Deacetylated GM3 Promotes uPAR-Associated Membrane Molecular Complex to Activate p38 MAPK in Metastatic Melanoma

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Abstract

GM3, the simplest ganglioside, regulates cell proliferation, migration, and invasion by influencing cell signaling at the membrane level. Although the classic N-acetylated form of GM3 (NeuAcLacCer) is commonly expressed and has been well studied, deacetylated GM3 (NeuN²⁴LacCer, d-GM3) has been poorly investigated, despite its presence in metastatic tumors but not in noninvasive melanomas or benign nevi. We have recently found that d-GM3 stimulates cell migration and invasion by activating urokinase plasminogen activator receptor (uPAR) signaling to augment matrix metalloproteinase-2 (MMP-2) function. However, the mechanisms by which d-GM3/ uPAR increase MMP-2 expression and activation are not clear. By modifying the expression of d-GM3 genetically and biochemically, we found that decreasing d-GM3 expression inhibits, whereas overexpressing d-GM3 stimulates, p38 mitogen-activated protein kinase (MAPK) activity to influence MMP-2 expression and activation. p38 MAPK (p38) activation requires the formation of a membrane complex that contains uPAR, caveolin-1, and integrin α5β1 in membrane lipid rafts. In addition, knocking down or inhibiting focal adhesion kinase (FAK), phosphoinositide 3-kinase (PI3K), or Src kinase significantly reduces d-GM3–induced p38 phosphorylation and activation. Taken together, these results suggest that d-GM3 enhances the metastatic phenotype by activating p38 signaling through uPAR/integrin signaling with FAK, PI3K, and Src kinase as intermediates. Elucidation of the mechanisms by which d-GM3, a newly discovered, potential biomarker of metastatic melanomas, promotes cell metastasis will help us to understand the function of d-GM3 in metastatic melanomas and may lead to novel GM3-based cancer therapies. Mol Cancer Res; 11(6); 665–75. ©2013 AACR.

Introduction

Although recent therapeutic progress for metastatic melanomas has significantly improved, including the more recent availability of small-molecule inhibitors targeting BRAF mutation or both BRAF mutation and MEK, or anti-CTLA4 antibody, survival is minimally prolonged and mortality rate of metastatic melanomas remains high (1–3). It is of utmost importance to discover specific molecular biomarkers that can distinguish melanomas with metastatic propensity from more indolent ones, which will improve prognostication, allow for earlier and more efficacious treatments, and provide additional targets for novel therapy.

Gangliosides are sialic acid-containing membrane glycosphingolipids, which regulate tumor invasion, metastasis, and growth by modulating signal transduction at the plasma membrane (4–6). Alterations of ganglioside expression and distribution in malignant tumors correlate with their features of abnormal cell growth, associated inflammation, and oncogenic transformation (4, 6, 7). These observations, combined with their accessibility on the cell membrane surface, make gangliosides ideal markers for diagnosis and targets of cancer therapy. GM3, the simplest ganglioside and precursor for all other gangliosides, is 1 of the major gangliosides in melanomas (8). GM3 overexpression in melanoma correlates with an increased risk of metastasis (9, 10), and therapies that target global GM3 reduce disease burden and increase overall survival (11, 12). However, these treatment modalities are not able to completely clear the tumor or prevent metastasis. The likely reason for this incomplete efficacy is that the common form of N-acetylated GM3 (classic GM3 or c-GM3) is also widely expressed in normal cells, including immune cells. Thus, globally targeting GM3 produces undesirable toxicity and limits the ability of innate immunity to combat melanoma. Therefore, distinguishing a specific metastatic-related form of GM3 could improve therapies that target GM3.
We have recently discovered that the expression of d-GM3, a specific form of GM3 generated by removing the acetyl group of the sialic acid residue on c-GM3, correlates with enhanced metastasis in melanomas. We detected d-GM3 only in vertical growth phase and metastatic melanomas with none evident in benign nevi or radial growth-phase melanomas (13). Hanai and colleagues had found that d-GM3 expression enhances EGF receptor (EGF) activity in epidermoid carcinoma A431 cells and B16 mouse melanoma cells (7). Our prior studies discovered that d-GM3 enhances melanoma metastasis by elevating levels of urokinase plasminogen activator (uPA) to trigger the activation of its receptor, uPAR. Through uPAR signaling activation, d-GM3 promotes the expression and activation of matrix metalloproteinase-2 (MMP-2; ref. 13). However, the signaling pathway between d-GM3/uPAR activation and MMP-2 expression/activation is unclear.

Lacking both transmembrane and intracrytoplasmic domains (14), uPAR-modulated signal transduction is thought to require transmembrane adaptors such as integrins (15, 16). We have previously shown that uPAR associates with integrin α5β1 to activate intracellular signaling and promote cell proliferation (17). uPAR/integrin activation triggers various intracellular signaling pathways, including focal adhesion kinase (FAK), Src kinase, phosphoinositide 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK; refs. 15, 18). The p38 MAPK (p38) belongs to the MAPK family, which also includes extracellular signal-regulated kinase (ERK). Four identified isoforms of p38 (p38α, p38β, p38γ, and p38δ) share more than 60% homology and differ in their expression patterns, substrate specificities, and sensitivities to pharmacologic inhibitors. Among the 3 isoforms of p38 (p38α, p38β, and p38γ) that are expressed in melanoma, p38α is both well characterized and the most abundant and widely expressed isoform (19). p38 activation is commonly known to trigger stress pathways that block cell proliferation or induce cell apoptosis in a variety of cells (20, 21). In contrast, p38α signaling has also been found to maintain the invasiveness of breast cancer by promoting the uPA/uPAR pathway (22).

In this current study, we show that, in the presence of uPA, d-GM3 triggers uPAR clustering at the cell surface where uPAR interacts or associates with integrin α5β1. Through integrin α5β1, d-GM3/uPAR activates intracellular p38 signaling with FAK, Src kinase, and PI3K as intermediates. p38 activation then stimulates MMP-2 expression and activation. The association of activated uPAR with integrin α5β1 requires the presence of caveolin-1, and is disrupted by cholesterol depletion with MβCD, suggesting that uPAR associates or interacts with integrins in specific membrane microdomain-lipid rafts, which are specific plasma membrane domains enriched in cholesterol and sphingolipids.

**Materials and Methods**

**Cells**

Human cutaneous melanoma A375P and C8161 cells (courtesy of Drs. A. Hess and M. Hendrix, Northwestern University, Chicago, IL) and SKMEL-28 cells (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% FBS (Invitrogen).

**Modulating d-GM3 expression**

The content of d-GM3 was increased by either pharmacologic addition of purified d-GM3 or overexpression of GM3S by stable transfection of human GM3S cDNA (GenBank accession no. AB018356, a generous gift from Dr. Colombo, University of Milan, Italy; ref. 23) and was decreased by knocking down GM3S with 3 siRNAs of GM3S (Table 1) or by inhibiting the biosynthesis of GM3 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 (24, 25). The content of ganglioside was detected by ganglioside ELISA (24–27), routine thin-layer chromatography (TLC), and TLC immunostaining (24, 28, 29) using antibodies directed against total GM3 (DH2, Glycotech) or d-GM3 (DH5, generous gift from Dr. Hakomori, Pacific Northwest Diabetes Research Institute, Seattle, WA; ref. 7). BM (3,3′,5,5′-tetramethylbenzidine, TMB) blue POD (peroxidase) substrate (Roche Applied Science) was used to detect the binding in ganglioside ELISAs, and 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 × 10⁶) using 10 volume of chloroform/methanol (2:1; ref. 28). The aqueous phase was separated and desalted, and the bands were separated by TLC in chloroform/methanol/water in 0.02% CaCl₂, 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. Bands were visualized by an ECL kit on X-Omat film. Band density was quantified using the NIH ImageJ program (28).

**Immunofluorescence staining**

Cells plated onto glass coverslips were fixed with 4% paraformaldehyde, blocked with 2% goat serum, and incubated with anti-uPAR antibody (Santa Cruz Biotechnology), followed by incubation with fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Images were captured under Zeiss Axioscope 2 Plus fluorescence microscope with AxioVision LE imaging software after counter staining with 4′,6-diamidino-2-phenylindole (DAPI; ref. 25).

**Gene regulation**

uPAR cDNA (courtesy of Dr. L. Osowski, Mount Sinai School of Medicine, New York, NY) in V5/His-pcDNA3.1 vector (Invitrogen), and its vector control (V5/His-pcDNA3.1) were stably transfected into cells to overexpress uPAR. To rule out the off-target effect of uPAR siRNAs on p38 MAPK signaling (30), 4 independent uPAR siRNAs were used (Table 1). To elucidate the signaling pathway from d-GM3/uPAR/integrin α5β1 to p38 activation, hemagglutinin-tagged wild FAK cDNA or FAK397 site mutation cDNA (Y397K) in a pcDNA vector (both a
generous gift from Dr. D. Schlaepfer, Scripps Research Institute, La Jolla, CA) were stably transfected into cells. FAK expression was also reduced by treatment of cells with 2 independent siRNAs directed against FAK (Table 1). PI3K and Src kinase were inhibited by their specific inhibitors, LY294002, wortarin in, or PP2. Caveolin-1 was knocked down by treating cells with different sequences of siRNA against caveolin-1 (Table 1) and GM3S was knocked down by either independent siRNA sequences transiently (Table 1) or stably introducing GM3S short hairpin RNA (shRNA)-lentivirus particles (Santa Cruz Biotechnology; 3 different sequences of shRNA pool). Integrin subunits α5, α3, and β1 were also knocked down using their specific siRNAs (Table 1). All siRNAs (10 nmol/L) were transfected into cells using DharmaFECT transfection reagents according to the manufacturer’s instruction.

### Table 1. siRNAs to specific human genes used in this report

<table>
<thead>
<tr>
<th>Gene</th>
<th>#1 Sense</th>
<th>#1 Antisense</th>
<th>#2 Sense</th>
<th>#2 Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM3S (ST3GAL5)</td>
<td>CCAGUUGCAUGGUUGAUAtt</td>
<td>UAUCAACAACUAUGACUGGtt</td>
<td>GAAACCAAGUUCUCUAAAtt</td>
<td>UUAAGGAGAACUUGGCUUAtt</td>
</tr>
<tr>
<td>Caveolin-1</td>
<td>AGACGAGCUGAGGAAGCA</td>
<td>GCUGAAGGAAAGGCGUCCAA</td>
<td>CAUAGCAGAGGUGAAGAA</td>
<td>GUAGUCAAGCUCUAGUAGGtt</td>
</tr>
<tr>
<td>uPAR (PLAUR)</td>
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<td>#1 siRNA</td>
<td>#2 siRNA</td>
<td>#2 siRNA</td>
</tr>
<tr>
<td>Integrin α5 (ITGA5)</td>
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<td>1 Sense</td>
<td>2 Sense</td>
<td>2 Sense</td>
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<tr>
<td>FAK</td>
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<td>2 Sense</td>
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</tr>
<tr>
<td>EGFR</td>
<td>1 Sense</td>
<td>1 Sense</td>
<td>2 Sense</td>
<td>2 Sense</td>
</tr>
<tr>
<td>Scrambled si</td>
<td>UUC UCR GAA CGU GUC AGC UTT</td>
<td>UACGUGAGGCAAGCTTACUCAUAUUGGtt</td>
<td>UAAUUGCAAGCCUCUGCACUGCUU</td>
<td>UGAUGGCUAUAUUGGCUU</td>
</tr>
</tbody>
</table>

**NOTE:** All sequences are provided in 5’ → 3’ orientation.

### Cell migration and invasion assay

Cell migration assays were conducted using both scratch analysis and chemotaxis migration assay (Transwell cell culture system, Becton Dickinson Biosciences; refs. 27, 31). The following cells were prepared for migration assays: parental nontreated control cells, vector (v5/His pcDNA3.1) stably transfected control cells, GM3S-over-expressing cells, GM3S shRNA-lentivirus particle–transduced cells or scrambled shRNA-transduced control cells, cells pretreated with DH5 antibody (to block d-GM3) or immunoglobulin G (IgG) antibody control, and cells treated with small-molecule inhibitors to inhibit p38 MAPK (SB202190, SB203580) or ERK (PD98059).

### Invasion assay

Cell invasion assays were conducted (27) using Becton Dickinson BioCoar Matrigel Invasion Chambers (Becton...
Dickinson Biosciences) according to the manufacturer’s instruction. Cells prepared as indicated above were plated onto the top surface of the filter and allowed to invade to the bottom level for 48 hours. Cells that invaded into the bottom level were collected and counted.

**Reverse transcription PCR**

Total RNA was isolated from cells using an RNeasy mini-Kit (Qiagen) according to the manufacturer’s instructions. cDNA were synthesized using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) with 2 μg of total RNA according to the manufacturer’s protocol. The expression of GM3S, caveolin-1, integrin α3, α5, and β1 in cells was examined by PCR with primers with 200 ng cDNA per reaction as described previously (27, 32, 33). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was detected as an internal control (33). PCR was carried out under the following conditions: 1 cycle at 42°C for 30 minutes; 1 cycle at 95°C for 5 minutes; 25 cycles at 95°C for 1 minute, and 72°C for 3 minutes; then 1 cycle at 72°C for 5 minutes (GeneAmp PCR system 2700; Applied Biosystems). The PCR products were detected on 1.5 or 2% agarose gels.

**Immunoblotting**

Total protein from the whole-cell lysate was prepared by treating cells with boiling lysis buffer (1% SDS, 1 mmol/L Na3VO4, 10 mmol/L Tris-HCl, pH 7.4) for 10 minutes. Protein concentration was measured using Coomassie Blue dye 3-phosphate dehydrogenase (GAPDH) expression was detected as an internal control (33). PCR was carried out under the following conditions: 1 cycle at 42°C for 30 minutes; 1 cycle at 95°C for 5 minutes; 25 cycles at 95°C for 1 minute, and 72°C for 3 minutes; then 1 cycle at 72°C for 5 minutes (GeneAmp PCR system 2700; Applied Biosystems). The PCR products were detected on 1.5 or 2% agarose gels.

**Immunoprecipitation**

Cells growing in 6-well plates were allowed to reach 80% confluence before harvesting as described previously (35). In brief, cells were lysed in cold radioimmunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology), and insoluble cell debris was removed by centrifugation (5,000 rpm for 5 minutes). The crude cell lysate was precleared by incubation with Agarose A-sepharose before incubating with polyclonal antibody of uPAR. After incubation with antibody directed against uPAR, magnetic Agarose A beads were added and then incubated for 3 hours before pulling down the uPAR-bound magnetic Agarose A beads. After a brief wash of the beads with RIPA buffer, laminin sample buffer (3 ×) was added and samples were heated for 5 minutes at 80°C to 85°C to dissociate uPAR from the beads.

**Zymography**

MMP-2 activation was assayed using conditioned medium (50 μg/lane) prepared as indicated earlier (13). To identify the bands ascribed to MMPs, duplicate gels were developed in the presence of 10 mmol/L EDTA (negative control for MMP activity; ref. 27).

**Results**

**Cell migration and invasion are impaired by decreasing the expression of d-GM3**

Two d-GM3–positive human metastatic melanoma cell lines (C8161 and SKMEL-28) and 1 d-GM3–null human primary melanoma cell line (A375P) were selected to study the effects of d-GM3 expression on melanoma metastases. C8161 cells were used because they express a high level of d-GM3 (95% of total GM3), proliferate rapidly, and are highly invasive; A375P cells do not express d-GM3, proliferate slowly, and are poorly invasive. Although knocking down GM3 synthase (GM3S) dramatically decreased d-GM3 level in C8161 cells, overexpression of GM3S could not further increase d-GM3 in C8161 cells, suggesting that d-GM3 has reached its maximal expression level in C8161 cells. SKMEL-28 cells, however, express medium levels of d-GM3 (65% of total GM3) and, therefore, d-GM3 level in SKMEL-28 cells can be easily increased or decreased by regulating GM3S expression.

Among the 5 siRNAs tested, we found that 3 sequences of siRNAs (Table 1) knocked down the expression of GM3S, an enzyme required for the synthesis of GM3, by 80% to 95% as examined by regular reverse transcription PCR (RT-PCR; Fig. 1A) and quantitative RT-PCR (qRT-PCR; data not shown). The expression of total GM3 was significantly reduced by 70% to 90%, with reduction of d-GM3 accounting for 80% to 88% as determined by TLC immunostaining and ganglioside ELISA (Fig. 1A). Knocking down GM3S decreased C8161 cell migration and invasion (Fig. 1B).

Total GM3 and d-GM3 are maximally expressed in C8161 cells; as shown in Fig. 1, GM3S overexpression was neither enhancing GM3 expression nor influencing C8161 cell migration and invasion. However, GM3S overexpression increased the expression of both total GM3 and d-GM3 by 3.3- to 4.2-fold in SKMEL-28 cells, in which 65% of the total GM3 is d-GM3. GM3S overexpression also promoted SKMEL-28 cell migration and invasion, and knocking down GM3S by 75% to 95% decreased the expression of d-GM3 in SKMEL-28 cells and slowed SKMEL-28 cell migration and invasion. A375P cells do not express d-GM3 (13). Manipulating GM3S expression in A375P cells neither induced d-GM3 expression nor changed cell migration and invasion (data not shown). Thus, A375P cells were used as a negative control.

**d-GM3 increases the expression and activation of MMP-2 by promoting the activation of p38**

To consider whether MAPK signaling participates in d-GM3–promoted MMP-2 expression and activation,
d-GM3–positive and d-GM3–negative melanoma cells were treated with uPA and small-molecule inhibitors to ERK (PD98059) or p38 (SB202190 and SB203580). Inhibition of p38, but not ERK, dramatically reduced the migration and invasion of d-GM3–positive C8161 cells (Fig. 2A, bars 1–6). In contrast, inhibition of ERK, but not p38, decreased d-GM3–null A375P cell migration and invasion (Fig. 2A, bars 7–12). Furthermore, increasing d-GM3 by overexpression of GM3S in SKMEL-28 cells significantly increased cell migration and invasion (Fig. 2A, bars 13–15). The phosphorylation of p38 was reduced 9- to 13-fold when uPAR was knocked down, and increased 2.9-fold when uPAR was overexpressed (Fig. 3A, third row and bar graph underneath). Manipulating uPAR expression, however, did not alter the expression of total p38 (Fig. 3A, fourth row).

d-GM3 enhances metastasis by activating p38 MAPK

We have previously shown that d-GM3 increases melanoma metastasis by activating uPAR signaling (13). To determine whether d-GM3 activates p38 through uPAR signaling, uPAR in d-GM3–positive C8161 cells was either knocked down or overexpressed, as previously described (13). uPAR siRNA knocked down uPAR by 9- to 18-fold (Fig. 3A, top row, lanes 3–6 and bar graph underneath). The phosphorylation of p38α was reduced 9- to 13-fold when uPAR was knocked down, and increased 2.9-fold when uPAR was overexpressed (Fig. 3A, third row and bar graph underneath). Manipulating uPAR expression, however, did not alter the expression of total p38α (Fig. 3A, fourth row). In contrast, modulating uPAR expression did not influence p38α phosphorylation and expression in d-GM3–null A375P cells (Fig. 3A). Furthermore, knocking down GM3S with its siRNA (sequence #1 and #3; Table 1) to reduce d-GM3 expression predominantly alters the level of deacetylated GM3 (d-GM3) in metastatic melanoma cells. A, the expression of GM3S in d-GM3–positive melanoma cells (C8161 and SKMEL-28) was knocked down by 3 siRNAs of GM3S (sequence #1, #2, and #3; Table 1) or increased genetically. The expression of GM3S was examined by RT-PCR, and GM3 variants were detected by TLC immunostaining and ganglioside ELISA. **, P < 0.01; ****, P < 0.001; B, alterations in d-GM3 content influence melanoma cell migration and invasion. All experiments were conducted 3 different times.
GM3 in C8161 cells prevented p38α activity (data not shown) and phosphorylation in cells with overexpressed uPAR (Fig. 3B). These findings suggest that activation of p38 signaling by uPAR requires the presence of d-GM3. Further studies revealed that pharmacologic addition of purified d-GM3, but not c-GM3, to cells depleted of all ganglioside by P4 treatment (24) promoted uPAR to cluster on the cell surface (Fig. 3C). This finding suggests that d-GM3 may stimulate uPAR to cluster in specific membrane microdomains. P4 treatment and pharmacologic addition of either d-GM3 or c-GM3 does not alter the expression of uPAR (Fig. 3D).

To further explore the role of d-GM3 in uPAR-activated p38 signaling, we investigated the impact of d-GM3 on membrane-based molecular association. In the presence of uPA, uPAR coimmunoprecipitated with caveolin-1 and...
integrin subunits (α5, α3, and β1) in d-GM3–positive C8161 cells, but not in d-GM3–deficient A375P cells (Fig. 4A). uPA treatment strengthened the association of uPAR with integrin subunits α5, β1, and caveolin-1, and enabled integrin α5 to join the membrane molecular complex (Fig. 4A). Decreasing d-GM3 by GM3S expression knockdown using shRNAs (Santa Cruz) weakened the association of uPAR with integrin subunits α5 and α3 and completely prevented the association of uPAR with β1 integrin subunit and caveolin-1 (Fig. 4A). The addition of purified d-GM3, not c-GM3, rescued the association of these molecules (Fig. 4A).

To examine the impact of integrins and caveolin-1 on the uPAR-associated membrane molecular complex, integrin subunits α5, α3, β1, and caveolin-1 in C8161 cells were knocked down by their specific siRNAs (Table 1). As determined by semi-quantitative RT-PCR, the knocking down efficacy for integrin subunit α5, α3, and β1 was more than >90%, 82%, and 75%, respectively, and for caveolin-1 it was more than 75% (Fig. 4B).

Coimmunoprecipitation studies revealed that knocking down caveolin-1 with the different sequences of siRNA (Table 1) all prevented or reduced the association of uPAR with integrin subunits (Fig. 4C, lanes 10–11), whereas knocking down integrin subunits did not influence the association of uPAR with caveolin-1 (Fig. 4C, lanes 4–9). Disrupting caveolar domains by treating cells with methyl-β-cyclodextrin (MβCD; ref. 25) prevented uPAR coimmunoprecipitation with both integrins and caveolin-1 (Fig. 4C, lane 13). These findings provide evidence that uPAR associates with integrins and caveolin-1 in lipid-enriched membrane microdomain rafts, and that the presence of caveolin-1 in the rafts is required for uPAR to associate with integrins. Knocking down integrin subunit α5 significantly reduced the association of integrin subunit β1 with uPAR (Fig. 4C, lanes 4–5). Knocking down integrin subunit α3 only slightly...
decreased the association of integrin subunit β1 with uPAR (Fig. 4C, lanes 6–7). Knocking down integrin subunit β1 also significantly reduced the association of α5 with uPAR (lanes 8–9, second row), but did not influence the association of α3 with uPAR (third row), suggesting that uPAR predominantly associates with integrin α5β1 complexes.

We then determined the impact of these alterations in the uPAR–integrin lipid rafts on p38 signaling. By kinase ELISA, we found that knocking down caveolin-1, integrin subunits α5, β1, and to a lesser extent α3, significantly decreased p38 activity (Fig. 4D; \( P < 0.05 \) for α3; \( P < 0.01 \) for α5; and \( P < 0.001 \) for β1 and caveolin-1). Treatment with MβCD to disrupt lipid rafts structure also dramatically reduced p38 activity (Fig. 4D; \( P < 0.001 \)), suggesting that lipid rafts, enriched in cholesterol and sphingolipids membrane domains, are important for uPAR-induced p38 signaling.

d-GM3/uPAR-activated p38 signaling uses FAK, Src, and PI3K as intermediates

To further elucidate the signaling pathways that lead to the activation of p38 by d-GM3/uPAR, d-GM3-positive C8161 metastatic melanoma cells were treated with small-molecule inhibitors for Src (PP2), PI3K (LY294002, wortmannin) and EGFR (AG1478), FAK siRNAs (Table 1), or stably transfected with FAK397 site mutation cDNA (Y397K) and their respective controls. uPA-induced p38 activity was decreased in Y397K cDNA-transfected cells and FAK siRNA-treated cells, compared with cells transfected with WT FAK cDNA or treated with scrambled siRNA (Fig. 5A, both \( P < 0.01 \)). In addition, inhibition of Src and PI3K independently reduced p38 activity when compared with vehicle-treated control cells (Fig. 5A, all \( P < 0.05 \) or \( < 0.01 \)). Inhibition of EGFR kinase (\( P = 0.23 \)) or EGFR knockdown with 2 independent siRNAs (data not shown; Table 1) did not reduce uPA-induced p38 activity (Fig. 5A). These data suggest that uPA activates p38 signaling in d-GM3–positive cells through FAK, PI3K, and Src kinase, but not EGFR signaling.

To further confirm the roles of d-GM3, caveolin-1, and uPAR in activating the expression and phosphorylation of FAK, Src, and PI3K, we suppressed the expression of d-GM3, caveolin-1, and uPAR in C8161 cells with their respective siRNAs (Table 1). Decreasing d-GM3 or...
caveolin-1 had no effect on the expression of FAK, Src, or PI3K, but did profoundly inhibit their phosphorylation (Fig. 5B, lanes 3–6). Knockdown of uPAR expression also reduced activation of FAK, Src, and PI3K, whereas overexpression of uPAR enhanced their phosphorylation (Fig. 5C). Expression of FAK, Src, or PI3K was unaffected by uPAR manipulation (Fig. 5C). Interestingly, modulation of uPAR expression in d-GM3–null A375P cells did not influence the expression or the phosphorylation of FAK, Src, or PI3K (Fig. 5C), just as it did not lead to the formation of a membrane-based uPAR–caveolin-1–integrin complex in these d-GM3–deficient cells. These findings suggest that FAK, Src, and PI3K are intermediates for uPA-induced activation of p38 in the d-GM3/uPAR signaling pathway.

Discussion

In prior studies, we have found that ganglioside GM3 deacetylation increases the expression and activation of uPA and MMP-2 to stimulate melanoma metastasis (13). In this study, we further delineated the mechanism by which deacetylated GM3 activates MMP-2 and increases melanoma cell invasiveness. Our results show that d-GM3 activates uPA receptor (uPAR) signaling by stimulating uPAR to cluster with other molecules on the cell surface in specific micromembrane domains known as "lipid rafts." In the presence of caveolin-1, d-GM3 promotes activated uPAR to associate with integrin α5β1. Through integrin α5β1, uPAR activates intracellular signaling of p38 MAPK (p38) via intermediate molecules, including FAK, Src kinase, and PI3K, but not EGFR. The activation of p38 signaling further stimulates MMP-2 expression and activation.

GM3 is synthesized by GM3 synthase (GM3S, CMP-NeuAc:lactosylceramide α 2,3-sialyltransferase: EC 2.4.99.9). GM3S catalyzes the transfer of a sialic acid residue from CMP-sialic acid to a nonreduced terminal galactose residue of lactosylceramide through an α-2,3 glycosyl bond to increase GM3 levels. GM3 is the simplest ganglioside and the precursor for other gangliosides, including tumor-related ganglioside markers (GM2, GD3, and GD2) in melanoma (8). Although gangliosides are expressed in the membranes of most normal cells, their content is often markedly increased in tumors, including melanomas. Although classic GM3 (c-GM3) is the predominant ganglioside in most normal cells, including melanocytes and immune cells, a deacetylated form of GM3 (d-GM3), which is immunologically distinct from c-GM3, is not found in normal cells. Furthermore, GM3 deacetylation correlates with enhanced metastatic potential in melanomas (13), with d-GM3 predominantly expressed in metastatic melanoma tissues, but not in normal melanocytes, benign nevi, or the majority of poorly invasive, primary melanomas (13). Thus, d-GM3, rather than c-GM3, is a promising target for immunotherapy of melanomas, especially metastatic forms, while avoiding the risk of toxicity to normal cells including immune cells that participate in melanoma clearance.

Understanding how d-GM3 regulates cell signaling to increase melanoma metastasis will allow us to target d-GM3 more efficiently. Tumor cells metastasize by producing and activating proteinases to degrade extracellular matrices or basement membranes. We have found that d-GM3 expression promotes melanoma migration and invasion via stimulation of uPA and MMP-2 expression and activation (13). However, the signaling pathways underlying MMP-2 expression and activation, as induced by d-GM3, have not been elucidated. Activation of p38 signaling mediates transcriptional activation of MMP-2 and MMP-9 (36, 37), but the role of p38 in tumor metastasis is controversial (for reviews, refs. 19, 21, 38). Although the activation of p38
pathway is likely involved in tumor suppression by negatively regulating cell survival and proliferation (21, 38). It has been suggested that p38 can positively regulate cell adhesion, invasion, and metastasis in tumors, including melanomas (32, 36, 37, 39–41).

uPAR, a glycosylphosphatidylinositol (GPI)-anchored glycoprotein located in the outer leaflet of the plasma membrane, has been shown to initiate signal transduction events and affects cell proliferation, adhesion, migration, and invasion (for review, ref. 42). p38α activation is known to increase uPA/uPAR expression in cancer cells (22). Whether uPA/uPAR activation influences p38 signaling is obscure. Without transmembrane and intracellular domains (14), uPAR cannot signal across cellular membrane without involving other transmembrane signaling molecules, such as integrins and EGFR (16, 17, 43, 44), with data on EGFR protein expression in melanomas conflicting (45, 46). On the other hand, various subunits of integrin are abundantly expressed in melanomas and seem to play critical roles for melanoma metastasis. For instance, uPAR is known to associate with integrin β1 to promote tumor metastasis (16, 31, 33). In the current study, we have shown that d-GM3 promotes the association of uPAR and integrin α5β1, and the downstream activation of p38α is a key in melanoma invasion.

Caveolin-1 is a 22 to 24 kDa protein, which was initially identified as a structural component of caveolae, plasma membrane invaginations where molecules associate to activate intracellular signaling pathways (47). Caveolin-1 is capable of regulating the communication of caveolin-associating signaling molecules, including uPAR, integrins, and Src kinase (48). Although the role of caveolin-1 expression on tumor progression is controversial, whether caveolin-1 is an oncogene or a tumor suppressor depends on the tumor stage and the cellular microenvironment (49). Studies have recently found that caveolin-1 expression promotes human melanoma progression and metastasis by increasing the expression of MMPs, including MMP-2 (50). Our studies suggest that the caveolin-1–enriched caveolar domain functions as a signaling platform to facilitate the association of GPI-linked uPAR with transmembrane protein integrin α5β1, leading to activation of intracellular p38 signaling through Src, FAK, and P38K.

Another molecule critical in facilitating proper cell signaling is cholesterol, a major component of lipid rafts, which stabilizes the structure of the lipid rafts. Using cholesterol-depleting agent MJβCD and siRNA, we found that disrupting raft structure attenuates p38 signaling through the d-GM3–stimulated uPAR/integrin α5β1 pathway. These results provide strong evidence that d-GM3 triggers uPAR signaling activation and promotes melanoma metastasis in lipid rafts.

In conclusion, deacetylated-GM3, found predominantly in metastatic melanomas, promotes tumor metastasis via the uPAR/integrin and p38 MAPK pathway. Furthermore, apart from stimulating uPAR translocation at the plasma membrane and uPAR clustering on the cell surface, we have also found that d-GM3 promotes uPAR to associate with integrin α5β1 through caveolin-1 in lipid rafts. Our studies suggest that d-GM3 is not just an antigenic marker, but also has great potential to be a therapeutic target. Furthermore, concurrent targeting of d-GM3 and uPAR optimizes the clinical outcome, and decreases the metastatic potential of melanoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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