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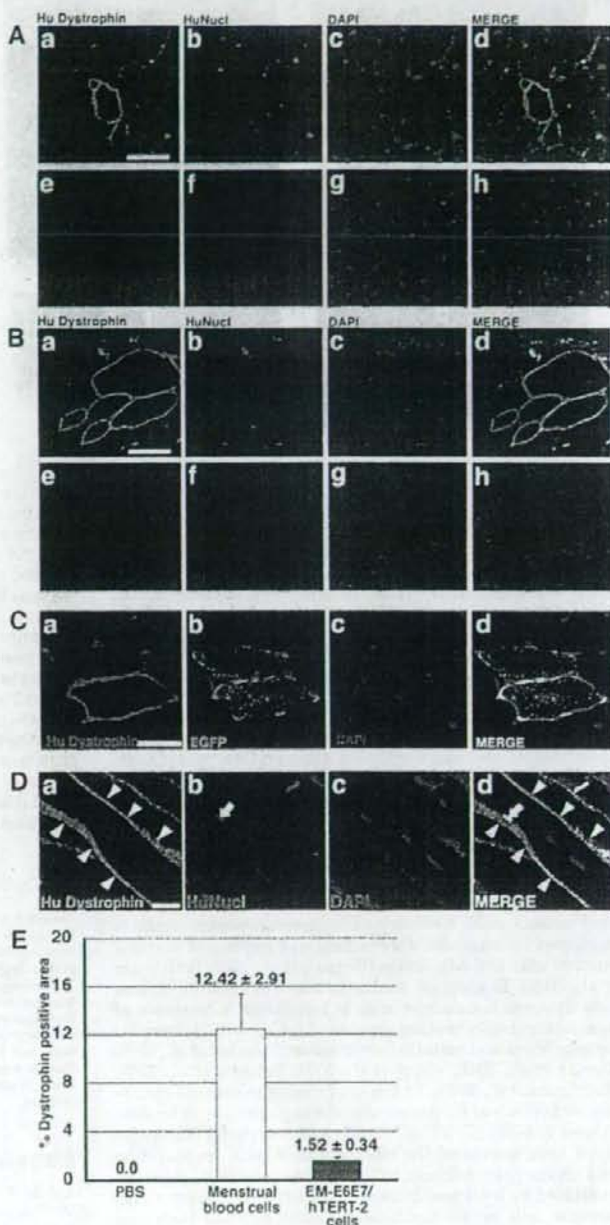

Figure 5. Conferral of dystrophin to mdx myocytes by human endometrial cells. (A and B) Immunohistochemistry analysis using an antibody against human dystrophin molecule (green), human nuclei (HuNucl, red), and DAPI staining (blue) on thigh muscle sections of mdx-scid mice after direct injection of EM-E6/E7/hTERT-2 cells (A) or menstrual blood–derived cells (B) without any treatment or induction. (C) EGFP-labeled EM-E6/E7/hTERT-2 cells without any treatment or induction were directly injected into the thigh muscle of mdx-scid mice. Immunohistochemistry revealed the incorporation of implanted cells into newly formed EGFP-positive myofibers, which expressed human dystrophin 3 wk after implantation. (A and B) As a methodological control, the primary antibody to dystrophin was omitted (e and f). (D) Immunohistochemistry analysis using an antibody against human dystrophin molecule (green, arrowheads), human nuclei (HuNucl, red, arrow), and DAPI staining (blue) on thigh muscle sections of mdx-scid mice after direct injection of human EM-E6/E7/hTERT-2 cells without any treatment or induction. (A and B) Merge of a–c is shown in d, and merge of e–g is shown in h. (C and D) Merge of a–c is shown in d. Scale bars, 50 μm (A and B), 20 μm (C and D). (E) Quantitative analysis of human dystrophin-positive myotubes. Menstrual blood–derived cells or EM-E6/E7/hTERT-2 cells without any treatment or induction were directly injected into thigh muscle of mdx-scid mice. The percentage of human dystrophin–positive-myofiber areas was calculated 3 wk after implantation of the EM-E6/E7/hTERT-2 cells or menstrual blood–derived cells. Injection of PBS without cells into mdx-scid myofibers was used as a control.

mal cells) at the start of cell cultivation, as is the case with hematopoietic stem cells (Suda *et al.*, 1984).

Menstrual blood–derived cells had a high replicative ability similar to progenitors or stem cells that display a long-term self-renewal capacity and had a much higher growth rate in our experimental conditions than marrow-derived stromal cells (Mori *et al.*, 2005). In addition, the myogenic potential of menstrual blood–derived cells, i.e., a high frequency of desmin-positive cells after induction, is much greater than expected. The higher myogenic differentiation ratio can be explained just by alteration of cell characteristics from epithelial and mesenchymal bipotential cells or heter-

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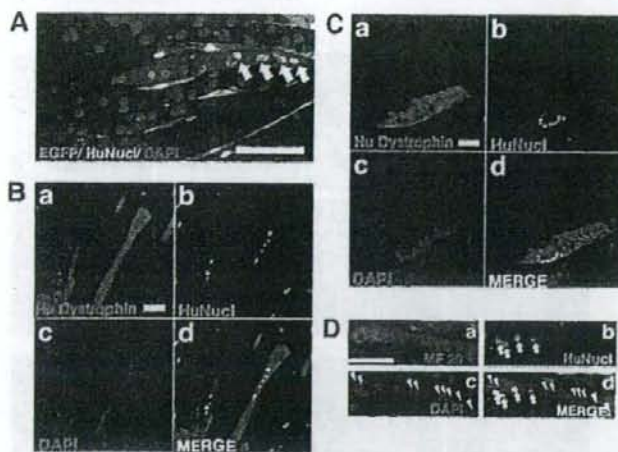

Figure 6. Detection of human endometrial cell contribution to myotubes in an in vitro and in vivo myogenesis model. EGFP-labeled EM-E6/E7/hTERT-2 cells (A) or EM-E6/E7/hTERT-2 cells (B) or menstrual blood-derived cells (C and D) were cocultured with C2C12 myoblasts for 2 d under conditions that favored proliferation. The cultures were then changed to differentiation media for 7 d to induce myogenic fusion. (A) Myotubes were revealed by EGFP (green); human nuclei were detected by antibody specific to human nuclei (HuNucl, red, arrows). (B–D) Myotubes were revealed by specific human dystrophin mAb NCL-DYS3 (B and C, red) or anti-myosin heavy chain mAb MF-20 (D, red). (D) Human nuclei were detected by antibody specific to human nuclei (HuNucl, green, arrows). Total cell nuclei in the culture were stained with DAPI (blue, arrowheads). (B–D) Merge of a–c are shown in d. The cultures were then changed to differentiation media for 7 d to induce myogenic fusion. Scale bars, 100 μm (A–D).

ogeneous populations of cells to cells with the mesenchymal phenotype in our cultivation condition, as determined by cell surface markers (Figure 1, C–E). MyoD-positive cells are present in many fetal chick organs such as brain, lung, intestine, kidney, spleen, heart, and liver (Gerhart *et al.*, 2001), and these cells can differentiate into skeletal muscle in culture. Constitutive expression of MyoD, desmin, and myogenin, all markers for skeletal myogenic differentiation in both immortalized EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells, implies either that most of these cells are myogenic progenitors or that these cells have myogenic potential. Expression of MyoD, one of the basic helix-loop-helix transcription factors that directly regulate myocyte cell specification and differentiation (Edmondson and Olson, 1993), occurs at the early stage of myogenic differentiation, whereas myogenin is expressed later, related to cell fusion and differentiation (Aurade *et al.*, 1994).

Acquisition or recovery of dystrophin expression in dystrophic muscle is attributed to two different mechanisms: 1) myogenic differentiation of implanted or transplanted cells and 2) cell fusion of implanted or transplanted cells with host muscle cells. Recovery of dystrophin-positive cells is explained by muscular differentiation of implanted marrow stromal cells and adipocytes (Dezawa *et al.*, 2005; Rodriguez *et al.*, 2005). In contrast, implantation of normal myoblasts into dystrophin-deficient muscle can create a reservoir of normal myoblasts that are capable of fusing with dystrophic muscle fibers and restoring dystrophin (Mendell *et al.*, 1995; Terada *et al.*, 2002; Wang *et al.*, 2003; Dezawa *et al.*, 2005; Rodriguez *et al.*, 2005). In this study using menstrual blood–derived cells, our findings—that the implantation of immortalized EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells improved the efficiency of muscle regeneration and dystrophin delivery to dystrophic muscle in mice—is explained by both possibilities or the latter possibility alone, because cells expressing human dystrophin had both murine and human nuclei, located in the center and periphery of dystrophic muscular fiber, respectively (Figures 5D, in vivo, and 6, A–D, in vitro).

DMD is a devastating X-linked muscle disease characterized by progressive muscle weakness attributable to a lack of dystrophin expression at the sarcolemma of muscle fibers (Mendell *et al.*, 1995; Rodriguez *et al.*, 2005), and there are no effective therapeutic approaches for muscular dystrophy at present. Human menstrual blood–derived cells are obtained by a simple, safe, and painless procedure and can be expanded efficiently in vitro. In contrast, isolation of mesenchymal stem cells/mesenchymal cells from other sources, such as bone marrow and adipose tissue, is accompanied by a painful and complicated operation. Efficient fusion systems of our immortalized human EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells with host dystrophic myocytes may contribute substantially to a major advance toward eventual cell-based therapies for muscle injury or chronic muscular disease. Finally, we would like to reemphasize that human menstrual blood–derived cells possess high self-renewal capacity, whereas biopsied myoblasts capable of differentiating into muscular cells are poorly expandable in vitro and rapidly undergo senescence (Cossu and Mavilio, 2000).

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オリゴ糖鎖合成／(株)グライコメディクス
―糖鎖プライマー法で生産した糖鎖の医療分野への応用―

中島英規

オリゴ糖鎖は糖脂質，糖タンパク質などさまざまな形態で存在し，重要な生体反応に関与している。糖鎖は構成単糖が多種であることに加え結合様式も多様であるため，さまざまなバリエーションが存在している。糖鎖は大量に入手することが困難であったため，生物学的・生理的に重要な役割を担っていると考えられながら医薬品や食品など産業分野への応用は，大量に得られる多糖を除けばほとんど例がない。これまでの糖鎖の入手方法は，生物材料からの抽出，有機合成，酵素を用いた方法などがおもに試みられてきたが，複雑な配列を有する糖鎖を効率良く得ることは熟練を要しコストや時間の面でも問題があった。

弊社が行う培養細胞を利用した糖鎖プライマー法による糖鎖の生産は，糖鎖生合成過程における糖鎖生合成中間体を模倣した人工的な構造の化合物（糖鎖プライマーと呼ぶ）を培養細胞の糖鎖生合成経路に割り込ませ，この糖鎖プライマー上に糖鎖を伸張させて入手困難であった複雑な構造をもつ糖鎖を大量に作り出す方法である。これまでに糖鎖プライマーの構造を工夫することによってさまざまな糖脂質糖鎖，または糖タンパク質O-結合型糖鎖を合成することに成功している。弊社糖鎖プライマー法では，アグリコン部分の構造を工夫することで他分子へ結合や基材への固定化などを非常に容易にすることができる。このような糖鎖プライマーを用いれば，複雑な糖鎖をもった糖鎖ポリマーもしくは糖鎖オリゴマーを作り出すのも容易である。

これまで複雑な構造をもつ疾患関連糖鎖を入手することは難しく，入手できたとしても微量で，化学的に修飾したオリゴマーなどを合成することは非常に困難であった。弊社糖鎖プライマー法で得られた糖鎖は，このような問題を解決しつつ，新たな糖鎖を基本とした予防・診断・治療薬を提案できると考えている。

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細胞シート工学による再生医療事業の創造／(株)セルシード

長谷川幸雄

バイオと工学の接点で生まれる新しい材料開発が，医療を大きく変えつつある。革新的な材料のシーズを実用化し，自分自身の細胞を用いて，機能障害に陥った組織，臓器の再生をはかる再生医療の実現が急がれている。筆者らは，新しい産学連携の強固な枠組みとしてベンチャー企業（セルシード）を立ち上げ，東京女子医科大学と連携して，インテリジェント材料を用いた細胞シート工学という新しいコンセプトと技術を基盤とした再生医療の早期実現を目指している。

温度応答性ポリマーを細胞培養器材表面にナノレベルで構築すると，温度により器材表面を親水性および疎水性に自由に可逆的に制御可能なインテリジェント表面を構築することができる。培養温度を下げると表面は親水性になるため，培養細胞が器材表面から簡単にはがれ細胞シートとして回収することができる。このシートは細胞自身がつくり出す接着因子「のり」を表面にもっており，縫合することなく移植が可能となる。

本技術を用いて，大阪大学と共同でヒト角膜輪部組織の微小切片から角膜上皮細胞の培養，上皮シートとして回収および移植が可能であることを実証した。さらに，角膜疾患の治療においては圧倒的なドナー不足であるため，患者自身の口腔粘膜より上皮細胞シートの培養，回収，自家移植も試み，成功した。現在，厚生労働省へ安全性の確認申請の準備を行い，早期に再生角膜上皮移植の治験開始を目指している。

インテリジェント材料に基づく細胞シート工学は組織の機能障害や機能不全の回復に有効であり，再生医療にきわめて有用である。今後も，バイオと工学の接点で生まれる新しい材料開発が21世紀の新しい医療において，大きな役割を果たすためにはさらに強固な産学連携が大きな鍵を握ると考えている。

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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.
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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.

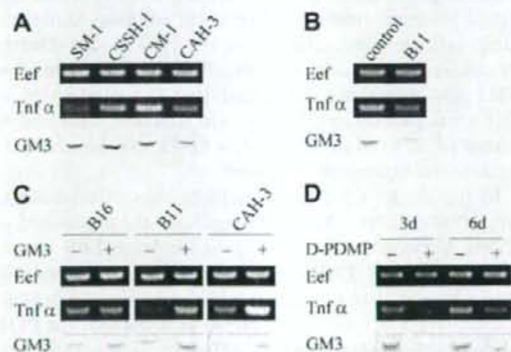

Fig. 1. The expression of TNFα is positively regulated by GM3. RT-PCR analysis of TNFα expression was carried out in (A) B16 and its variants that were generated by transfecting B4GalT-6 cDNA in a sense or antisense direction (CSSH-1, SM-1, CAH-3, and CM-1 are B16 cells transfected with sense B4GalT-6 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

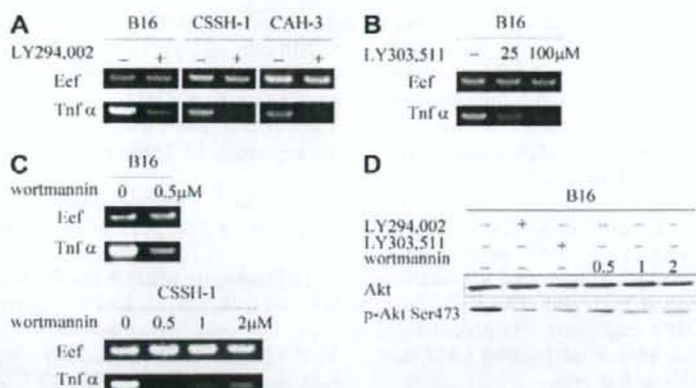

Fig. 2. Effect of PI3K inhibitors, LY294,002, LY303,511 and wortmannin on the expression of TNFα. In (A), B16, CSSH-1 and CAH-3 cells were treated with LY294,002 (25 μM) for 24 h. B16 cells were treated with LY303,511 (25 or 100 μM) (B) or wortmannin (0.5–2 mM) for 24 h (C). In (D), phosphorylation of 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.

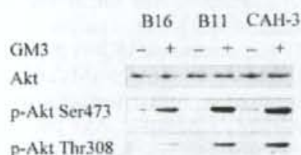

Fig. 3. Phosphorylation of Akt at the Ser^{473} and Thr^{308} sites was stimulated by GM3. Cells were starved for 6 h followed by incubation with GM3 (10 μM) under serum-free conditions for 10 min, lysed in 0.5 ml lysis buffer then analyzed by SDS–PAGE and Western blotting. Similar results were obtained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

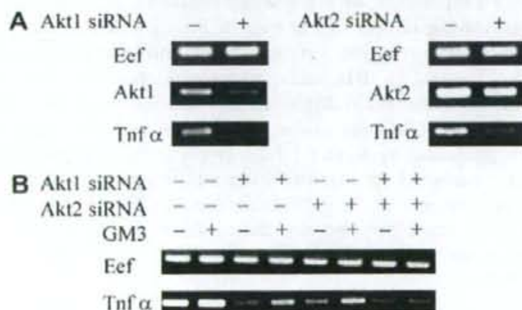

Fig. 4. Akt is essential for GM3-stimulated TNFα expression in B16 cells. TNFα was effectively suppressed in B16 cells treated with Akt1 and Akt2 siRNAs (A). Akt1, Akt2, or Akt1 plus Akt2 siRNAs transfected cells were screened with G418 (1.5 mg/ml) and the resistant cells were incubated with GM3 (25 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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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.
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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 *Not*I and *Sal*I 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.

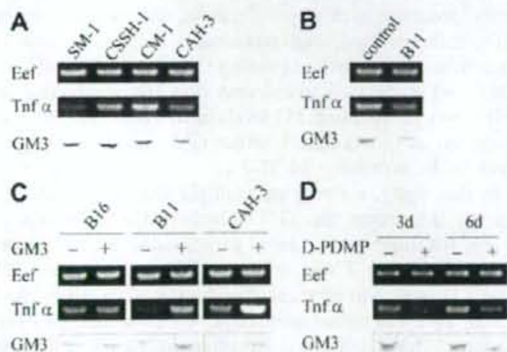

Fig. 1. The expression of TNFα is positively regulated by GM3. RT-PCR analysis of TNFα expression was carried out in (A) B16 and its variants that were generated by transfecting B4GalT-6 cDNA in a sense or antisense direction (CSSH-1, SM-1, CAH-3, and CM-1 are B16 cells transfected with sense B4GalT-6 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

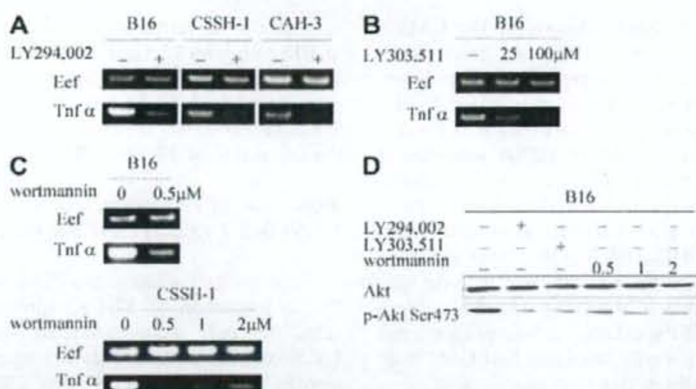

Fig. 2. Effect of PI3K inhibitors, LY294,002, LY303,511 and wortmannin on the expression of TNFα. In (A), B16, CSSH-1 and CAH-3 cells were treated with LY294,002 (25 μM) for 24 h. B16 cells were treated with LY303,511 (25 or 100 μM) (B) or wortmannin (0.5–2 mM) for 24 h (C). In (D), phosphorylation of 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.

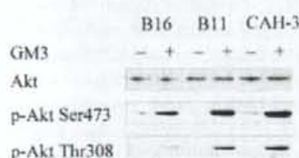

Fig. 3. Phosphorylation of Akt at the Ser^{473} and Thr^{308} sites was stimulated by GM3. Cells were starved for 6 h followed by incubation with GM3 (10 μM) under serum-free conditions for 10 min, lysed in 0.5 ml lysis buffer then analyzed by SDS–PAGE and Western blotting. Similar results were obtained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

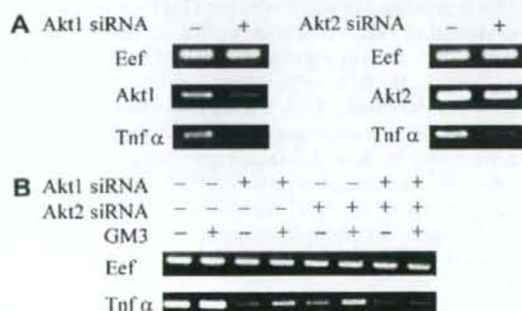

Fig. 4. Akt is essential for GM3-stimulated TNFα expression in B16 cells. TNFα was effectively suppressed in B16 cells treated with Akt1 and Akt2 siRNAs (A). Akt1, Akt2, or Akt1 plus Akt2 siRNAs transfected cells were screened with G418 (1.5 mg/ml) and the resistant cells were incubated with GM3 (25 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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Keywords D-PDMP, Ganglioside, GD1a, Metastasis, MMP-9, SiRNA, St3gal2

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

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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 $\pm$ S.E.).

siRNA Transfection

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

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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 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.

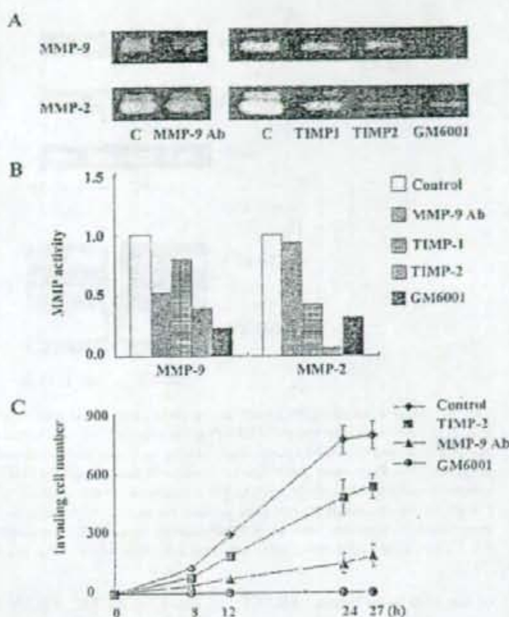

FIG. 1. MMP-9 responsible for metastaticity of FBJ-LL cells. To demonstrate that MMP inhibitors suppress MMP-9 and/or MMP-2 activity, (A) FBJ-M5 cells were incubated in serum-free medium from which an aliquot was mixed with an equal volume of double-strength Laemmli's sample buffer with no reducing reagent. 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.

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

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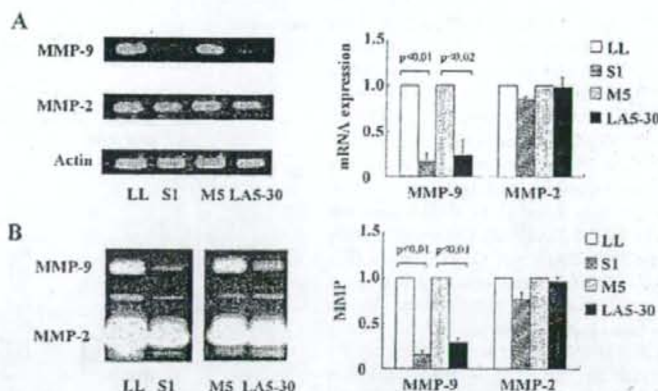

FIG. 2. MMP-9 but not MMP-2 levels are inversely proportional to GD1a expression at the level of mRNA and protein. FBJ-S1 (rich in GD1a), FBJ-LL (devoid of GD1a), FBJ-M5 (vector control of GM2/GD2 synthase cDNA transfectant originating from FBJ-LL), and FBJ-LA5-30 (GD1a rich due to GM2/GD2 synthase cDNA 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