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I. 知的財産権の出願・登録状況

1) 特許取得

なし

2) 実用新案登録

なし

3) その他

なし

別紙３
厚生労働科学研究費補助金（創薬基盤推進研究事業：ヒトゲノムテーラーメード研究）

分担研究報告書

発現糖鎖解析、糖鎖修飾分子合成および抗糖鎖抗体作製

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研究要旨：分化・がん化により細胞に発現する糖鎖は多様に変化する。その発現糖鎖の変化を明らかにするためには糖蛋白質から効率よく糖鎖のみを切り出して分離し、微量で高感度に検出して構造解析をする手法を確立する必要がある。糖鎖を糖蛋白質より切り出す手法としてヒドラジン分解法を採用し、当研究室でグラファイトカーボン固相を利用して糖鎖遊離反応で使用した余剰ヒドラジンを安全に除去すると共に糖鎖を効率よく回収する方法を確立した。またゲル電気泳動をして単一バンドとなったゲル中の糖蛋白質から同様にヒドラジン分解し、糖鎖を回収する手法を確立した。糖鎖を高精度に分離・構造解析するため、遊離した糖鎖を2-アミノピリジンで修飾し、キャピラリー高速液体クロマトグラフィー（Cap LC）と構造解析が高感度に行うことが可能なマススペクトロメトリー（MS）を組み合わせたLC/MSで同時に解析する手法を確立した。今後これらの手法を利用して、患者検体や未分化細胞のように非常に微量な試料を用いた発現糖鎖の解析に着手している。

A．研究目的

糖鎖は核酸・蛋白質に続く第３の生命鎖といわれ、細胞に発現する糖鎖は、細胞の成熟やがん化により発現パターンが大きく変化することが知られている。これまでこの糖鎖の解析は薄層クロマトグラフィー（TLC）やレクチンなどを用いて行われてきたが、感度や特異性が充分ではなかったため、詳細な解析は比較的大量に存在するものに限られていた。一方マススペクトロメトリーは微量な生体分子を網羅的に高感度で検出できる手段であり、近年蛋白質を網羅的に解析するプロテオミクスに大いに利用されている。また糖鎖プライマー法は細胞をいわば糖鎖工場と見立てて培養液中に糖鎖を大量に生産させる技術である。

本研究ではこのマススペクトロメトリーと糖鎖プライマー法を網羅的発現糖鎖解析（グライコミクス）に利用して、ヒト未分化細胞（白血病細胞やヒト間葉系幹細胞）に発現している糖鎖を網羅的に解析し、これまで微量しか発現していなかったため同定されていなかった糖鎖を同定し、細胞の糖鎖発現パネルを作製すると共に、その発現糖鎖の中から診断に有用な糖鎖を検索し、将来的に診断・治療応用することを目指す。昨年度はLC/MSを用いて糖脂質糖鎖を分離・同定・構造解析する系を構築した。本年度は糖蛋白質糖鎖を同定・構造解析する実験系の構築を行った。

糖蛋白質糖鎖は、一種類の蛋白質であっても多様な糖鎖構造が存在する。このような糖鎖構造を明らかにするためには、糖蛋白質から糖鎖のみを切り離し、多様な糖鎖をクロマトグラフィーで分離することでその糖鎖構造を推定されてきた。糖鎖を切り出す方法としてヒドラジン分解という方法があるが、ヒドラジンは非常に扱いづらいことに加え、試料から除去するために特殊な装置を用意しなければならないなど非常に困難であった。池中一裕教授（生理研）らはグラファイトカーボン固相を用い、非常に簡便で安全に余剰ヒドラジンを除去すると共に遊離糖鎖を効率よく回収する方法を開発した。(Glycobiology 17, 261-276, 2007) またこの方法は蛋白質ゲル電気泳動で単一バンドとなった糖蛋白質に対しても適用可能で、たった１種類の蛋白質糖鎖の持つ糖鎖の解析も可能である。本研究ではこの手法を採用し、白血病等の未分化細胞の糖蛋白質N結合型糖鎖発現糖鎖パネルの作製を行う技術を確立する。

B．研究方法

1．ヒドラジン分解法による糖蛋白質からの糖鎖の遊離とグラファイトカーボンカートリッジによる糖鎖精製

糖蛋白質10 μgまたは1×10^7細胞のペレットを調製し、クロロフォルム：メタノール=2 ： 1に続き、クロロフォルム：イソプロパノール：水=7 ： 11 ： 2で総脂質を抽出した。ここで抽出した総脂質はODSの固相抽出で部分精製し、LC/MSによる発現糖脂質解析に使用した。糖蛋白質の含まれる脂質抽出残渣に対してヒドラジン分解法による糖蛋白質糖鎖検体とした。即ち、充分乾燥した残渣に対して無水ヒドラジン200 μlを加え、100°C, 10時間反応させ、糖蛋白質N結合型糖鎖を遊離させた。反応終了後充分量の酢酸アンモニウム水溶液を添加し、グラファイトカーボンカラムに通して遊離糖鎖を吸着させた。酢酸アンモニウムで充分カラムを洗浄し、無水酢酸を添加したアセトニトリル/水混合溶液で糖鎖を溶出すると共にヒドラジン分解により遊離した糖鎖中の再アセチル化を行った。

ゲル電気泳動を行った糖蛋白質については、ゲルをクマシー染色してバンドを切り出し、凍結乾燥をして水分を充分除去した後、同様にヒドラジンを添加してヒドラジン分解を行った。遊離反応終了後、過剰量の酢酸アンモニウム水溶液を添加して反応終了すると共に超音波照射してゲル中の遊離糖鎖を充分抽出し、同様にグラファイトカーボンカラムで遊離糖鎖を精製した。

2．遊離糖鎖のPA化と固相抽出による精製

親水性の高い遊離糖鎖はLC/MSにおいて分離後のイオン化が非常に弱いため、検出・構造解析が困難である。また糖は分子自身に特異的な吸収を持たないため、一般に蛍光団による分子修飾を行い、クロマトグラフィーによって分離しながら蛍光をモニターして分離・検出を行うのが一般的である。このような蛍光団による糖鎖の修飾で使用される蛍光団はいくつか存在するが、本研究では2-aminopyridine (2-AP), 2-aminobenzamide (2-AB), 2-aminobenzoic Acid (2-AA) による糖鎖修飾を行い、ESIによるイオン化効率の検討を行った。即ち遊離糖鎖に標識分子の酢酸溶液を添加して90°C, 60分反応させ、更にジメチルアミノボランを添加して標識分子と糖鎖間に安定な結合を形成させる。反応終了後、セルロースカラムを用いて糖鎖誘導体を吸着させ、余剰標識試薬を除去してESI/MSによる分析サンプルとした。

3．LC/MSによる糖鎖の分離と構造解析

LC/MSにおけるクロマトグラフィーの分離モードは、(i) イオン交換、(ii) HILIC、(iii) 逆相による分離を試みた。

(i) イオン交換クロマトグラフィー

イオン交換クロマトグラフィーでは、酸性糖鎖に含まれるシアル酸の数に従って分離するために行った。カラムにはMonoQ HR5/5 カラム(GE Health Science)を使用し、X%アンモニア水 (pH9.0) と0.5M 酢酸アンモニウム (pH9.0)の混合比率を変化させてイオン強度を増すことにより溶出した。

(ii) HILIC [Hydrophilic Interaction Chromatography]

カラムには昭和電工社製ShodexNH2P50を使用し、（アセトニトリル：水）の混合溶媒系で水の混合比をリニアグラディエントで増加させ、試料を分離・溶出した。

C．研究結果

1．遊離糖鎖蛍光分子修飾のESIイオン化効率に及ぼす影響

ヒドラジン分解法によって得られた遊離糖鎖をPA化する前に、市販のoligo Glcを蛍光分子で標識したものをそれぞれ調製し、ESI/MSで分析してイオン化効率を検討した。結果、PA化したものが最もイオン化効率が高く、高いシグナルが得られた。2AB修飾のものはPA化に比べ半分程度、2AAでは10%程度のシグナルしか得ることができなかった。これらの結果から以後、糖鎖の修飾はPA化を採用した。

2．LC/MSによる糖蛋白質糖鎖の分離と構造解析条件の確立

細胞から抽出した糖鎖の解析を解析する前に、PA化された標準糖鎖、精製糖蛋白質（ヒトIgG、フェチュイン等）から調製したPA化糖鎖を利用し、分離条件検討を行った。

イオン交換クロマトグラフィーでは、素通り画分からシアル酸のない糖鎖を回収することが可能であった。移動相中の酢酸アンモニウム濃度を上げるに従って糖鎖に含まれるシアル酸の数、すなわちモノシアロ、ジシアロ、トリシアロの順で分離・分画することが可能であった。素通り画分についてはそのまま、各シアロ糖鎖画分についてはシアリダーゼでシアル酸を切り離した糖鎖についてHILICモードによる糖鎖分離を行った。

HILICモードによる糖鎖分離では、まず標

準PA化糖鎖（M2A, M3B, M4B, M5A, M6B, M7A, M8A, M9A)を用いて分離条件検討した。結果我々の条件ではおよそ１５分間隔で糖鎖の数が増えるに従い後に溶出され、充分な分離能を得ることができた。この分離条件でヒトIgGから得られた糖鎖を解析したところ、アシアロ画分で１６種類の糖鎖ピークが確認された。現在MS/MSスペクトルから糖鎖構造を明らかにすることに着手している。

D．考察

これまで糖脂質を含む糖鎖の分析にはTLCやレクチンなどをプローブとした分析法が多用されてきたが、感度に大きな問題点があった。中でも糖脂質分析に用いられるTLCでは、細胞から有機溶媒で抽出した総脂質を直接載せてして分離し、糖のみを検出する試薬を噴霧して検出する方法が採られてきたが、この方法ではリン脂質のTLC上での挙動が糖脂質と非常に近いため、しばしば結果に影響を与えることがあることに加え、感度的にも問題があった。昨年度はLC/MSを使用することにより糖脂質糖鎖を網羅的に解析する方法を確立した。

一方糖蛋白質糖鎖の分析では従来、糖蛋白質から一旦糖鎖のみを遊離させ、クロマトグラフィーで分離して分析する方法で行われてきた。糖鎖を糖蛋白質から遊離させる方法としては、化学的方法、酵素学的方法の２種類があり、それぞれ一長一短がある。酵素学的方法では、非常に簡便で緩和な条件で行うことができるが、酵素の基質特異性に依存して遊離できない糖鎖があることに加え、糖鎖の回収率が低いという欠点がある。一方化学的方法ではヒドラジン分解法という非常に優れた方法があるが、毒性・爆発性のある無水ヒドラジンを使用しなければならないことに加え、反応終了後にそれを除くのに特殊な装置を使わなければならないなどの問題点があった。我々が今回導入した方法は、無水ヒドラジンにより糖鎖を遊離させた後、爆発の危険無く安全にカーボングラファイト固相を使用してヒドラジンを除くことができることが優れている。またこの方法は溶液状態の糖蛋白質ばかりでなく、SDSゲル電気泳動で分離した蛋白質のバンドについても適用可能である点も優れている。

遊離糖鎖のクロマトグラフィーによる解析については従来、2AP, 2AB, 2AAによって蛍光標識を導入し、HPLCで分離しつつ蛍光検出器で溶出パターンを観察する方法で糖鎖構造の決定が行われてきた。この方法はHPLCからの溶出時間を基準に行われ、２種類のカラム（逆相およびアミド若しくはアミノカラム）を用いてそれぞれ２次元に展開し、２次元糖鎖マップを描いて構造決定がなされてきた。この方法では２次元糖鎖マップから糖鎖構造を非常に効率よく推定することができるが、カラムからの溶出時間に基づいて行うため、装置やカラムが異なったものでは全く同じ結果を容易に得ることは困難であり、ある程度標準糖鎖を用意しなくては完全なる構造決定は困難であった。我々のLC/MSの分析系では、標準糖鎖を用意しなくてもMS/MSのスペクトルを得ることで糖鎖の配列や分枝などの構造を得ることが可能であり、２次元糖鎖マップにでていない未知の糖鎖についても対応が可能であると考えられる。

MSを用いた糖鎖の検出と構造解析ではクロマトグラフィーからの溶出物をいかに効率よくイオン化するかが高感度に検出するのに重要な要因となる。遊離糖鎖の2AP, 2AB, 2AAによる蛍光標識について、ESI/MSによるシグナル強度を比較したところ、2APによる標識（PA化)が最も強いシグナルを得ることができた。本邦ではPA化糖鎖のHPLC２次元マップが充実しており、HPLC溶出の順序などの情報が得やすい。我々は検出系にIT/MSを使用したことで、MS/MSのスペクトルから糖鎖配列構造を推定することができる点に優れている。蛍光検出器を使用したPA化糖鎖は十分に吟味された実験系ではおよそ10fmol程度まで検出可能であるといわれている。我々の質量分析装置を使用した検出系では、感度的にはそれ以上の潜在能力を持っていると言われるが、本年度確立した条件では同程度の検出感度にとどまっている。一般にnano ESI MSでは単純ペプチドなどの分子の検出感度はアトモルレベルでも可能だといわれているが、親水性が高い糖鎖はESIでのイオン化効率が悪いため、MSの強力な利点である高感度分析を行うためには更なる条件検討が必要である。

E．結論

細胞の成熟やがん化により発現パターンが大きく変化する糖鎖を持つ分子としては細胞表面に存在する糖脂質と糖蛋白質が知られている。本年度の検討により昨年度の糖脂質糖鎖のLC/MSを使った分離・分析および構造解析法に加え糖蛋白質N結合型糖鎖の解析の体制が整いつつある。国立成育医療センター研究所では白血病等患者検体を多く保有することに加え、ヒト正常骨髄CD

34+細胞やヒト間葉系前駆細胞などの未分化細胞およびその分化誘導系を有している。このような系で発現糖鎖の網羅的解析はほとんどなされていないのが実情である。本年度確立したLC/MSを使用した糖鎖解析系と糖鎖プライマー方を適時応用することで発現量が他の糖鎖に比べて極端に少ないために同定が難しかった糖鎖についても分析及び構造解析が出来るよう体制が整いつつある。来年度以降は、LC/MSで得られたそれぞれの細胞の糖脂質糖鎖、糖蛋白質糖鎖のマススペクトルを得て、多変量解析・主成分分析等の統計学的手法で診断に有用な発現糖鎖パターンを選び出すことを目指す。同時にそれら糖鎖を抗原として、抗体作製を行う技術の確立を目指し、簡便に未分化細胞を選別する手法に応用する。

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G．知的財産権の出願・登録状況

（予定を含む。）

1．特許取得

該当なし

2．実用新案登録

該当なし

3.その他

該当なし

III　研究成果に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
佐藤　智典	グライコチップ（糖鎖アレイ）		ナノバイオ計測の実際	講談社サイエンティフィク	東京	2007	42-51

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Pu Wang, Peixing Wu, Jinghai Zhang, Toshinori Sato, Sadako Yamagata and Tatsuya Yamagata	Positive regulation of tumor necrosis factor-alpha by ganglioside GM3 through Akt in mouse melanoma B16 cells	Biochem, Biophys. Res. Commun.	356	438-443	2007
D. Hu, Z. Man, P. Wang, X. Tan, X Wang, S. Takaku, S. Hyuga, T. Sato, X-S. Yao, S. Yamagata, T. Yamagata	Ganglioside GD1a negatively regulates matrix metalloproteinase-9 (MMP-9) expression in mouse FBJ cell lines at the transcriptional level	Connective Tissue Research	48	198-205	2007
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IV　研究成果の刊行物・別冊

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Positive regulation of tumor necrosis factor-α by ganglioside GM3 through Akt in mouse melanoma B16 cells

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Abstract

GM3 has been shown to suppress TNFα expression in blood monocytes. However, we found that GM3 and TNFα were expressed in parallel in mouse melanoma B16 cells that were transfected with UDP-Gal:glucosylceramide β-1,4-galactosyltransferase cDNA in a sense or antisense direction or CMP-NeuAc:lactosylceramide α-2,3-sialyltransferase siRNA. TNFα expression was increased by addition of GM3 to the B16 transfectants and decreased after treatment with D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, an inhibitor of glucosylceramide synthesis. These results clearly indicate that GM3 positively regulates TNFα expression in B16 cells. Phosphoinositide 3-kinase inhibitors, wortmannin and LY294,002, suppressed TNFα expression and Akt phosphorylation. GM3 was shown to increase phosphorylation of Akt in B16 cells and the B16-derived transfectants. Treatment of B16 cells with siRNA targeted to Akt1/2 resulted in TNFα suppression, indicating that Akt plays an important role in regulation of TNFα expression. Suppression of Akt1/2 rendered cells insensitive to GM3, suggesting that the GM3 signal may be transduced via Akt.

Keywords: Ganglioside GM3; TNFα; PI3K; Akt; LY294,002; Wortmannin

Glycosphingolipids are ubiquitous components of the outer leaflet of plasma membranes in vertebrate tissue [1]. In particular, ganglioside GM3, one of the sialylated glycosphingolipids, has been implicated in differentiation [2], growth regulation [3] and cell adhesion [4]. Large amounts of GM3 are expressed in certain kinds of animal tumors, such as murine B16 melanoma [5] and Cloudman S91 melanoma [6]. In these cells, GM3 is the dominant glycosphingolipid [7]. GM3 has also been implicated in signal transduction in B16 cells [8]. Gangliosides including GD3, GD1a, GM3, GM2, and GM1 reportedly decrease TNFα gene expression induced by different types of stimuli, especially lipopolysaccharides (LPSs), in monocytes [9]. However, the effects of gangliosides on various inflammatory mediators, such as cytokines [10] and inducible nitric oxide synthase (iNOS), in brain microglia and astrocytes [11] are controversial. Gangliosides as well as LPSs significantly increase TNFα by rapidly changing the cell surface expression of toll like receptors (TLR) in microglia and astrocytes [12]. TLRs mediate signaling responses elicited by various exogenous and endogenous molecules, including LPSs.

It has been suggested that phosphoinositide 3-kinase (PI3K) is involved in TLR signaling [13]. However, it has been shown that the PI3K inhibitors, wortmannin and LY294,002, have different effects on TNFα expression. Wortmannin greatly enhanced TLR-mediated iNOS expression and TNFα production in the mouse macrophage cell line, Raw264.7. The effect of wortmannin occurred in cells expressing TLR-2, -3, -4, and -9 and

Abbreviations: PBS(-), phosphate-buffered saline without Ca and Mg cations; HPTLC, high performance thin layer chromatography.

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was accompanied by activation of NF-κB, leading to up-regulation of cytokine mRNA production in Raw264.7 [13]. In support of this notion, a constitutively active PI3K negatively regulated induction of iNOS in murine peritoneal macrophages and C6 glial cells [14]. Another PI3K inhibitor, LY294,002, has been shown to strongly suppress TNFα production [13]. However, since both wortmannin and LY294,002 inhibit Akt phosphorylation, it is believed that the PI3K-Akt pathway negatively regulates the expression of TNFα via TLR stimulation. LY294,002 is an inhibitor of not only PI3K but also casein kinase II and estrogen receptors [15,16].

In the present study, we found that GM3 and TNFα were expressed in parallel in mouse melanoma B16 cells stably transfected with the UDP-Gal:glucosylceramide β-1, 4-galactosyltransferase (B4Gal-T6) cDNA in a sense or anisense direction or siRNA targeted to CMP-NeuAc:lactosylceramide α-2,3-sialyltransferase (St3gal5) mRNA. Addition of GM3 to the B16 transfectants increased TNFα expression, while D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) treatment decreased expression. Akt was shown to be involved in regulation of TNFα and to be the molecule through which the GM3 signal is transduced. These results clearly indicate that GM3 positively regulates TNFα expression through Akt in B16 cells.

Materials and methods

Cell lines and culture. Mouse melanoma B16 cells were kindly provided by Dr. Kiyoshi Furukawa at Nagaoka University of Technology, Japan. The mouse melanoma cell lines, CSSH-1 and CAH-3, were produced from B16 by transfection with a vector containing UDP-Gal:glucosylceramide β-1,4-galactosyltransferase (B4Gal-T6) cDNA in a sense or antisense direction, respectively, as reported elsewhere (manuscript in preparation). Briefly, a full length B4Gal-T6 cDNA was inserting to a pCMV-SPORT2 expression vector between *NotI* and *SalI* for sense expression. An antisense expression vector was constructed by introducing the B4Gal-T6 cDNA to a pCMV5 vector in an antisense direction. Mock transfectant cell lines, SM-1 and CM-1, were used as controls. A sense-transfectant, CSSH-1, expressing about 2-fold higher mRNA of B4Gal-T6 than mock SM-1 cells, and an antisense transfectant, CAH-3, whose B4Gal-T6 mRNA expression was decreased to 50% than mock CM-1 cells, were used in this study. Lactosylceramide content of the transfectants remained the same as that of the control. The metastatic ability of these cell lines is inversely related to the GM3 content in the cells. The cells were maintained in media containing DMEM (Gibco, Invitrogen Corporation, NY, USA) supplemented with 10% fetal bovine serum (FBS) (TBD-TianJin Hao Yang Biological Company, TianJin, China), 100 U/ml penicillin, and 100 μg/ml streptomycin, and were incubated in a humidified (37 °C, 5% CO_2 and 95% air) incubator (Sanyo, Tokyo, Japan). To examine the effects of gangliosides on TNFα expression, the cells were incubated with 25 μM GM3 in the absence of serum for 4 h and cultured with media containing 5% serum for an additional 20 h [17], or cells were starved for 6 h followed by incubation with 10 μM GM3 under serum-free conditions for 10 min. To suppress glycolipid synthesis, the cells were cultured in the presence of 12.5 μM D-PDMP for up to 6 days, with fresh media containing the inhibitor being added everyday. To determine the effects of PI3K inhibitors on TNFα production, the cells were seeded at a density of 1×10^6 cells/dish in a 60 mm dish in DMEM/FBS. After 24 h, the media was replaced with media containing the indicated concentrations of inhibitor. After a further 24 h, RNA was extracted from the cultured cells and TNFα expression was determined by RT-PCR.

Chemicals and antibodies. Ganglioside GM3 from bovine brain was obtained from Wako (Tokyo, Japan). LY294,002, LY303,511 and wortmannin were purchased from Sigma (USA). D-PDMP was from Matreya (USA). Rabbit anti-Akt, antiphospho-Akt (Ser^{473}), antiphospho-Akt (Thr^{308}) antibodies and horseradish peroxidase (HRP)-linked anti-rabbit secondary antibody were obtained from Cell Signaling (MA, USA). The RNeasy Mini kit used to extract total RNA was from Qiagen (Hilden, Germany). The RT-PCR kit was from the TAKARA Biotechnology Corporation (Dalian, China).

RNA extraction and RT-PCR. RNA extraction and analysis of amplified DNA have been described previously [18]. The primers used in this study were designed by Primer 3 software and synthesized by Invitrogen (Shanghai, China). Primer sequences were as follows: for eukaryotic elongation factor (Eef), sense: 5′-CGCTGCTGGAAGCTTTGGAT-3′ and antisense: 5′-GGGGCCATCTTCCAGCTTCT-3′; for TNFα, sense: 5′-TCCAGGCGGTGCCTATGTCT-3′ and antisense: 5′-GTTTGAGCT CAGCCCCCTCA-3′; for Akt1, sense: 5′-AGGAACGGCCTCAGGAT GTG-3′ and antisense, 5′-TAAGCGTGTGGGCAACCTCA-3′; for Akt2, sense: 5′-GGATGCGGGCTATCCAGATG-3′ and antisense: 5′-TCACC CCCGTTGGCATACTC-3′. RT-PCR was used to semi-quantitatively determine the levels of mRNA of the genes under consideration. Eef mRNA was used as a control [17].

Western blotting analysis. 2×10^6 cells were lysed in 1 ml sample buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerine, 5% β-mercaptoethanol, and 0.01% bromophenol blue) at 37 °C for 30 min and boiled at 100 °C for 5 min. An aliquot of the lysate was loaded onto a 10% SDS–polyacrylamide gel. After electrophoresis, the gel was blotted onto nitrocellulose membrane (Protran BA85, Schleicher and Schuell, Germany). The membrane was incubated with the antibody at 1/5000 dilution, followed by incubation with the HRP-conjugated anti-rabbit IgG secondary antibody (1/5000 dilution). Western blots were visualized by ECL.

SiRNA. Target sequences and the scrambled sequence of CMP-NeuAc:lactosylceramide α-2,3-sialyltransferase (St3gal5) encoding mouse ganglioside GM3 synthase were selected using a Genscript program, Mulfold software and the assistance of Dr. N. Ota of Riken (Yokohama, Japan). The sequences were inserted into a retroviral vector with Neomycin resistance at TAKARA Biotechnology Corporation. Stable transfection was carried out with Fugene reagent (Roche, USA), essentially following to instructions of the manufacturer. Briefly, cells were seeded at a density of 20% confluency in a 60 mm dish and then transfected with St3gal5 siRNA vector for 3 days; stably transfected cells were selected by G418. The expression of St3gal5 mRNA and GM3 content were analyzed by RT-PCR and HPTLC, respectively. One of the monoclonal transfectants, B11, showed a 35% decrease in St3gal5 expression. The most effective target was found to be the St3gal5 siRNA sequence 2, 5′-AGAC GGCTATGGCTCTGTTAT-3′. Other siRNA sequences used were as follows: Akt1 siRNA, 5′-CAACTTCTCAGTGGCACAATG-3′ and Akt2 siRNA, 5′-GGTCATTCTGGTTCGAGAGAA-3′. The control siRNA contained the sequence 5′-CGAAGTTCGTTGCACTATGGT-3′.

Ganglioside extraction and HPTLC. B16 cells were grown to ~90% confluence in 10 cm dishes, harvested, and washed three times with PBS(-). GM3 was extracted once with 1 ml of chloroform/methanol (2:1, v/v) and once with chloroform/isopropanol/methanol (7:11:2, v/v/v) with sonication for 1 h. The supernatants were evaporated at 60 °C, and lipid fractions were dissolved in chloroform/methanol (2:1, v/v), developed in chloroform/methanol/0.25% KCl (5:4:1, v/v/v), and stained with orcinol/sulfuric acid reagent.

Results

Positive regulation of TNFα expression by GM3

During the course of our previous study implicating B4GalT-6 in lactosylceramide synthesis, we obtained cells (CSSH-1) that overexpressed B4GalT-6 cDNA and cells (CAH-3) that suppressed its expression. In the CSSH-1

cells, GM3 expression doubled, whereas in the CAH-3 cells, GM3 expression was halved (Fig. 1A). Since GM3 has previously been reported to suppress TNFα expression [9], expression of TNFα mRNA was determined in the B4GalT-6 cDNA sense- and antisense-transfected cells. RT-PCR revealed that the B4GalT-6 cDNA sense-transfected cell line (CSSH-1, rich in GM3) had a 3-fold increase in TNFα expression compared with the vector control transfected cells (SM-1). However, cells transfected with the antisense cDNA of B4GalT-6 (CAH-3, poor in GM3) decreased TNFα expression by half compared with the vector control transfected cells (CM-1) (Fig. 1A). This observation indicated that TNFα expression was proportional to the GM3 content in the cells, implying that GM3 may possibly up-regulate TNFα in B16 cells.

SiRNA targeted against St3gal5 suppressed mRNA expression of sialyltransferase, an enzyme responsible for the synthesis of GM3 by 35%, leading to suppression of GM3 as revealed by HPTLC (Fig. 1B). Furthermore, it significantly suppressed TNFα expression (Fig. 1B). In order to know whether exogenous addition of GM3 would give rise to the same results, B16 and the above-mentioned transfectants derived from B16 cells were incubated with GM3 (25 μM) for 4 h without serum, supplemented with serum to a final concentration of 5% and cultured the cells for an additional 20 h [17]. In B16 cells, TNFα mRNA expression was increased by addition of GM3 (Fig. 1C). However, the increase in TNFα expression was much more significant in CAH-3 and B11 cells with lower GM3 expression than in the B16 and control cells (Fig. 1C). Treatment of B16 cells with 12.5 μM D-PDMP (an inhibitor of glucosylceramide synthesis) [18] for 3 or 6 days resulted in suppression of both GM3 and TNFα expression (Fig. 1D). These results clearly indicate that GM3 positively regulates the expression of TNFα in B16 cells.

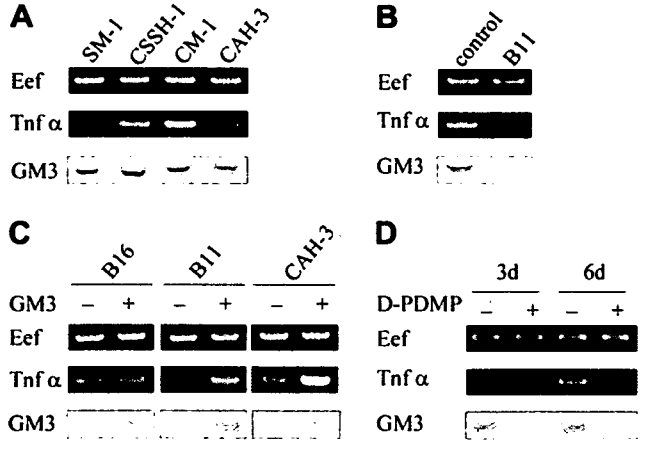

Fig. 1. The expression of TNFα is positively regulated by GM3. RT-PCR analysis of TNFα expression was carried out in (A) B16 and its variants that were generated by transfecting B4GalT-6 cDNA in a sense or antisense direction (CSSH-1, SM-1, CAH-3, and CM-1 are B16 cells transfected with sense B4GalT-6 cDNA, its vector control, antisense B4GalT-6 cDNA and its vector control cells, respectively), (B) B16 cells transfected with St3gal5 siRNA (B11 cell line is one of the stable monoclonal transfectants) or a scrambled siRNA sequence, (C) B16, B11 and CAH-3 cells treated with GM3 (25 μM) for 24 h as described in Materials and methods, and (D) B16 cells treated with D-PDMP (12.5 μM) for 3 or 6 days. Each panel shows a representative result of the RT-PCR of TNFα with Eef serving as the house keeping gene and the GM3 content determined by HPTLC. Similar results were obtained in three independent experiments.

Suppression of TNFα expression by PI3K inhibitors, LY294,002, LY303,511 and wortmannin

To investigate whether the PI3K pathway is involved in the up-regulation of TNFα expression by GM3 in B16 cells, the cells were incubated with PI3K inhibitors. LY294,002 (25 μM) significantly suppressed TNFα expression in parental B16, GM3-rich CSSH-1 and GM3-poor CAH-3 cells (Fig. 2A). LY303,511 was developed as a negative control reagent to LY294,002 [19], but, as shown in Fig. 2B, expression of TNFα was suppressed by 25 μM as well as 100 μM LY303,511. Wortmannin suppressed TNFα at 0.5 μM in B16 cells and between 0.5 and 2 μM in CSSH-1 cells (Fig. 2C). Phosphorylation of Akt at Ser^{473} was investigated using Western blot analysis (Fig. 2D). Phosphorylated Akt was suppressed to 20% of the original levels by incubation with LY294,002 (25 μM) and approximately halved by incubation with wortmannin (0.5–2 μM). LY303,511 (100 μM) suppressed Akt phosphorylation to the same level as wortmannin. All these data indicate that the PI3K-Akt pathway positively regulates TNFα expression.

Elevated phosphorylation of Akt by GM3

Since Akt was shown to be involved in the regulation of TNFα expression, we asked whether GM3 affects the phosphorylation of Akt. As shown in Fig. 3, GM3 increased Akt phosphorylation significantly at both the Ser^{473} and Thr^{308} sites. In B16 cells, phosphorylation of Akt at Thr^{308} was increased 2-fold. In B11 and CAH-3 cells, with suppressed GM3 expression, Thr^{308} Akt phosphorylation was increased by 4- and 7-fold, respectively, compared to the control. Akt phosphorylation was completely inhibited in the presence of the PI3K inhibitors, LY294,002 or wortmannin (data not shown), indicating that Akt plays an important role in the induction of TNFα expression by GM3.

Involvement of Akt in GM3 signaling that regulates TNFα expression

To further confirm the involvement of Akt in TNFα expression, B16 cells were treated with siRNA against Akt1 and Akt2. In cells with suppressed Akt1 levels (after siRNA treatment), TNFα expression was significantly suppressed. A similar result was observed after suppression of Akt2 (Fig. 4A). Addition of GM3 to B16 cells results in an increased expression of TNFα. Thus, we asked whether GM3 exerts this effect via Akt1 or Akt2 by examining

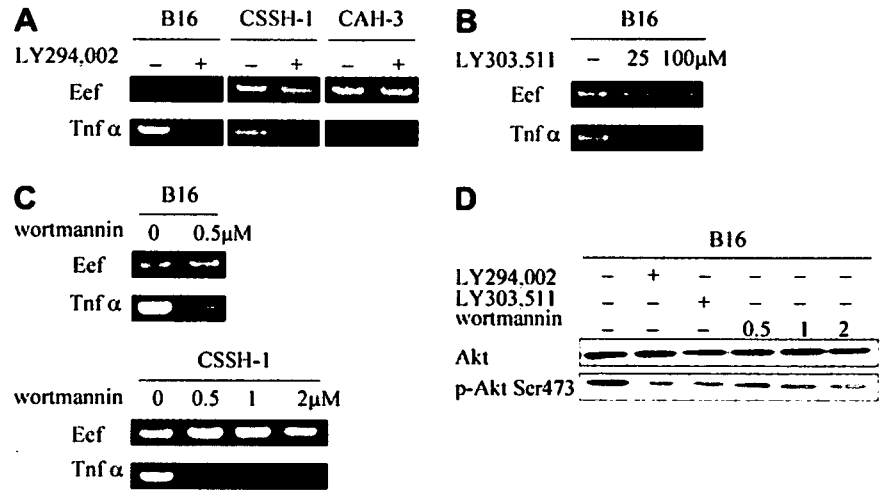

Fig. 2. Effect of PI3K inhibitors, LY294,002, LY303,511 and wortmannin on the expression of TNFα. In (A), B16, CSSH-1 and CAH-3 cells were treated with LY294,002 (25 μM) for 24 h. B16 cells were treated with LY303,511 (25 or 100 μM) (B) or wortmannin (0.5–2 mM) for 24 h (C). In (D), phosphorylation of Akt at Ser^{473} in B16 cells treated with LY294,002 (25 μM), LY303,511 (100 μM) and wortmannin (0.5–2 μM) for 24 h. Similar results were obtained in three independent experiments.

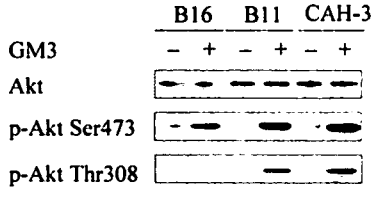

Fig. 3. Phosphorylation of Akt at the Ser^{473} and Thr^{308} sites was stimulated by GM3. Cells were starved for 6 h followed by incubation with GM3 (10 μM) under serum-free conditions for 10 min, lysed in 0.5 ml lysis buffer then analyzed by SDS–PAGE and Western blotting. Similar results were obtained in two independent experiments.

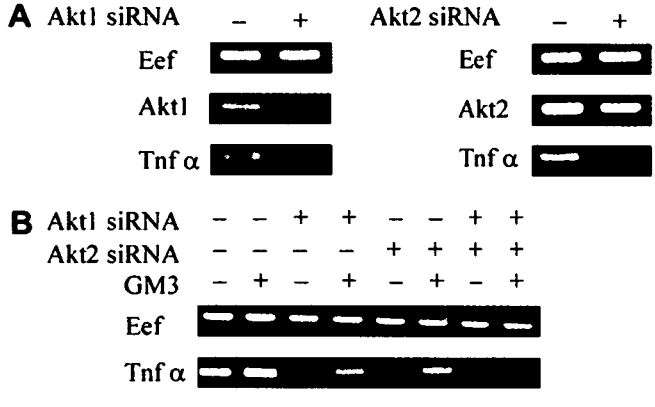

Fig. 4. Akt is essential for GM3-stimulated TNFα expression in B16 cells. TNFα was effectively suppressed in B16 cells treated with Akt1 and Akt2 siRNAs (A). Akt1, Akt2, or Akt1 plus Akt2 siRNAs transfected cells were screened with G418 (1.5 mg/ml) and the resistant cells were incubated with GM3 (25 μM) for 24 h and assayed for TNFα expression (B). Similar results were obtained in two independent experiments.

TNFα expression in cells treated with Akt1 or Akt2 siRNA. As shown in Fig. 4B, suppression of either isoform of Akt by RNA silencing did not affect the ability of GM3 to stimulate TNFα expression. However, suppression of both Akt1 and Akt2 by RNA silencing resulted in impairment of GM3 function: addition of GM3 failed to up-regulate TNFα expression in both Akt1 and Akt2 suppressed cells (Fig. 4B). These observations strongly indicate that the GM3 signal may be transduced via Akt1/2.

Discussion

Gangliosides are not only passive structural components of cell membranes but rather modulators of important biological processes such as proliferation, adhesion, differentiation, inflammation, and metastasis [2–4,20–22]. During the course of our work examining the relationship between GM3 and metastasis, we found that the production of TNFα was parallel to GM3 levels in B16 cells. This finding prompted us to examine whether GM3 has the ability to regulate the expression of TNFα.

In this study, we provide multiple lines of evidence to support the notion that GM3 regulates the expression of TNFα. Although, a mixture of gangliosides or LPS induces the expression of TNFα in microglia and astrocytes [12], little is known as to how gangliosides stimulate the production of TNFα in tumor cells. Here, we show that the PI3K inhibitors, LY294,002 and wortmannin, significantly suppressed TNFα transcription as well as phosphorylation of Akt at the Ser^{473} site in B16 cells. GM3 was found to stimulate phosphorylation of Akt at both the Ser^{473} and Thr^{308} sites. Akt/PKB is a member of the AGC kinase family (named for the similar Ser/Thr kinases, cAMP-dependent protein kinase, cGMP-dependent protein kinase and protein kinase C), including p70 S6 kinase (S6K), p90 S6K (RSK), and PKC [23,24]. Most of the family members, including Akt, are phosphorylated at two key residues located at the catalytic (activation loop or T-loop) and the C-terminal hydrophobic motif (HM) sites. Phosphorylation of the HM site promotes docking of the 3′-phosphoi-

nostide-dependent kinase 1 (PDK1) interacting fragment (PIF) pocket of PDK1 to the HM site and concomitantly leads to the phosphorylation of the T-loop site upon growth factor stimulation and PI3K activation [25]. HM phosphorylation of Akt at Ser^{473} and Thr^{308} phosphorylation has been proposed to be interdependent on each other [26,27]. Upon stimulation by GM3, phosphorylation of Akt at the Thr^{308} site was shown to proceed in a similar way to phosphorylation at the Ser^{473} site, indicating that TNFα production is parallel to Akt activity.

Mammalian cells express three Akt isoforms (Akt1-3) encoded by three separate genes. The amino acid sequences of the three isoforms are almost identical. Relative expression of these isoforms, however, differs in various mammalian tissues [28]. B16 cells express Akt1 and Akt2 predominantly, with Akt1 comprising two variants. Involvement of Akt in the synthesis of TNFα was shown using siRNAs that were designed to knock down the respective genes. However, it still remained unclear if Akt was the key molecule through which GM3 regulates the synthesis of TNFα. Therefore, we generated Akt1 and Akt2 double knocked down cells. The results support the notion that Akt is the key molecule through which GM3 up-regulates TNFα expression. Although Akt is shown to play a pivotal role in GM3 regulation of TNFα, it remains to be elucidated if Akt exerts its function through iNOS or NF-κB. In the following paper [29], we will report the involvement of mTOR and Rictor in the pathway through which GM3 regulates TNFα expression.

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Ganglioside GD1a Negatively Regulates Matrix Metalloproteinase-9 Expression in Mouse FBJ Cell Lines at the Transcriptional Level

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Mouse FBJ virus-induced osteosarcoma FBJ-S1 cells rich in GD1a are not readily metastatic, whereas FBJ-LL cells with low levels of GD1a are highly metastatic. GD1a was previously shown to suppress metastasis of mouse FBJ cells and to upregulate caveolin-1 and stromal interaction molecule 1 expression. The present study demonstrates that matrix metalloproteinase-9 (MMP-9) expression renders FBJ-LL cells invasive. MMP-9 is inversely regulated by GD1a, based upon four observations: MMP-9 mRNA content was 5 times higher in FBJ-LL cells than FBJ-S1 cells; a GD1a-re-expressing FBJ-LL cell variant produced through β1,4GalNAcT-1 cDNA transfection expressed lower levels of MMP-9; exogenous addition of GD1a to FBJ-LL cells decreased MMP-9 production in a dose- and time-dependent manner; and treatment of GD1a-rich cells with D-PDMP or siRNA targeting St3gal2 decreased GD1a expression, but augmented MMP-9 expression. This is the first report demonstrating that GD1a negatively regulates expression of MMP-9 at the transcriptional level.

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INTRODUCTION

The spread of cells from primary tumors results in metastasis to secondary sites and is the most life-threatening aspect of most cancers. Cell lines that have the greatest metastatic potential in the lung and liver secrete the most matrix metalloproteinase-9 [MMP-9, 1]. A number of reports have therefore suggested the possible involvement of MMPs in extracellular matrix degradation during tumor cell migration, particularly in metastasis [2–4].

Metastasis takes place in several discreet steps including invasion, intravasation, extravasation, and angiogenesis to bring about colonized tumor growth [5]. Recent work on metastasis has led to the new concept that under the direction of tumor cells, normal host cells select a microenvironment suitable for metastasis [6]. The notion that MMPs are essential for the infiltration of tumor cells into surrounding tissue is important, particularly since malignant tumors continue to be leading

causes of death in developed countries. Along with other family members, MMP-9 is critical for cell migration and invasion [7, 8] and is always highly expressed by malignant tumor cells.

Gangliosides, or sialic acid–containing glycosphingolipids, are present on the outer leaflet of the lipid bilayer of the plasma membrane [9]. Gangliosides are thought to play roles in cell growth, adhesion, differentiation, tumorigenesis, and tumor metastasis. Change in ganglioside expression in tumor cells also has been implicated in the metastatic potential of tumors [10]. In previous studies, poorly metastatic mouse osteosarcoma FBJ-S1 cells obtained from osteosarcoma induced by FBJ virus in mice were noted to express GM3 and the complex ganglioside GD1a, whereas highly metastatic FBJ-LL cells contained essentially the same GM3 content though only very low levels of GD1a [11]. FBJ-LL cells transfected with β1,4GalNAcT-1 (GM2/GD2 synthase) cDNA showed no sign of metastasis following the subcutaneous transplantation, thus showing GD1a likely suppresses the metastasis of FBJ osteosarcoma cells [12].

To clarify the mechanism by which GD1a controls cell metastatic capacity, we have looked for molecules responsible for metastasis and have identified caveolin-1 and stromal interaction molecule 1 (Stim1) whose expression was positively regulated by GD1a [13]. The present study clearly implicates MMP-9 as a major factor in the malignant potential of FBJ-LL cells, as determined by Matrigel invasion assay. Furthermore, MMP-9 but not MMP-2 expression was found to be negatively regulated by GD1a at the transcriptional level.

MATERIALS AND METHODS

Cell Lines and Culture

The highly metastatic mouse osteosarcoma cell line, FBJ-LL, and poorly metastatic cell line, FBJ-S1, were produced from a FBJ virus-induced osteosarcoma of the BALB/c mouse [1]. FBJ-S1 cells expressed GM3 and GD1a, whereas FBJ-LL cells expressed GM3 and had only low levels of GD1a. The capacity for FBJ-LL cells to migrate was ten times greater than that of FBJ-S1 cells, but decreased by half on treatment with GD1a [11]. FBJ-LL cells metastasized into the liver and lung, but not FBJ-S1 [12]. FBJ-LA5-22 and FBJ-LA5-30 cells were obtained by transfection of FBJ-LL cells with β1-4GalNAcT-1 (GM2/GD2-synthase) and mock-transfection of FBJ-M5 cells, as a control. GD1a expression in LA5-22 and LA5-30 cells was five-times greater than that of FBJ-M5 cells. Migration capacity of LA5-22 and LA5-30 cells was about one tenth that of FBJ-M5, comparable to the capacity of FBJ-S1 cells.

When FBJ-M5 cells were inoculated into mice, metastatic nodules were observed in liver, lung, kidney, and adrenal glands within 4 to 5 weeks, while LA5–22 cell transplantation did not show any sign of metastasis [12]. The FBJ cells were maintained in medium containing RPMI-1640 (GIBICO, Invitrogen Corporation, NY, USA) supplemented with 10% fetal bovine serum (TianJin HaoYang Biological Company, TianJin, China), 100 units/ml penicillin, and 100 μg/ml streptomycin and incubated in a humidified (37°C, 5% CO_2 and 95% air) incubator (Sanyo, Tokyo, Japan).

For ganglioside treatment, the cells were seeded and cultured overnight, washed with serum free RPMI-1640, and incubated with ganglioside at the concentration specified in the absence of serum for the period of time indicated. To suppress glycosphingolipid biosynthesis, the cells were cultured in the presence of 12.5 μM D-PDMP and provided with fresh medium containing the inhibitor daily.

Chemicals and Antibodies

Ganglioside GD1a from bovine brain was purchased from Wako (Osaka, Japan). D-PDMP was from Matreya. The RNeasy Mini Kit to extract total RNA was purchased from Qiagen. The RT-PCR kit was from Takara Biotechnology Corporation (Dalian, China).

Cell Invasion Assay Using Matrigel

First, 100 μl 1 mg/ml Matrigel matrix (BD Bioscience, USA) were poured into an upper chamber of a Transwell (8 μm in pore size, Corning Coaster Corporation, USA) and incubated at 37°C for 5 hr to gel. Second, 600 μl RPMI 1640 supplemented with 0.5% FBS were placed in a lower chamber and 1×10^5 cells in 100 μl serum-free medium were added onto the gel. After incubation in the CO_2 incubator for the time indicated, the number of cells that had transmigrated to the lower chamber were counted under a Nikon TMD microscope.

RNA Extraction and RT-PCR

RNA extraction and analysis of amplified DNA have been detailed previously [13]. The primers used in this study were designed with Primer 3 software and synthesized by Invitrogen (Shanghai, China). Primer sequences used for PCR in this study were as follows: for β-Actin, sense 5′-ACACTGTGTGCCCATCTACGAGG-3′ and antisense 5′-AGGGGCCGGACTCGTCGTCATACT-3′; for MMP-9, sense 5′-CTGACTACGATAAGGACGGCAA-3′ and antisense 5′-ATACTGGATGCCGTCTATGTCG-3′; for MMP-2, sense 5′- ACCTGGATGCCGTCGTGGAC-3′ and antisense 5′-TGTGGCAGCACCAGGGCAGC-3′; antisense 5′-AGGGGCCGGACTCGTCGTCATACT-3′; for St3gal2, sense 5′-GTGACGCCAGCACCTCTGAA-3′ and antisense 5′-GGACCAGCACGAAGCTGACA-3′; for Rpl13, sense 5′-CATCAGGCCCATCGTGAGGT-3′ and antisense 5′-GCAG CTTCCTTCGCCCTTTT-3′. RT-PCR was used to semiquantitatively determine the levels of mRNA of the genes under consideration and β-actin mRNA as control [13]. MMP mRNA values are expressed as a ratio of MMP to β-actin mRNA and usually expressed as 1 for control experiments (mean ± S.E.).

siRNA Transfection

Target three sequences and the scrambled sequence of St3gal2 encoding mouse ganglioside GD1a synthase (SAT-IV,

Siat5) were selected using a Genscript program, Mulfold software, and with the assistance of Dr. N. Ota of Riken (Yokohama, Japan). The sequences were made to constitute a retroviral vector with neomycin resistance at Takara Biotechnology Corporation. Plasmids were transfected into FBJ-S1 cells in the presence of Fugene (Roche, USA), as specified by the manufacturer. After 3 days transfection, RNA was extracted and assayed for expression of St3gal2 and MMP-9. The most effective target was found to be siRNA sequence 1, 5′-ACCAGGCTATTCAGGACTACA-3′. The control siRNA contained the sequence 5′-CAGAGCAATGTATCAATCCGC-3′. Stable transfections were carried out with Fugene reagent, essentially following instructions of the manufacturer.

Briefly, cells were seeded at a density of 50% confluency in a 60 mm dish and then transfected with St3gal2 siRNA plasmid in a 60 mm dish for 3 days; stably transfected cells were selected by G418. The expression of St3gal2 mRNA and GD1a content as revealed by HPTLC (high performance thin layer chromatography) [13] were suppressed to 75% and 20%, respectively.

Gelatin Zymography and Statistical Analysis

Gelatinase activity was determined as described previously [1, 14]. Data were analyzed using Microsoft Excel. All values are given as mean ± S.E and levels of significance are indicated in figures.

RESULTS

Role of MMP-9 in FBJ-LL Cell Metastasis

MMP-9 and MMP-2 are closely associated with tumor malignancy, i.e., invasion and metastasis. To determine whether MMP-9 and MMP-2 of FBJ cells are responsible for cell metastasis, invasion assays using Matrigel in the absence or presence of MMP inhibitors or antibody were carried out. Inhibitors that suppress MMP-9 and MMP-2 activity as indicated by gelatin-zymography were evaluated (Figure 1A). TIMP-1 (0.2 μg/ml), TIMP-2 (0.2 μg/ml) and GM6001 (25 μM) significantly suppressed MMP-9 and MMP-2 activity. Anti-MMP-9 antibody (0.2 μg/ml) specifically suppressed MMP-9 to a minor extent, but not MMP-2 activity. MMP-9 of FBJ-LL cells was evaluated by Matrigel invasion assays in the absence or presence of inhibitors or the antibody.

The number of FBJ-LA5–30 cells that transmigrated to the lower chamber in 24 hr (5±10) was much less compared with FBJ-M5 cells (820 ± 50), indicating the latter to be highly invasive, consistent with previous data [12]. TIMP-2 and GM6001 treatment decreased the number of cells transmigrating to the lower chamber to 60% and 0%, respectively (Figure 1B). TIMP-2 and GM6001 suppressed MMP-9 and MMP-2, while anti-MMP-9 antibody specific to MMP-9 suppressed the extent of cell invasion capacity to 15%, suggesting MMP-9 to be responsible for metastasis. GD1a-deficient FBJ-M5 cells

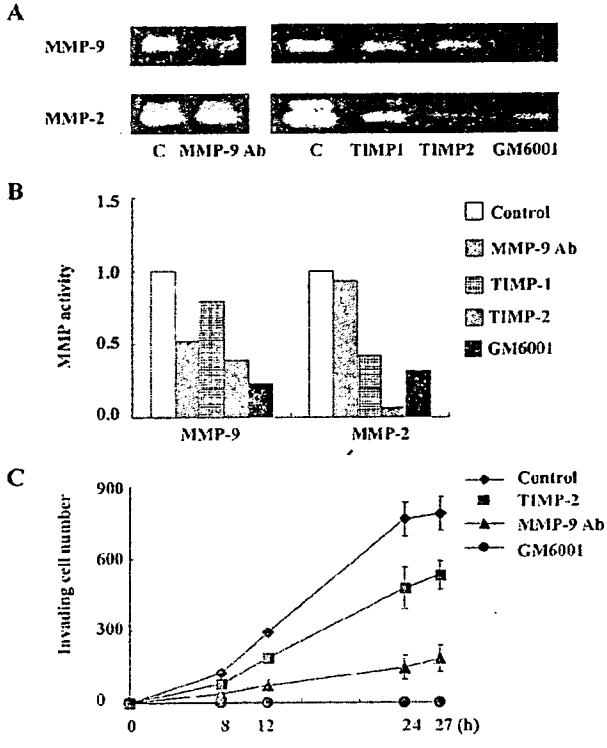

FIG. 1. MMP-9 responsible for metastaticity of FBJ-LL cells. To demonstrate that MMP inhibitors suppress MMP-9 and/or MMP-2 activity, (A) FBJ-M5 cells were incubated in serum-free medium from which an aliquot was mixed with an equal volume of double-strength Laemmli's sample buffer with no reducing reagent. Without heating this was applied to electrophoresis on a gel containing 0.3 mg/ml gelatin. After electrophoresis, each lane was cut out and rinsed with 2.5% Triton X-100 for 1 hr at room temperature followed by incubation in the reaction buffer (10 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.4, 0.02% NaN_3) in the presence or absence of 0.2 μg/ml TIMP-1, 0.2 μg/ml TIMP-2, 25 μM GM6001, or 0.2 μg/ml anti-MMP-9 antibody for 16 hr at 37°C and stained with CBB [14]. A panel in (B) shows densitometric analysis of the zymogram given in (A). Assays were performed twice and representative data are shown. In (C), using a Transwell overlayed with Matrigel, an invasion assay was carried out in the presence or absence of 0.2 μg/ml TIMP-2, 25 μM GM6001, or 0.2 μg/ml anti-MMP-9 antibody. Cell numbers were determined in triplicate. Each assay was conducted twice and mean values are presented ± SE.

were actively invasive due to MMP-9 expression, though not GD1a-rich FBJ-LA5-30, and so consequently MMP-9 would appear related to GD1a content in the cells.

GD1a-Deficient FBJ-LL and FBJ-M5 Cells Express More MMP-9 than GD1a-Rich Cells

To determine whether MMP-2 and MMP-9 expression is related to GD1a levels, RNA was extracted from FBJ-LL, -S1, -M5, and -LA5-30 cells that had reached late logarithmic growth phase and assayed for MMP-2 and MMP-9 expression by RT-PCR, using β-actin as the standard. The expression of MMP-9 of highly-metastatic FBJ-LL cells was five times greater than that

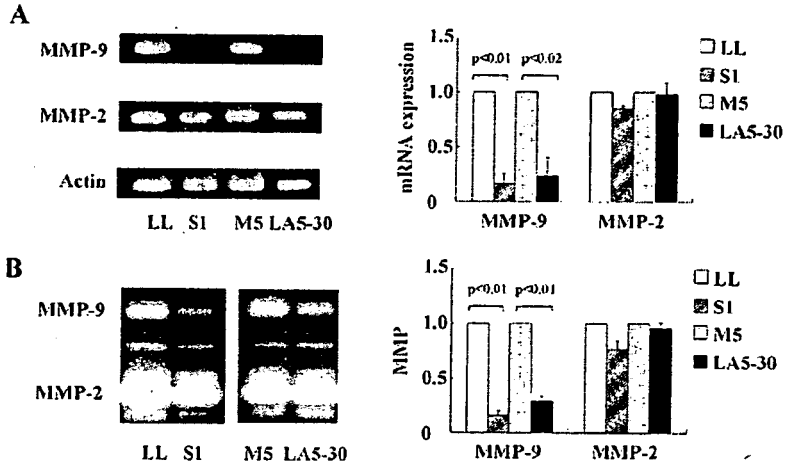

FIG. 2. MMP-9 but not MMP-2 levels are inversely proportional to GD1a expression at the level of mRNA and protein. FBJ-S1 (rich in GD1a), FBJ-LL (devoid of GD1a), FBJ-M5 (vector control of GM2/GD2 synthase cDNA transfectant originating from FBJ-LL), and FBJ-LA5-30 (GD1a rich due to GM2/GD2 synthase cDNA transfection into FBJ-LL) cells were cultured under standard conditions. RNA was extracted to assay MMP-9 and MMP-2 mRNA expression using β-actin as standard (A). Right-hand panel shows densitometric analysis of MMP mRNA expression in FBJ-S1 compared with FBJ-LL cells ($p < 0.01$) and FBJ-LA5-30 compared with FBJ-M5 cells ($p < 0.02$). For the purpose of normalization, DNA content was divided by that of β-actin and that for the control was expressed as 1 in all the experiments. In B, cells were incubated in serum-free medium for 24 hr and aliquots of conditioned medium used for gelatin zymography. Right-hand panel shows densitometric analysis of MMP expression in FBJ-S1 compared with FBJ-LL cells ($p < 0.01$) and FBJ-LA5-30 compared with FBJ-M5 cells ($p < 0.01$). Three separate determinations were made and mean values ± S.E. are shown.

of the poorly metastatic FBJ-S1 cell line (Figure 2A). FBJ-M5 cells were the vector control when FBJ-LL was transfected with GM2/GD2 synthase cDNA, and FBJ-5-30 was a transfectant producing GD1a to the same extent as FBJ-S1 cells [12]. MMP-9 expression of FBJ-M5 was as high as in FBJ-LL cells, in contrast to FBJ-5-30 cells whose MMP-9 expression was as low as in FBJ-S1 cells.

No significant difference in the expression of MMP-2 could be detected among these cell lines, as also was confirmed by gelatin-zymography (Figure 2B). The four cell lines were incubated in serum-free culture medium for 24 hr and aliquots of the culture medium were assayed for MMPs by zymography. MMP-9 production by FBJ-LL cells exceeded that in FBJ-S1 cells 5-fold, as indicated by densitometric analysis. MMP-9 activity of FBJ-M5 was 2.5 times greater than FBJ-5-30 cells. The activity of FBJ-LL and -M5 was noted to be saturated in gelatin zymograhy and thus possibly may be higher by one order of magnitude.

Expression of MMP-9 is Downregulated by Exogenous GD1a Addition

The above findings indicate MMP-9 expression to be inversely proportional to GD1 content in the cells, implying GD1a to possibly downregulate MMP-9 expression in FBJ cells. To determine whether GD1a regulates MMP-9 expression, the effects of exogenous GD1a addition on MMP-9 expression were investigated. MMP-9 expression of FBJ-M5 was suppressed by incubation with 50 μM and 100 μM GD1a in the absence of serum for 6 hr (Figure 3A). No definite suppression of MMP-9 expression of FBJ-LL cells was evident, after incubation with 50 μM GD1a but was suppressed with 100 μM GD1a in the absence of serum for 6 hr (Figure 3B). FBJ-M5 or FBJ-LL cells were incubated with 50 μM GD1a in the absence of serum for the time indicated (Figures 3C and 3D). MMP-9 mRNA production was significantly reduced in FBJ-M5 cells incubated with 50 μM GD1a for 3 hr and continued to be so with further incubation with GD1a (Figure 3C).

Suppression was evident with FBJ-LL cells treated with 50 μM GD1a for 12 hr, but additional incubation led to no additional decrease. Figure 3E shows zymography of FBJ-M5 cells treated with 50 μM GD1a in the absence of serum. MMP-9 activity of cells incubated with GD1a decreased in a time-dependent manner. Decrease in activity was evident even after 6 hr incubation and had reached statistical significance by 12 hr. The exogenous addition of GD1a to FBJ-M5 or FBJ-LL cells therefore resulted in the suppression of MMP-9 production and/or secretion, while MMP-2 mRNA and activity were not affected by GD1a addition. GD1a thus appears likely to bring about suppression of MMP-9 expression in mRNA and protein.

Increase in MMP-9 Expression by Depletion of Endogenous GD1a

The effects of depleting GD1a also were examined by treating FBJ-5 30 cells with or without 12.5 μM D-PDMP, an inhibitor of glucosylceramide synthase [16] in the presence of serum for 6 days followed by extraction of RNA from the cells. This reduces