 Plus fluorescence microscope with AxioVision LE imaging software (11).

Gene transfection. uPAR cDNA (courtesy of Dr. L. Ossowski, New York, NY) in V_5/His-pcDNA3.1 vector (Invitrogen), its vector control (V_5/His-pcDNA3.1), uPAR small interfering RNA (siRNA), or a scrambled oligomer (negative control) in a pSilencer cytomegalovirus hygro-vector (Ambion, Inc.) were transfected into melanoma cells using Lipofectamine 2000 reagent (Invitrogen; refs. 11, 36). Stably transfected cells were selected using hygromycin (50 μg/mL) for 4 to 6 wk.

Migration assay. Cell migration assays were performed using both scratch analysis and chemotaxis migration assay (Transwell cell culture system, Becton Dickinson Biosciences; refs. 9, 10). The following cells were prepared for migration assays: parental control cells, P4-treated cells, uPAR-overexpressing cells without or with pharmacologic addition of purified c-GM3 or d-GM3, v5/His pcDNA3.1 vector stably transfected control cells, uPAR knockdown cells, and its scrambled control cells. d-GM3 at 6 μg/mL maximally stimulated cell chemotactic migration (1–10 μg/mL tested), whereas c-GM3 did not influence melanoma cell migration even at a concentration as high as 100 μg/mL. As a result, 6 μg/mL of d-GM3 and 10 μg/mL of c-GM3 were used.

Invasion assay. Cell invasion assays were performed (9) using Becton Dickinson BioCoat Matrigel Invasion Chambers (Becton Dickinson Biosciences) per manufacturer's instruction. Cells prepared as indicated above were plated onto the upper surface of the filter and allowed to invade to the lower level for 48 h; cells that invaded into the lower level were collected and counted.

Reverse transcription-PCR. Total RNA was isolated from cells using an RNeasy mini-Kit (Qiagen) per manufacturer's instruction. cDNAs were prepared using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) with 2 μg of total RNA according to the manufacturer's protocol. The expression of uPA, uPAR, MMP-9, and MMP-2 in these human cells was examined by PCR using primers as described elsewhere (9, 37, 38). Glyceraldehyde 3-phosphate dehydrogenase expression was detected as an internal control (38). PCR was carried out under the following conditions: 1 cycle at 42°C for 30 min; 1 cycle at 95°C for 5 min; 25 cycles at 95°C for 1 min, and 72°C for 3 min; then 1 cycle at 72°C for 5 min (GeneAmp PCR system 2700, Applied Biosystems). The PCR products were detected using 1.2% agarose gel.

uPA activity assay. The uPA activity was assessed using a uPA activity ELISA kit per manufacturer's instruction (LOXO). Cells (2×10^7) were grown for 48 h in 20 mL of serum-free medium containing 0.1% bovine serum albumin in 150-mm Petri dishes. Conditioned medium (20 mL) was dialyzed 2 h against cold water, concentrated to 1 mL by lyophilization, and used (200 μL) for uPA activity assay. The final absorbance values were measured at 450 nm. uPA activity was also measured by casein/plasminogen zymography as follows: concentrated conditioned medium (10 μL) was loaded onto a 10% polyacrylamide gel containing 1 mg/mL casein and 5 units/mL plasminogen (American Diagnostica); after electrophoresis, the gel was developed (39). To confirm the identity of the bands ascribed to uPA, duplicate gels were developed in the presence of 5 mmol/L amiloride, an inhibitor of uPA.

Immunoblotting. Total protein from the whole-cell lysate was prepared by treating cells with boiling lysis buffer [1% SDS, 1 mmol/L Na_3VO_4, 10 mmol/L Tris-HCl (pH 7.4)] for 10 min. Protein concentration was measured using Coomassie blue R250 (11, 40). Total protein (10 μg) was applied onto a 10% SDS-PAGE mini gel. After electrophoresis, the migrated protein was transferred onto a nitrocellulose membrane. Expression of uPAR

Table 1. The percentage of DH5-stained tissues/tumor tissues

Groups (intensity of d-GM3 staining)	0 (0%)	1 (<25%)	2 (25-50%)	3 (50-75%)	4 (>75%)
Metastatic melanomas (54 cases)	—	—	4/54	21/54	29/54
Primary melanomas (38 cases)					
VGP (10 cases)	2/10	6/10	2/10	—	—
HGP and non-VGP (28 cases)	27/28	1/28	—	—	—
Normal nevi (8 cases)	8/8	—	—	—	—

NOTE: The percentage of pixels representing the positive DH5 immunofluorescence–stained tumors versus the pixels representing the pigmented or nonpigmented tumor tissues from either nonstained same sections or H&E-stained serial sections was graded as 0 to 4+.
Abbreviations: VGP, vertical growth phase; HGP, horizontal growth phase.

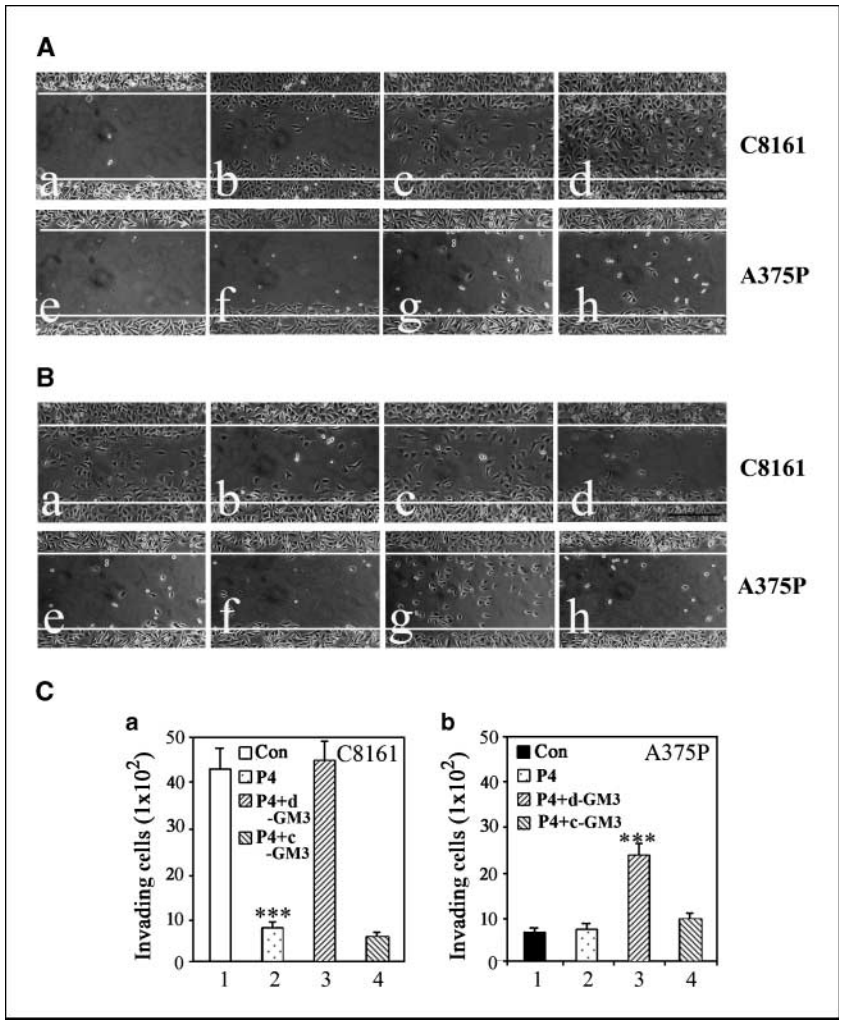

Figure 2. Increasing d-GM3 facilitates, whereas depletion d-GM3 inhibits, melanoma cell migration. Cells pretreated without (*A, a, e* of *B*) or with P4 in the absence (*b, f* of *B*) or presence of pharmacologically added d-GM3 (c, *g* of *B*) or c-GM3 (*d, h* of *B*) were prepared for scratch migration assay (9). Plates were photographed (*A,* 0, 2, 4, and 8 h; *B,* 4 h) at 200× magnification after the scratch was made. *Bar,* 200 μm. *C,* Matrigel invasion assay was performed using a Transwell system (BD Biosciences; ref. 9). Cells prepared as described in Materials and Methods were starved of serum, growth factors, and extracellular matrices overnight before plating onto the upper surface of the filter. Cells were allowed to invade for 48 h at 37°C. Cells that invaded into the lower level were collected and counted. *Columns,* mean from three different experiments with triplicate wells per experiment; *bars,* SD. ***, $P < 0.001$. All experiments were performed at least six times.

was detected by immunblotting the membrane with anti-uPAR antibody (Santa Cruz Biotechnology). Actin expression was used as a loading control.

Zymography. Conditioned medium (50 μg/lane) prepared as indicated above was used to analyze MMP-2 and MMP-9 activation. To confirm the identity of the bands ascribed to MMPs, duplicate gels were developed in the presence of 10 mmol/L EDTA (negative control for MMP activity; ref. 9).

Results

The expression of d-GM3 characterizes the melanoma metastatic phenotype *in vitro* and *in vivo*. In this study, we detected the expression of total GM3, *N*-glycolyl GM3, and d-GM3 in normal human melanocytes (NHM), poorly invasive melanoma cells (A375P and Wm3211), and aggressive melanoma cells (C8161 and Lu1205) by ganglioside ELISA (6, 7, 9). In comparison with NHMs, the total GM3 content was 1.7- to 2.2-fold greater in poorly invasive melanoma cells ($P < 0.01$) and 4.1- to 4.6-fold greater in aggressive metastatic melanoma cells ($P < 0.001$; Fig. 1*A, a*). In the poorly invasive primary melanoma cells, the increase in GM3 content resulted from increased *N*-glycolyl GM3 ($P < 0.01$; Fig. 1*A, b*), whereas the increase in GM3 reflected a marked expression of d-GM3 in the aggressive metastatic melanoma cells ($P < 0.001$; Fig. 1*A, c*). In addition to increased GM3, using routine TLC, we also observed a similar ganglioside expression pattern (GM2, GD3, GD2) with markedly reduced GT1b in metastatic melanoma cells when compared with NHMs (Fig. 1*B, a*). Results from TLC immunostaining corroborated the findings in ELISA.

To further evaluate whether d-GM3 expression correlates with a melanoma metastatic phenotype *in vivo*, we examined the expression of d-GM3 in patient specimens of paraffin- or OCT-embedded normal nevi (8 cases), primary melanomas (38 cases), and metastatic melanomas (54 cases). The intensity of d-GM3 staining was quantified by converting the digital images (10 images per section, 200×) to grayscale using the histogram application in Adobe Photoshop CS 8.0 (41). The percentage of pixels representing the positive DH5 immunofluorescence–stained tumor tissues and pixels representing pigmented or nonpigmented tumor tissues (from unstained same sections or H&E-stained serial sections) was graded 0 to 4+. As shown in Fig. 1*C* and Table 1, d-GM3 was predominantly expressed in metastatic melanomas (54 of 54; *a*, cutaneous metastatic melanomas; *b*, lymph node) with 7.4% at 2+, 38.9% at 3+, and 53.7% at 4+. Although some cases of vertical growth phase primary melanomas (8 of 10; *c*, cutaneous primary melanoma) showed d-GM3 staining intensity with 60% at 2+ and 20% at 3+, and one case of the nonvertical growth phase melanomas (1 of 28) was d-GM3 positive, most nonvertical growth phase primary melanomas (27 of 28; *d*, primary cutaneous melanoma) and benign nevi (8 of 8; *e*) did not express d-GM3.

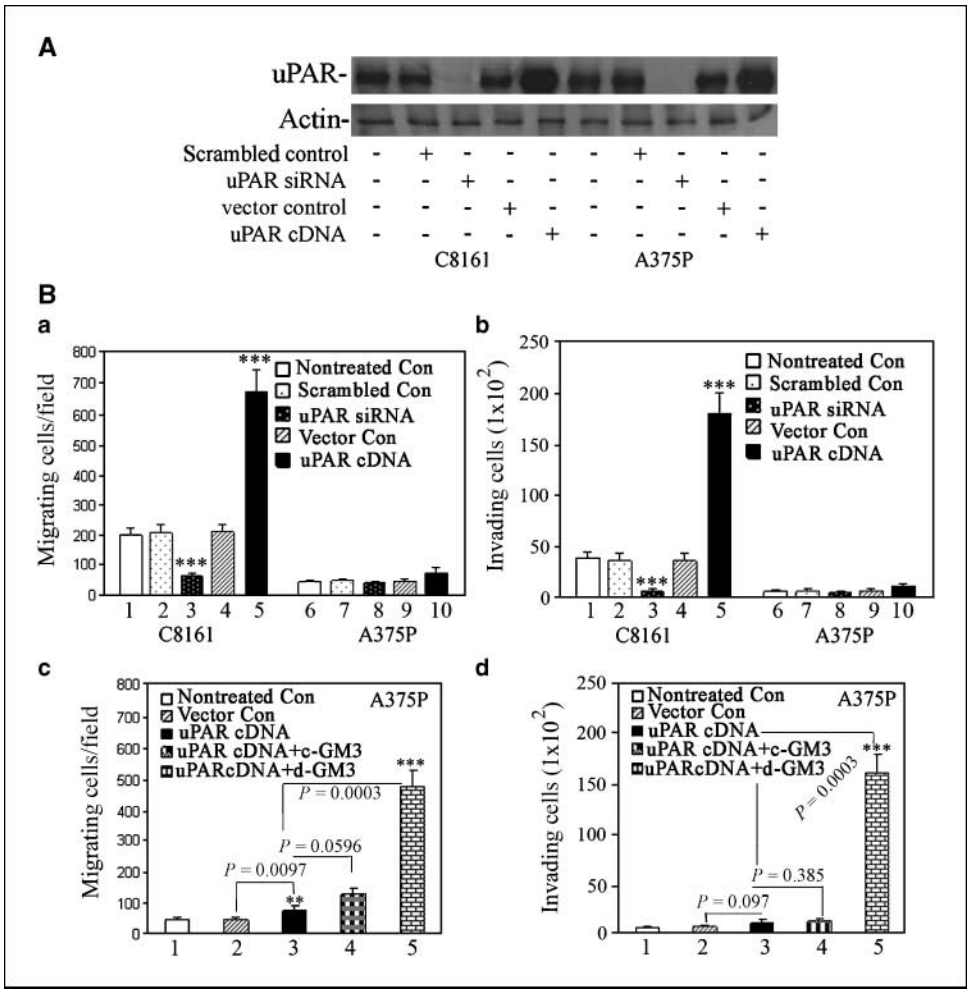

Figure 3. d-GM3 regulates cell migration and invasion through uPAR signaling. *A,* uPAR expression was either knocked down or overexpressed, respectively, by treatment with uPAR siRNA or stable transfection of human uPAR cDNA. Cells treated with scrambled siRNA or stably transfected with a pcDNA vector were prepared in parallel as controls. uPAR expression was detected by immunoblotting (*top row*). Equal loading was confirmed by probing the same membrane with actin (*bottom row*). *B,* the impact of d-GM3 and uPAR on cell migration (*a, c*) and invasion (*b, d*) was detected by chemotaxis migration and Matrigel invasion assay as described in Materials and Methods. *Columns,* mean from three different experiments performed in duplicate; *bars,* SD. **, $P < 0.01$; ***, $P < 0.001$.

d-GM3 expression enhances melanoma cell migration and invasion. Scratch assay showed that the d-GM3–positive, more aggressive melanoma C8161 cells migrated 3.76 to 4.39 times faster than the d-GM3–negative, poorly invasive A375P cells (Fig. 2*A*). Depletion of GM3 by treating cells with P4 (4) inhibited the migration of metastatic C8161 melanoma cells in both scratch (Fig. 2*B, b* versus *a*) and chemotaxis (not shown) assays; the addition of purified d-GM3 reversed the inhibitory effect of P4 on cell migration (Fig. 2*B, c* versus *b* and *a*), whereas the addition of c-GM3 had no effect (Fig. 2*B, d* versus *b* and *a*). Neither depletion of GM3 by P4 treatment nor addition of c-GM3 to d-GM3–negative, poorly invasive A375P melanoma cells altered their already poor migration (Fig. 2*B, f* and *h* versus *e*); however, addition of purified d-GM3 dramatically stimulated cell migration of the poorly invasive melanoma cells (Fig. 2*B, g* versus *e* and *f*). Boyden chamber Matrigel invasion assays were also used to examine the effect of d-GM3 on cell invasion. Consistently, depletion of d-GM3 by P4 treatment dramatically reduced cell invasion of aggressive metastatic C8161 melanoma cells (*lane 2* versus *1*, Fig. 2*C, a*), whereas increases in d-GM3, but not c-GM3, stimulated cell invasion in both P4-treated aggressive metastatic C8161 cells and poorly invasive A375P melanoma cell invasion (*lane 3* or *4* versus *2* and *1*, Fig. 2*C*). The effects of P4, d-GM3, and c-GM3 on cell migration and invasion in Lu1205 and Wm3211 cells closely resembled those of the C8161 and A375P cells, respectively (not shown).

Activation of uPAR signaling is required for the biological effects of d-GM3. To study the potential role of uPAR signaling in d-GM3–enhanced melanoma cell migration and invasion, uPAR expression was either overexpressed or knocked down (Fig. 3*A*). Knockdown of uPAR inhibited, whereas uPAR overexpression facilitated, C8161 melanoma cell migration (Fig. 3*B, a, lane 3* versus *2,* $P = 0.004$; *lane 5* versus *4,* $P = 0.002$) and invasion (Fig. 3*B, b, lane 3* versus *2,* $P = 0.007$; *lane 5* versus *4,* $P = 0.0009$). Knockdown of uPAR expression did not influence A375P cell migration (*a, lane 8* versus *7,* $P = 0.0536$), but overexpression of uPAR enhanced it (*lane 10* versus *9,* $P = 0.00907$). In addition, d-GM3, but not c-GM3, dramatically increased A375P cell migration (*c*) and invasion (*d*) in cells that overexpressed uPAR (Fig. 3*B, lane 5* versus *3,* $P = 0.0003$). These results indicate that d-GM3, rather than c-GM3, drives uPAR signaling activation to stimulate melanoma migration and invasion.

d-GM3 expression augments uPA expression and activity. To consider how d-GM3 affects uPAR activation, the expression of uPA and uPAR was examined. Although uPAR expression itself did not correlate with d-GM3 expression (Fig. 4*A*), uPA expression was 2.6 to 2.9 times higher in d-GM3–positive metastatic melanoma cells (Fig. 4*B*). Casein/plasminogen zymography (Fig. 4*C*) showed that uPA activity was significantly elevated in d-GM3–positive C8161 (12.10-fold, *bottom row, lane 1*) and Lu1205 (9.05-fold, *bottom row, lane 2*) cells compared with d-GM3–negative A375P (*bottom row, lane 3*) and Wm3211 (*bottom row, lane 4*) cells. To further verify

the correlation of uPA activity and d-GM3 expression, d-GM3 was first depleted by treatment with P4 and then repleted by the addition of purified d-GM3. d-GM3 was also used to treat A375P and Wm3211 cells. By uPA activity ELISA (Fig. 4*D*), uPA activity was 8.8- to 10-fold higher in C8161 (*lane 5*) and Lu1205 cells (*lane 8*) than in A375P (*lane 1*) and Wm3211 cells (*lane 3*). uPA activity was increased 6.6- to 6.7-fold in d-GM3–treated A375P (*lane 2* versus *1*) and Wm3211 cells (*lane 4* versus *3*). Consistently, depletion of d-GM3 significantly decreased uPA activity by 3.6- to 4.6-fold in C8161 (*lane 6* versus *5*, $P < 0.01$) and Lu1205 cells (*lane 9* versus *8*, $P < 0.01$). uPA activity was recovered by d-GM3 repletion (*lane 7* versus *6* and *lane 10* versus *9*, $P < 0.001$).

d-GM3 expression promotes MMP-2 expression and activation through uPAR signaling. Given the role of MMP activation in melanoma metastasis, we compared MMP expression and activation in d-GM3–positive C8161 cells and d-GM3–negative A375P cells. By gelatin zymography, the expression and activity of MMP-2 were dramatically elevated in C8161 cells compared with A375P cells (Fig. 5*A, a, middle* and *bottom rows*). These cells showed no difference in MMP-9 expression (Fig. 5*A, a, top row*) and no detectable activation of MMP-9. Consistently, reverse transcription-PCR (RT-PCR) showed that the expression of MMP-2 (*bottom row*), but not MMP-9 (*top row*), was increased in C8161 cells (Fig. 5*A, b*). Although P4 treatment did not alter MMP-2 in A375P cells (Fig. 5*B, lane 2* versus *1*), it reduced MMP-2 in C8161 cells (*lane 5* versus *4*). Although d-GM3 repletion recovered MMP-2 in C8161 cells (*lane 6* versus *5*), the addition of d-GM3 increased MMP-2 in A375P cells (*lane 3* versus *1*). None of these treatments altered MMP-9 expression (Fig. 5*B, top row*). To assess whether an increase in d-GM3–induced MMP-2 requires uPAR signaling, uPAR expression was increased or decreased in d-GM3–positive C8161 cells. Increases in uPAR expression augmented MMP-2 expression (4.5-fold) and activation (9.6-fold, Fig. 5*C, lane 2* versus *1*). Depletion of d-GM3 eliminated the increase in MMP-2 activity induced by uPAR overexpression (*lane 3* versus *2* and *1*), whereas d-GM3 repletion increased it (3.9-fold increase in expression and 3.6-fold increase in activity, *lane 4* versus *3*). These data suggest that the uPAR-induced increase in MMP-2 at least partially depends on d-GM3 expression (*lane 4* versus *2* and *3*), although the MMP-2 expression and activation recovered by d-GM3 repletion were 1.68-fold and 2.67-fold less, respectively (*lane 4* versus *2*). Knocking down uPAR expression reduced MMP-2 expression and prevented its activation (*lane 7* versus *6*), whereas d-GM3 repletion only slightly increased MMP-2 expression (1.7-fold, *lane 8* versus *7*). Although uPAR signaling activation elevated MMP-2 expression and activation only partially depends on d-GM3 expression (Fig. 5*C, lane 4* versus *2*, $P = 0.0235$), our results suggest that d-GM3 facilitated MMP-2 activation predominantly through uPAR signaling (Fig. 5*C, lane 8* versus *7* and *6*).

Discussion

Several gangliosides, including GM3, GM2, GD3, and GD2, are recognized as tumor-related markers for melanomas (15). Low expression levels of these gangliosides are also found in most normal cells, thus revealing that rare and/or unusual variations of conventional gangliosides may be promising targets for immunotherapy. In this report, we have distinguished metastatic melanoma cells from poorly invasive melanoma cells and normal melanocytes by the de-*N*-acetylation of GM3 (d-GM3). d-GM3 expression correlates with the melanoma metastatic phenotype both *in vitro* and *in vivo*. The mechanism involves activation of MMP-2 and requires the presence of uPAR and elevated uPA activity. When d-GM3 is absent, increases in uPAR expression stimulate melanoma migration but have no effect on invasion. In contrast, when d-GM3 is present, uPAR overexpression increases both melanoma cell migration and invasion. Taken together, these results suggest that d-GM3 is a novel marker for melanoma metastasis and plays a critical role in regulating cell invasion through activation of uPAR signaling and MMP-2.

The impact of tumor-related gangliosides on metastasis has been studied for over a decade (12, 16–18, 23). By TLC, we found four gangliosides, GM3, GM2, GD3, and GD2, that are expressed in metastatic melanoma cells as well as in normal melanocytes. We also noted a marked diminution in expression of GT1b. The influence of GD3 and GD2 on melanoma metastasis has been extensively studied (for reviews, see refs. 12–14, 23, 42). In addition, de-*N*-acetylation of GD3 has been described as a potential tumor

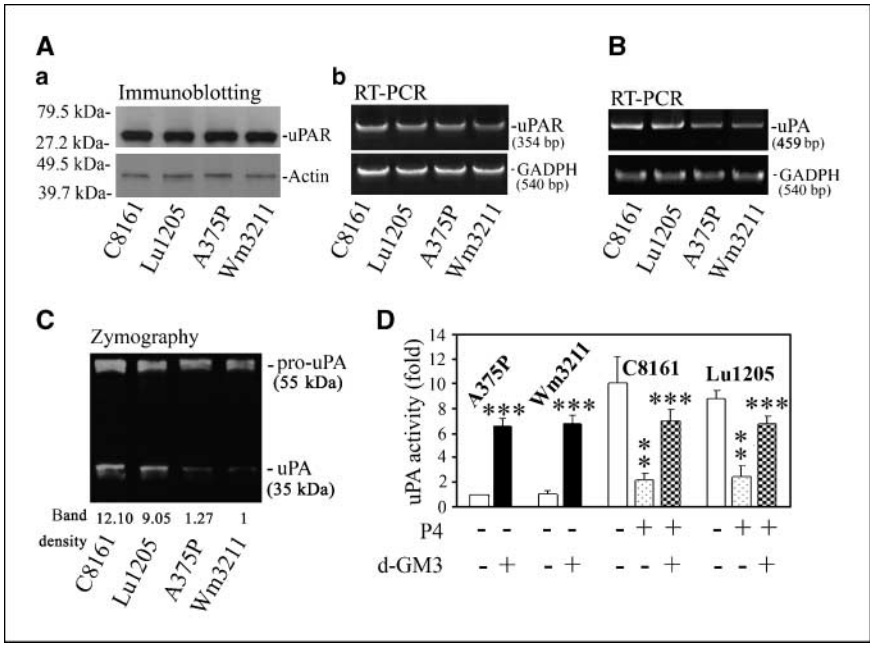

Figure 4. The expression and activity of uPA, but not uPAR expression, are elevated in d-GM3–positive melanoma cells, and depletion of d-GM3 prevents uPA activation. The expression of uPAR (*A*) and uPA (*B* and *C*) was assessed by RT-PCR (*A, b* and *B*) and immunoblotting (*A, a*) or zymography (*C*) as described in Materials and Methods. *D*, uPA activity was examined using a uPA activity ELISA kit. d-GM3 in metastatic melanoma cells (*C8161* and *Lu1205*) was depleted by PPPP treatment for 3 d before c-GM3 or d-GM3 was added in the presence of PPPP for another 2 d. Conditioned cell culture medium was concentrated for uPA activity analysis as described in Materials and Methods. ***, $P < 0.001$.

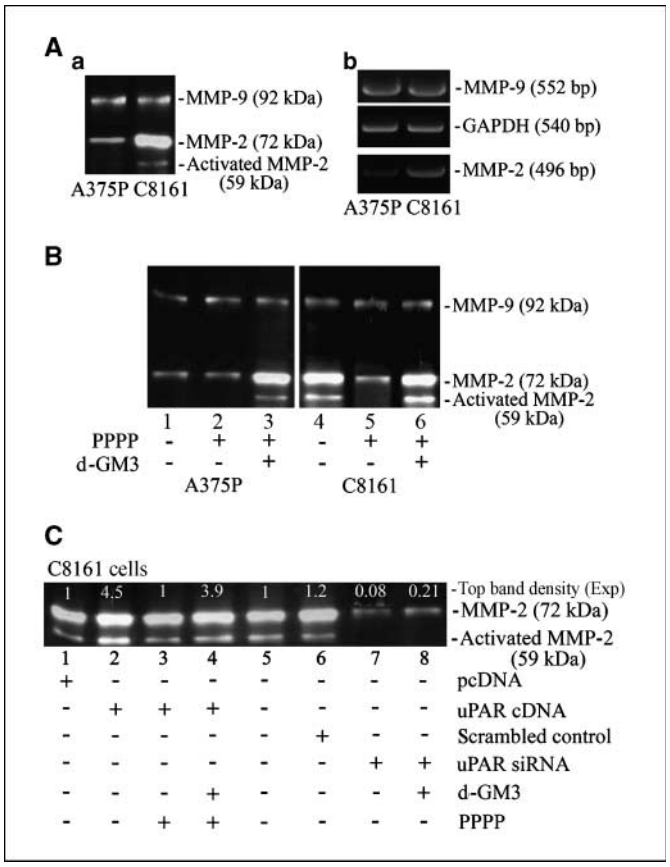

Figure 5. The expression and activity of MMP-2, but not MMP-9, require d-GM3–induced uPAR overexpression. *A*, gelatin zymography (*a*) and RT-PCR (*b*) were performed to detect the expression and activity of both MMP-2 and MMP-9 as described in Materials and Methods. The influence of d-GM3 on both MMP-2 and MMP-9 in A375P and C8161 cells (*B*) and the expression of uPAR and d-GM3 on MMP-2 expression and activity in d-GM3–positive C8161 cells (*C*) were assessed by gelatin zymography.

marker (43). Our studies noted more than one band of GM3 in melanoma cells but not in normal melanocytes. We thus focused our investigation on the identity of these bands of GM3 in an effort to define unusual ganglioside variants for immunotargeting.

Among different variants of GM3, *N*-glycolyl GM3 has been found in cancers, including melanomas and gliomas (19, 20, 44), but not in normal human tissues. *In vitro* studies and clinical trials attempting to target *N*-glycolyl GM3, however, have had limited success. Although active vaccination and antibody administration targeting *N*-glycolyl GM3 shrunk melanomas and increased survival, neither therapy completely cleared melanoma or prevented its metastasis in patients or mice (45, 46). *N*-glycolyl GM3 differs from c-GM3 by substitution of a glycolyl group ($CH_2OHCONH$) for the acetyl (CH_3CONH). This structural similarity prevents specific antigenic distinction (34). In addition, we have found that *N*-glycolyl GM3 is predominantly expressed in poorly invasive primary melanoma cells but not in aggressive metastatic melanoma cells. d-GM3, in contrast, is a more important target than *N*-glycolyl GM3 because d-GM3 is found in highly metastatic melanomas and is also structurally and antigenically distinct from c-GM3 (the acetyl group in c-GM3 is cleaved to NH_2 in d-GM3). Therefore, targeting d-GM3 should spare normal melanocytes and immune cells that mediate tumor clearance.

Pharmacologic and genetic manipulation of the expression of specific gangliosides has been used to study the influence of gangliosides on cell biological behavior, including on tumor metastasis (for reviews, see refs. 12, 13, 23, 42, 47). PPPP, an inhibitor of glucosylceramide synthase that does not cause ceramide accumulation, can totally deplete d-GM3 *in vitro* (4, 6, 9–11). Pharmacologic addition of purified d-GM3 repletes cell surface content to 80% of the baseline level[5] and drives cell migration and invasion.

By producing and activating proteinases that degrade extracellular matrices or basement membranes, tumor cells metastasize. Among different proteinases, uPA is a widely acting serine protease. uPA degrades the extracellular matrix components and plays a pivotal role in cancer progression (for reviews, see refs. 24, 25). GM3 inhibits squamous carcinoma SCC12 cell proliferation through suppressing epidermal growth factor receptor (EGFR) signaling (3, 8). Paradoxically, when the SCC12 cells are grown in the presence of pro-uPA, increasing the concentration of GM3 leads to an increase in cell proliferation (by activation of uPAR and p70S6 kinase signaling) despite the continued suppression of EGFR signaling (4). This stimulation of cell growth requires the presence of uPA, which deacetylates c-GM3.[6] Many melanoma cells do not express EGFR but express a considerable amount of uPAR as well as high concentrations of uPA. Binding of uPA with its receptor, uPAR, not only elevates uPA/uPAR proteolysis activity but also triggers uPAR intracellular signaling (25, 27, 48). We have recently shown that d-GM3 colocalizes with uPAR at the cell membrane and promotes clustering of uPAR at the membrane in glycosphingolipid-depleted cells,[5] further suggesting the importance of the d-GM3/uPAR association on melanoma metastasis.

MMP-2 is a key mediator of melanoma invasion and metastasis (49). We have found that uPAR signaling activation stimulates extracellular signal-regulated kinase–independent phosphoinositide 3-kinase (PI3K) and PKC-ζ signaling to activate p70S6 kinase (4). In addition, blocking PI3K, PKC-ζ, or p70S6 kinase inhibits uPA-induced MMP-2 expression and activation (50). Our studies revealed that d-GM3 stimulates uPAR signaling to activate MMP-2, thereby promoting melanoma metastasis.

Taken together, these results suggest that d-GM3 is not just an antigenic marker but also a valid therapeutic target. Targeting both d-GM3 and uPAR signaling would lead to a more favorable clinical outcome than targeting uPAR or d-GM3 alone. In addition, elimination of d-GM3 could likely decrease the metastatic potential of melanoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

[5] J.W. Liu, P. Sun, Q. Yan, A. Hui, and X.Q. Wang, unpublished data.
[6] Q. Yan, P. Sun, J.W. Liu, A.S. Paller, and X.Q. Wang, unpublished data.

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